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BIOCHEMICAL AND MOLECULAR STUDIES OF NORMAL AND MUTANT HUMAN HEME BIOSYNTHETIC ENZYMES

City University of New York PH.D. 1986

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BIOCHEMICAL AND MOLECULAR STUDIES OF NORMAL AND MUTANT HUMAN HEME BIOSYNTHETIC ENZYMES

by

Ludmila Teresa Ostasiewicz

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

1986

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BIOCHEMICAL AND MOLECULAR STUDIES OF NORMAL AND MUTANT HUMAN HEME BIOSYNTHETIC ENZYMES

by: Ludmila Teresa Ostasiewicz

Advisor: Professor Robert J. Desnick

ABSTRACT

PBG-deaminase was studied in erythrocyte lysates from 165 AIP heterozygotes from 92 unrelated families. Immunologic, physical, and kinetic characterization revealed four classes of PBG-deaminase mutations. In the majority of families, the amount of immunoreactive enzyme protein corresponded to enzymatic activity (CRIM-negative Type 1). In three families, patients had normal levels of erythrocyte PBG-deaminase activity (CRIM-negative Type 2). Two types of CRIM-positive mutations were identified: the Type 1 mutation had a CRIM/activity ratio of≃1.7 and a crossed-immunoelectrophoretic profile in which all the enzyme intermediates were increased with the B intermediate predominant (B>A≫C=D>E). The mutation altered both the kinetics and stability of the noncatalytic immunoreactive enzyme protein. The second CRIM-positive mutation, Type 2, had a CRIM/activity ratio of ≃5.7. Crossed-immunoelectrophoresis revealed markedly increased amounts of substrate

bound intermediates (B>C>D>E»A) with increased resistance to intraerythrocyte proteolysis.

Three monoclonal antibodies were produced to human ALA-D and used to develop a rapid immunoaffinity purification procedure for the enzyme. Crude enzyme preparations were applied to the column and the enzyme eluted with 3 M KSCN resulting in a 19,500-fold increase in purity, an overall recovery of activity of 26% and enzyme which was greater than 95% pure. Three human ALA-D isozymes 1-1, 1-2, and 2-2 were affinity purified and characterized. The isozymes exhibited similar pH optima, apparent k_m values, stabilities, and effects of activators and inhibitors. They were distinguishable by starch gel electrophoresis, isoelectric focusing (pI 1-1 = 5.31; pI 1-2 = 5.17-5.31; pI 2-2 = 5.17), and DEAE-cellulose chromatography. In the presence of 0.05 mM zinc, the 1-1, 1-2, and 2-2 isozymes eluted from DEAE-cellulose at 0.180, 0.205, and 0.230 M KCl, respectively.

Human erythrocyte ALA-D was purified to homogeneity and the N-terminal amino acid sequence determined. Mixed oligonucleotide probes corresponding to this sequence, as well as to bovine sequences, were synthesized and used, along with monospecific polyclonal rabbit anti-human ALA-D antibodies in the isolation of an 827 base pair clone for ALA-D from a pEX human liver cDNA expression library. The authenticity of this cDNA was demonstrated by colinearity of

the predicted amino acid sequence with peptide sequences from the purified enzyme.

FOREWORD

Portions of this thesis have been presented in the following publications:

Desnick, R.J., Ostasiewicz, L.T., Tishler, P.A., and Mustajoki, P.: Acute intermittent porphyria: Characterization of a novel mutation in the structural gene for porphobilinogen deaminase. Demonstration of non-catalytic enzyme intermediates stabilized by bound substrate. J. Clin. Invest. 76: 865-874, 1985.

Wetmur, J.G., Bishop, D.F., Ostasiewicz, L., and Desnick, R.J.: Molecular cloning of a cDNA for human δ-aminolevulinate dehydratase. Gene 43: 123-130, 1986.

Ostasiewicz, L.T., Bishop, D.F., Quinn, M., and Desnick, R.J.: Immunoaffinity purification and characterization of δ-aminolevulinic acid dehydratase isozymes from human erythrocytes. J. Biol. Chem., in review.

