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Biochemical and molecular genetic studies of uroporphyrinogen III synthase

Tsai, Shih-feng, Ph.D.

City University of New York, 1987

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Biochemical and Molecular Genetic Studies of Uroporphyrinogen III Synthase

by

Shih-feng Tsai, M.D.

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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May 14, 1987
Date

Robert J. Desnick
Chair of Examining Committee

May 14, 1987
Date

Executive Officer

James L. Wehmun

Gregory A. Grabowski

David F. Bishop
Supervisory Committee

The City University of New York

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List of Abbreviations

ALA-S	δ-aminolevulinate synthase
ALA-D	δ-aminolevulinate dehydratase
HMB-S	hydroxymethylbilane synthase
URO-S	uroporphyrinogen III synthase
URO-D	uroporphyrinogen decarboxylase
ALA	δ-aminolevulinic acid
PBG	porphobilinogen
HMB	hydroxymethylbilane
URO'gen	uroporphyrinogen
URO	uroporphyrin
CEP	congenital erythropoietic porphyria
EPP	erythropoietic protoporphyria
pCMB	para-chloromercuribenzoate
PMSF	phenylmethylsulfonyl fluoride
HPLC	high pressure liquid chromatography
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
cDNA	complementary DNA

INTRODUCTION

A. Human Heme Biosynthesis and the Porphyrias

The heme biosynthetic pathway and the associated "experiments of nature", the porphyrias, provide a unique system to investigate 1)the molecular pathology of human inherited diseases, and 2) the genetic regulation and control of vital human process.

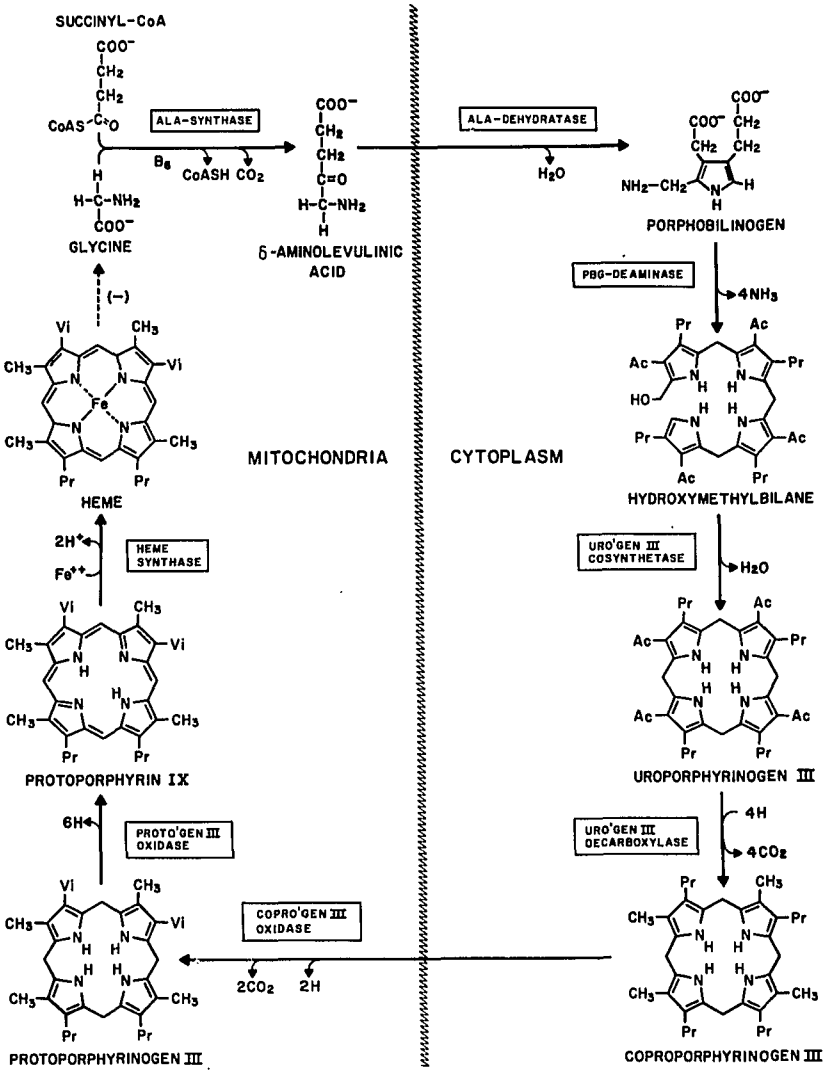
The human porphyrias are a heterogeneous group of diseases characterized by excessive production of porphyrins or their precursors. The clinical manifestations of the porphyrias vary, reflecting the biochemical consequences of specific enzyme deficiencies in the heme biosynthetic pathway, as well as the interaction of environmental factors.

1. Heme Biosynthetic Pathway

The biosynthesis of heme was not understood until the late 1940's when the application of radiotracer technology and the pioneering work of Shemin and others led to the elucidation of the heme biosynthetic pathway (1). Although the porphyrias were recognized early in the 20th century as diseases of abnormal heme metabolism characterized by specific patterns of porphyrin excretion, the enzymatic deficiencies in these disorders were not identified until the 1970's. Figure 1 shows the current concept of mammalian heme biosynthesis. Eight enzymes are required for the synthesis of heme. Interestingly, four are localized in the mitochondria while the other four are cytocylic. In mammals, δ-aminolevulinic acid (ALA) is synthesized in the mitochondria by condensation of succinyl CoA and glycine. This molecule is then transported into the cytosol, where the sequential and concerted action

Figure 1. Current concepts of heme biosynthetic pathway.

CURRENT CONCEPT OF HEME BIOSYNTHESIS



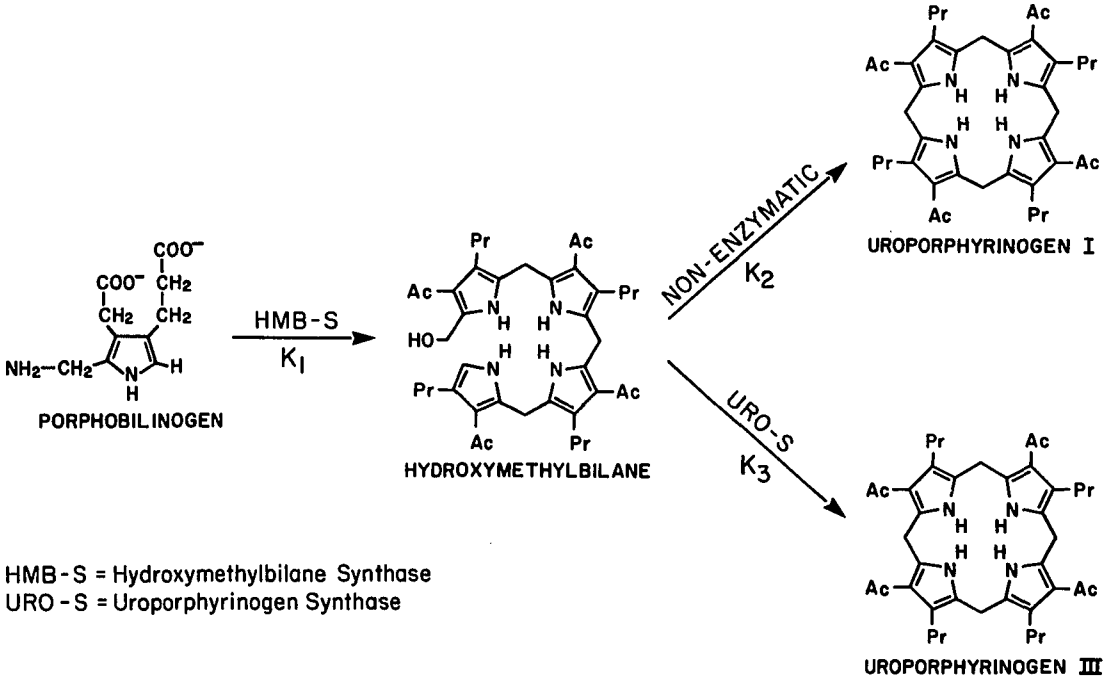
of δ-aminolevulinic dehydratase (ALA-D) acid, hydroxymethylbilane synthase (HMB-S), uroporphyrinogen III synthase (URO-S), and uroporphyrinogen decarboxylase (URO-D) converts ALA into a tetracarboxylic porphyrin, coproporphyrinogen III (COPRO'gen III). Subsequent oxidative reactions in the mitochondria by coproporphyrinogen oxidase (COPRO-oxidase) and protoporphyrinogen oxidase (PROTO-oxidase) result in the formation of protoporphyrin, which is chelated with iron to complete the biosynthesis of heme.

2. Biology of Porphyrins and Role of Uroporphyrinogen III and Uroporphyrinogen III Synthase in Heme Biosynthesis

Metalloporphyrins are tetrapyrrole compounds that are the essential prosthetic groups of hemoglobin, chlorophylls, cytochromes and cobalamins. Porphyrins consist of a cyclic tetrapyrrole ring with a metal atom in its center. Different metal atoms are present in hemoglobin and cytochromes (Fe), chlorophyll (Mg), cobalamin (Co). Under different oxidation-reduction states, these metal ions change their valency, and therefore, the metalloporphyrins function in electron transfer reactions. Because of their functional importance and universal existence, these metalloporphyrins have been collectively referred to as the pigments of life (2).

It is notable that the biosynthesis of the metalloporphyrins occurs through a common tetrapyrrole intermediate, uroporphyrinogen III (URO'gen III). Early work by various investigators (e.g., Bogorad, Granick, Neuberger, Remington and Shemin) have shown that URO'gen III is assembled from four molecules of porphobilinogen (PBG) (for review, see ref. 3). The pioneering work of Bogorad in 1953 (4,5) clearly demonstrated that two enzymes were involved in the synthesis of URO'gen III

Figure 2. Reaction scheme for the conversion of PBG to URO'gen I and III.



HMB-S = Hydroxymethylbilane Synthase
URO-S = Uroporphyrinogen Synthase

from porphobilinogen (PBG), namely, hydroxymethylbilane synthase (HMB-S; E. C. 4.3.1.8) and uroporphyrinogen III synthase (URO-S) (E.C. 4.2.1.75).

Since these seminal studies, the physical and kinetic properties of HMB-S and URO-S, their functional relationships (e.g., enzyme complex; co-factor, etc) and the chemical nature of their metabolic intermediates have been the subject of intense interest. More than 40 different hypotheses have been proposed for the reaction mechanism for URO'gen III formation (6). In 1979, evidence was reported indicating that HMB-S catalyzed the formation of an unstable linear tetrapyrrole from four molecules of PBG (7). Two groups reported different structures for the linear tetrapyrrole intermediate which presumably was converted to URO'gen III by URO-S. Scott and his colleagues referred to this intermediate as pre'urogen (8). It was Battersby et al., also using NMR spectroscopy and synthesis techniques, who suggested that the intermediate was hydroxymethylbilane (HMB) (9). The fact that HMB is a substrate for URO-S was subsequently demonstrated when the kinetic properties of synthetic HMB were shown to be identical to that of the substrate prepared by enzymatic conversion of PBG to the tetrapyrrole intermediate (9). The current concept of URO'gen III formation is shown in Fig. 2. HMB-S converts four molecules of PBG into the linear tetrapyrrole, HMB. In the presence of URO-S, the linear tetrapyrrole is cyclized and the D-ring is rearranged to form URO'gen III. In the absence of URO-S, HMB is non-enzymatically cyclized to form the URO'gen I isomer.

3. The Human Inherited Porphyrias

Of the eight enzymes of the heme biosynthetic pathway, inherited enzyme deficiency disorders have been reported for each with the exception of the first and rate-limiting enzyme, δ-aminolevulinic acid synthase (ALA-S). Collectively, these inherited disorders are referred to as the porphyrias. Table I lists the different types of porphyrias and their associated enzyme defects. These diseases are classified as either hepatic or erythroid depending on their tissue origin or major site of aberrant gene expression. With the exception of the two autosomal recessive disorders, congenital erythropoietic porphyria (CEP) and and δ-aminolevulinic acid dehydratase (ALA-D) deficiency, the porphyrias are transmitted as autosomal dominant traits.

a. Hepatic Porphyrias:

Hepatic prophyrias are particularly interesting in that individuals affected with these disorders may not manifest disease symptoms except when exposed to various environmental factors (e.g., sunlight, certain drugs, diet, hormones, etc). Such individuals may experience acute attacks of abdominal pain, neurological dysfunction, neuropsychiatric symptoms and paresis when exposed to these environmental factors. Excessive urinary excretion of δ-aminolevulinic acid (ALA) or PBG during acute attacks is a cardinal biochemical finding in the hepatic porphyrias (with the exception of porphyria cutanea tarda). Drugs such as alcohol, certain antibiotics and phenobarbitol, as well as physiological changes (hormones, starvation) have been shown to induce the activity of hepatic ALA-S, and therefore to precipitate acute attacks of the hepatic porphyrias. In each of these disorders, the deficiency of a specific heme biosynthetic enzyme renders the pathway

Table I

THE INHERITED HUMAN PORPHYRIAS

Porphyria	Enzymatic Defect	Mode of Inheritance*
Erythropoietic Porphyrias:		
Congenital Erythropoietic Porphyria	URO III Synthase	AR
Protoporphyria	Ferrochelatase	AD
Hepatic Porphyrias:		
ALA-Dehydratase Deficiency	ALA-Dehydratase	AR
Acute Intermittent Porphyria	HMB-Synthase	AD
Porphyria Cutanea Tarda	URO-Decarboxylase	AD
Coproporphyria	COPRO-Oxidase	AD
Variegate Porphyria	PROTO-Oxidase	AD

*AR = Autosomal Recessive; AD = Autosomal Dominant

ineffective, since the porphyrin precursors, ALA and PBG cannot be metabolized efficiently, and therefore they accumulate. These compounds are believed to mediate the clinical manifestations of an acute attack. Concommitent with the accumulation of these porphyrin precursors, the amount of heme produced by the pathway is markedly decreased. The lack of a sufficient free heme pool to feedback or repress the synthesis of ALA-S results in the further induction of hepatic ALA-S and the production of additional ALA and PBG. This cycle of events can only be reversed by the repression of ALA-S synthesis. Thus, investigators have intravenously administered hematin or heme-conjugates with dramatic biochemical and clinical improvement (10).

It is noteworthy that ALA-S isozymes occur in the liver and bone marrow (11). However, only hepatic ALA-S is subject to the feedback regulation of heme (12). Yamamoto et al. have reported that different mRNA forms may exist in chick liver and bone marrow (13). Maguire and colleagues reported that only one gene is present in chicken (14). These studies suggest that differential mechanism, either due to specific tissue factors, selective useage of promoters or alternate splicing of the introns from a single gene may be responsible for the tissue specific expression of the ALA-S isozymes. Thus it is likely that the tissue specific expression of ALA-S provides the molecular basis for the observed differences in the differential kinetic properties of the hepatic and erythroid forms. This subject is an active area of current research.

b. Erythropoietic Porphyrias :

Erythropoietic protoporphyria (EPP) and CEP are the two erythropoietic porphyrias. They are characterized by the over-production of porphyrins in erythrons. The deficient activity of ferrochelatase (heme synthase) in EPP results in accumulation of the free protoporphyrin and subsequent diffusion of the porphyrin into the plasma and then into the skin and other tissues. In the presence of sunlight, photo-oxidative cell damage occurs, leading to skin blistering, infection,and scarring in the affected individuals. Animal models have been described in cattle (15).

Among the human porphyrias, CEP has probably been studied the least. The enzymatic defect in this erythropoietic prophyria was first proposed by Bogorad in the course of his classic studies of URO'gen III formation. CEP appears to be the most infrequent of the porphyrias. By 1972, only 60 cases were reported in the literature (16). The clinical manifestations of CEP include photosensitivity, hemolytic anemia, erythrodontia, and porphyrinuria. These symptoms result from the deficient activity of URO-S and the resultant over-production in the bone marrow of URO'gen I and its decarboxylated derivative, coproporphyrinogen I (COPRO I). Accumulated URO'gen I is oxidized to uroporphyrin I (URO I) in erythrocytes and this compound is toxic to these cells resulting in their lysis. The erythrocyte URO I is then released into the plasma and is taken up by the tissues and excreted. Porphyric animals with similar biochemical and clinical features have been reported in cattle (17) and the red squirrel (18).

Bogorad first suggested that deficient URO-S activity, or increased HMB-S activity, or a combination of both, might be the biochemical defect in CEP (5). Subsequently, Romeo and Levin demonstrated low levels of erythrocyte URO-S activity in five patients with CEP (19). Watson and his coworkers, on the other hand, postulated that a primary increase of ALA-S or HMB-S activity, either due to a hyperactive variant allele or secondary to a mutation involving a negative regulator mechanism, was responsible for the observation that CEP patients excreted both URO I and III in excessive amounts (20). Watson et al. observed increased HMB-S activity in CEP erythrocytes (21), but this was probably secondary to the reticulocytosis in response to hemolysis in these patients. Moreover, the HMB-S activity was normal whereas reduced URO-S activity was observed in cultured fibroblasts from CEP homozygotes (22). Finally, evidence for deficient URO-S as the primary enzymatic defect in CEP was the fact that obligate heterozygote for CEP had half-normal levels of URO-S in cultured fibroblasts (23). Thus, it is currently accepted that deficient URO-S activity is the enzymatic defect responsible for this inborn error of heme biosynthesis.

B. Molecular Studies of Human Heme Biosynthesis

In order to characterize the structure, genomic organization, and the tissue specific expression of the heme biosynthetic genes, as well as to investigate the nature of the molecular lesions which cause the different porphyrias, recent efforts have been directed to study the human heme biosynthesis at the molecular level.

To date, 5 genes (cDNAs) have been cloned (Table II). The first gene isolated was ALA-S from chick embryo liver (Borthwick et al (24). The cloning of this gene was facilitated by the fact that ALA-S activity could be markedly induced by the administration of either AIA or DDC. Thus, the enrichment of ALA-S mRNA in the chick embryo liver permitted the isolation of the message by hybrid selected translation and immuno precipitation. Also in 1984, HMB-S and URO-D cDNAs were isolated from rat splenic tissue; these clones were confirmed by hybrid selected translation with the respective monospecific antibodies (25, 26). These were the first mammalian cDNAs for heme biosynthetic enzyme isolation. Subsequently, cDNAs were also isolated from other species (Table III).

Investigators were intrigued by the possibility that the genes encoding the heme biosynthetic enzymes were linked in a gene family so that they could be efficiently regulated. In several bacteria and in yeast, these genes are next to each other or closely linked (27). The first human heme biosynthetic gene assigned to a chromosome was HMB-S (chromosome 11) (28). The subsequent reports that ALA-D was on chromosome 9 (29) and that the gene for URO-D was on chromosome 1 (30) laid to rest the concept that the human genes were linked and indicated that the coordinate regulation of the pathway presumably involved trans-activating factors. Table III summarizes the current status of the chromosomal assignments of the human hemebiosynthetic genes. Note that five of the eight gene loci have been assigned and that these genes are dispersed on chromosomes 1,3,9 and 11.

Table II

MOLECULAR CLONING OF cDNAs ENCODING THE HEME BIOSYNTHETIC ENZYMES
(STATUS 5/1/87)

cDNA	Source	Reference
ALA-Synthase	Chick Embryo Liver	Borthwick et al. 1984
	Chicken Erythrocytes	Yamamoto et al. 1985
	Yeast	Urban-Grimal et al. 1986
	Murine Liver/Spleen	Schoenhaut et al. 1986
	Human Liver	Bishop et al. Unpublished
ALA-Dehydratase	Human Liver	Wetmur et al. 1986
	Rat Liver	Bishop et al. 1986
HMB-Synthase	Rat Spleen	Grandchamp et al. 1984
	Human Spleen	Raich et al. 1986
	Human Liver	Wetmur et al. Unpublished
URO III Synthase	Human Liver	Tsai et al. Unpublished
URO-Decarboxylase	Rat Spleen	Romeo et al. 1984
	Human Spleen	Romeo et al. 1986
	Human Liver	Tsai et al. Unpublished

Table III

CHROMOSOMAL LOCALIZATION OF THE HEME BIOSYNTHETIC GENES

Structural Gene	Chromosomal Assignment	Method	Reference
ALA-Synthase	3	Hybrid Panel*	Bishop et al. unpublished
ALA-Dehydratase	9q34	In Situ**	Potluri et al. 1986
HMB-Synthase	11q23-ter	Hybrid Panel	Wang et al. 1981
URO-Decarboxylase	1p34	In Situ	Romeo et al. 1986
COPRO-Oxidase	9	Hybrid Panel	Grandchamp et al. 1983

*Hybrid Panel = Analysis of Somatic Cell Hybrid Cell Panel
**In Situ = Hybridization with cDNA Probe.

Specific Aims of this Research

In order to characterize the property of URO-S, and study the genetic basis of CEP, the following objectives were defined:

1) Development of assay methods for URO-S.

The assay methods published previously require substantial efforts to separate and quantitate the URO I and III isomers. (For review, see ref. 31). Therefore, a simple and reliable assay method was needed to accurately measure URO-S activity. This method would be required for biochemical confirmation of CEP homozygotes and for the detection of carriers of the CEP gene. In addition, this assay should provide an efficient method for monitoring enzyme activity during protein purification, particularly since URO-S is unstable.

2) Purification and Characterization of URO-S.

Because of the difficulty associated with the assay methods and the exquisite instability of URO-S, the enzyme from animal sources has never been purified sufficiently to reliably determine its physical and kinetic properties. In fact, limited physicokinetic data are available for this enzyme. For example, the molecular weight determinations reported in literature range from 10,000 to 210,000 (32). In order to accurately characterize the enzyme's physical and kinetic properties, the enzyme must be purified to near homogeneity. However, the lack of an reliable assay and the fact that the enzyme is so unstable has stymied previous purification efforts. To date the URO-S has not been purified from a mammalian source more than several hundred fold.

3) Isolation and sequencing of a full-length cDNA for human URO-S.

In order to study the molecular genetic basis of CEP, cDNA clones encoding human URO-S must be isolated and characterized. One approach for isolating specific cDNAs involves the use of expression libraries which have been constructed to produce fusion proteins containing the amino acid sequences in the cDNA inserts (33). Expression of the cDNA of interest is detected using the specific antibody (34). Another approach for isolating a specific cDNA employs the principle of hybridization to synthetic oligonucleotide mixtures (35). When the amino acid sequence of the expressed protein is known, synthetic oligonucleotide mixtures can be constructed to correspond to a portion of the amino acid sequence with minimal codon redundancy. Appropriate hybridization and washing conditions can then be chosen so that hybridization will occur when a sequence in the oligonucleotide probe mixture is correctly matched by a complementary sequence in a cDNA insert. The latter technique has been employed for cloning the cDNA for human URO-S.

The isolated cDNA clone can be used to study the molecular nature of CEP mutations in unrelated families, to study the organization and chromosomal localization of URO-S, and to investigate the molecular basis of metabolic regulation of the URO-S gene. As noted above, this is particularly important since the heme biosynthetic enzyme genes are distributed at different chromosomes (Table III), yet, the regulation of these genes seem to be concerted with a specific temporal scheme during induction or differentiation (36). Moreover, cDNA clones have been isolated for several of the heme biosynthetic genes from different species (Table III). To date, human cDNA clones have been isolated for

the four cytosolic enzymes, that is, ALA-D (37), HMB-S (38), URO-S (see Chapter three), and URO-D (39). To facilitate the cloning of each of these genes, the respective enzymes were purified from human erythrocytes and their properties were compared (40). In all cases, peptide sequences derived from the purified erythrocyte enzyme were used to confirm the authenticity of the putative clones. Human cDNAs for ALA-D and URO-S were isolated from an adult liver library (41), while clones for HMB-S and URO-D were initially isolated from a library constructed from splenic tissue of an anemic rat (25-26). The murine clones were then used to screen a human library of erythroid (HMB-S and URO-D) or hepatic origin (HMB-S). It is noteworthy that cDNAs encoding hepatic and erythroid HMB-S differ only in their 5' sequences (42). Moreover, it was proposed that two different promoters exist in a single gene, and preferential utilization of promoters may explain the tissue-specific expression of HMB-S.