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LIST OF ABBREVIATIONS

AIA	2-allylisopropylacetamide
AIP	acute intermittent porphyria
ALA	δ-aminolevulinic acid
ALA-D	δ-aminolevulinic acid dehydratase
ALA-S	δ-aminolevulinic acid synthase
BSA	bovine serum albumin
bp	base pair
CoA	coenzyme A
CRIM	cross reacting immunologic material
DDC	1,4-dihydro 3,5-dicarbethoxycollidine
DEAE	diethylaminoethyl
DTE	dithioerythritol
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
EtdBr	ethidium bromide
HAT	hypoxanthine-aminopterine-thymidine
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
HRP	horseradish peroxidase
IEF	isoelectric focusing
Lv	levulinate locus
nt	nucleotide
PAGE	polyacrylamide gel electrophoresis
PBG	porphobilinogen
PBG-D	porphobilinogen deaminase

RIA	radioimmunoassay
SDS	sodium dodecyl sulfate
UROCos	uroporphyrinogen cosynthase

I. BACKGROUND

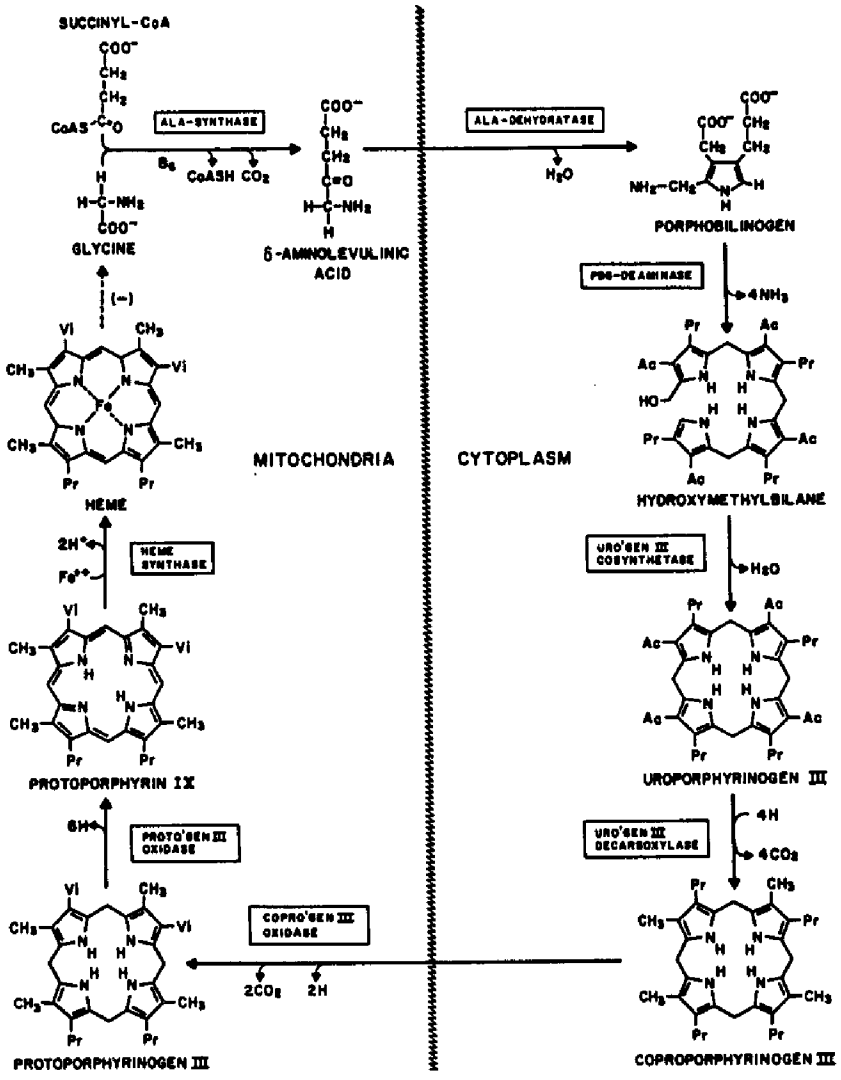
A. Heme Biosynthesis Historical Overview

Although the diseases associated with defects of the heme biosynthetic pathway have intrigued investigators since the end of the nineteenth century, the biosynthesis of heme was not understood until the mid-1940's and the enzymatic deficiencies in these disorders were not identified until the 1970's.

In 1946, Shemin and Rittenberg (1) performed radioactive tracer experiments to show that glycine and succinic acid acted as precursors of the porphyrins. Subsequently, they proposed that the 5-carbon amino ketone δ-aminolevulinic acid (ALA) was the product of the condensation of glycine and succinate (2). The demonstration in 1975 that two enzymes, PBG-deaminase (PBG-D) and uroporphyrinogen cosynthase (UROCos), were required to catalyze the conversion of four molecules of the monopyrrole porphobilinogen (PBG) into uroporphyrinogen III (3) opened the door for the elucidation of the heme biosynthetic pathway. Today it is known that the action of eight distinct enzymes is required for the conversion of eight moles of glycine and eight moles of succinyl CoA to form the porphyrin precursors, porphyrins and, finally, one mole of heme. The current concept of heme biosynthesis is illustrated in Figure 1. δ-aminolevulinic acid synthase

Figure 1. Current concept of heme biosynthesis. ALA-S, the first enzyme of the pathway, and coproporphyrinogen oxidase, protoporphyrinogen oxidase, and ferrochelatase (heme synthase), the last three enzymes, function in the mitochondria; the four intermediate enzymes are located in the cytosol. In liver, ALA-S is the the rate limiting enzyme of the pathway and is negatively feedback regulated by the intracellular concentration of heme.

CURRENT CONCEPT OF HEME BIOSYNTHESIS



(ALA-S), the first enzyme of the pathway, and coproporphyrinogen oxidase, protoporphyrinogen oxidase and ferrochelatase, the last three enzymes, function in the mitochondria; the four intermediate enzymes are located in the cytosol.

Heme biosynthesis is modulated by genetic and metabolic mechanisms, primarily through the control of ALA-S, the rate limiting enzyme in the pathway. In liver, ALA-S is negatively feedback regulated by the intracellular concentration of heme which influences the transcription of mRNA for the enzyme (4) and the translocation of the enzyme from the cytosol to the mitochondria (5-7). That ALA-S is an inducible enzyme is supported by the finding that certain drugs, such as 2-allylisopropylacetamide (AIA) and 1,4-dihydro 3,5-dicarbethoxycollidine (DDC), cause an increase in ALA-S activity by increasing the level of ALA-S protein (4). AIA is thought to induce ALA-S by enhancing the destruction of heme by hepatic cytochrome P-450, while DDC has been shown to inhibit the activity of ferrochelatase, resulting in a decreased amount of heme formed from protoporphyrin (9). Because it can be prevented by inhibitors of RNA synthesis (4,10), the induction of ALA-S results, in part, from the transcriptional induction of the mRNA for ALA-S.