Interesting insights into the evolution of the hemebiosynthetic genes have come from molecular studies of ALAS. cDNA clones for ALA-S have been isolated from yeast (43), chick embryo liver (25), mouse liver and erythroid sources (44), and most recently from human liver (Dr. David F. Bishop, unpublished data). These cDNAs represent the only sequences available for a mitochondrially compartmentalized enzyme of the heme biosynthetic pathway. A gradient of protein sequence homology was found in the deduced amino acid sequence from different species (44). Portions of the amino acid sequence which appear to be unessential for catalytic function have been shown to diverge, while other parts of

the sequence were highly conserved (90% amino acid homology), suggesting that a common structural conformation has been preserved through evolution.

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Chapter One

COUPLED-ENZYME AND DIRECT ASSAYS FOR UROPORPHYRINOGEN III SYNTHASE ACTIVITY IN HUMAN ERYTHROCYTES AND CULTURED LYMPHOBLASTS

Enzymatic Diagnosis of Heterozygotes and Homozygotes with Congenital Erythropoietic Porphyria

Shih-feng Tsai, David F. Bishop, and Robert J. Desnick

From The Division of Medical Genetics
Mount Sinai School of Medicine
New York, New York 10029

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ABSTRACT

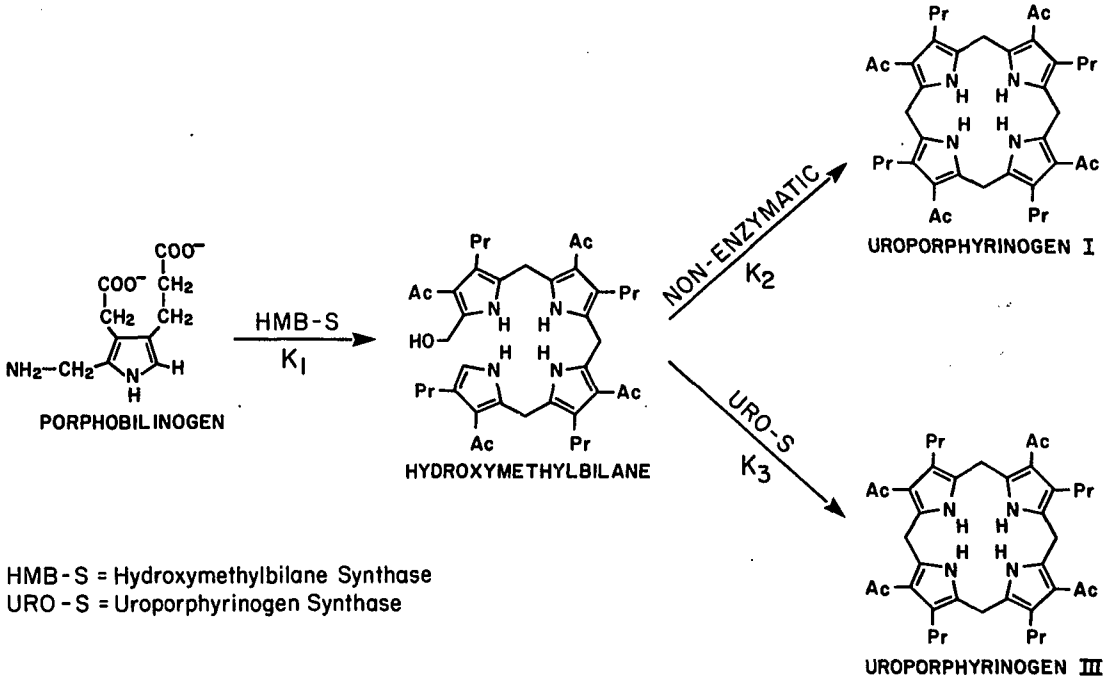
Rapid and reproducible assays for uroporphyrinogen III synthase (URO-S; EC 4.2.1.75) have been developed and used to determine the enzymatic activity in human erythrocytes and cultured lymphoid cells. In the coupled-enzyme assay, porphobilinogen was first converted to hydroxymethylbilane, the natural substrate for URO-S, by hydroxymethylbilane synthase (HMB-S) which was conveniently obtained from heat-treated erythrocyte lysates. In the direct assay, synthetic hydroxymethylbilane was used as substrate. In both assays, the uroporphyrinogen reaction products were oxidized to their respective uroporphyrin isomers, which were then resolved and quantitated by reversed-phase high pressure liquid chromatography. Both assays were optimized for pH, substrate concentration and linearity with time and protein concentration. The mean URO-S activities in normal human erythrocyte lysates determined by the coupled-enzyme and direct assays were 7.41 ± 1.35 and 7.64 ± 1.73 units/mg protein, respectively. In normal human cultured lymphoid cells, the mean activities were 13.7 ± 1.39 and 17.6 ± 1.15 units/mg protein for the coupled-enzyme and direct assays, respectively. In four families with congenital erythropoietic porphyria, both assays reliably identified the markedly decreased URO-S activities in erythrocytes and cultured lymphoid cells from affected homozygotes and the half-normal activities in these sources from obligate heterozygotes. The coupled-enzyme assay was easier to perform and was suited for clinical diagnostic assays and for monitoring of enzyme purification procedures, while the direct assay, which required substrate preparation and technical dexterity, was best for kinetic studies of URO-S.

INTRODUCTION

The conversion of the monopyrrole, porphobilinogen (PBG), to uroporphyrinogen III (URO'gen III), the tetrapyrrole that is the physiological precursor of protoporphyrin IX and heme, requires the sequential action of two enzymes: hydroxymethylbilane synthase (HMB-S; EC 4.3.1.8; previously known as porphobilinogen deaminase) and uroporphyrinogen III synthase (URO-S; EC 4.2.1.75; formerly designated uroporphyrinogen III cosynthase) (1-4). As shown in Figure 1, HMB-S catalyzes the head to tail condensation of four molecules of PBG to form the linear tetrapyrrole, hydroxymethylbilane (HMB) (5-9). In the presence of URO-S, HMB is rapidly converted to URO'gen III by a reaction mechanism which involves the intramolecular rearragement of the D pyrrole group and ring closure (10-12). In the absence of URO-S, HMB is non-enzymatically cyclized to form uroporphyrinogen I (URO'gen I), an isomer which has no known physiologic function. In fact, the deficient activity of URO-S in homozygotes with congenital erythropoietic porphyria (CEP) (13-15) results in the accumulation of URO'gen I, the primary pathologic compound in this inherited human disorder.

The characterization of URO-S has been limited primarily by the lack of a rapid, accurate and reproducible assay. Early assays were based on the rate of PBG consumption (e.g., 3,16,17) and/or the ratio of URO'gen III and I formation as estimated following their conversion to and separation as porphyrins (e.g., 3,13-21). These assays were extremely laborious and at best semi-quantitative due to the difficulty in reproducibly separating and quantitating the porphyrin isomers (for review, see 22). In 1980, Jordan et al. introduced an assay (23) based

Figure 1: Reaction scheme for the conversion of PBG to URO'gen I and III.



HMB-S = Hydroxymethylbilane Synthase
URO-S = Uroporphyrinogen Synthase

on the fact that HMB (which they designated "preuroporphyrinogen") was rapidly cyclized to URO'gen III by URO-S whereas it was non-enzymatically converted to URO'gen I at a markedly slower rate. Although this assay did not require URO isomer separation, it was limited by the need for large amounts of purified HMB-S (to generate HMB) as well as by the manual dexterity required to reproducibly initiate, mix and terminate the reactions in exactly 1 min in order to minimize the formation of URO'gen I.

The recent availability of HPLC methods to separate and quantitate uroporphyrin isomers without derivatization has facilitated the development of more reliable URO-S assays (e.g., 24-26). Wright and Lim (27) recently reported a novel method for the simultaneous determination of HMB-S and URO-S activities in human erythrocytes. In their procedure, HMB was generated by HMB-S which was conveniently obtained after denaturation of the remarkably thermolabile URO-S activity in the hemolysate. Following addition and incubation with the enzyme source (untreated hemolysate), the reaction products were oxidized to uroporphyrin (URO) I and III and then separated and quantitated directly (without esterification of the URO isomers) by high pressure liquid chromatography (HPLC) using a C_{18} reversed-phase HPLC column. The HMB-S actvitity was determined from the total URO (I plus III) formed, while the URO-S activity was calculated from the amount of URO III produced. This method used an aliquot of the hemolysate to generate HMB; however, no provisions were made to determine the URO-S activity in enzyme sources which did not contain HMB-S. Thus, this assay could not be used for monitoring the purification of URO-S. In addition, since the HMB-S activity in the reaction mixture was not standardized, the amounts of

HMB, URO I and III produced would vary with each sample, thereby limiting the usefulness of this method for the enzymatic diagnosis of individuals with CEP. In fact, when erythrocytes from a homozygote with CEP were assayed, the URO-S activity was about 50% of the mean level in normal hemolysates (27), a value expected for heterozygous carriers of the CEP gene.

More recently, Battersby and coworkers reported the development of a direct assay for URO-S based on their previous finding that HMB was a substrate for this enzyme (11). Employing synthetic HMB, this assay has been used to determine the kinetic properties of purified URO-S from Euglena (12) and from human erythrocytes (28). These studies definitively demonstrated that URO'gen III was rapidly formed from HMB by URO-S in the absence of HMB-S. However, the direct assay has never been used for the determination of URO-S activity in human erythrocytes or lymphoblasts for clinical diagnostic studies or other mammalian sources for research purposes.

In this communication, we describe modified coupled-enzyme and direct assays for the determination of URO-S activity in human erythrocytes and cultured lymphoid cells. Under these optimized conditions, accurate detection of affected homozygotes and obligate heterozygotes in families with CEP was achieved. In addition, the K_m values for URO-S in human erythrocytes were determined using both assay systems.

METHODS AND MATERIALS

Materials:

Ammonium acetate (HPLC grade) was obtained from Fisher Scientific Company (Springfield, NJ). Acetonitrile (Burdick and Jackson Laboratories, Inc.) was purchased from American Scientific Products, (Edison, NJ). HMB octamethyl ester and URO I and III were purchased from Porphyrin Products (Logan, UT). HMB octamethyl ester also was generously provided by Dr. A.R. Battersby, Cambridge, England. PBG hydrate was synthesized as described (29).

Specimen Collection and Preparation:

Blood (10 ml) from normal individuals and from heterozygotes and homozygotes with CEP was collected in heparinized tubes (Vacutainers, Becton and Dickinson, Rutherford, NJ). Following centrifugation at 800 x g, the plasma and buffy coat were removed. The packed erythrocytes were resuspended and washed twice in 0.15 M NaCl and either frozen at -70°C or used immediately for assay. For preparation of hemolysates (1:19 dilution) in the coupled-enzyme assay, 50 µl of packed, washed erythrocytes and 950 µl of lysis buffer (0.1 M HEPES buffer, pH 8.2, containing 0.1% Triton X-100 and 0.02% sodium azide) were pipetted into 1.5 ml Eppendorf tubes, vortexed, centrifuged in a microfuge for 15 min and then the supernatants were removed for assay. For preparation of hemolysates (1:3 diluted) in the direct assay, 250 µl of packed, washed erythrocytes were lysed in 750 µl of buffer and the supernatants were removed for assay as above.

Another 10 ml of heparinized blood from each patient was used to establish cultured lymphoid lines as previously described (30,31). The cultured cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (GIBCO, Grand Island, NY). When the culture reached a density of 10^6 cells/ml, 50 ml of the suspension was centrifuged at 800 x g for 10 min. The cells were resuspended and washed twice with cold 0.15 M NaCl. The cell pellet was then suspended in 100 µl of lysis buffer, subjected to one cycle of freeze-thaw, microfuged at 4°C for 15 min and then the supernatant was removed for assay.

Uroporphyrin Separation and Quantitation:

URO isomers were separated and quantitated by HPLC as previously described (26,27), except that the column was placed in a water-jacket which was maintained at 20°C using a constant temperature circulating bath (Haake Model FE2). The HPLC system consisted of a Waters Associates 720 controller, dual model 6000A pumps, a model 730 data integrator and a WISP automatic sample processor. A Shandon 0.46 x 25 cm ODS-Hypersil column was used. Porphyrins were resolved using a 15 min 13-30% concave (Water's curve No. 8) acetonitrile gradient in 1.0 M ammonium acetate (1.0 M as ammonium ion), pH 5.16, at a flow rate of 1 ml/min. Re-equilibration was accomplished with a 5 min reverse gradient and a 15 min isocratic step at 13% acetonitrile. Detection with a minimum of 1.0 pmol was accomplished with a Kratos model FS 950 filter fluorimeter equipped with a 28 µl flowcell, a red-sensitive photomultiplier tube and a Waters Associates UV lamp (No. 78245). The excitation wavelength was 395 nm using an interference filter (LAG 11-10); the emission wavelength was selected with a 530 nm cutoff

filter (Series D, S-3-133-8). Uroporphyrin standard curves were established using URO I and III dissolved in 1 N HCl. Injection of solutions containing 10 to 400 pmol URO I or III established a linear range for 10 to 150 pmol of either URO isomer (Fig. 2).

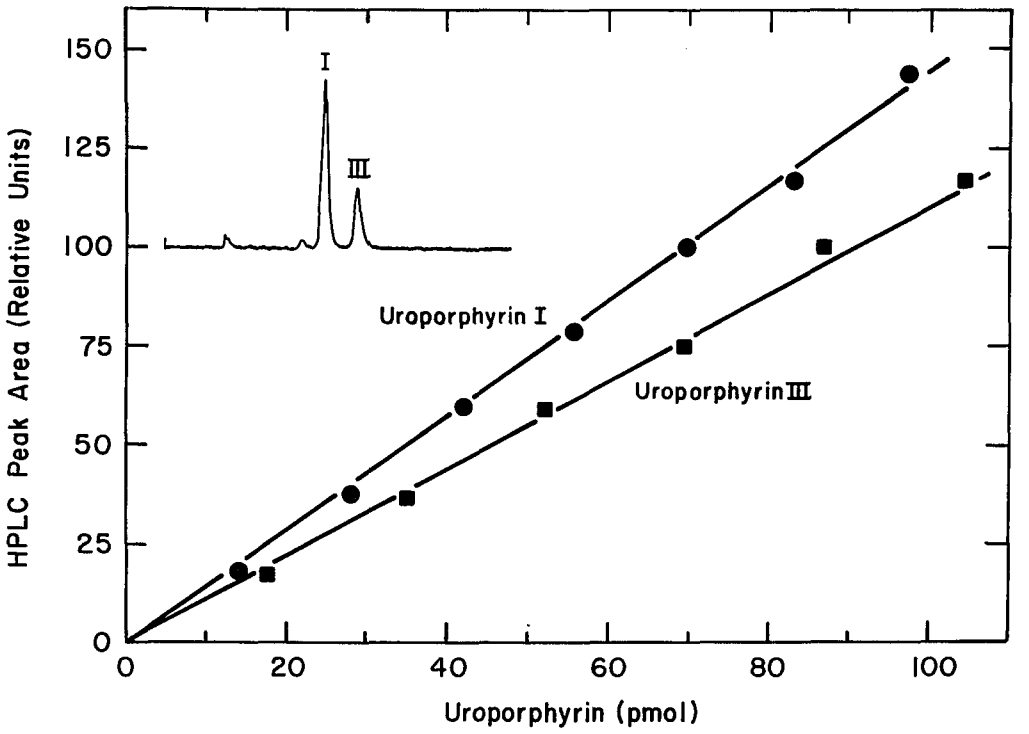
Assays for HMB-S Activity and Protein Concentration:

HMB-S activities were determined as previously described (29). One unit (U) of HMB-S activity equals that amount of enzyme required to form one nanomole of HMB (determined as URO I) per hour. Protein concentrations were measured by the fluorescamine method (32).

URO-S Coupled-Enzyme Assay:

Preparation of HMB-Generating Hemolysate. Outdated packed erythrocytes (obtained from the Greater New York Blood Center) were washed twice with cold 0.15 M NaCl. For hemolysis, 100 ml of packed erythrocytes were mixed at 4°C for 1 h with 3 volumes of lysis buffer. URO-S activity was completely inactivated by heating the hemolysate at 60°C in a waterbath for 2 h. The hemolysate was centrifuged at 10,000 x g for 15 min, and then the supernatant was removed and diluted with lysis buffer to 5 U of URO-S activity per ml (designated the HMB-generating hemolysate). The HMB-S activity in the hemolysate remained active when stored in 20 ml aliquots at -70°C for several months. Hemolysate from 100 ml of washed packed erythrocytes was prepared in one day and provided HMB-generating hemolysate for more than 800 assays. In each preparation of the HMB-generating hemolysate, the absence of URO-S activity and endogenous URO I and/or III was routinely demonstrated prior to use.

Figure 2: Resolution and quantitation of URO I and III by reversed phase HPLC. Although the integrated area was linear for either isomer up to at least 150 pmol, only the range of 0 to 100 pmol was used for determination of conversion factors for URO I and III quantitation. The HPLC resolution of the URO I and III isomers is shown in the inset.


HPLC Peak Area (Relative Units)
150
125
100
75
50
25
0
0
20
40
60
80
100
Uroporphyrin (pmol)
I
III
Uroporphyrin I
Uroporphyrin III

Assay. Aliquots (500 µl) of the HMB-generating hemolysate and 10 µl of 1:19 diluted hemolysate or lymphoid cell lysate were prewarmed at 37°C for 5 min in 13 x 100 mm disposable culture tubes. The reaction was initiated by the addition of 50 µl of 3.4 mM PBG hydrate and the mixture was incubated for 15 min at 37°C. The reaction was terminated by adding 440 µl of 10% trichloroacetic acid (TCA; w/v) containing 0.5% (w/v) iodine. Following centrifugation at 4000 x g for 20 min, 250 µl of the supernatant was transferred to an HPLC autosampler vial which was wrapped with aluminum foil to prevent photo-oxidation. Duplicate 100 µl aliquots were analyzed by HPLC to determine the amounts of URO I and III formed. One unit (U) of URO-S activity is defined as that amount of enzyme required to form one nmol of URO'gen III per hour. For example, the URO-S activity/ml for 10 µl of enzyme source = nmol URO III formed x 1000/ 100 x 1000/10 x 60/15.

K_m Determination. The coupled-enzyme assay was modified to determine the apparent K_m for URO-S from normal human erythrocytes. The HMB-generating hemolysate was diluted with the lysis buffer to obtain a series of concentrations ranging from 0.5 to 5.0 U/ml HMB-S. An aliquot (500 µl) of each dilution was prewarmed at 37°C for 5 min, and then 50 µl of 3.4 mM PBG hydrate was added at zero time. The mixture was preincubated for 13 min to obtain steady-state HMB levels, at which time, 10 µl of purified URO-S (40 U/ml) were added. The reaction mixture was incubated for an additional 15 min and terminated by the addition of 440 µl of 10% TCA (w/v) containing 0.5% (w/v) iodine. The velocity was expressed as nmol/min.

Direct URO-S Assay:

Hydrolysis of HMB Octamethyl Ester. Due to its instability, HMB was prepared and stored as an octamethyl ester and hydrolyzed to HMB immediately prior to use (11,12). To maintain anerobic conditions, 10 µl of 2.0 N KOH (saturated with N_2) was added with a Hamilton syringe to a screw-capped reaction vial, fitted with a teflon septum, containing 34 nmol of HMB octamethyl ester. The reaction mixture was vortexed vigorously for 2 min. The HMB octamethyl ester was hydrolyzed for 16 h by incubation in a 16°C or 23°C waterbath, or by incubation for 15 min in a 70°C waterbath. Freshly prepared HMB was then diluted with 690 µl of cold, deionized water for a final HMB concentration of 48 µM, assuming 100% yield from the hydrolysis. For the standard assay, hydrolysis was performed at 70°C for 15 min, which resulted in about a 60% conversion of HMB octamethyl ester to HMB (see Table 1). Thus, the standard hydrolysate contained about 30 µM HMB.

Assay. The assay was performed at room temperature (23°C). To a 1.5 ml microcentrifuge tube, 430 µl of 50 mM Tris-HCl buffer, pH 7.5, containing 0.15 M KCl and 50 µl of the hemolysate (1:3 diluted) or lymphoid cell lysate were added. For routine clinical diagnosis, the reaction was initiated by the addition of 20 µl of 30 µM HMB with immediate vortexing. The reaction was terminated precisely 30 sec later by the addition of 500 µl of 10% TCA (w/v) containing 0.5% iodine (w/v). The reaction mixture was centrifuged at 10,000 x g for 5 min, the supernatant removed and a 250 µl aliquot was transferred into an HPLC autosampler vial as above and then up to 200 µl were injected on to the HPLC column to determine the URO I and III content.

K_m Determination: The K_m was determined at pH 8.2 and 37°C in order to compare the result with that obtained using the coupled assay. After hydrolysis of the HMB octamethyl ester, the following incubations were performed for each HMB concentration: 1) Assay: enzyme (11 U) was incubated for 20 sec in 0.5 M Tris-HCl buffer, pH 8.2, containing 150 mM KCl. 2) Enzyme blank: to correct for spontaneous conversion of HMB to URO I, lysis buffer was added in place of enzyme. 3) Zero time: to correct for the amount of URO I and III in the substrate solution, the reaction was terminated at T_0. 4) To determine the HMB concentration in the reaction mixture, another zero time sample was terminated with TCA (to convert all the HMB to URO'gen) followed by oxidation with I_2 (to form URO from URO'gen). From this value, the amount of URO in the zero time blank was subtracted, giving the HMB concentration. The velocity was expressed as μmol/hr. At an HMB concentration equivalent to that in the standard assay, the URO III concentration in the enzyme blank was 0 to 0.2% of the total URO detected. At 4-fold greater HMB concentrations, the URO III content in the enzyme blank was 2% of the total URO detected. Thus, enzyme blanks should be performed, particularly when using higher HMB concentrations for assay.

RESULTS

Separation and Quantitation of the URO Isomers:

Since the accuracy of both the coupled-enzyme and direct assays depend on the ability to reproducibly separate the URO I and III isomers for quantitation, initial efforts were directed to optimize the HPLC method of Rideout et al. (26) for the resolution of the URO isomers as their free acids. The life of the column was prolonged (~ 3000 cycles)

by eluting the other porphyrin free acids (without impairing isomer resolution) with a concave gradient of 13-30% acetonitrile in 1.0 M ammonium acetate in place of the 13% isocratic elution used by Rideout et al. (26). Use of the acetonitrile gradient also permitted the elution and quantitation of the URO'gen decarboxylation intermediates (hepta-, hexa-, and penta-carboxylic acid porphyrins) and coproporphyrin without additional elution time. In addition, both URO isomer separation and run to run reproducibility were improved by maintaining the column at a lower constant temperature using a 20°C water-jacket. Finally, evaluation of several commercially available C_{18} columns found the Shandon C_{18} reversed-phase column gave the best URO isomer resolution. Using these conditions, excellent separation of the URO I and III isomers was obtained with baseline resolution (Fig. 2, inset), facilitating accurate quantitation. As shown in Fig. 2, increasing amounts of URO I and III standards gave slightly different, but linear responses permitting the calculation of conversion constants for URO I and III quantitation, respectively.

Optimization of the Coupled-Enzyme Assay:

The coupled-enzyme assay was optimized for 1) preparation and standardization of the HMB-generating hemolysate, 2) pH and buffer, and 3) linearity with time and enzyme concentration.

Preparation and Standardization of the HMB-Generating Hemolysate. Since the HMB-S generating hemolysate must be devoid of URO-S activity, initial studies were conducted to determine the optimal conditions for heat-inactivating URO-S in normal erythrocytes. Aliquots of a hemolysate containing 7.0 U/ml of HMB-S activity were heated at 56°C or 60°C

for up to 5 h, incubated with PBG at 37°C for 20 min (29), and then the reaction products were oxidized and the URO isomer content was determined by HPLC. Thermal inactivation of URO-S at 56°C for 1 h as previously described (27) did not reproducibly inactivate all of the endogenous URO-S activity; up to 5% of the total URO formed was URO III under these conditions. At 60°C, URO-S was totally inactivated by 15 min, while HMB-S was stable for at least 4 h (data not shown).