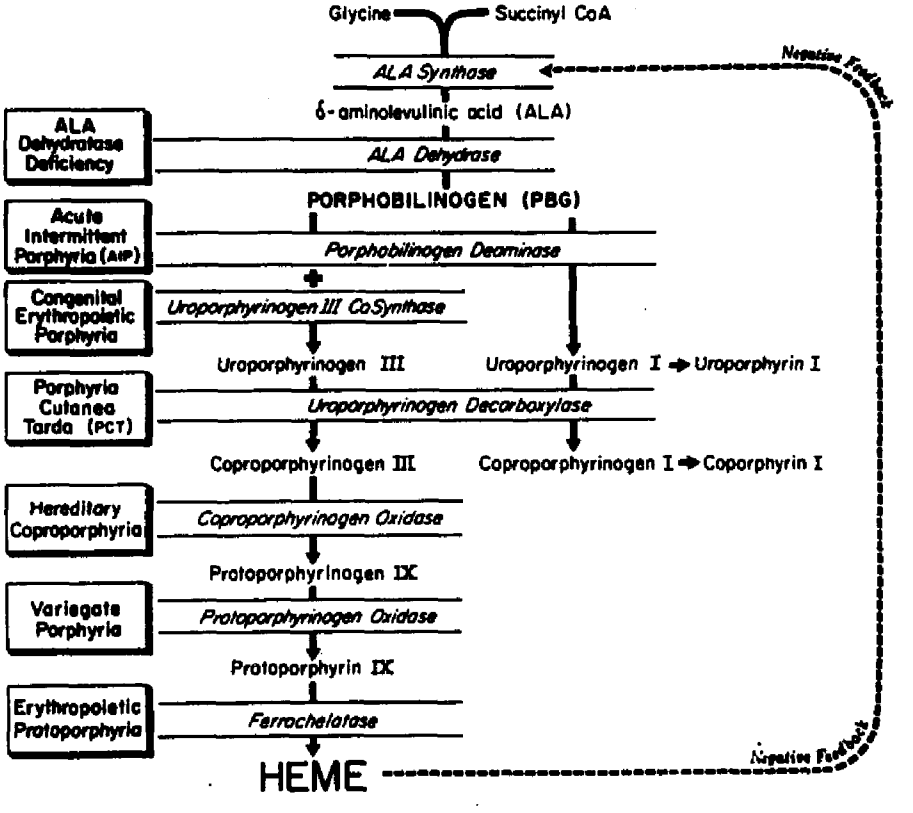
B. The Porphyrias

Deficiencies in the enzymes of the heme biosynthetic pathway affect the regulation of the pathway and result in the disorders known as the porphyrias. These are a group of related, but nonetheless distinct, diseases. To date, seven porphyrias are known (Figure 2) and, depending on the organ system in which defects in porphyrin synthesis are expressed, are classified as either erythropoietic or hepatic (11,12). Although the modes of inheritance and the patterns of excretion of porphyrins vary with the disease, symptoms in the porphyrias are characterized by two important clinical features. Neurologic manifestations, accompanied by accumulation and excretion of ALA and PBG, are common in the acute porphyrias: δ-aminolevulinic acid dehydratase (ALA-D) deficiency, acute intermittent porphyria (PBG-deaminase deficiency), hereditary coproporphyria (coproporphyrinogen oxidase deficiency), and variegate porphyria (protoporphyrinogen oxidase deficiency) (Figure 2). Cutaneous photosensitivity is characteristic of all porphyrias except ALA-D deficiency and acute intermittent porphyria.

The acute porphyrias are of particular interest in that clinical expression of the porphyria is highly variable and affected individuals may be asymptomatic for most of their lives. Acute episodes characterized by abdominal pain, severe neurologic dysfunction and increased excretion of ALA and PBG, can be precipitated by environmental and chemical

Figure 2. Inborn errors of heme biosynthesis. An enzyme defect has been identified for each step in the pathway, excluding the first. Of the seven known porphyrias, only congenital erythropoietic porphyria and ALA-D deficiency are characterized by autosomal recessive inheritance.

Inborn Errors of HEME Biosynthesis



factors. As shown in Table 1, these include steroid hormones, alcohol, reduced caloric and carbohydrate intake, and a variety of drugs which are believed to act adversely by inducing the cytochrome P-450 system (13). Among the erythropoietic porphyrias, sunlight is a major influence of clinical expression. With the exception of congenital erythropoietic porphyria and the recently discovered ALA-D deficiency (14), which are autosomal recessive disorders, all porphyrias are characterized by autosomal dominant inheritance.

Table 1. Examples of Environmental Factors which Precipitate Attacks of Acute Intermittent Porphyria

Drugs	Heavy Metals	Miscellaneous
Amphetamines	Arsenic	Alcohol
Barbiturates	Lead	Infection
Chloroquin	Zinc	Low food intake
Estrogens		
Eucalyptol		
Griseofulvin		
Oral contraceptives		
Phenytoin (Dilantin)		
Sulfonamides		

C. ALA-D

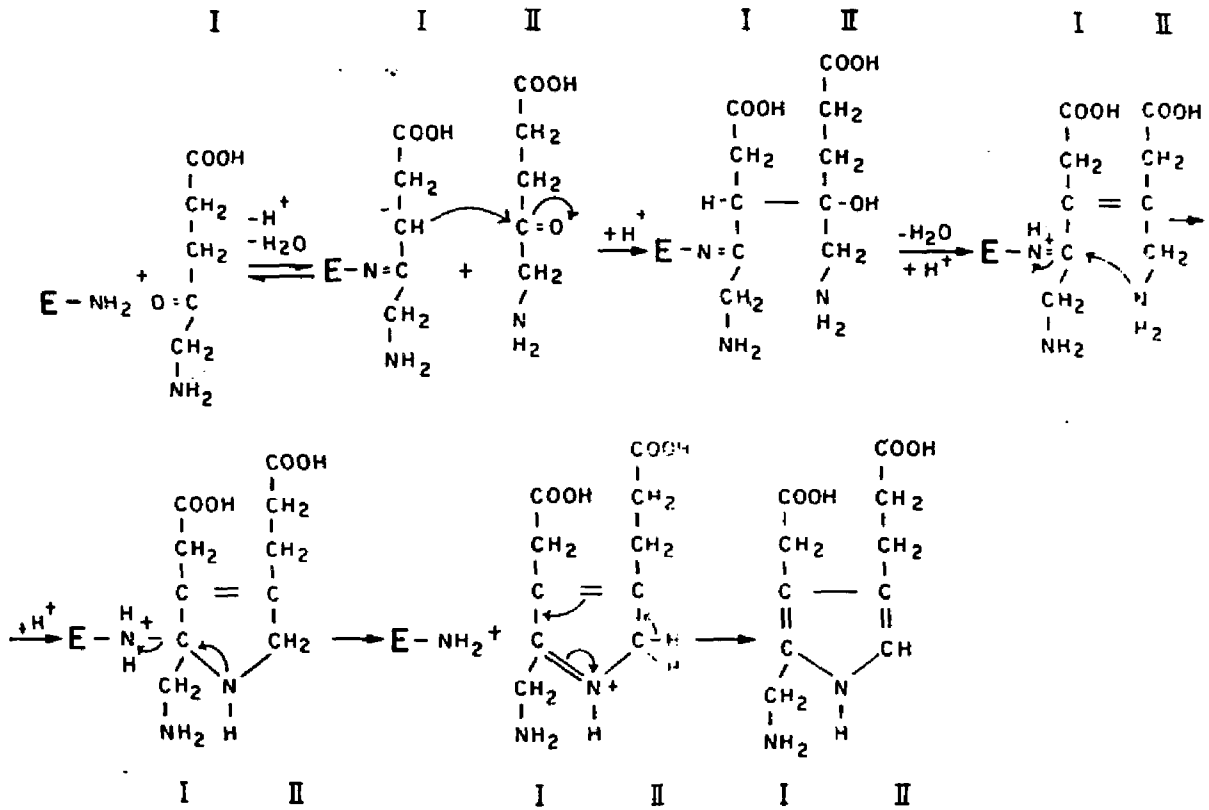
ALA-D (EC 4.2.1.24), the second enzyme of the heme biosynthetic pathway, is responsible for the formation of PBG by the condensation of two molecules of ALA. The

reactions mechanism of prokaryotic ALA-D is summarized in Figure 3.

Based on studies by Nandi and Shemin (15) using ALA-D obtained from the photosynthetic bacterium Rhodopseudomonas spheroides, it has been proposed that the keto group of ALA forms a Schiff base with the epsilon-amino group of lysine on the enzyme. A second ALA molecule condenses with the imine and the resulting aldol loses one molecule of water. Through a transamination reaction, the free amino group of the second ALA molecule displaces the epsilon-amino group of lysine on the enzyme, forming PBG. Through the use of ^{14}C labeling studies, Jordan and Seehra (16) have shown that, in bovine liver, the ALA which participates in the formation of the Schiff base ultimately forms the propionic acid side chain of PBG, while the second ALA gives rise to the acetic acid moiety of PBG.

To date, ALA-D has been purified from murine (17) and bovine liver (18, 19) and human erythrocytes (20), as well as from plant (21) and bacterial sources (22). The purified enzyme has a native molecular weight of 250,000-280,000, depending on the source, and consists of multiple subunits of identical molecular weight. It has been shown that the purified enzymes obtained from bovine liver and human erythrocytes are homooctomeric with subunits of molecular weight 35,500 (18) and 31,000 (20), respectively, and contain eight Zn^{2+} molecules per octamer (18,20,23,24).

Figure 3. The reaction catalyzed by ALA-D from Rhodopseudomonas spheroides (15).



The enzymes from these two sources are maximally active in the presence of zinc and sulfhydryl compounds such as 2-mercaptoethanol, cysteine, reduced glutathione, or dithiothreitol (DTT). Removal of zinc by EDTA or other chelators, or oxidation or inhibition of sulfhydryl groups, results in rapid loss of activity (18, 25-27). Interestingly, under anaerobic conditions, removal of zinc does not cause loss of activity, implying that zinc may not be required for catalytic activity, but may act to protect essential sulfhydryl groups from oxidation (18). It has recently been shown that, in the bovine enzyme, four of the eight Zn^{2+} molecules are sufficient for full catalytic activity; the remaining four molecules may be involved in maintaining the stability of the enzyme (24). It has been proposed that lead, a potent inhibitor of ALA-D (28, 29), inactivates thiol groups by displacing zinc from the enzyme (18), and inhibition of the enzyme is a sensitive diagnostic indicator of lead poisoning. (30). In vivo and in vitro, the inhibition is reversible and the enzyme can be reactivated by the addition of 0.1 mM zinc or 10 mM DTT (23,25,26,28,31).

Other inhibitors of ALA-D include levulinic acid, an analogue of ALA, hemin, and succinylacetone. As a structrural analogue of ALA, succinylacetone is a potent, competitive inhibitor of ALA-D from bovine liver or human erythrocytes. The K_i of succinylacetone for the bovine

enzyme is 1/10,000 the K_m of the enzyme for ALA. As demonstrated by Tschudy et al. (32), it acts by binding to the enzyme at the site normally involved in the formation of a Schiff base with ALA. Interestingly, patients with hereditary tyrosinemia, who excrete large quantitities of succinylacetone, exhibit severe secondary ALA-D deficiency, and, therefore, excrete large quantities of ALA into their urine (33).