The preparation and activity of the HMB-generating hemolysate were standardized in order to maximize assay reproducibility and to extend the use of the coupled-enzyme assay to enzyme sources with little or no endogenous HMB-S activity. Routinely, 100 ml of packed, normal human erythrocytes were lysed, the endogenous URO-S was heat-inactivated at 60°C for 2 h and the hemolysate was diluted to give a final HMB-S activity of 5 U/ml.

pH and Buffer. Figure 3 shows the pH versus activity profile which was determined using several different buffers. The activity of URO-S in the coupled-enzyme assay was maximal at pH 8.2 with either Tris-HCl or HEPES buffer; HEPES buffer was chosen for the standard assay since the protein concentration was determined by the fluorescamine method (32). $MgCl_2$ was eliminated from the buffer since it inhibited HMB-S activity 30% and 55% at 14 mM and 50 mM concentrations, respectively (28). In addition, sodium azide was added to the buffer to prevent microbial growth.

Linearity with Time and Enzyme Concentration. Under the conditions defined above, total URO (I+III) production was linear with time for at least 1 h, while URO III production was linear for only 15 min (Fig. 4). Therefore, all subsequent assays were 15 min incubations. The coupled-

Figure 3: Effect of pH on URO-S activity in the coupled enzyme assay. HEPES, Tris, and borate buffers were used to adjust the pH of each incubation mixture (0.1M buffer salt and 0.1% Triton X-100, final concentrations). After incubation for 15 min, an aliquot was removed from each reaction mixture to determine the actual pH value. The URO-S activity was expressed as the percentage of maximal activity in HEPES buffer, pH 8.2.

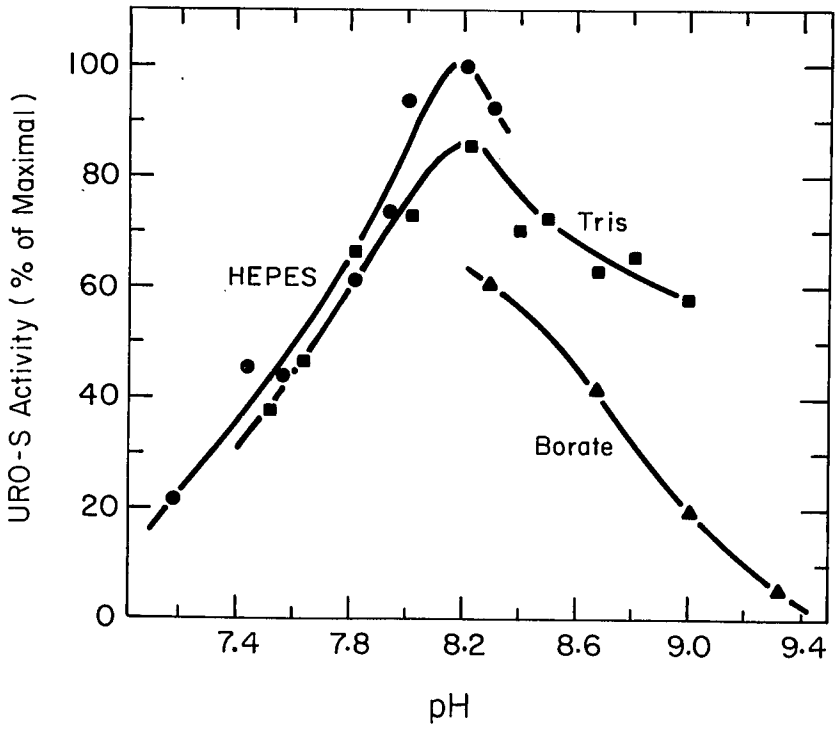
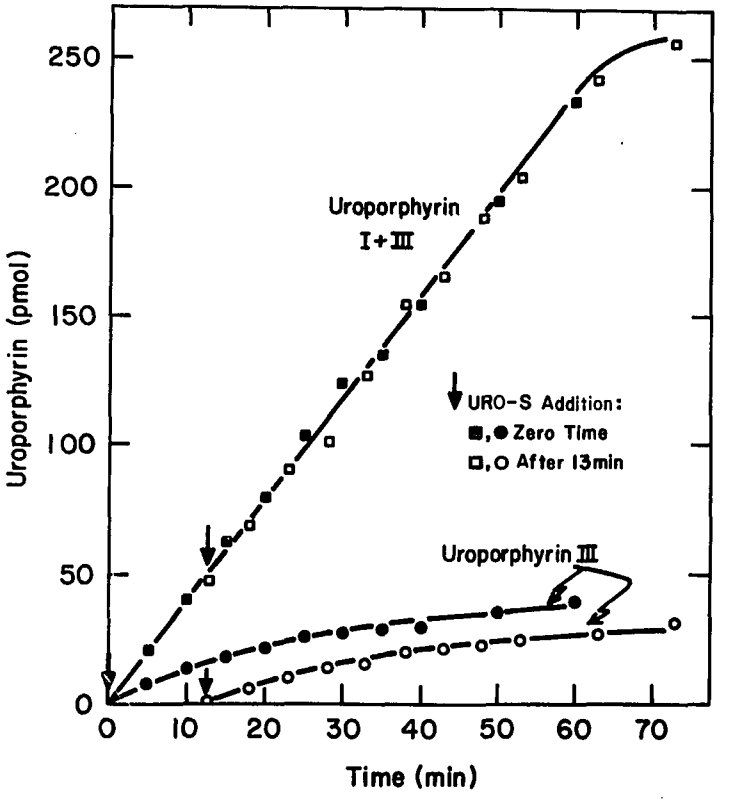

URO-S Activity (% of Maximal)
100
80
60
40
20
0
7.4
7.8
8.2
8.6
9.0
9.4
pH
HEPES
Tris
Borate

Figure 4: Linearity with time in the coupled-enzyme assay. Each point represents the pmole URO detected following injection of 100 µl of reaction mixture. URO-S was added either at zero time (URO I + URO III, ■ ; URO III, ●) or at 13 min after the 37°C incubation had been initiated (URO I + URO III, □ ; URO III, ○). Note that addition of URO-S after 13 min did not alter the linearity of URO I + III formation with time.


250
200
150
100
50
0
Uroporphyrin (pmol)
0
10
20
30
40
50
60
70
Time (min)
Uroporphyrin I+III
URO-S Addition:
■,● Zero Time
□,○ After 13min
Uroporphyrin III

enzyme assay was linear with enzyme concentrations from 0.1 (limit of sensitivity) to 1 U per assay (Fig. 5). A 15-fold increase in enzyme concentration could be achieved by using an empirical index based on the ratio of URO III to URO I produced; note that the ratio of URO III/URO I formed (Fig. 5) was linear from 0 to 15 U of URO-S activity per assay. This ratio provided an estimate of the URO-S activity as well as the dilution factor required to adjust the activity into the linear range for precise quantitation of enzymatic activity. In practice, URO-S activity could be estimated within 20% using URO III/URO I ratios as high as 30. For example, the ratio proved extremely useful in URO-S purification for monitoring column fractions for enzyme activity (28).

Optimization of the Direct Assay:

To optimize the direct assay, the conditions for the hydrolysis of HMB octamethyl ester were evaluated. As shown in Table 1, hydrolysis at either 70°C for 15 min or 16°C for 16 h produced more HMB than overnight hydrolysis at 23°C. The amount of URO'gen I contamination in the hydrolysates varied from 1.0 to 5.5 nmoles. Essentially no URO'gen III was detected in any hydrolysate after oxidative destruction of HMB with iodine (23) (data not shown). When the hydrolysates were acidified with TCA (which is used to terminate the standard reaction by converting free HMB to URO'gen I and by precipitating protein) and then oxidized with iodine to convert URO'gen to URO, the amount of HMB formed (detected after oxidation as URO) ranged from 15.1 to 20.5 nmol. Since hydrolysis at 70°C for 15 min produced essentially the same amount of HMB as hydrolysis overnight at 16°C, the former and more convenient conditions were used routinely for HMB hydrolysis in the direct assay.

Figure 5: Linearity with enzyme concentration in the coupled-enzyme assay. URO-S was assayed for enzyme activities ranging from 0.4 to 36 U under standard reaction conditions. For the 100 µl of reaction mixture analyzed by HPLC, URO-S activity was expressed as 1) pmoles URO III (■); 2) URO III as a percentage of the total URO I and URO III (▲); and 3) the ratio of URO III/URO I (●). For example, a URO III/URO I ratio of about 5 corresponded to a URO-S activity of 9 U of enzyme in the assay. Note that U/ml could be approximated by doubling the URO III/URO I ratio.

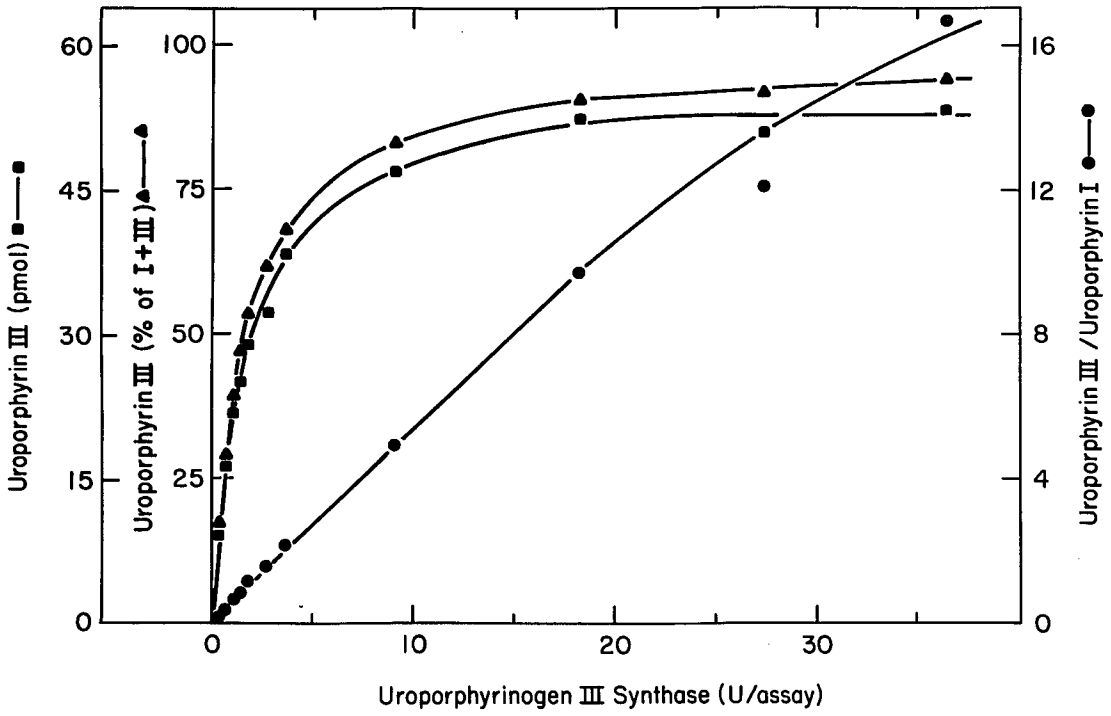

Uroporphyrin III (pmol) ■—■
Uroporphyrin III (% of I+III) ▲—▲
Uroporphyrin III / Uroporphyrin I ●—●
60
45
30
15
0
100
75
50
25
16
12
8
4
0
0
10
20
30
Uroporphyrinogen III Synthase (U/assay)

Table 1. HMB and URO'gen I Formed Following Hydrolysis of HMB Octamethyl Ester Under Different Conditions*

Hydrolysis Conditions	Reaction Products	
	URO'gen I**	HMB***
	(n m o l)	
16°C; 16h	1.0	20.5
23°C; 16h	2.1	15.1
70°C; 15 min	5.5	20.4

* HMB octamethyl ester (34 nmol) was hydrolyzed under the indicated conditions. The values represent the means of two independent experiments, each of which was performed in duplicate, and the URO isomer concentration in each duplicate was determined by HPLC in triplicate.

** The amount of URO'gen I was determined by oxidizing an aliquot of the hydrolysate and analyzing the URO I content by HPLC as described in the text. Essentially no URO'gen III was detected.

*** An aliquot of the hydrolysate was acidified with 10% TCA (which cyclized the free HMB to URO'gen I), oxidized with iodine, and then the URO I and URO III content was determined by HPLC. The HMB concentration was determined by subtracting the URO I present before acidification from the total URO I and III (the URO III isomer was about 10% of the total URO) in the hydrolysate after

acidification. For example, in the hydrolysate prepared at 70°C for 15 min, there was 24.2 nmol of URO I and 1.7 nmol of URO III, the total amount of URO being 25.9 nmol. Therefore, the available HMB for reaction was 25.9 nmol minus the 5.5 nmol of URO I formed during the hydrolysis of HMB octamethyl ester, or 20.4 nmol.

In addition, the assay was performed at pH 7.5, the optimum of the purified erythrocyte enzyme (28), and 0.15 M KCl was added to the buffer since K^+ ions stimulated the purified enzyme about 2-fold (28,33). Under these conditions, the direct assay was linear with time for about 1 min with a hemolysate or with pure enzyme protein. When incubation was routinely performed for exactly 30 sec, the assay was linear with enzyme concentration up to 15 U/assay of hemolysate or purified enzyme protein (data not shown).

Determination of the K_m Values for URO-S:

Coupled-Enzyme Assay. The K_m for URO-S toward HMB was determined by the coupled-enzyme assay with the following considerations. If the amount of HMB-S present in the HMB-generating hemolysate is varied, the steady-state HMB concentration (34) can be estimated and the K_m of URO-S can be calculated (35). In the conversion of PBG to URO I and III as shown in Figure 1, the first step of the reaction (K_1) is zero order since PBG is saturating. For K_2, the reaction is first order, as it involves a non-enzymatic, intramolecular cyclization (4). Since the half-life of HMB at pH 8.3 and 37°C has been estimated experimentally to be 4.0 min (23), K_2 can be calculated as following derivatives:

$$-d[S]/dt = K_2[S]$$

or: $$-d[S]/S = K_2 dt$$

integrating: $$\ln([S]_o/[S]) = K_2 t$$

in one half-life: $$\ln(1/0.5) = K_2 t_{1/2}$$

or: $$K_2 = 0.173\ min^{-1}$$

The integrated rate equation (35) for the coupled-enzyme assay is:

$$\ln(1-K_2[S]/K_1) = -K_2t$$

at steady state: $K_1 = K_2[S]_{ss}$

Therefore: $\ln(1-[S]/[S]_{ss}) = -K_2t$

if $[S]$ = 90% of $[S]_{ss}$: $t = -\ln(1-0.9)/0.173\ min^{-1}$

or: $t = 13$ min

Thus, for K_m determinations, the assay was preincubated for 13 min prior to URO-S addition in order to achieve the assumed steady-state concentration of HMB. The addition of URO-S to the reaction mixture did not alter the rate of HMB production by HMB-S since the total URO I + III production was linear with time (see Fig. 4). Therefore, the concentration of HMB in the K_m experiment was established by varying the HMB-S activity in the assay to produce various steady-state concentrations of HMB. Since $[HMB]_{ss} = K_1/K_2$ and since K_1 equals the velocity (nmol/min/ml) for a zero order reaction:

$$[HMB]_{ss} = \frac{\text{nmol URO I + III/min}}{0.173\ min^{-1}} \text{ / ml}$$

Figure 6A shows the Lineweaver-Burk double reciprocal plot based on the above derivation, from which the apparent K_m of URO-S for the coupled-enzyme assay was estimated to be 0.13 μM.

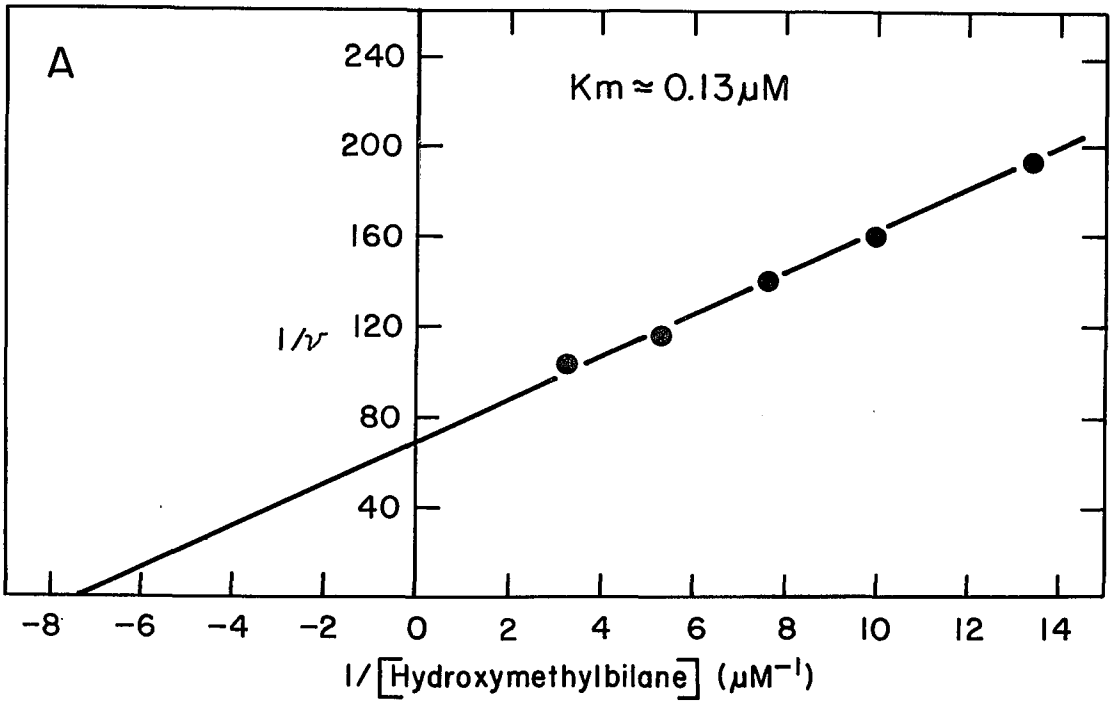
Direct Assay. The K_m of URO-S for HMB also was determined using the direct assay. Using the standard assay conditions (pH 7.5; 23°C), the apparent K_m determined for the purified enzyme was 7-20 μM (28). For

comparison with the coupled-enzyme assay, the K_m determinations were performed at pH 8.2 and 37°C; the apparent K_m derived from a Lineweaver-Burke plot was 1.9 μM (Fig. 6B).

URO-S Activities in Normal Individuals and in Homozygotes and Obligate Heterozygotes for CEP:

Figure 7 compares the URO-S activities in erythrocytes and cultured lymphoid cells from homozygotes and obligate heterozygotes from four unrelated CEP families as determined by the optimized coupled-enzyme and direct assays. Due to the ± 10% variability observed for the activity in repeated samples from a normal individual, the activities were expressed as percent of normal mean values for each enzyme source. This was particularly the case for the direct assay in which the total HMB produced depended on the percent HMB octamethyl ester hydrolyzed and the variability inherent in a 30 sec assay. The mean activities (± 1 standard deviation) for normal erythrocytes were 7.41 ± 1.16 (n = 28) for the coupled-enzyme assay and 7.64 ± 1.73 (n = 20) for the direct assay. The mean normal lymphoblast activities (± 1 standard deviation) were 13.7 ± 1.39 (n = 6) and 17.6 ± 1.15 (n = 6) for the coupled-enzyme and direct assays, respectively. In either assay system, erythrocytes from homozygotes and obligate heterozygotes for CEP had activities ranging from 8 to 36% and 33 to 107% of the normal mean values, respectively. In cultured lymphocytes, the CEP homozygotes and obligate heterozygotes had activities in both assay systems that were about 0 to 16% and about 18 to 76% of normal mean activity, respectively. One CEP homozygote who was being treated with periodic exchange transfusions

Figure 6: Lineweaver-Burke plot for URO-S from human erythrocytes (A) using the coupled-enzyme assay and (B) the direct assay. See text for details.


A
Km ≃ 0.13 μM
240
200
160
120
80
40
1/v
-8
-6
-4
-2
0
2
4
6
8
10
12
14
1/[Hydroxymethylbilane] (μM⁻¹)

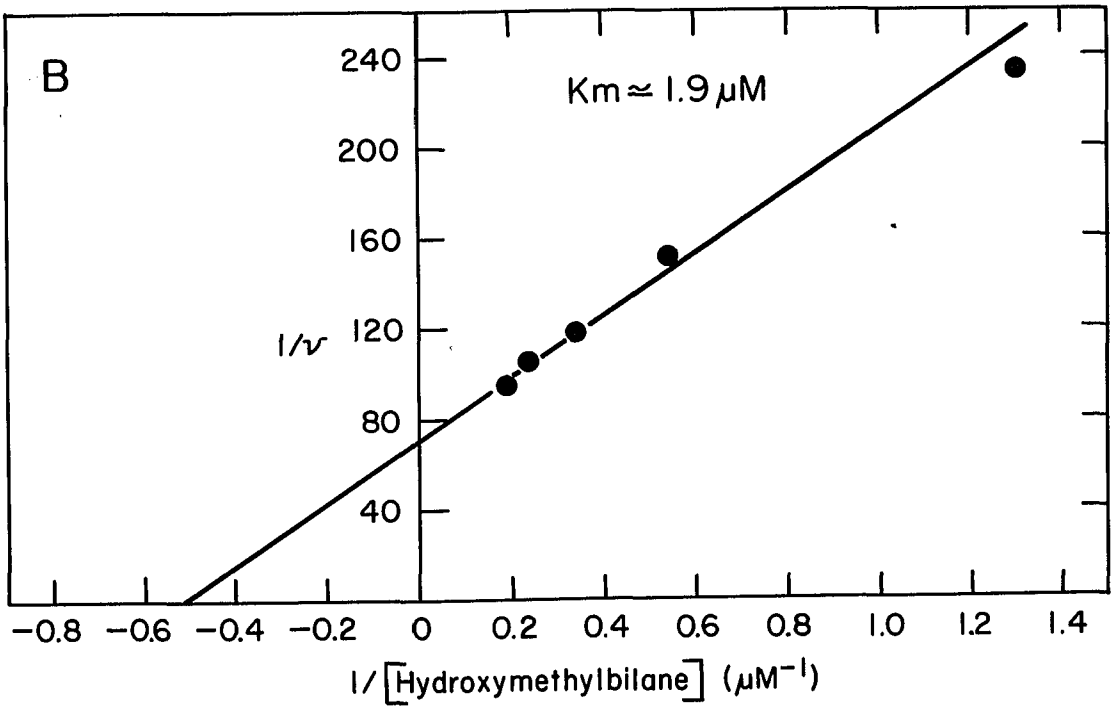
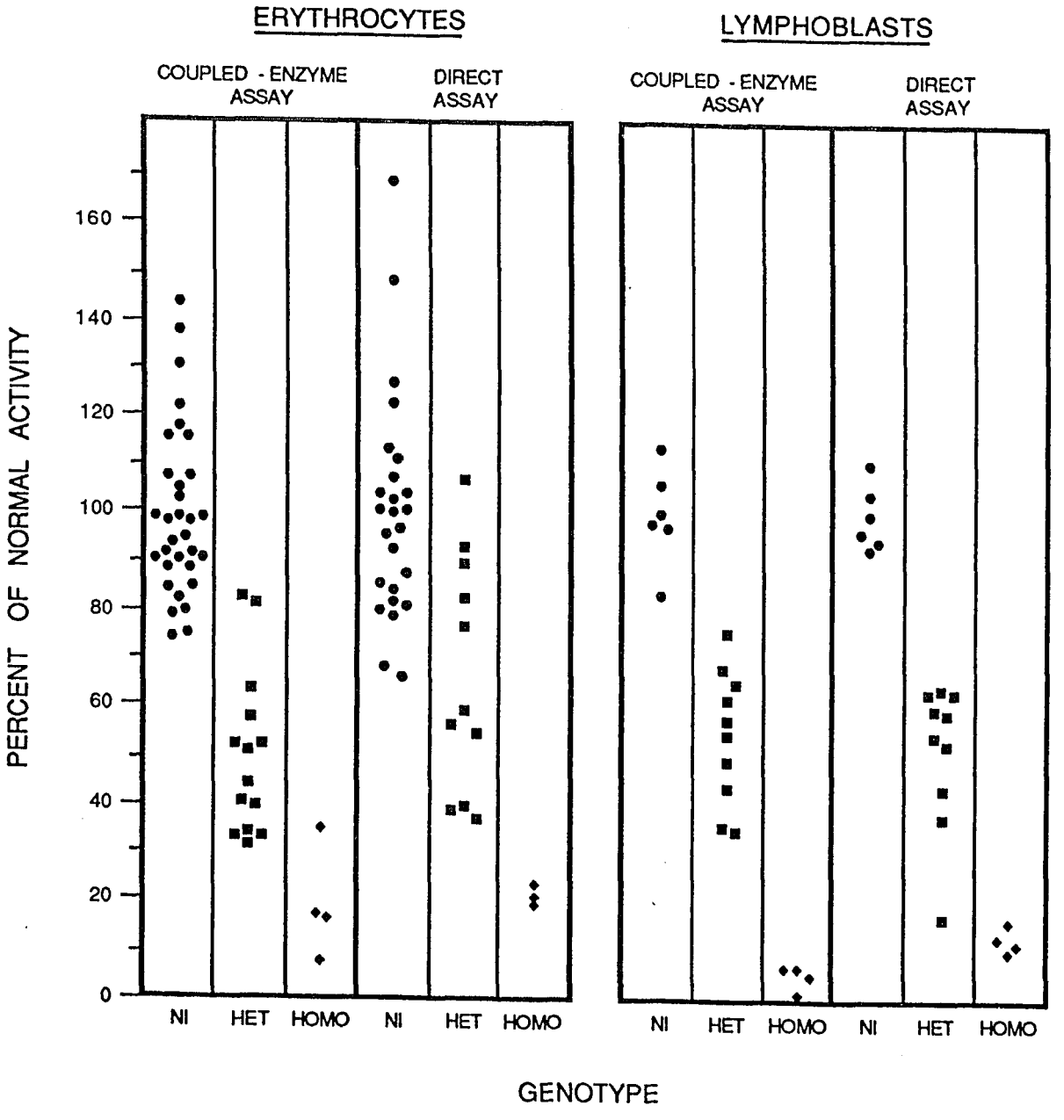

B
Km ≃ 1.9 μM
240
200
160
120
80
40
1/v
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1/[Hydroxymethylbilane] (μM⁻¹)

Figure 7: Comparison of the activities of URO-S in erythrocytes and cultured lymphoblasts from normal individuals (●) and from obligate heterozygotes (■) and homozygotes with CEP (◆). The values are expressed as percent of the mean normal activity which was determined in the same set of assays for the respective enzyme source. See text for details.