Several assays for the determination of ALA-D activity have been developed (20,34,35). Generally, the enzyme is incubated with ALA in the presence of sulfhydryl compounds at a pH of 6.3-6.8 and the enzymatically generated PBG is determined colorimetrically after its condensation with Ehrlich's reagent (p-dimethylaminobenzaldehyde, mercuric chloride, and perchloric acid) to the red colored compound p-N,N-dimethylpyrryl-toluidine. Recently, a more sensitive, fluorescent assay which allows accurate measurement of ALA-D activity in small amounts of tissue homogenates or in cultured cells with low specific activity has been developed (36). This coupled enzyme assay uses PBG-deaminase to enzymatically convert PBG (produced from ALA by ALA-D) to uroporphyrinogen I which nonenzymatically oxidizes to the fluorescent compound uroporphyrin I. The sensitivity of this assay is 10 times greater than that of conventional colorimetric methods.

Studies of the genetic regulation of ALA-D in mice indicate that the enzyme activity is under the control of at least two codominant alleles at a single genetic locus, the Lv or levulinate locus (17). Synthesis of ALA-D is more than three times greater in mice homozygous for the Lv^a allele than in those strains homozygous for the Lv^b allele. Physicokinetic studies revealed no differences between ALA-D from the two loci. However, it has been shown that, although the rate of ALA-D degradation in the two strains is identical, Lv^a mice have higher rates of ALA-D synthesis than do Lv^b mice (37). Gene regulation has also been implicated in the control of the rate of synthesis of the human enzyme. In the human population, ALA-D shows a 3- to 4-fold range of enzyme activity, with a much closer enzyme activity distribution among sibs than nonsibs and with very similar values for monozygotic twins (38).

Studies of liver and erythrocyte ALA-D have indicated that the enzymatic activity changes during liver development and erythropoietic maturation. ALA-D activity in mouse and erythroid cells increases sharply during erythroid differentiation, then falls with the maturation of reticulocytes into mature erythrocytes, with the activity in erythrocytes being approximately 15-fold less than in reticulocytes (39). Similarly, the specific activity of ALA-D in fetal rodent liver has been found to be 3-fold greater than in adult liver; the enzymatic activity was

lowest at, or soon after, birth and gradually rose to adult levels during the first month of life (37).

D. ALA-D Mutations

In 1977, Doss et al. discovered two unrelated German patients who had less than 3% of normal ALA-D activity (14). These individuals exhibited symptoms of acute hepatic porphyria, severe neurologic involvement, and urinary excretion of large quantities of ALA, PBG, and porphyrins. The possibility of lead poisoning was ruled out on the basis that activity could not be restored with added zinc or DTT; mixing experiments with normal blood indicated no evidence of an inhibitor. The parents and other family members were asymptomatic but exhibited only 50% of normal ALA-D activity implying an autosomal recessive mode of inheritance. The discovery of this new hepatic porphyria due to deficient ALA-D activity is intriguing in that it implicates ALA accumulation as a cause of neurologic manifestations of this disease and provides insight into the understanding of the neurologic manifestations of lead poisoning, which often resemble those of acute intermittent porphyria (40).

Subsequently, Bird et al. (41) reported an inherited deficiency of erythrocyte ALA-D activity occurring over three generations in an autosomal dominant pattern. The family consisted of eleven normal individuals and ten members with 22-41% of normal mean erythrocyte activity.

The deficiency, however, was not associated with symptoms of porphyria, and urinary levels of ALA and other porphyrin precursors were normal. In addition, physicokinetic studies of enzymes from normal and affected individuals revealed no differences. Added zinc was found to stimulate normal and deficient erythrocytes equally (20% mean increase). It appears that these individuals represent the heterozygotes for the defect reported earlier by Doss, although the correlation between the two forms of deficiency needs to be more firmly established. Since ALA-D is normally produced in great excess, only a marked deficiency of the enzyme would be associated with disease. This would explain the asymptomatic nature of the condition described by Bird.

Recently, immunologic studies have indicated normal levels of enzyme protein in the erythrocytes of ALA-D deficient heterozygotes (Desnick et al., unpublished data). These findings suggest that the gene defect in ALA-D deficient individuals is structural rather than regulatory and that the mutant gene product is stable and immunologically active. It would be intriguing to speculate that the mutation causing decreased ALA-D activity could result in an individual's increased sensitivity to environmental lead exposure. Indeed, there have been reports of heterozygotes who have developed acute lead poisoning upon exposure to low levels of lead (42,43).

TABLE 2

HUMAN ALA-D POLYMORPHISM:
PHENOTYPE AND GENE FREQUENCIES

Sample	N	Phenotype Frequency 1-1	2-1	2-2	Gene Frequency ALA-D-1	ALA-D-2
Italian Normals (44):	762	0.81	0.17	0.02	0.89	0.11
German Normals (45):	144	0.81	0.15	0.03	0.89	0.11
Japanese Normals (45):	121	0.90	0.08	0.02	0.94	0.06
Liberian Normals (45):	296	1.00	--	--	1.00	0.00
Mount Sinai (NYC)						
Total Normal Population:	954	0.71	0.22	0.01	0.88	0.12
Ashkenazi Jewish	872	0.76	0.22	0.02	0.87	0.13
Caucasian, Non-Jewish	62	0.82	0.18	--	0.91	0.09
Black	15	1.00	--	--	1.00	0.00
Asian	5	0.80	0.20	--	0.89	0.11
Hispanic	6	0.83	0.17	--	0.91	0.09

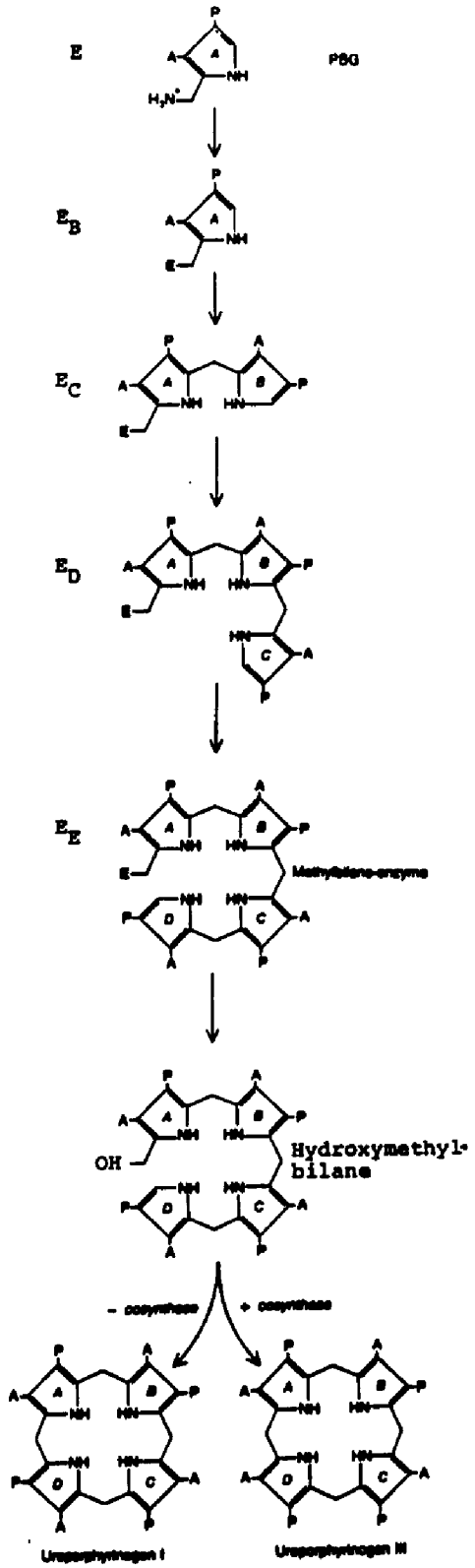
In 1982, Petrucci et al. (44) used starch gel electrophoresis to identify three isozymes encoded by two codominant alleles for ALA-D. These were designated as ALA-D 1-1, 1-2, and 2-2. In the Italian population studied, the phenotype frequencies were 81% for 1-1, 17% for 1-2, and 2% for 2-2, corresponding to gene frequencies of 0.90 for the 1 allele and 0.10 for the 2 allele. Table 2 summarizes the distribution of ALA-D phenotypes in some of the populations studied. Normal ALA-D activity is associated with all three isozymes and to date, the only distinguishing feature of the three enzyme forms is their differential electrophoretic mobility.

E. PBG-Deaminase

PBG-deaminase (EC 4.3.1.8), the third enzyme of the heme biosynthetic pathway, catalyzes the condensation of four molecules of the monopyrrole, PBG, to form ammonia and hydroxymethylbilane which nonenzymatically cyclizes to form uroporphyrinogen I, the first heterocyclic intermediate of the pathway. The reaction mechanism is summarized in Figure 4.