ERYTHROCYTES
LYMPHOBLASTS
COUPLED - ENZYME ASSAY
DIRECT ASSAY
COUPLED - ENZYME ASSAY
DIRECT ASSAY
PERCENT OF NORMAL ACTIVITY
0
20
40
60
80
100
120
140
160
NI
HET
HOMO
NI
HET
HOMO
NI
HET
HOMO
NI
HET
HOMO
GENOTYPE

(36) had 36% of normal mean erythrocyte activity (coupled-enzyme assay) but only 5.6 and 13.1% of normal mean activity in his cultured lymphocytes in the coupled-enzyme and direct assays, respectively.

Sensitivity of the URO-S Assays:

Under the optimized conditions, using either crude enzyme or highly purified URO-S (28) as enzyme source (~ 300,000 U/mg), the coupled-enzyme and direct assays could detect 0.1 and 2 U of enzyme per assay, respectively (data not shown).

DISCUSSION

Coupled-enzyme and direct assays for URO-S have been optimized and evaluated for the diagnosis of affected homozygotes and obligate heterozygotes for CEP, as well as for use in the purification and characterization of this heme biosynthetic enzyme. The coupled-enzyme assay of Wright and Lim (27) was modified by the use of a standardized HMB-generating hemolysate, and by optimizing the reaction for pH, buffer and linearity with time and enzyme concentration. By standardizing the amount of HMB-S in the HMB-generating hemolysate, the assay could be extended to URO-S sources which contained little, or no, HMB-S activity. For example, the coupled-enzyme assay permitted the convenient and relatively simple monitoring of column fractions during the purification of URO-S from human erythrocytes (28). In addition, the standardized HMB-generating hemolysate allowed the more reliable comparison of URO-S activities in assays performed at different times and with different enzyme sources. The direct URO-S assay of Battersby and colleagues (11,12) was modified by determining the conditions for maximal HMB octamethyl ester hydrolysis and for optimal pH, salt concentration and linearity with time and enzyme concentration. The finding that HMB octamethyl ester hydrolyzed at 70°C for 15 min contained the same amount of HMB as when hydrolyzed at 16° or 23°C overnight facilitated same day substrate preparation and enzyme assay. The fact that greater amounts of URO I were present in the hydrolysate prepared at 70°C did not alter the sensitivity or accuracy of the assay since the enzymatic activity was based solely on the URO III detected. Moreover, the reliable quantitation of the URO isomers produced in both assays was optimized by the

improved and reproducible separation obtained by modifying the HPLC gradient for removal of other porphyrins and by maintaining the C_{18} reversed-phase column at a lower, constant temperature.

Various properties of the two URO-S assays are compared in Table 2. Note that the coupled-enzyme assay was performed at pH 8.2, whereas the direct assay was carried out at pH 7.5, the pH optimum of URO-S (28). The higher pH in the coupled-enzyme assay apparently was required by HMB-S for the efficient production of HMB. However, it should be noted that the pH optimum for purified URO-S from human erythrocytes was broad (28); at pH 8.2 in the direct assay, the human enzyme had 90% of maximal activity at pH 7.5. Compared to the direct assay, the coupled-enzyme assay was linear over a greater time period (1 vs. 15 min) and over a greater range of URO-S concentration (15 vs. 30 U/assay). Both assays were able to reliably detect low levels of URO-S activity; however, the coupled-enzyme assay was 20 times more sensitive.

The apparent K_m values for URO-S also were determined. For the coupled-enzyme assay, it was presumed necessary to preincubate the HMB-generating hemolysate with 3.4 mM PBG for 13 min to saturate HMB-S in order to maintain a steady-state HMB concentration. However, the observed findings suggested that the preincubation was not required since the rates of URO'gen III production were essentially the same whether the coupled-enzyme reaction was carried out with or without the preincubation (Fig. 4). The apparent K_m values for URO-S from a normal human hemolysate were 0.13 μM and 1.9 μM for the coupled-enzyme and direct assays, respectively. The lower K_m observed for the coupled-enzyme assay may reflect the fact that HMB-S generates HMB (or a form of HMB) which is rapidly converted by URO-S to URO'gen III without release

Table 2. Comparison of the Assays for URO-S Activity

Property/Features	Coupled-Enzyme Assay	Direct Assay
Substrate	PBG	HMB
pH Optimum	8.2	7.5
Linearity, Time (min)	15	1
Linearity, Enzyme (U/assay)	30*	15
Sensitivity (U/Assay)	0.1	2

* When using the URO III/URO I ratio to estimate activity (see text).

of the HMB intermediate into the solution. In addition, the HMB-S and URO-S may be in a complex (as previously proposed, e.g. 28) which facilitates URO'gen III formation. Thus, the lower K_m of the coupled-enzyme assay may reflect more closely the in vivo kinetics of URO'gen III formation.

Using the optimized conditions for each assay, the URO-S activities were determined in erythrocytes and cultured lymphoid cells from affected homozygotes and obligate heterozygotes from CEP families. The finding of markedly deficient activities in affected homozygotes confirmed the fact that URO-S is the primary enzymatic defect in this porphyria as previously demonstrated in erythrocytes (13,15) and cultured fibroblasts (14).

The presence of residual activity in CEP homozygotes was consistent with the fact that they must have sufficient enzyme to produce URO'gen III or the patients could not survive. That obligate heterozygotes had levels of enzymatic activity intermediate between those of affected homozygotes and normal individuals was consistent with the autosomal recessive transmission of the enzymatic defect (15,37). Note that one homozygote had an erythrocyte level in the heterozygote range. This individual was receiving chronic exchange transfusions as therapy for this disease (36). For such transfusion-dependent patients, the diagnosis of CEP can be made enzymatically by the use of cultured lymphoid cells, as demonstrated in these studies. In addition, cultured lymphoid cells should provide an excellent source for future studies of the physical and kinetic properties of the residual enzyme from CEP homozygotes.

In routine use, the coupled-enzyme assay was easier to perform than the direct assay. This was particularly true when a large quantity of the HMB-generating hemolysate had been prepared, aliquoted and stored. In contrast, the direct assay required hydrolysis of the HMB octamethyl ester immediately prior to assay. Based on experience with both assays, it is recommended that the coupled-enzyme assay be employed for determining the URO-S activity in diagnostic samples as well as for enzyme purification. Although the direct assay may be used reliably for diagnostic purposes, it is valuable for studies of the enzyme's kinetic properties (including the effects of inhibitors and activators), since the URO-S activity can be determined in the absence of HMB-S. Nonetheless, the direct assay requires extreme care due to its short incubation time. Furthermore, a high degree of accuracy is necessary in pipetting the substrate since it is present at levels approximating its K_m. The availability of these two assays should facilitate the purification and characterization of URO-S from a variety of enzyme sources and should provide for the reliable diagnosis of homozygotes and heterozygotes for CEP as well as permit studies of the residual mutant enzyme in affected individuals.

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Chapter Two

PURIFICATION AND PROPERTIES OF UROPORPHYRINOGEN III SYNTHASE FROM HUMAN ERYTHROCYTES

Shih-Feng Tsai, David F. Bishop and Robert J. Desnick

Division of Medical Genetics

Mount Sinai School of Medicine

Fifth Avenue at 100th Street

New York, New York 10029

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SUMMARY

Uroporphyrinogen III synthase (hydroxymethylbilane hydro-lase, cyclizing; EC 4.2.1.75), the fourth enzyme in the heme biosynthetic pathway, was purified to homogeneity from human erythrocytes. For enzyme purification and characterization, a sensitive coupled-enzyme assay was used which generated the substrate, hydroxymethylbilane; the oxidized product, uroporphyrin III, was quantitated by high pressure liquid chromatography. Uroporphyrinogen III synthase was initially separated from δ-aminolevulinate dehydratase and hydroxymethylbilane synthase by a preparative anion exchange chromatographic step. Subsequent chromatography on hydroxyapatite, phenyl-Sepharose and Sephadex G-100 purified the enzyme about 70,000-fold with an 8% yield. Homogeneous enzyme was obtained following a final C_4-reversed phase high pressure liquid chromatographic step which removed a single major and several minor protein contamnants from the enzyme. The purified enzyme had a specific activity of over 300,000 U/mg, an isoelectric point of 5.5 and was thermolabile ($t_{1/2}$ at 60°C ∿ 1 min). Molecular weight studies by gel filtration ($M_r \cong 30,000$) and analytical SDS polyacrylamide gel electrophoresis ($M_r \cong 29,500$) were consistent with the enzyme being a monomer. Using hydroxymethylbilane as substrate, the purified enzyme formed uroporphyrinogen III in the absence of hydroxymethylbilane synthase or other cofactors. The pH optimum was 7.4 and the K_m for hydroxymethylbilane was 5 to 20 μM. The enzyme was activated by Na^+, K^+, Mg^+, and Ca^{++}, and was inhibited by Cd^{++}, Cu^{++}, Hg^{++} and Zn^{++}. Amino acid composition analysis was performed and the N-terminal sequence, Met-Lys-Val-Leu-Leu-Leu, was determined by microsequencng. The availability of the purified enzyme should permit investigation of its reaction mechan-

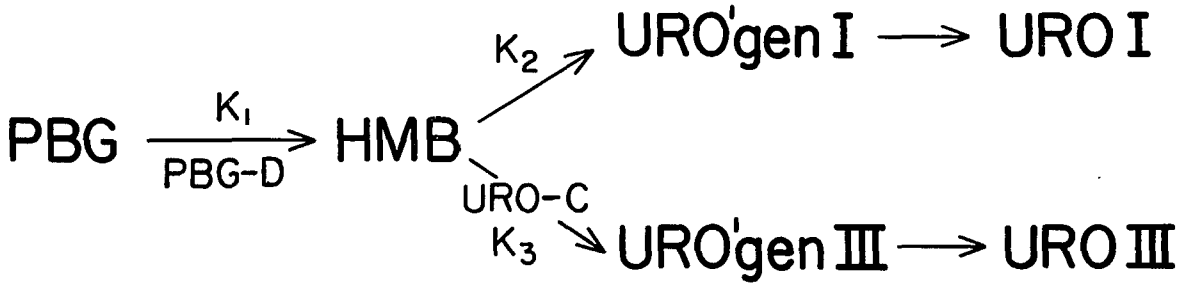
ism as well as facilitate biochemical and molecular studies of the genetic defect in congenital erythropoietic porphyria.

In the heme biosynthetic pathway, uroporphyrinogen III is the first cyclic tetrapyrrole and the physiologic precursor of vital heme containing macromolecules including hemoglobins, cytochromes, chlorophylls, and cobalamins (1). Bogorad first recognized that uroporphyrinogen III was synthesized from four molecules of porphobilinogen (PBG[1]) by two enzymes, hydroxymethylbilane synthase (EC 4.3.1.8, HMB-S, previously designated PBG-deaminase or uroporphyrinogen I synthase) and uroporphyrinogen III synthase (EC 4.2.1.75; URO-S) (2-5). This finding stimulated studies of the molecular mechanism and reaction intermediates involved in uroporphyrinogen III biosynthesis as well as efforts to characterize the two enzymes and determine if they were associated in a complex or functioned independently. Although the mechanism of uroporphyrinogen III formation was the subject of intense investigation and controversy (for review, 1,6,7), it is now recognized that HMB-S catalyzes the head to tail condensation of four molecules of PBG to form the linear tetrapyrrole, hydroxymethylbilane (HMB) (8-13). In the presence of URO-S, HMB is rapidly converted to uroporphyrinogen III by ring closure and intramolecular rearrangement of ring D, whereas in the absence of URO-S, HMB non-enzymatically cyclizes to uroporphyrinogen I (8-13) (Fig. 1). In support of this mechanism, Battersby and coworkers have shown that partially purified URO-S from Euglena gracilis forms uroporphyrinogen III from HMB in the absence of HMB-S (14).

Although mammalian URO-S was first separated from HMB-S in 1968 (15), the enzyme has not been purified more than 200-fold from any animal source (15-24). Moreover, only limited studies of the physical and kinetic properties have been described using these crude or partially purified preparations. The mammalian enzyme has been reported to have a pH

Figure 1: Reaction scheme for the conversion of PBG to uroporphyrinogen isomers I and III.

Reaction Scheme for the Conversion of PBG to Uroporphyrins



optimum of 7.4 (18), 7.7 to 7.9 (19) and 7.8 (22), and apparent K_m values of 5 μM (22) and about 26 μM (24). Estimates of the enzyme's molecular weight have ranged from 10,000 to 210,000 (7,23,24). The enzyme has been shown to be extremely heat labile, stabilized by thiol reagents and inhibited by hydroxylamine and various heavy metals, including zinc, copper and cadmium (15,18,21-24). In addition, a dialyzable protein factor (18) and a folate derivative (23) have been implicated as cofactors for the partially purified enzyme from bovine and rat liver, respectively. However, recent studies have clearly shown that a folate derivative is not a cofactor for URO-S from Euglena (14). Finally, several investigators, using crude or partially purified preparations, have suggested that URO-S and HMB-S interact directly, possibly being associated in a complex in the cytosol (4,7,14,18,20,21,25). However, further studies of possible cofactor requirements, characterization of the reaction mechanism and interaction(s) with other cytosolic heme biosynthetic enzymes will require future evaluation with purified enzymes.

Since δ-aminolevulinate dehydratase (EC 4.2.1.24; ALA-D; also called porphobilinogen synthase) and HMB-S from human erythrocytes were previously purified to homogeneity in this laboratory (10,26), efforts have been directed to purify URO-S from human erythrocytes in order to determine the enzyme's physicokinetic properties, its possible interaction with HMB-S and for molecular genetic studies of the defective URO-S activity in congenital erythropoietic porphyria (2,27). In this communication, a reproducible method for the purification of URO-S to homogeneity is described and the major physicokinetic properties of the enzyme from human erythrocytes are reported.

EXPERIMENTAL PROCEDURES

Materials

Outdated human erythrocytes were obtained from the Greater New York Blood Center. Phenyl-Sepharose CL-4B, Sephadex G-100 superfine, low molecular weight gel filtration standards, and Pharmalytes were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Ammonium sulfate ultrapure, special enzyme grade was from Schwarz/Mann Research Laboratories, Cambridge, MA. Hydroxyapatite and materials for polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories, Richmond, CA. Sodium dodecylsulfate (specially pure) was purchased from BDH Chemicals Ltd., Poole, England. Porphyrin standards were from Porphyrin Products, Logan, UT. Acetonitrile was obtained from American Burdick and Jackson, Muskegon, MI. Fluorescamine and DEAE-cellulose (Whatman DE-52) were purchased from Pierce Chemical Co., Rockford, IL. TPCK-Trypsin was from Cooper Biomedical, Malvern, PA. The Vectastain ABC kit was obtained from Vector Laboratories, Inc., Burlingame, CA. Centricon 10 microconcentrators, and Amicon ultrafiltration membranes and concentration chambers were purchased from Amicon Corp., Lexington, MA. Nitrocellulose filters (BA85 0.45 μm) were from Schleicher and Schuell, Keene, NH. Shandon 5 μ ODS-Hypersil columns (4.6 mm x 25 cm) were obtained from Rainin Instrument Co. Inc., Woburn, MA. Vydac 5 μ C_4 reversed phase columns (4.6 mm x 25 cm) were purchased from The Separations Group, Hesperia, CA.

Methods

Assays of heme biosynthetic enzymes. ALA-D and HMB-S activities were determined as previously described (26,28). One unit (U) of ALA-D or HMB-S activity equaled that amount of enzyme required to form one micromole of PBG or one nanomole of uroporphyrinogen, respectively, per hour at 37°C. URO-S activity was determined essentially by the coupled-enzyme assay of Wright and Lim (29) with the following modifications. Briefly, a human erythrocyte lysate was heated at 60°C for 120 min (to inactivate URO-S) and used as a source of HMB-S to generate HMB. An aliquot of enzyme source was then incubated with 0.5 ml of the heat-treated lysate (containing 2.5 U of HMB-S) and 183 nmol of PBG for 15 min at 37°C in a reaction volume of 560 µl. The reaction was terminated by the addition of 440 µl of 10% trichloroacetic acid saturated with iodine. The oxidized uroporphyrin isomers forfor incubation time and enzyme concentration. One U of URO-S activity equaled that amount of enzyme which formed one nanomole of uroporphyrinogen III per hour at 37°C. The ratio of uroporphyrin III/uroporphyrin I was used to monitor enzymatic activity during URO-S purification; the assay was linear up to 15 U of URO-S activity. A uroporphyrin III/uroporphyrin I ratio of 1.0 was equivalent to 1.3 U of URO-S activity.

For studies of enzyme kinetics and effects of various activators and inhibitors, the purified enzyme was assayed with the synthetic substrate HMB. Hydroxymethylbilane octamethyl ester was hydrolyzed at 15°C overnight in 2N KOH under nitrogen at concentrations of 1-4 mg/ml. URO-S (20 µl) was added to 460 µl of 0.05 M Tris-HCl buffer, pH 7.2, containing 0.15 M KCl at 25°C. The reaction was initiated while mixing by the addition of 20 µl of 50 µM HMB. The pH of the Tris buffer was chosen

such that the final assay pH was 7.4. The reaction was terminated 20 s later with 400 µl of 0.5% I_2 in 1.0% KI to destroy any remaining HMB. After 3 min, 100 µl of 1% $Na_2S_2O_5$ and 250 µl of 50% trichloroacetic acid were added. Controls included 0 and 20 s assays minus enzyme as well as HMB controls where the trichloroacetic acid was added before the iodine to convert all of the HMB to uroporphyrin I. Uroporphyrin isomers were separated and quantitated by reversed phase HPLC as described above.

Protein assay. Protein concentration was determined by the fluorescamine method described previously (26).

SDS-PAGE and electroelution of stained proteins. Samples were electrophoresed by the method of Laemmli and Favre (31). For analytical gels, a minigel apparatus (Hoefer, San Francisco, CA) was run at constant voltage (150 V) for 60 min. Electroelution from preparative gels was essentially as described by Hunkapiller et al. (32) using a Studier type gel apparatus (33) and 12.5% gels. Protein (25 µg) was applied to each 1.5 mm x 3 cm sample well and electrophoresed at 120 V for 6 h (or 50 V for 15 h). After lightly staining the gel with Coomassie Blue R250 for about 15 min and destaining with methanol/acetic acid/water (10:33:157; v/v/v) for 1 h, the protein bands were excised with a scalpel, soaked in distilled water for 60 min, and then in elution buffer (0.1% SDS in 0.05 M ammonium bicarbonate) for 10 min. The gel strips were placed in dialysis tubing filled with elution buffer and then were electroeluted at 10 V for 12 to 16 h in a Trans-Blot apparatus (Bio-Rad, Richmond, CA) containing 500 ml of the elution buffer. This buffer was replaced with 5-fold diluted elution buffer and elution/dialysis was continued at 20 V

for 24 h. The protein solution in the dialysis tubing was lyophilized in RBS 35-washed (Pierce Chemicals, Rockford, IL) and siliconized glass tubes followed by reconstitution with distilled water to approximately 1 mg/ml.

HPLC of tryptic peptides. Trypsin digestion of homogeneous URO-S was performed as follows. One-ninth volume of ice cold 100% trichloroacetic acid was added to 1.5 ml microfuge tubes containing 5-10 nmol of electroeluted enzyme. After 30 min at 4°C, the samples were centrifuged for 10 min at 10,000 x g, and the precipitates washed twice with 200 µl cold acetone. After drying under a gentle stream of nitrogen, the protein was dissolved in 50 µl of fresh 8 M urea and adjusted to pH 8 by the addition of approximately 150 µl of 0.2 M NH_4HCO_3. TPCK-Trypsin (1 mg/ml in 1.0 mM HCl) was mixed with URO-S (1 µg trypsin/25 µg URO-S), the reaction mixture was incubated at 37°C for 24 h and then terminated by freezing at -20°C. The resultant peptides were separated by reversed phase HPLC as described in the legend for Fig. 6.

Isoelectric focusing. Flatbed isoelectric focusing was performed on an LKB Multiphor apparatus using Pharmalyte, with a pH range of 4 to 6.5 in agarose gels as previously described (34). About 50 µg of post G-100 sample was applied to the gel surface on a 7 x 7 mm Whatman No. 17 paper square. The enzyme was initially electrofocused at 10 w constant power for 1 h, then the paper square was removed and the power was adjusted periodically to maintain the voltage at 1000 V for an additional 150 min. URO-S activity was measured after homogenizing 0.5 x 0.7 cm gel strips in 500 µl of the heat-treated erythrocyte lysate of the coupled-

enzyme assay as described above. To determine the pH gradient, adjacent agarose strips were soaked in 5 ml of water overnight, and the pH of each solution was measured.

Molecular weight determinations. The native molecular weight of URO-S was determined by gel filtration. The post-phenyl-Sepharose fraction of URO-S was applied to a 2.5 x 80 cm column of Sephadex G-100. Molecular weight standards (2 mg each) were applied in two consecutive runs to calibrate the column: bovine serum albumin (M_r = 67,000) and chymotrypsinogen A (M_r = 25,000) were chromatographed first followed by ovalbumin (M_r = 43,000) and ribonuclease A (M_r = 13,700). The molecular weight was obtained from a plot of the partition coefficient K_{av} versus log molecular weight. K_{av} was calculated as $(V_E-V_O)/ V_T-V_O)$, where V_E represented the elution volume of the solute, V_T and V_O were the total volume and the void volume of the column, respectively. The molecular weight of the denatured URO-S was determined by analytical SDS-PAGE according to the method of Weber and Osborn (35).

Amino acid composition and microsequencing. Aliquots of URO-S (0.14 nmol) were hydrolyzed for 24, 48 and 72 h in 6 M HCl at 110°C. The amino acid concentrations were then determined with a modified Beckman model 121 amino acid analyzer. The values represent averages of the 48 and 72 h hydrolyses except as follows: Performic acid oxidation (in the 24 h hydrolysate) was used for analysis of cysteine as cysteic acid. Threonine and serine values were obtained from extrapolation to zero time. Valine, isoleucine and leucine values were from the 72 h hydrolysis. The tryptophan concentration was obtained from the ratio of its

absorbance to that of tyrosine in homogeneous URO-S as determined by the spectrophotometric method of Edelhoch (36). The N-terminal amino acid sequence was determined by gas-phase microsequencing of the purified enzyme and HPLC identification of the phenylthiohydantoin amino acids (37).

Immunologic studies.

Mouse anti-human antibodies were produced against homogeneous URO-S obtained from gel slices following SDS-PAGE of the post-Sephadex G-100 enzyme preparation. The gel slices were pulverized and suspended in an emulsion with Freund's complete adjuvant. Four injections, each containing about 2 μg of enzyme protein, were administered subcutaneously at two week intervals and then the mice were bled the following week. For immunoblot studies, aliquots of the post-Sephadex G-100 preparation were subjected to SDS-PAGE in 1.5 mm slab gels (31). After electrophoresis, the proteins were electroblotted (70 volts, 90 min) onto a nitrocellulose membrane in transfer buffer, 0.025 M Tris-0.19 M glycine, pH 8.3, containing 20% methanol (38). The nitrocellulose membrane was blocked with PBS-BLOTTO (39) and incubated for 16 h at 4°C with the mouse anti-human URO-S antiserum (2 μl in 1 ml of PBS-BLOTTO). The bound antibody was detected with Vectastain ABC.

Purification of URO-S.

All purification procedures were carried out at 4°C. Potassium phosphate buffers (molarities specified in the text) of pH 7.0 (A buffers) or pH 8.0 (B buffers) all contained 1 mM dithiothreitol (DTT), 0.1 μM phenylmethylsulfonylfluoride (PMSF), and 0.02% sodium azide. Outdated

human red blood cells were prepared and hemolyzed as described previously (26). The resultant supernatant from 5 liters of packed cells was dialyzed against 25 liters of water followed by 25 liters of 5 mM buffer A (i.e., potassium phosphate buffer, pH 7.0, containing 1 mM DTT, 0.1 μM PMSF, 0.02% sodium azide).

DEAE-cellulose chromatography. A 10 x 25 cm column of DEAE-cellulose was used for the batch separation of three cytosolic enzymes in the heme biosynthetic pathway. The column containing approximately two liters of DEAE-cellulose was thoroughly equilibrated with 5 mM buffer A and then the dialyzed erythrocyte hemolysate was pumped onto the support at a flow rate of 10 ml/min. The column was washed until the eluate was clear (two bed volumes of 5 mM buffer A). HMB-S, URO-S, and ALA-D were eluted batchwise by 2.5, 6, and 2 bed volumes of 5 mM buffer A containing 0.08 M, 0.12 M and 0.24 M NaCl, respectively.

Hydroxyapatite chromatography. The fractions containing URO-S activity were pooled and pumped at 2 ml/min onto a hydroxyapatite column (5 x 40 cm) equilibrated with 5 mM buffer A containing 0.12 M NaCl. After washing with two bed volumes of 5 mM buffer A, the enzyme was eluted with a 4 liter, 5 to 100 mM buffer A gradient. Those fractions that had URO-S activity were pooled and concentrated to about 100 ml by ultrafiltration using an Amicon YM-10 membrane.

Phenyl-Sepharose chromatography. The above concentrated solution was adjusted with an appropriate volume of saturated ammonium sulfate (SAS) solution so that the final solution contained ammonium sulfate at 45%

(v/v) of saturation. The resultant protein precipitate was removed by centrifugation at 12,000 x g for 30 min, and the supernatant was pumped (1.0 ml/min) onto a phenyl-Sepharose column (1.5 x 40 cm) equilibrated with 45% SAS/20 mM buffer B (i.e., 20 mM potassium phosphate buffer, pH 8.0, containing 1 mM DTT, 0.1 μM PMSF, and 0.02% sodium azide) (v/v). The enzyme was eluted with a 1,400 ml gradient of 45% to 0% SAS/20 mM buffer B. Fractions containing URO-S activity (about 5% of saturation) were pooled and concentrated to 2 ml as described above.

Gel filtration chromatography. The concentrated enzyme solution was applied to a Sephadex G-100 superfine column (2.5 x 80 cm) which had been equilibrated with 20 mM buffer B containing 0.1 M KCl. A slow flow rate (0.1 ml/min) was controlled by a peristalic pump, and fractions containing the enzymatic activity were pooled and concentrated immediately.

Reversed phase high performance liquid chromatography. A Vydac C_4 reversed phase column was used to separate tryptic peptides on a Waters Associates 840 HPLC system which included a Digital PRO 350 system controller, WISP sample processor, model 6000A HPLC pumps and a model 441 UV spectrophotometer. Proteins were separated by a H_2O/acetonitrile gradient as described in the legend for Fig 5. Aliquots of the concentrated post-Sephadex G-100 enzyme (50 to 1000 μg) were injected and UV absorbance was monitored at 214 and 280 nm. The peak fractions were concentrated by evaporation of the acetonitrile under a stream of dry nitrogen and centrifugal ultrafiltration using a Centricon 10 microconcentrator.

RESULTS

Purification of Uroporphyrinogen III Synthase

Table I summarizes the results of a representative purification of URO-S from 5 liters of outdated human erythrocytes. The enzyme was purified about 70,000-fold with a yield of 8% after gel filtration. The initial preparative DEAE-cellulose chromatographic step efficiently removed the hemoglobin and separated the three cytosolic heme biosynthetic enzymes, ALA-D, HMB-S and URO-S (Fig. 2). By a minor modification of this step, uroporphyrinogen decarboxylase (URO-D) also can be isolated from the erythrocyte lysate (40), thereby providing a common source of all four human cytosolic heme biosynthetic enzymes. URO-S was purified approximately 75-fold with a recovery of about 40%. Absorption chromatography on hydroxyapatite permitted direct application, without prior concentration, of the 10 liter DEAE-cellulose eluate containing URO-S activity. Saturated ammonium sulfate was added to the post-hydroxyapatite fraction to 45% of saturation (v/v) to remove lower molecular weight proteins and to promote binding to the hydrophobic phenyl-Sepharose support. The supernatant was then chromatographed on phenyl-Sepharose and eluted with a decreasing gradient of ammonium sulfate (peak eluted at about 5% of saturation), resulting in a 6-fold purification of URO-S with over 80% recovery. The gel filtration step, shown in Fig. 3, removed a major protein contaminant of $M_r \sim 67,000$, resulting in a 40-fold purification of URO-S with a specific activity of 338,000 U/mg, as determined by the coupled-enzyme assay.

For the last six enzyme purifications, the specific activities of the post-Sephadex G-100 preparations ranged from 145,000 to 350,000 U/mg with yields of approximately 8 to 40%. In each of the final prepara-

TABLE I

Purification of URO-S from human erythrocytes

The results represent typical values for purification of the enzyme from 5 liters of packed erythrocytes. See text for details.

Step	Volume	Total Activity	Specific Activity	Purification	Yield
	ml	U	U/mg	fold	%
Erythrocyte Hemolysate	19,500	4,310,000	4.9	1	100
DEAE-Cellulose	10,000	1,710,000	363	74	40
Hydroxyapatite	565	603,000	1,420	290	14
$(NH_4)_2SO_4$/ Phenyl-Sepharose	166	497,000	8,810	1,800	12
Sephadex G-100	26	352,000	338,000	69,000	8

Figure 2: Resolution of ALA-D, HMB-S and URO-S from human erythrocytes by DEAE-cellulose chromatography. Fractions (400 ml) were collected and assayed for protein concentration (■ --- ■) and each enzymatic activity: ALA-D (▲ —— ▲), HMB-S (■ —— ■), and URO-S (● —— ●).

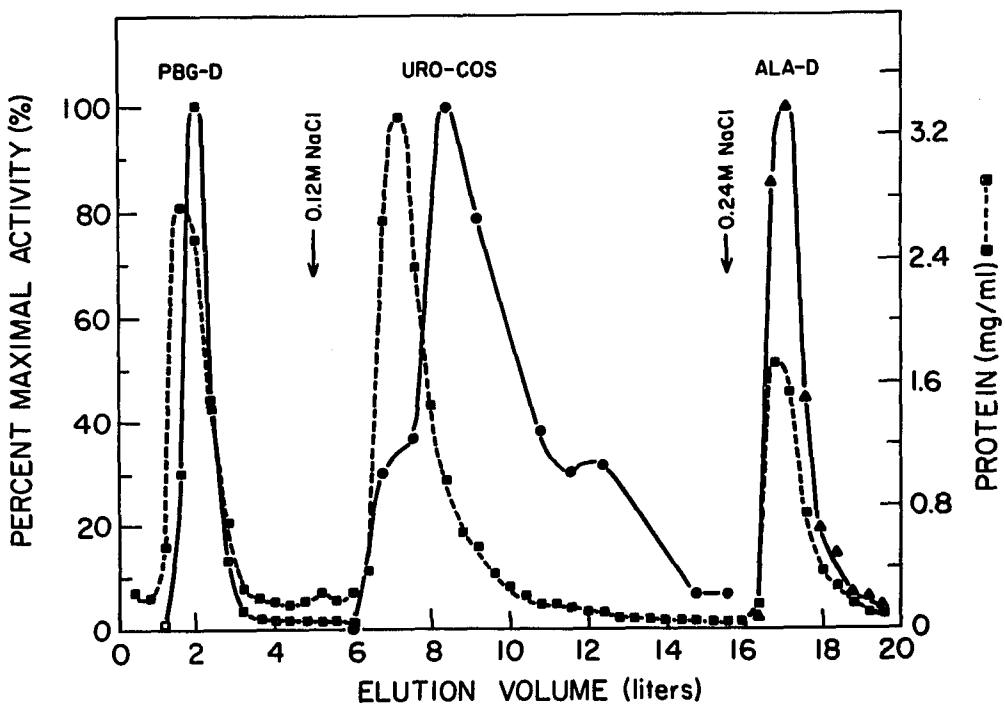
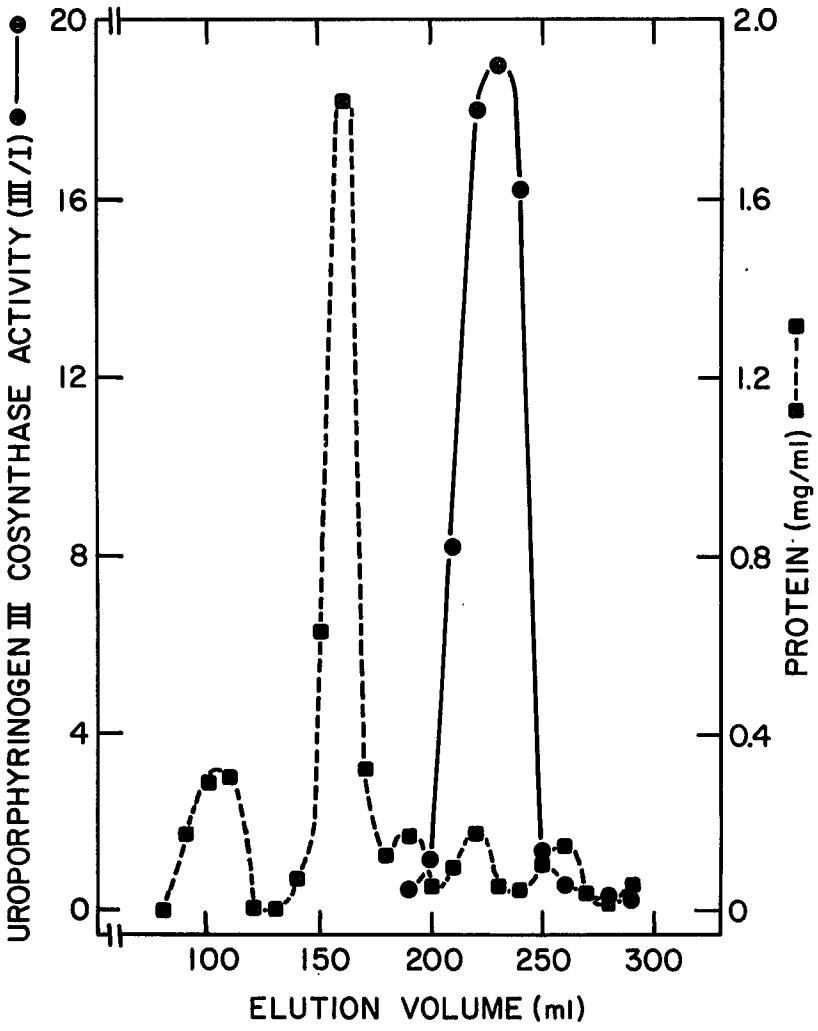

PBG-D
URO-COS
ALA-D
0.12M NaCl
0.24M NaCl
PERCENT MAXIMAL ACTIVITY (%)
PROTEIN (mg/ml)
ELUTION VOLUME (liters)
0
20
40
60
80
100
0.8
1.6
2.4
3.2
0
2
4
6
8
10
12
14
16
18
20

Figure 3: Elution profile of URO-S from human erythrocytes on Sephadex G-100. Note that a major protein contaminant ($M_r \sim 67,000$) was separated from URO-S. Protein concentration (■ --- ■); URO-S activity (●——●).


UROPORPHYRINOGEN III COSYNTHASE ACTIVITY (III/I)
PROTEIN (mg/ml)
ELUTION VOLUME (ml)
0
4
8
12
16
20
0
0.4
0.8
1.2
1.6
2.0
100
150
200
250
300

tions, two protein bands ($M_r \cong$ 32,000 and 29,500) with similar intensties were consistently observed by analytical SDS-PAGE (Fig. 4, lane 2). When these two proteins were resolved by C_4-reversed phase HPLC (Fig. 5), one (peak 2) retained about 20% of the applied URO-S activity and migrated as a single species on analytical SDS-PAGE with a $M_r \cong$ 29,500 (Fig. 4, lane 4). These two proteins also were carefully electroeluted after SDS-PAGE, digested with TPCK-trypsin, and the resultant peptides separated by C_4-reversed phase HPLC. As shown in Fig. 6, the peptide profiles of the two proteins were distinctly different. Immunoblots of the post-Sephadex G-100 preparation incubated with mouse anti-human URO-S antibodies raised against the electroeluted 29.5K protein did not show cross-reactivity with the 32K protein (Fig. 7). Furthermore, the same 29.5K protein was recognized by anti-URO-S in crude extracts, demonstrating the lack of proteolytic processing of this enzyme (data not shown).

Purity.

The post Sephadex G-100 preparation was completely free of HMB-S activty. The specific activity of this material was 338,000 U/mg (coupled-enzyme assay) when the contaminating 32K band was present in a similar concentration. Thus, the estimated specific activity of URO-S in the post-Sephadex G-100 preparation was about 675,000 U/mg. Analytical SDS-PAGE of the post-HPLC peak 2 (Fig. 5) revealed only one band (29.5K) when stained with Coomassie Blue R250 (Fig. 4, lane 4).

Figure 4: SDS-PAGE of peaks resolved by reversed phase HPLC. Lane 1, molecular weight standards: phosphorylase b (M_r = 94,000), bovine serum albumin (M_r = 67,000), ovalbumin (M_r = 43,000), carbonic anhydrase (M_r = 30,000), soybean trypsin inhibitor (M_r = 20,100), and α-lactalbumin (M_r = 14,400). Lane 2, post-Sephadex G-100 preparation of URO-S (4 μg; 97,000-fold purified); Lanes 3 to 6, HPLC peaks 1, 2, 3, and 4, respectively (2 μg each). See text for details.

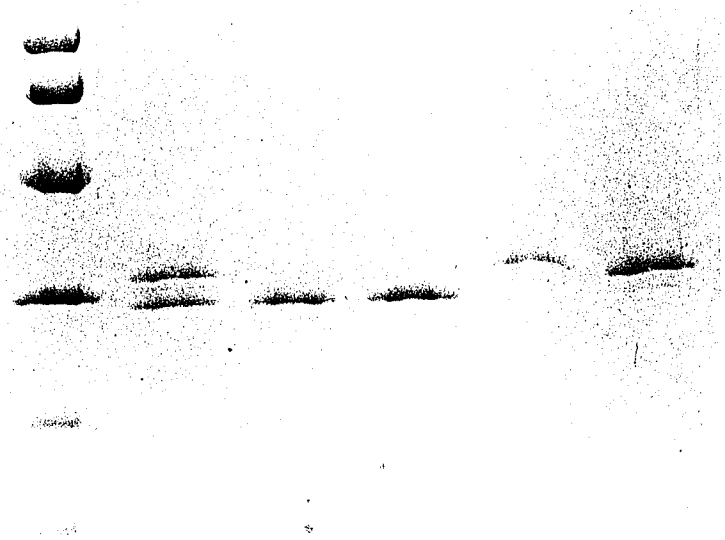


Figure 5: Resolution of URO-S by reversed phase HPLC. URO-S (400 µg of the post-Sephadex G-100 preparation) was chromatographed on a Vydac C_4 reversed phase column which had been equilibrated with a mobile phase of 70% solution A (100% water with 0.05% trifluoroacetic acid) and 30% solution B (80% acetonitrile, 20% water with 0.05% trifluoroacetic acid). Sequential gradients were applied at a flow rate of 0.7 ml/min: 30 to 45% solution B over 20 min, 45 to 55% solution B over 60 min, and 55% to 60% solution B over 15 min. Protein concentration was monitored by the absorbance at 214 nm and peak fractions were assayed for URO-S activity. Note that only peak 2 had URO-S activity.

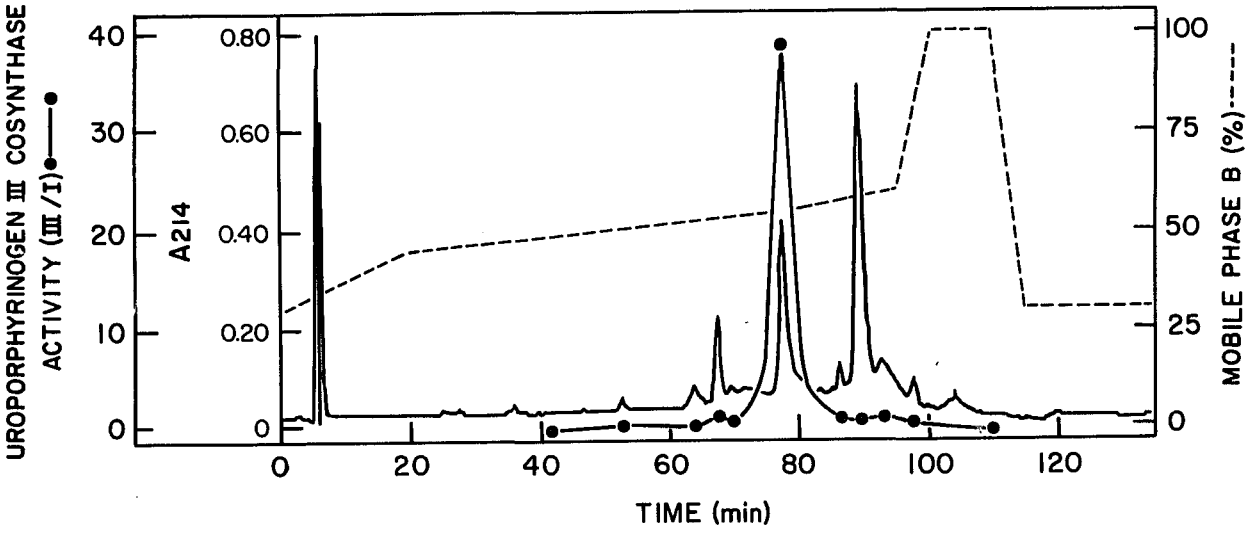

UROPORPHYRINOGEN III COSYNTHASE ACTIVITY (III/I)
A214
MOBILE PHASE B (%)
TIME (min)
0
10
20
30
40
0.20
0.40
0.60
0.80
25
50
75
100
0
20
40
60
80
100
120

Figure 6: Profiles of tryptic peptides resolved by reversed phase HPLC. The 29.5K and 32K proteins in the post-Sephadex G-100 fraction were individually electroeluted after preparative SDS-PAGE, digested with trypsin and chromatographed on a Vydac C_4 reversed phase column which had been equilibrated with solution A (100% water with 0.05% trifluoroacetic acid). A 2 h linear gradient from 0 to 60% solution B (80% acetonitrile, 20% water with 0.05% trifluoroacetic acid) at a flow rate of 0.7 ml/min was used to separate the peptides. The eluate was monitored by absorbance at 214 nm. (A) 32K protein, 0.55 mg/ml, 200 µl applied; (B) 29.5K protein, 0.30 mg/ml, 200 µl applied; and (C) control tryptic digestion without added protein, 200 µl applied.

Reverse Phase HPLC Profiles of Tryptic Peptides of Electroeluted Uroporphyrinogen III Cosynthase

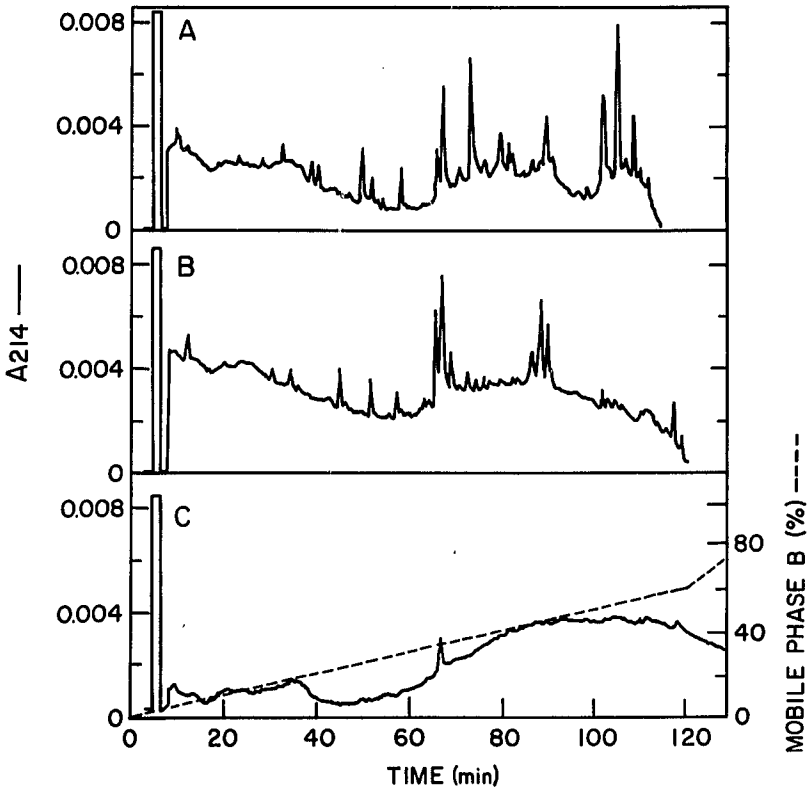
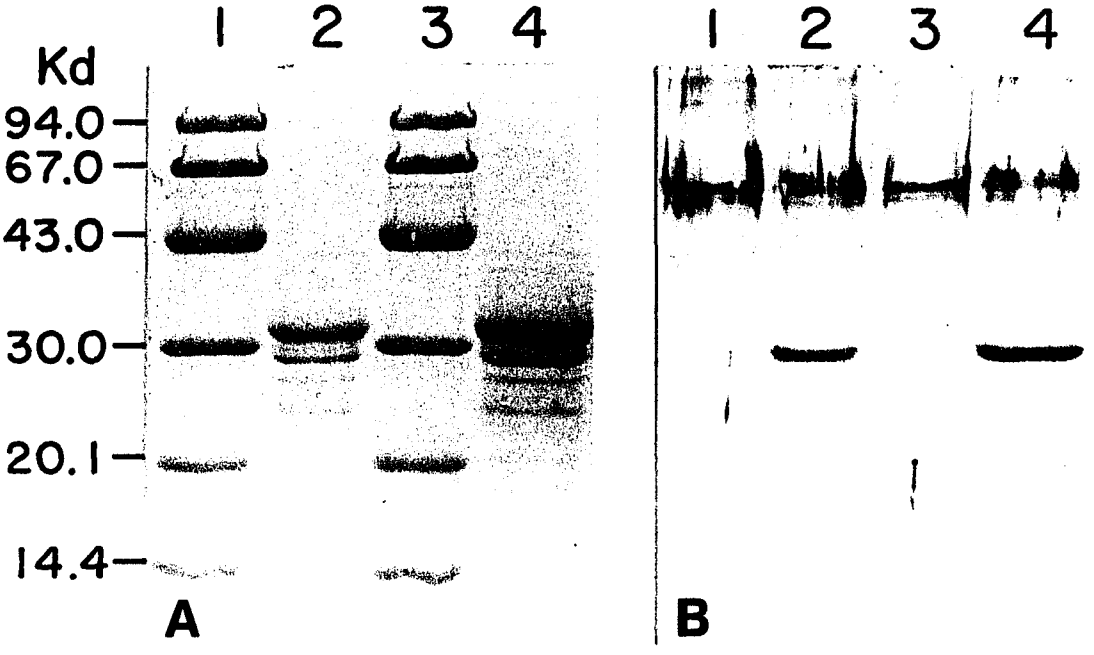


Figure 7: Immunoblot of purified URO-S. (A) SDS-PAGE of purified URO-S. Lanes 1 and 3, molecular weight standards (M_r values indicated); lanes 2 and 4 contained 2.5 and 5 µg of post-Sephadex G-100 URO-S, respectively. (B) Following electrophoresis, the proteins were transferred to nitrocellulose, treated with mouse anti-human URO-S antiserum, and then visualized with a biotinylated second antibody-avidin peroxidase complex. Note that only the 29.5K protein was visualized. Non-specific bands (~ 55 to 60K) were observed in all four lanes.


1 2 3 4
Kd
94.0
67.0
43.0
30.0
20.1
14.4
A
1 2 3 4
B

Molecular Weight Determinations.

The apparent molecular weight of the native enzyme was 30K as determined by gel filtration on Sephadex G-100 (Fig. 8A). When subjected to SDS-PAGE, the denatured enzyme had a molecular weight of 29.5K (Fig. 8B). These results are consistent with URO-S being a monomer.

Amino Acid Composition and N-Terminal Amino Acid Sequence.

Table II summarizes the results of the amino acid composition analysis of the homogeneous enzyme. Hydrophobic residues (aromatic, Ile, Leu, Met and Val) constituted about 30% of the amino acid composition. When the enzyme was subjected to gas-phase microsequencing, the N-terminus was not blocked and the first six cycles revealed the sequence, Met-Lys-Val-Leu-Leu-Leu.

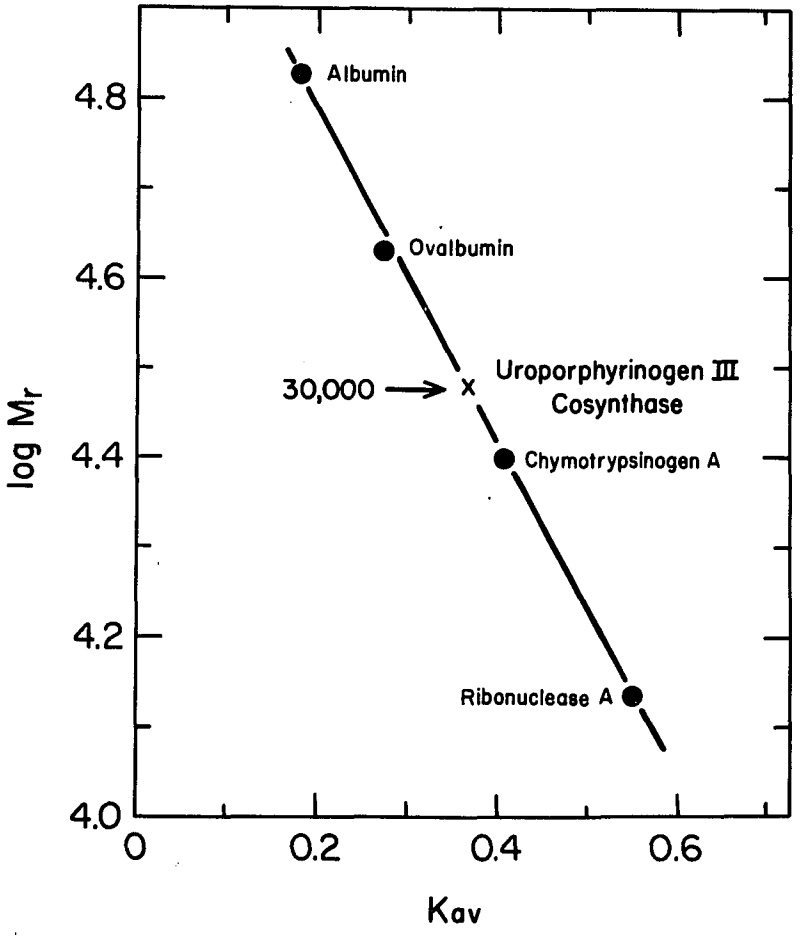
Isoelectric Focusing.

A single isoelectric point of 5.5 was obtained when the enzyme was subjected to flatbed isoelectric focusing using pH 4.0 to 6.5 Pharmalyte (data not shown). This result was consistent with the enzyme's behavior on DEAE-cellulose, eluting between HMB-S and ALA-D which have isoelectric points of 6.2 to 6.8 and 4.9, respectively (10,26).

Thermostability of URO-S.

Inactivation of URO-S was carried out in 1 mg/ml bovine serum albumin to avoid URO-S denaturation due to low protein concentration. The half-lives of inactivation at 37°, 45° and 60°C were 30, 4 and 1 min, respectively (Fig. 9).

Figure 8: Determination of the native and subunit molecular weights of URO-S from human erythrocytes by (A) gel filtration chromatography and (B) SDS-PAGE. Note that URO-S had essentially the same native and subunit molecular weights. See text for details.


Albumin
Ovalbumin
30,000
Uroporphyrinogen III
Cosynthase
Chymotrypsinogen A
Ribonuclease A
4.8
4.6
4.4
4.2
4.0
log M_r
0
0.2
0.4
0.6
Kav

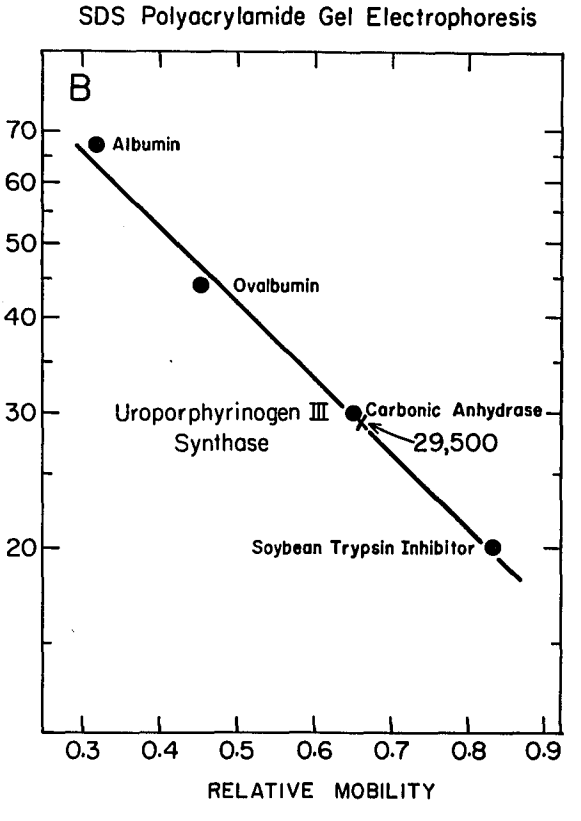

SDS Polyacrylamide Gel Electrophoresis
B
70
60
50
40
30
20
Albumin
Ovalbumin
Uroporphyrinogen III Synthase
Carbonic Anhydrase
29,500
Soybean Trypsin Inhibitor
0.3
0.4
0.5
0.6
0.7
0.8
0.9
RELATIVE MOBILITY

TABLE II

Amino acid composition of URO-S from human erythrocytes

Amino Acid	mol/29,500 g of protein[a]	Nearest interger/ 29,500 g of protein
Asx	20.4	20
Thr	15.3[b]	15
Ser	26.5[b]	27
Glx	35.0	35
Pro	17.8	18
Gly	21.6	22
Ala	26.1	26
Val	13.5[c]	14
Met	1.7	2
Ile	15.0[c]	15
Leu	34.1[c]	34
Tyr	9.2	9
Trp	4.6	5
Phe	8.3	8
His	4.3	4
Lys	16.7	17
Arg	7.1	7
Cys	7.3[d]	7

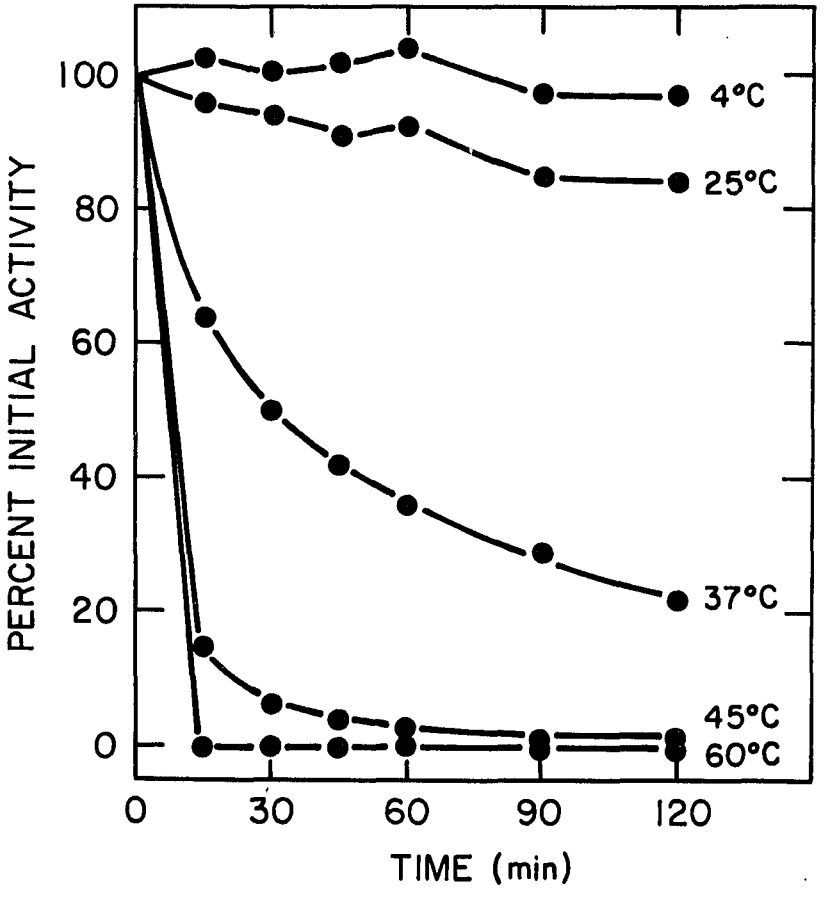
[a]Average of 48 and 72 h hydrolyses.

[b]Extrapolated to t_o from 24, 48 and 72 h hydrolyses.

[c]72 h hydrolysis value only.

[d]24 h performic acid oxidation.

Figure 9: Thermostability of purified URO-S from human erythrocytes. The remaining activities were expressed as percent of initial activity. Aliquots of post-Sephadex G-100 purified URO-S (180 U) were incubated in the presence of 1 mg/ml of bovine serum albumin at various temperatures. Aliquots were removed at timed intervals for assay.


PERCENT INITIAL ACTIVITY
100
80
60
40
20
0
0
30
60
90
120
TIME (min)
4°C
25°C
37°C
45°C
60°C

Effect of pH on Enzyme Activity.

The pH curves in 0.05 M Tris-malate containing 0.1 M NaCl, 0.05 M Tris-HCl containing 0.15 M KCl and 0.05 M Bis-Tris-propane containing 0.15 M KCl were broad, with an optimum at about pH 7.4 (Fig. 10). The absence of 0.15 M KCl or 0.1 M NaCl did not alter the pH curve (data not shown).

Monovalent and Divalent Cation Activation of URO-S.

The monovalent cations, Na^+ and K^+, activated URO-S activity more than 2-fold (Table III). Both ions exhibited similar hyperbolic saturation curves with maximal activation at about 150 mM, the concentration of potassium in erythrocytes. Mg^{++} and Ca^{++} also significantly activated URO-S; however, additional activation did not occur in the presence of 150 mM K^+ (Table III). Pb^{++}, Sn^{++}, and Co^{++} had little effect on enzymatic activity at the concentrations tested. Notably, Pb^{++} had no effect on URO-S activity at a concentration which markedly inactivated purified HMB-S from human erythrocytes (10).

Inhibition of URO-S by Metals.

URO-S activity was markedly inhibited by 1 μM $HgCl_2$. At 10 μM concentrations, $CdCl_2$, $CuCl_2$, and $ZnCl_2$ also were strong inhibitors (Table III). $FeCl_2$, $FeCl_3$ and $MnCl_2$ appeared to be inhibitory, however analysis of the HPLC assay profiles revealed decreased or absent uroporphyrin I and uroporphyrin III, suggesting that these metals either directly altered HMB or the uroporphyrin(ogen) isomers. EDTA (1 mM) slightly activated URO-S activity.

Figure 10: Effect of pH. URO-S activity was determined as described under "Experimental Procedures" over the pH range from 5.8 to 9.5 using 0.05 M Tris-malate containing 0.1 M NaCl (■), 0.05 M Tris containing 0.15 M KCl (▲) and 0.05 M Bis-Tris-propane containing 0.15 M KCl (●). The pH was measured at 25°C in the final assay mixture. The activities are expressed as percent of the maximal value for each buffer.

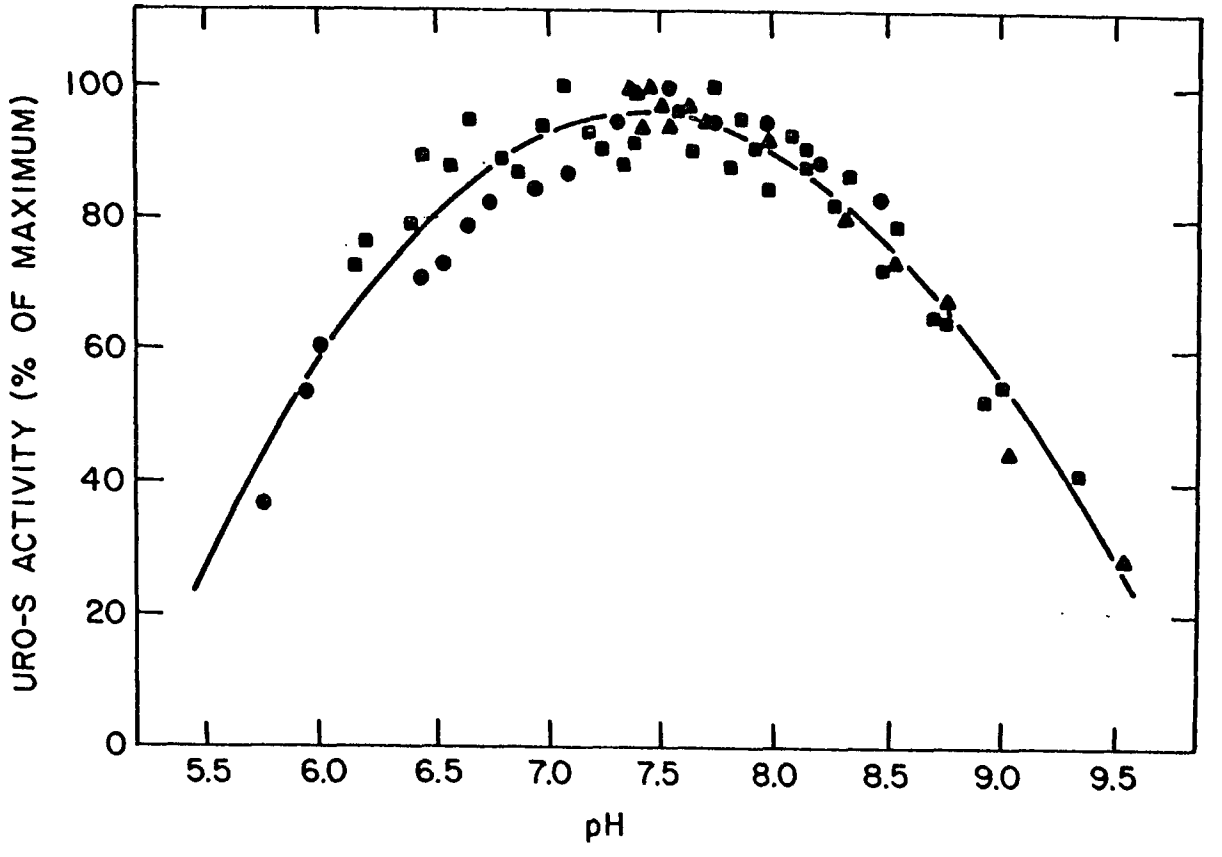

URO-S ACTIVITY (% OF MAXIMUM)
100
80
60
40
20
0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
pH

TABLE III

Effects of various compounds on URO-S activity

Compound	Concentration	Percent of Initial Activity[a]
	(mM)	(%)
KCl	25	137
	50	167
	100	210
	150	216
	200	216
NaCl	25	129
	100	209
	200	221
$MgCl_2$	10	172
	40	162
$KCl/MgCl_2$	150/5	194
	150/20	170
$CaCl_2$	1	116
	10	141
	40	92
$PbCl_2$	0.005	101
$SnCl_2$	0.01	102
$CoCl_2$	0.01	101
	0.10	112
EDTA	1.0	113
$HgCl_2$	0.001	30
$CdCl_2$	0.005	87
	0.01	49
$CuCl_2$	0.01	72
$ZnCl_2$	0.01	74
$FeCl_2$	1.0	0
	0.1	98
$FeCl_3$	1.0	19
	0.5	72
$MnCl_2$	10.0	6
$NiCl_2$	0.10	91

[a]Each assay contained 50 mM Tris-HCl, pH 7.4, 2 μM HMB, and 100 U of purified URO-S.

Effect of Thiol Reagents and Hydroxylamine.

p-Chloromercuribenzoate (20 μM; pCMB) inhibited enzymatic activity by about 80% while the presence of 1 mM DTT prevented pCMB inhibition (Table IV). DTT alone activated the enzyme about 15%. However, in the presence of 0.15 M KCl, DTT did not increase the activity further. Hydroxylamine slightly increased URO-S activity at high concentrations (20 mM).

K_m for HMB.

URO-S activity was determined by the direct HMB assay in the presence of 0.15 M KCl, 0.5 M Tris-HCl and varying concentrations of freshly prepared synthetic HMB. The apparent K_m was 5 to 20 μM. The turnover number estimated from the purified preparation with the highest specific activity was 350 min^{-1}.

TABLE IV

Effect of hydroxylamine and thiol reagents on URO-S activity

Effector	Concentration	Percent of Initial Activity[a]
	(mM)	(%)
Hydroxylamine	20.0	110
DTT	1.0	117
DTT/KCl	1.0/150	216[b]
β-Mercaptoethanol	1.0	107
N-Ethylmaleimide	5.0	63
N-Ethylmaleimide/DTT	5.0/2.5	136
pCMB	0.02	19
pCMB/DTT	0.02/1.0	110

[a]Each assay contained 50 mM Tris-HCl, pH 7.4, 2 μM HMB, and 100 U of purified URO-S.

[b]150 mM KCl alone resulted in 216% of initial activity.

DISCUSSION

The isolation of URO-S from human erythrocytes represents the first purification of this heme biosynthetic enzyme to homogeneity from any source. To facilitate enzyme purification and characterization, it was necessary to optimize assays for URO-S. The recently described coupled-enzyme assay of Wright and Lim (29) was modified by increasing the HMB-S concentration and shortening the incubation period in order to achieve linearity with time and protein concentration. This assay provided reliable monitoring of URO-S activity during enzyme purification. For enzyme characterization, the direct HMB assay (12) was optimzed for pH, buffer, ionic strength, and the HPLC separation and quantitation of uroporphyrin III. This assay permitted the accurate determination of the kinetic properties of human URO-S in the absence of HMB-S.

In all purification procedures performed to date, analytical SDS-PAGE revealed the presence of two major proteins of 29.5 and 32K in the post-Sephadex G-100 URO-S peak. In order to determine which had URO-S activity as well as the possible relatedness (e.g., precursor or modified enzyme) of these co-purified proteins, they were separated and characterized. Reversed phase HPLC clearly resolved the two proteins; only the 29.5K protein had URO-S activity (Figs. 4 and 5). When these proteins were separated by SDS-PAGE, individually electroeluted and then digested with trypsin, distinct peptide profiles were observed (Fig. 6), and immunoblots using mouse anti-human antibodies raised against the electroeluted 29.5K enzyme protein did not cross-react with the 32K protein (Fig. 7). Thus, the peptide mapping and immunologic studies demonstrated that the co-purified proteins did not contain common protein sequences or antigenic determinants.

The purification of URO-S provided the opportunity to compare its physicokinetic properties to those of the other cytosolic heme biosynthetic enzymes, since all four now have been purified to homogeneity from human erythrocytes (Table V). Molecular weight estimates of URO-S were ∿ 30 and 29.5K by gel filtration and analytical SDS-PAGE, respectively, indicating that URO-S was a monomeric enzyme like HMB-S and URO-D. The exception was ALA-D, which is a homooctamer, one of the few known in humans (41). URO-S has been shown to be remarkably thermolabile, a property recognized first by Bogorad and Granick (2) and subsequently used as the basis to distinguish and assay both HMB-S and URO-S (9,12,29,42). It is notable that among the four human cytosolic heme biosynthetic enzymes, only URO-S is extremely thermolabile, whereas the other three, particularly HMB-S, are relatively thermostable even at 60°C. The pI of URO-S was similar to those of ALA-D and URO-D, but HMB-S had a value about 1 to 1.5 pH units higher.

Investigators have recognized the rapidity by which URO-S converts HMB to uroporphyrinogen III (1,8,9,42). However, kinetic studies of the enzyme were limited by the lack of homogeneous enzyme and substrate (HMB) for assays in the absence of HMB-S. These obstacles have been overcome, permitting comparison of the kinetic properties of URO-S with those of the other enzymes, ALA-D, HMB-S and URO-D. The pH vs activity curve for URO-S was broad, with optimal activity observed at about pH 7.4. The optima for ALA-D and URO-D were about 6.3 to 6.7 and 6.8, respectively, whereas that for HMB-S was 8.2. At their respective pH optima, the K_m values for URO-S and HMB-S were similar; in contrast, ALA-D and URO-D had K_m values which were an order of magnitude higher and lower, respectively. However, the turnover number for URO-S was

TABLE V

Comparison of the physicokinetic properties of the human cytosolic heme biosynthetic enzymes

Property	ALA-D (26)	HMB-S (10)	URO-S	URO-D (40)
Subunit Structure	Homooctamer	Monomer	Monomer	Monomer
Subunit Molecular Weight	35,000	37,000	29,500	46,000
K_m (μM)	270	6	5-20	0.35
Turnover Number (mol/mol monomer/min)	11	1.4	350	7.6
pH Optimum	6.3-6.7	8.2	7.4	6.8
Thermostability ($T_{1/2}$ at 60°; min)	30	> 120	1	22 (52°C)
pI	4.9	6.2-6.8	5.5	4.6
Hydrophobic Residues %	32	27	30	33
Inhibitors	Fe,Hg,Pb	Ca,Cu,Fe	Cu,Cd Hg,Pb	Cu,Hg,Pt Hg,Zn
Activators	Zn	--	Na,K	--
Thiol Activation	+	+	+	+

about 30 to 250 times greater than those for ALA-D, HMB-S and URO-D. The activities of all the human cytosolic heme biosynthetic enzymes were strongly inhibited by pCMB and enhanced by the presence of thiol reducing agents such as DTT, implicating the importance of reduced cysteine residue(s) in the active sites. Although ALA-D is a zinc metalloenzyme whose activity is enhanced by this divalent metal cation, zinc did not increase the activity of URO-S, HMB-S or URO-D. However, URO-S activity was marked enhanced by the monovalent cations, sodium and potassium, consistent with the previously reported effect of sodium on the partially purified enzyme from human erythrocytes (16) and rat liver (23). All four enzymes were inhibited by Hg. That hydroxylamine and lead did not inhibit URO-S activity as previously reported (e.g., 16,22) emphasizes the fact that previous studies of URO-S may be erroneous if assays were employed which used HMB-S to generate HMB as substrate for URO-S. This problem has been obviated by the use of HMB in the direct assay.

The only other source from which URO-S has been purified is Euglena gracilis (14). Interestingly, this enzyme also was a monomeric protein with a similar molecular weight (31K by SDS-PAGE and 38.5K by gel filtration), a similar K_m value (12 to 40 μM HMB) and a slightly lower isoelectric point (4.8 to 5.1).

The fact that the HMB, generated by HMB-S, is rapidly metabolized to uroporphyrinogen III suggests that HMB-S and URO-S may be physically adjacent. In fact, several investigators have suggested that the two enzymes exist in a complex (4,7,14,18,20,21,25). Mutations which alter the interaction of HMB-S and URO-S may result in congenital erythropoietic porphyria. The availability of purified URO-S and HMB-S should

permit definitive investigation of this possibility as well as the characterization of the precise mechanism by which PBG is converted to uroporphyrinogen III in humans. Moreover, it is intriguing to speculate that all four cytosolic enzymes may function in a complex, thereby permitting the rapid and efficient conversion of δ-aminolevulinic acid, synthesized in the mitochondria and transported to the cytosol, to coproporphyrinogen, which then returns to the mitochondria for conversion to heme. Such a cytosolic complex would facilitate the efficiency of the sequential reactions and protect the porphyrinogen intermediates from being oxidized to non-metabolizable porphyrins.

The availability of purified URO-S and the fact that monospecific antibodies have been produced should facilitate efforts to obtain cDNA clones encoding URO-S for characterization of the structure, organization, chromosomal localization, and expression of this gene, as well as investigation of the molecular defects in unrelated families with congenital erythropoietic porphyria.

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Chapter Three

MOLECULAR CLONING AND SEQUENCING OF A FULL-LENGTH cDNA ENCODING HUMAN UROPORPHYRINOGEN III SYNTHASE

ABSTRACT

Uroporphyrinogen III synthase (URO-S) catalyzes the formation of the cyclic tetrapyrrole, uroporphyrinogen (URO'gen) III, from the linear tetrapyrrole, hydroxymethylbilane (HMB). The deficiency of URO-S is the biochemical defect in congenital erythropoietic porphyria (CEP). URO-S from human erythrocytes has been purified to homogeneity (Tsai et al. J. Biol. Chem. 262:1268-1273, 1987) and, amino acid sequences for the N-terminal and tryptic peptides were determined. Synthetic oligonucleotide mixtures were constructed to regions of amino acid sequence with minimal codon redundancy and were used to screen a human adult liver cDNA library. Eight positive clones were isolated from a total of 1.2×10^6 recombinants screened. Of these, one clone, designated pURO-S 2, had an insert of $\cong$ 1.3 kb which contained 5' and 3' untranslated sequences of 196 and 284 bp, respectively. An open reading frame of 798 bp was identified, which encoded a protein of 265 amino acids, with a molecular weight of 28,607. The fact that these clones contained the complete message encoding URO-S was supported by colinearity of the predicted amino acid sequence with 86 residues determined by microsequencing the purified enzyme, including 41 N-terminal amino acids. Northern hybridization of poly(A^+) RNA revealed a 1.3 kb transcript in normal human cultured lymphoblasts. The molecular cloning and sequencing of a full-length cDNA for URO-S should facilitate the studies of the structure, organization and expression of this human heme biosynthetic gene. In addition, the availability of the URO-S cDNA will permit localization of the structural gene for URO-S as well as the investigation of the molecular nature of the genetic lesions in CEP.

INTRODUCTION

Heme is an essential pigment of life and forms the prosthetic group of the hemoglobin and cytochromes. An important step in the formation of heme is the synthesis of URO'gen III, which is catalyzed by URO-S, the fourth enzyme in the heme biosynthetic pathway. This enzyme is responsible for ring closure of the linear tetrapyrrole, hydroxymethylbilane (HMB) and the rearrangement of the D-ring in the cyclic tetrapyrrole (1). It has been shown that HMB is a substrate for URO-S purified from Euglena gracilis (2) and from human erythrocytes (3,4).

The deficient activity of URO-S is the primary enzymatic defect in CEP,an erythropoietic porphyria which is transmitted as an autosomal recessive trait. The deficiency of URO-S activity in CEP homozygotes can be detected in erythrocytes (4,5), fibroblasts (6), and cultured lymphoid cells (4). Since HMB can not be converted to URO'gen III, the metabolic defect leads to the non-enzymatic formation and accumulation of URO'gen I, which is subsequently oxidized to URO I. Tissue deposition of URO I leads to the disease manifestations including porphyrinuria, erythrodontia, photosensitivity and hemolytic anemia. Severely affected patients are transfusion dependent, often become disfigured due to the chronic blistering and scaring of skin exposed to sunlight. Recently patients have been treated by chronic transfusions (7) with clinical benefit. Milder cases have been described which are not transfusion dependent, have attenuated manifestations and experience a near normal lifespan. The marked clinical variation in the disease phenotype emphasizes the genetic heterogeneity in this disorder.

To date, studies of CEP have been limited to the demonstration of the enzymatic defect in affected homozygotes and trials of various therapeutic strategies. No studies have been performed to investigate the nature of the enzymatic defect in affected homozygotes. The levels of residual URO-S activity have only recently been determined by reliable assays (4), however the physicokinetic properties of the residual activity in unrelated patients with CEP have not been characterized. In part, these studies were not performed in the past since sensitive enzyme assays were not available until recently (4) and since the study of the enzyme has been difficult due to its extreme lability. However, the recent successful purification of the human erythrocyte enzyme should facilitate further biochemical and genetic studies of this heme biosynthetic enzyme and the porphyria that results from its deficiency.

Since the availability of the cDNA encoding URO-S would permit studies of the structure, chromosomal localization, genomic organization, and expression of this enzyme, efforts were undertaken to clone the cDNA for this heme biosynthetic enzyme. A cDNA could be expressed and large amounts of the purified recombinant protein would permit studies of the kinetic properties of the enzyme and the possible interaction of URO-S with other heme biosynthetic enzymes. Moreover, the cDNA could be used to investigate the molecular defects in unrelated families with CEP. In this communication, the isolation and complete DNA sequence of a full-length cDNA clone encoding human URO-S is described.

MATERIALS AND METHODS

Materials

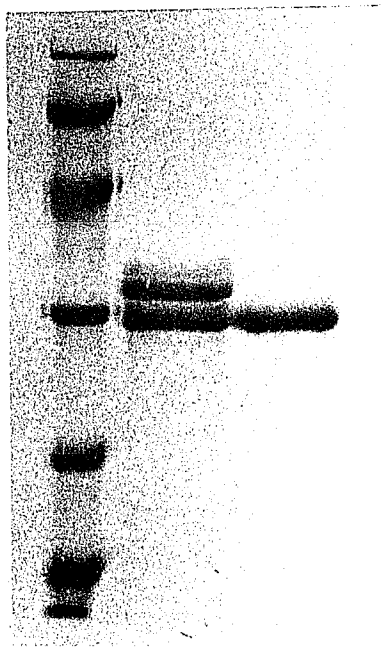
The column for solid phase support and all the reagents for oligonucleotide synthesis were purchased from American Bionuclear. Nitrocellulose filters were obtained from either Millipore or Schleischer and Schuell. Restriction endonucleases were from Boehringer Mannheim Biochemicals. Materials for DNA sequencing were from New England Biolabs (γ-^{32}P-deoxyadenosine, α-^{32}P-deoxyadenosine, α-^{32}P-deoxycytidine triphosphate, α-^{35}S-deoxyadenosine and α-^{35}S-deoxycytidine triphosphate) were purchased from Amersham.

Amino Acid Sequencing of N-terminal and Tryptic Peptides. URO-S from human erythrocytes was purified through the gel filtration step as previously described (3). The nearly homogeneous enzyme was then subjected to preparative SDS-PAGE and the enzyme protein was electroeluted, digested with trypsin, and the resulting peptides were resolved by C_4 reversed phase HPLC as shown in Fig 1 (3). The N-terminal and tryptic peptide sequences were determined by gas-phase microsequencing and HPLC identification of the phenylthiohydantoin amino acids (8).

Synthesis of Oligonucleotides. A Sam One oligonucleotide synthesizer (Biosearch) was used to construct synthetic oligomers and oligonucleotide mixtures using β-cyanoethylphosphoramidite chemistry. Mixed oligonucleotide probes (17 bases long) were synthesized to correspond to amino acid sequences with minimal codon redundancy (Fig 2). Oligonucleotides also were constructed as primers for DNA sequencing. Each of the oligonucleotides was purified by electrophoresis on a 20% polyacrylamide/8 M urea gel.

Figure 1: (A) SDS-PAGE of electroeluted URO-S. Lane 1, molecular weight standards: phosphorylase b (Mr = 94,000), bovine serum albumin (Mr = 67,000), ovalbumin (Mr = 43,000), carbonic anhydrase (Mr = 30,000) soybean trypsin inhibitor (Mr = 20,000), and α-lactalbumin (Mr = 14,400). Lane 2, post-Sephadex G-100 preparation of URO-S (4 µg, 97,000 fold purified); Lane 3, electroeluted protein (2 µg). (B) Profile of tryptic peptides resolved by reversed phase HPLC. The electroeluted URO-S protein was digested with trypsin and chromatographed on a Vydax C_4 reversed phase column equilibrated with solution A (100% water with 0.05% trifluoroacetic acid). A 3h gradient from 0 to 60% solution B (80% acetonitrile with 0.05% trifluoroacetic acid) was used to separate the peptides. The eluate was monitored by absorbance at 214 nm.

A



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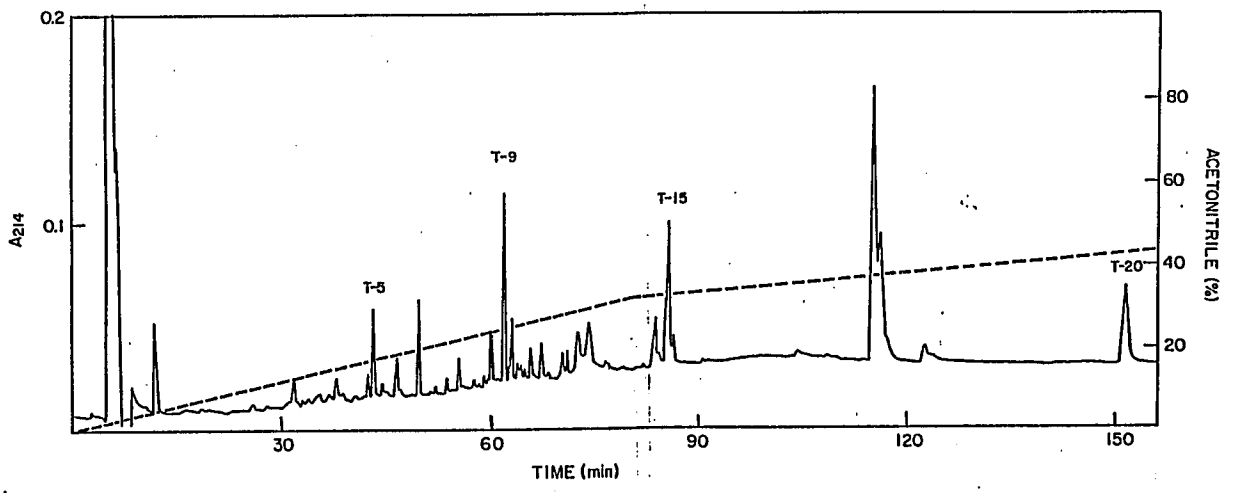
0.2
0.1
A214
T-5
T-9
T-15
T-20
30
60
90
120
150
TIME (min)
80
60
40
20
ACETONITRILE (%)

Figure 2: Amino acid sequence of N-terminal and tryptic peptides of uroporphyrinogen III synthase. Homogeneous URO-S was isolated from post-Sepadex G-100 preparation by reversed phase HPLC as previously described (3) Selected tryptic peptides were isolated as in Fig. 1. Amino acid microseqeucing was performed as described in the text.

Native Enzyme:

```
    1       5         10          15        20
NH2-M-K-V-L-L-L-K-D-A-K-E-D-D-(C)-G-Q-D-P-Y-I-

                                  25        30          35        40
                        -R-E-L-G-L-Y-G-L-E-A-T-(L)-I-P-V-L-S-F-A-T-L-
```

Tryptic Peptides:

```
                                  25        30
T-20                       -E-L-G-L-Y-G-L-E-A-(S)-X-I

T-5      -F-A-A-I-G-P-T-T-A-R-  (10)

T-9      -L-S-H-P-E-D-Y-G-G-L-I-(F)-X-T-S-P-  (16)

T-15     -G-I-A-M-(E)-S-I-T-V-Y-(Q)-T-V-A-X-P-G-I-X-G-  (20)
```

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*Microsequencing performed by Dr. K. Williams, Yale University.

Sequence data spanning 86 amino acids (or 32%) of 265 total residues estimated from the amino acid composition.

Isolation and Characerization of cDNA Clones. A human adult liver cDNA library (9) kindly provided by Dr. Stuart Orkin was screened with the mixed oligonucleotide probes. Colony hybridization was performed essentially as described by Hanahan and Meselson (10). Oligonucleotide probes were labelled with [δ-^{32} P] ATP (5000 Ci/mmol; Amersham) by T4 polynucleotide kinase (Bethesda Research Laboratory) (11). A total of 1.2 x 10^6 recombinants were screened at a density of about 50,000 per plate (150 mm). The filters were hybridized at 50° C with 6X SSC, 5X Denhardt's, 100 ug/ml denatured salmon sperm DNA (1X SCC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0; 1X Denhardt's = 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin). Stringent washing was done at the same temperature with 6X SSC/0.1% SDS for 3 h. Positive colonies were identified by autoradiography after exposing film (Kodak, XAR-2) with two intensifying screens (Cronex, Lightning-Plus) for 16 h. Plasmid DNAs from positive clones were prepared by the alkaline lysis method of Brinboim and Doly (12). After digesting with Pst I, insert fragments were analyzed by a 1% agarose gel and DNA was transferred to nitrocellulose filters as described (13). Hybridization and washing conditions were identical to the methods used for screening. Positive clones were initially grouped according to their insert fragment size. Representative clones were selected from each group and plasmid DNAs were nick-translated (14) for cross hybridization experiments.

DNA Sequencing. The dideoxy chain termination method of Sanger (15) was used for DNA sequencing. The Pst I insert from pURO-S 2 was either subcloned directly into M13 mp18, or subcloned into mp18 and mp19 after digesting the insert with Sau3A I or Hind III. 7-deaza-dGTP was used to

resolve ambiguities in G-C rich regions (16). A synthetic oligonucleotide [CTGCAG-$(G)_8$] was used as the primer to determine sequence adjacent to the Pst I cloning site generated by the G-C tailing technique (17).

RNA Hybridization Analysis. Transformed lymphoid cells were grown in RPMI 1640 medium until the cells reached a density of 10^6 cells/ml. About 1000 ml was centrifuged at 4000 g for 10 min and then washed with normal saline. RNA was isolated by the guanidine isothiocyanate method (18). Poly (A^+) RNA was selected by passage through oligo(dT) cellulose columns (19). About 3 µg of poly(A^+) RNA was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde and the RNA was transferred to nitrocellulose filters as described (20). The filters were then hybridized with a 968 bp Ava II insert from pURO-S 2, which was radiolabelled by the random primer method (21) to 10^9 cpm/µg. Stringent washing conditions were employed (2X SSC/0.1% SDS at 55° C for 30 min, followed by 0.1 X SSC/0.1% SDS at 55° C for 60 min).

RESULTS

Amino Acid Sequencing and Oligonucleotide Synthesis. Amino acid sequence was obtained for both N-terminal and selected tryptic peptides (Fig 2). One of the tryptic peptides (T27) is located at the N-terminal end of the protein and overlapped with the N-terminal amino acid sequence. This finding confirmed that the electroeluted protein was identical to that purified by HPLC. Altogether, a total of 86 nonoverlapping amino acid residues, or about one third of the total protein, was determined. Oligonucleotide mixtures (Fig. 3) were constructed to include all possible codon combinations corresponding to the N-terminal amino acid and an internal tryptic peptide sequence (T9). Both oligo

Figure 3: URO-S oligonucleotide sequences. Two regions of the URO-S protein tht contain minimal codon redundancy were chosen for the synthesis of oligonucleotides. Probe 1 consists of 64 different 17 mer species corresponding to amino acids 8-13. Probe 2 is also a pool of 64 different 17 mers whose sequene were from tryptic peptide (T-9) and correspond to amino acids 52-57 of the predicted sequence

N-Terminal Amino Acid Sequence:

```
  1         5         10        15        20        25
NH2-M-K-V-L-L-L-K-D-A-K-E-D-D-(C)-G-Q-D-P-Y-I-P-E-L-G-L-
                   Probe 1
```

```
   7   8   9   10  11  12
5' GA(T/C) GCN AA(A/G) GA(A/G) GA(T/C) GA 3'
```

Probe 1
17 mer; 64 mix

Internal Peptide (T-9) Amino Acid Sequence:

```
    1         5         10        15
-L-S-H-P-E-D-Y-G-G-L-I-(F)-X-T-S-P
      Probe 2
```

```
   3   4   5   6   7   8
5' CA(T/C) CCN GA(A/G) GA(T/C) TA(T/C) GG 3'
```

Probe 2
17 mer; 64 mix

nucleotide probes (1 and 2) are 17 mers and each is a mixture of 64 oligonucleotide species. The lowest dissociation temperatures (T_d) for probes 1 and 2 are 46 and 48 degrees, respectively, and these two probes were combined in the hybridization solution for screening.

Isolation and Characterization of Positive cDNA Clones. From the 1.2 x 10^6 recombinants screened, 28 putative candidates were selected initially. Upon purification, eight clones remained positive and were isolated. Plasmid DNA was prepared and digested with Pst I to identify their inserts. Three clones had three insert fragments of 840, 395, and 99 bp. Another two clones had two fragments of 750 and 500 bp, and three clones had a single insert fragment of 800 bp. In all cases, both probes 1 and 2 hybridized to the largest Pst I fragment of each clone (data not shown). One clone was selected from each group and the insert was radiolabelled by nick translation. Cross hybridization studies revealed that these three clones were related (data not shown).

Nucleotide Sequence Analysis. One clone with the largest insert (pURO-S 2) was completely sequenced on both strands as shown in Fig 4. The complete nucleotide sequence of 1296 bp of pURO-S is shown in Fig 5. An open reading frame of 795 bp was identified between nucleotide 1 corresponding to the first amino acid, methionine, and the termination codon at nucleotide 796. It encodes a protein of 265 amino acid residues with a predicted molecular weight of 28,607, which is in good agreement with the molecular weight of 29,500 previously estimated by SDS-PAGE (3). The amino acid sequences of all four tryptic peptides was colinear with the deduced amino acid sequence. In addition, two potential sites for N-linked glycosylation(Asn-X-Thr) (22) were identified. Of the 195 bp 5' untranslated sequence, there is no other in phase ATG. Of the

Figure 4: Restriction endonuclease map and sequencing strategy for the human uroporphyrinogen III synthase cDNA clone pURO-S 2. The position of three restriction endounculease (Pst I, Hind III, and Sau 3A I) in the insert are indicated. The open box represents the amino acid coding region. Except for two fragments (solid square) which were generated by deletion subcloning. All the other fragments correspond to the restriction map above. DNA sequence was determined by dideoxy chain termination method (15) using M13 universal primer (vertial bar) or URO-S sequence specific primer (solid circle)

Sequencing Strategy for Uroporphyrinogen III Synthase

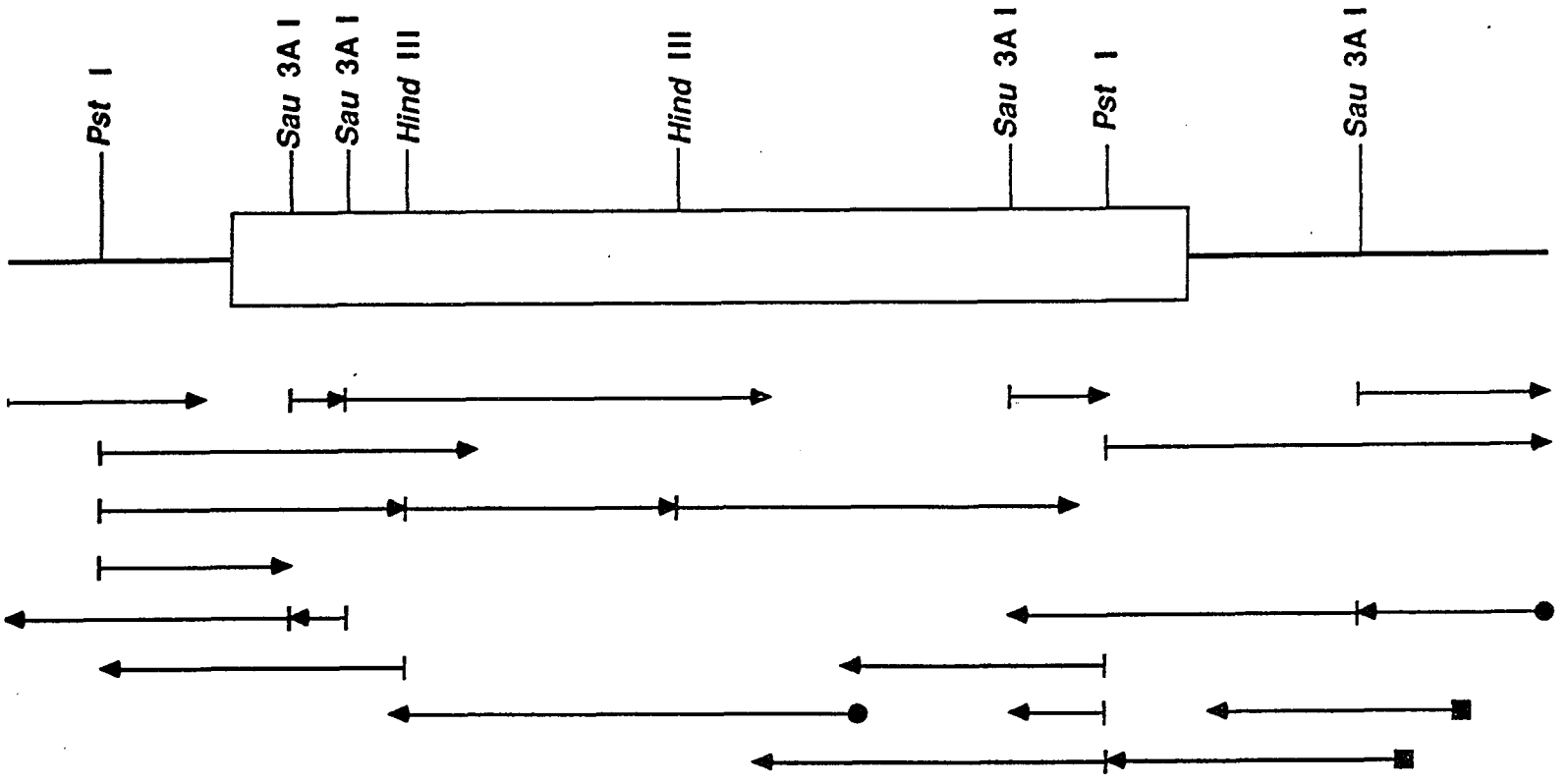
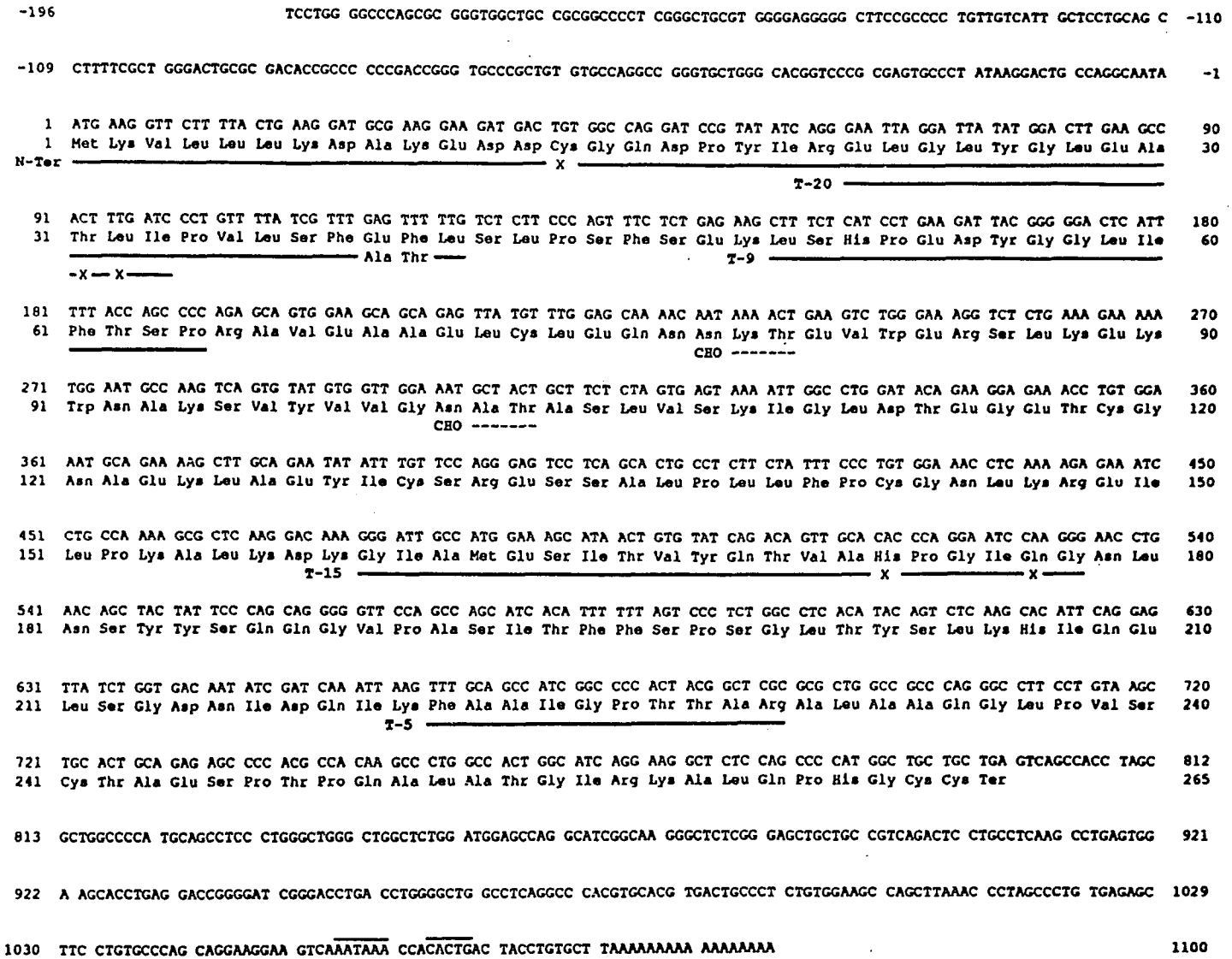


Figure 5: Nucleotide and predicted amino acid sequences of the pURO-S 2 insert. Amino acid 1 is the N-terminal residue. Bold underlines indicate confirmed amino acid sequence obtained by microsequencing of native protein (N-Ter) and tryptic peptides (T). Difference between microsequenced and predicted amino acids are shown; X denotes unassigned amino acids. Potential N-glycosylation sites are indicated by CHO. Overlines indicate polyadenylation signal AATAAA and the CACTG site recognized by U4 snRNP.


-196 TCCTGG GGCCCAGCGC GGGTGGCTGC CGCGGCCCCT CGGGCTGCGT GGGGAGGGGG CTTCCGCCCC TGTTGTCATT GCTCCTGCAG C -110
-109 CTTTTCGCT GGGACTGCGC GACACCGCCC CCCGACCGGG TGCCCGCTGT GTGCCAGGCC GGGTGCTGGG CACGGTCCCG CGAGTGCCCT ATAAGGACTG CCAGGCAATA -1
1 ATG AAG GTT CTT TTA CTG AAG GAT GCG AAG GAA GAT GAC TGT GGC CAG GAT CCG TAT ATC AGG GAA TTA GGA TTA TAT GGA CTT GAA GCC 90
1 Met Lys Val Leu Leu Leu Lys Asp Ala Lys Glu Asp Asp Cys Gly Gln Asp Pro Tyr Ile Arg Glu Leu Gly Leu Tyr Gly Leu Glu Ala 30
N-Ter
X
T-20
91 ACT TTG ATC CCT GTT TTA TCG TTT GAG TTT TTG TCT CTT CCC AGT TTC TCT GAG AAG CTT TCT CAT CCT GAA GAT TAC GGG GGA CTC ATT 180
31 Thr Leu Ile Pro Val Leu Ser Phe Glu Phe Leu Ser Leu Pro Ser Phe Ser Glu Lys Leu Ser His Pro Glu Asp Tyr Gly Gly Leu Ile 60
Ala Thr
T-9
-X— X—
181 TTT ACC AGC CCC AGA GCA GTG GAA GCA GCA GAG TTA TGT TTG GAG CAA AAC AAT AAA ACT GAA GTC TGG GAA AGG TCT CTG AAA GAA AAA 270
61 Phe Thr Ser Pro Arg Ala Val Glu Ala Ala Glu Leu Cys Leu Glu Gln Asn Asn Lys Thr Glu Val Trp Glu Arg Ser Leu Lys Glu Lys 90
CHO
271 TGG AAT GCC AAG TCA GTG TAT GTG GTT GGA AAT GCT ACT GCT TCT CTA GTG AGT AAA ATT GGC CTG GAT ACA GAA GGA GAA ACC TGT GGA 360
91 Trp Asn Ala Lys Ser Val Tyr Val Val Gly Asn Ala Thr Ala Ser Leu Val Ser Lys Ile Gly Leu Asp Thr Glu Gly Glu Thr Cys Gly 120
CHO
361 AAT GCA GAA AAG CTT GCA GAA TAT ATT TGT TCC AGG GAG TCC TCA GCA CTG CCT CTT CTA TTT CCC TGT GGA AAC CTC AAA AGA GAA ATC 450
121 Asn Ala Glu Lys Leu Ala Glu Tyr Ile Cys Ser Arg Glu Ser Ser Ala Leu Pro Leu Leu Phe Pro Cys Gly Asn Leu Lys Arg Glu Ile 150
451 CTG CCA AAA GCG CTC AAG GAC AAA GGG ATT GCC ATG GAA AGC ATA ACT GTG TAT CAG ACA GTT GCA CAC CCA GGA ATC CAA GGG AAC CTG 540
151 Leu Pro Lys Ala Leu Lys Asp Lys Gly Ile Ala Met Glu Ser Ile Thr Val Tyr Gln Thr Val Ala His Pro Gly Ile Gln Gly Asn Leu 180
T-15
X
X
541 AAC AGC TAC TAT TCC CAG CAG GGG GTT CCA GCC AGC ATC ACA TTT TTT AGT CCC TCT GGC CTC ACA TAC AGT CTC AAG CAC ATT CAG GAG 630
181 Asn Ser Tyr Tyr Ser Gln Gln Gly Val Pro Ala Ser Ile Thr Phe Phe Ser Pro Ser Gly Leu Thr Tyr Ser Leu Lys His Ile Gln Glu 210
631 TTA TCT GGT GAC AAT ATC GAT CAA ATT AAG TTT GCA GCC ATC GGC CCC ACT ACG GCT CGC GCG CTG GCC GCC CAG GGC CTT CCT GTA AGC 720
211 Leu Ser Gly Asp Asn Ile Asp Gln Ile Lys Phe Ala Ala Ile Gly Pro Thr Thr Ala Arg Ala Leu Ala Ala Gln Gly Leu Pro Val Ser 240
T-5
721 TGC ACT GCA GAG AGC CCC ACG CCA CAA GCC CTG GCC ACT GGC ATC AGG AAG GCT CTC CAG CCC CAT GGC TGC TGC TGA GTCAGCCACC TAGC 812
241 Cys Thr Ala Glu Ser Pro Thr Pro Gln Ala Leu Ala Thr Gly Ile Arg Lys Ala Leu Gln Pro His Gly Cys Cys Ter 265
813 GCTGGCCCCA TGCAGCCTCC CTGGGCTGGG CTGGCTCTGG ATGGAGCCAG GCATCGGCAA GGGCTCTCGG GAGCTGCTGC CGTCAGACTC CTGCCTCAAG CCTGAGTGG 921
922 A AGCACCTGAG GACCGGGGAT CGGGACCTGA CCTGGGGCTG GCCTCAGGCC CACGTGCACG TGACTGCCCT CTGTGGAAGC CAGCTTAAAC CCTAGCCCTG TGAGAGC 1029
1030 TTC CTGTGCCCAG CAGGAAGGAA GTCAAATAAA CCACACTGAC TACCTGTGCT TAAAAAAAAA AAAAAAAA 1100

305 bp 3' untranslated sequence, a conserved polyadenylation signal AATAAA (23), was present 27 nucleotides prior to the poly (A) tract. The conserved recognition sequence (CACTG) for the binding of the small nuclear ribonucleoprotein (SnRnp) U4 (24) was located between the polyadenylation signal and the poly (A) tract.

DISCUSSION

Human cDNA clones encoding URO-S, the fourth enzyme in the heme biosynthetic pathway, have been isolated and characterized. The fact that pURO-S 2 is full-length is supported by the following: First, the predicted amino acid sequence from pURO-S 2 is colinear with the N-terminal and tryptic peptide sequence obtained from purified protein. Second, the predicted amino acid composition is consistent with the composition data derived from the amino acid analysis of the purified protein. Table I compares the deduced amino acid composition with that obtained from the purified protein (3). Except for two amino acids (Trp and Ser), there is excellent correspondence between the predicted and determined amounts of each amino acid. Although the exact length of the 5' untranslated region has not been determined yet, it is likely that pURO-S 2 is nearly full-length since very few eukaryotic mRNAs have 5'untranslated sequences of more than 100 bp (25). Moreover, Northern blot analysis (Fig. 6) revealed a message size of about 1300 nt.

The successful identification of cDNA clones for URO-S represents the fourth human cDNA isolated for a heme biosynthetic enzyme (27, 29, 30, 31, 36) (Table III). cDNA clones for ALA-D have been isolated from mouse (26) and human origin (27). cDNA clones for HMB-S have been isolated from rat (28) and two human tissue sources (liver and spleen)

AMINO ACID COMPOSITION OF UROPORPHYRINOGEN III SYNTHASE

Amino Acid	Deduced from URO-S cDNA	Purified URO-S*
Ala	25	26
Val	12	14
Leu	32	34
Ile	15	15
Pro	16	18
Met	2	2
Phe	8	8
Trp	2	5
Gly	20	22
Ser	23	27
Thr	15	15
Cys	8	7
Tyr	9	9
Asx	18	20
Glx	32	35
Lys	17	17
Arg	7	7
His	4	4

*Residues/29,500 g Protein

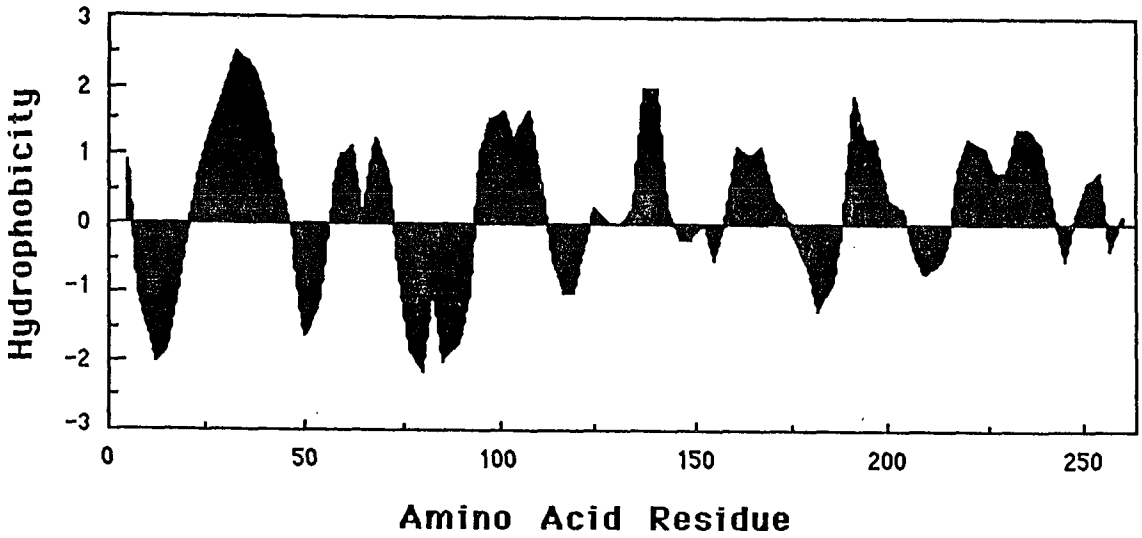
Figure 6: Blot analysis of human uroporphyrinogen III synthase mRNA. Poly (A^+) RNA isolated from two human lympholbast lines were electrophoresed on a 1% formaldehyde agarose gel; the RNA was transferred, hybridized, and washed as described in the text. The relative mobility of 18 S and 28 S RNAs are indicated.


28 S—
18 S—

(29,30). cDNA clones for URO-D also were isolated from both human and murine libraries (31,32) Compared to these cytosolic heme biosynthetic enzymes, less has been achieved in the molecular cloning for the four mitochondrial enzymes of heme biosynthesis. To date, only ALA-S cDNAs have been isolated from yeast (33), chicken embryo liver (34), mouse liver and erythroid cells and, most recently, from human liver (36). This may be due to the difficulty associated with the isolation of homogeneous enzymes from the mitochondrion. Although the amino acid sequence that was used to construct oligonucleotide probes 1 and 2 was derived from protein purified from erythrocytes, they permitted the successful isolation of cDNA clones from a human adult liver library, suggesting that both the erythroid and hepatic enzymes are encoded by a single gene. This finding does not exclude the possibility that tissue specific regulation may exist for URO-S. In fact, different regulatory mechanisms for heme biosynthesis may exist for hepatic and erythroid tissues. For example, ALA-S, the first enzyme in the pathway, is feed-back regulated by heme only in liver, and different messages have been identified for chicken liver and bone marrow (37). Ferrochelatase and iron, on the other hand, may play a key role of metabolic regulation of heme biosynthesis in erythroid tissue (38). Recently, Grandchamp et al. reported that a single HMB-S gene is transcribed from two different promoters (39), giving rise to two mRNAs. Furthermore, the apparent difference of molecular weight between liver and erythroid specific isoenzymes could be accounted for by the difference in mRNA sequences. The availability of predicted amino acid sequence from pURO-S 2 provides an opportunity to study the structural basis of its physical properties. One of the hallmarks of URO-S is its instability. Researchers noted that

the catalytic function of HMB-S and URO-S could be differentiated simply by thermal inactivation of URO-S (40). In contrast, HMB-S is resistant to heat denaturation at 60° C; HMB-S activity can be retained up to 4 h at this temperature (4). URO-S, on the other hand, loses its activity appreciably at room temperature. The T $1/_2$ of URO-S at 60° C is about 1 min (3). Two different aspects of URO-S instability have to be considered. First, the structural integrity of URO-S has to be maintained to preserve its catalytic function. The tertiary and quaternary structure of URO-S is critical for its enzymatic activity and sensitivity to heat denaturation. Figure 7 shows the hydropathy plot of URO-S. Notice that hydrophobic peaks alternate with hydrophilic valleys throughout the entire polypeptide sequence. This is typical of a globular protein, with its hydrophobic side chains buried in the interior of the protein. One may assume that the folding of URO-S, although essential for its function, is not accompanied by a favorable free energy gain, and any thermal insult would therefore result in an irreversible denaturation of the URO-S protein. A different condition presumably exists for the rapid loss of enzymatic activity without heat denaturation. It is plausible that proteolytic digestion of a specific amino acid sequence may be the mechanism underlying its rapid degradation. Two hypotheses recently have been put forward to explain the rapid degradation of protein in eukaryotic cells. Varshavsky and his colleagues have advanced the N-end rule from their studies of ubiquitin (41). In a test system with β-galactosidase expressed in yeast, they found that the N-terminal amino acid apparently determined the intracellular half-life of the protein. For example, methionine, serine, alanine, threonine, valine, and glycine at the N-terminus resulted in half-lives of more than 20 h

Figure 7: Hydropathy plot of human URO-S amino acid sequence predicted from cDNA clone pURO-S 2. The hydrophocity values (+ for hydrophobic, - for hydrophilic) were determined by the algorithm of Kyte and Doolittle (47).


Hydrophobicity
3
2
1
0
-1
-2
-3
0
50
100
150
200
250
Amino Acid Residue

for the protein, whereas arginine at the terminus had the shortest half-life of 2 min. To extend this observation, data were analyzed for a total of 208 proteins whose N-terminal amino acid and intracellular half-lives were known. No exception for the N-end rule was found for all these relatively long-lived proteins. Only one protein with short half-life was included in this study because the N-terminal amino acid sequence of an unstable protein is inherently difficult to determine. Another hypothesis, with a acronym of PEST, was proposed by Rogers et al. to account for the short half-life of rapidly degraded proteins (42). PEST regions can be recognized by examining amino acid residues adjacent to proline (P) for enrichment of glutamic acid (E), serine (S), and threonine (T). Localization of PEST regions can be assisted by the hydropathy plot. Typically, they appear in a hydrophilic valley followed or preceded by a hydrophobic peak. PEST regions are usually flanked by positively charged amino acids, but internal lysine, arginine, and histidine residues never occur inside the sequence. Of the ten proteins studied that have intracellular half-lives of less than 2 hours, all of them have at least one PEST region, whereas only 3 out of 35 proteins that have longer intracellular half-lives (20 to 220 hours) contain a PEST region. The mechanism by which PEST containing protein is degraded is not known. However, this mechanism is clearly mediated by specific proteolysis only in eukaryotic cells. Since some PEST containing proteins (E1A, and Myc protein) can be stably overexpressed in E. coli (43, 44), in contrast to its rapid degradation in cultured eukaryotic cells. Furthermore, prokaryotic proteins with short half-lives, (e.g., lambda protein N and C II) do not contain any PEST region (45). Although the precise intracellular half-life of URO-S has not been determined.

Methionine is the first amino acid residue obtained for the N-terminus of URO-S. If the N-end rule is operational, methionine should have conferred intracellular stability to URO-S. A "PEST-like" region can be identified from the predicted amino acid sequence (238-248) and hydropathy plot. A stretch of sequence, P-V-S-C-T-A-E-S-P-T-P occurs in a typical hydrophilic valley, without any internal, positively-charged amino acid. Whether this really represents a true PEST sequence and its significance in terms of proteolytic degradation will require further studies using expressed protein with site-specific modification of this region. A useful target for site-specific mutagenesis may be the proline residue, which may be critical to the sequence recognized by a specific proline endopeptidase (46).

In summary, the molecular cloning of a full-length human URO-S cDNA should facilitate the characterization of the genomic organization of this heme biosynthetic enzyme and should permit the elucidation of the molecular defect(s) in unrelated families with CEP. In addition, the isolation of this full-length clone should be useful for the investigation of the structural basis of the enzyme's unique physical and kinetic properties.

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SUMMARY

1. Reliable assays were developed to quantitate URO-S activity. C_{18} reversed phase HPLC was used to resolve and quantitate the URO I and III isomers without prior derivatization. Using either PBG (coupled-enzyme assay) or HMB (direct assay) as substrate, URO-S activity was measured by the amount of URO III formed, as analyzed by HPLC.

2. The enzymatic diagnosis of homozygotes with CEP can be achieved reliably by the direct or the coupled-enzyme assay. In most cases, the URO-S assays distinguished between normal individuals, obligate heterozyotes and homozygous patients with CEP using erythrocytes or, preferably cultured lymphoblasts as enzyme source. Cultured lymphoblasts were useful for the diagnosis of suspect patients who previously received blood transfusions for their hemolytic anemia.

3. The coupled-enzyme assay is easier to perform than the direct assay because there is no need for substrate preparation before incubation. This assay also is useful for monitoring enzymatic activity during purification of URO-S. However, the direct HMB assay is most reliable for kinetic studies of URO-S.

4. A purification scheme has been devised for URO-S which yields several milligrams of highly purified URO-S from five liters of human erythrocytes. Subsequent purification by either reversed phase HPLC or electroelution permits the isolation of homogeneous URO-S protein.

5. URO-S from human erythrocytes is a monomer with a molecular weight of 29,500. The purified protein has a pI of 5.5, a pH optimum of 7.4 and a Km for HMB of 5-20 uM. Na^{+}, K^{+}, Mg^{+2}, and Ca^{+2} are activators of URO-S, while the enzymatic activity is inhibited by Cd^{+2}, Cu^{+2}, Hg^{+2} and Zn^{+2}.

6. The amino acid sequence for N-terminal and tryptic peptides of URO-S was determined. Synthetic oligonucleotide mixtures were synthesized corresponding to amino acid sequences with minimal codon redundancy. Radiolabeled oligonucleotide probes were used for screening a human adult liver cDNA library.

7. Of the eight positive clones which had URO-S sequences, one clone (pURO-S-2) was full-length having an insert of 1296 bp, which included a 196 bp 5' untranslated region, an 85 bp 3' untranslated sequence and a 798 bp coding sequence (265 amino acid residues and the stop codon). The nucleotide sequence was determined for the entire insert. The authenticity of the URO-S cDNAs were established by the demonstration of colinearity of the deduced sequence with amino acids determined by microsequencing the homogeneous protein. Northern blot analysis revealed a single message of 1.3 kb, further indicating that pURO-S-2 contains the complete processed transcript.

8. The availability of the full-length cDNA encoding human URO-S will be useful for the investigation of the molecular defect(s) in CEP from unrelated families, and will facilitate studies of the structure, genomic organization, chromosomal localization and expression of human URO-S. Such studies also should provide insight into the regulation of the human heme biosynthetic pathway. In addition, the availability of

this cDNA will permit the development and evaluation of future trials of gene transfer into hematopoietic stem cells for the treatment of severely affected hemozygotes with CEP.