Hydroxymethylbilane is synthesized by a reaction mechanism involving the step-wise formation of stable enzyme-substrate intermediates (46-48). Five enzyme-substrate intermediates, A through E, have been identified by chromatographic and electrophoretic

Figure 4. Formation of uroporphyrinogen I and III from PBG. PBG-deaminase catalyzes the condensation of four PBG molecules in a head to tail fashion to yield a linear unrearranged bilane. This is released from the enzyme as hydroxymethylbilane which cyclizes nonenzymatically to form uroporphyrinogen I. In the presence of UROCos, hydroxymethylbilane is converted to uroporphyrinogen III (12). E represents the native enzyme, and E_B, E_C, E_D, and E_E the mono-, di-, tri-, and tetra-pyrrole enzyme intermediates, respectively.


E
PBG
E_B
E_C
E_D
E_E
Hydroxymethyl-
bilane
- cosynthase
+ cosynthase

techniques, the A form being the free enzyme and the B, C, D, and E forms representing the mono-, di-, tri-, and tetra-pyrrole substrate-enzyme intermediates, respectively (46,49). Kinetic studies have indicated that the C-intermediate, the dipyrrole-enzyme complex, is either the most stable intermediate or the rate-limiting step in the conversion of the monopyrrole to the tetrapyrrole (46,49).

Through the action of UROCos, the next enzyme in the pathway, hydroxymethylbilane is rapidly transformed into uroporphyrinogen III which, unlike the I isomer, can act as substrate for the following enzyme in the series. As shown in Figure 4, uroporphyrinogen III differs from uroporphyrinogen I by an inversion of the D ring (catalyzed by UROCos) with subsequent reversal of the acetyl and propionyl side chains.

Because of the extremely rapid enzymatic formation of uroporphyrinogen III [turnover number for UROCos = 350 mol product/min, (50)], much speculation exists as to the possible interaction of PBG-deaminase with UROCos. Frydman and Feinstein (51) have provided evidence for a strong association of the two enzymes which was independent of the presence of substrate. Studies by Higuchi and Bogorad (52) which indicate

that, in sucrose density gradients, PBG-deaminase from spinach leaf sedimented more rapidly in the presence of UROCos than in its absence also support this hypothesis.

To date, PBG-deaminase has been purified from a variety of sources including Rhodopseudomonas spheroides (53), bovine liver (54), human erythrocytes (46), and spinach (52). The purified enzymes have been shown to be monomeric with molecular weight estimates ranging from 36,000 to 40,000. Human erythrocyte PBG-deaminase has been purified to homogeneity and in its native, free enzyme, form has a molecular weight of 36,000 (46).

PBG-deaminase is maximally active in the presence of sulfhydryl compounds and is inhibited by oxidizing agents and heavy metals such as lead, mercury, and iron (46). The enzyme is characterized by its remarkable heat stability, exhibiting no loss of enzymatic activity after incubation at 56°C for 2h (46).

Two different principles have been utilized for the determination of PBG-deaminase activity. The classical method of Mauzerall and Granick (34) measures the disappearance of PBG substrate. The pyrrole reacts with Ehrlich's reagent (p-dimethylaminobenzaldehyde, mercuric chloride, and

perchloric acid) to form a pink colored product which is quantitated colorimetrically. A second approach measures the fluorescent compound uroporphyrin I which is formed stoichiometrically by the spontaneous oxidation of uroporphyrinogen I and can be quantitated spectrophotometrically (55) or fluorometrically (56). This assay has a sensitivity of up to 0.005 nmoles of uroporphyrin/ml in comparison to a sensitivity of 2.5 nmoles of uroporphyrin/ml for the colorimetric assay (56).

F. PBG-Deaminase Mutations

Acute intermittent porphyria (AIP) is a dominantly inherited inborn error of heme biosynthesis which results from the half-normal activity of PBG-deaminase (57-60). Clinical expression of the disease is highly variable, determined in part by environmental, metabolic, and hormonal factors which induce hepatic δ-aminolevulinic acid synthase activity and the subsequent increased production of heme precursors (11,12). Although the disease is clinically latent in many heterozygous individuals, those who are symptomatic usually have elevated levels of urinary PBG and its precursor, ALA, particularly during acute attacks. Most affected individuals can be diagnosed enzymatically (i.e., decreased

erythrocyte PBG-deaminase activity), however, kindreds have been described recently in which symptomatic patients have normal levels of the erythrocyte PBG-deaminase activity and elevated urinary PBG and ALA levels (61,62).

Recently, the first immunologic characterization of the enzymatic defect in unrelated heterozygotes with AIP was reported (49). Monospecific anti-human PBG-deaminase antibodies, which uniformly recognized each of the enzyme intermediates, were used to quantitate and characterize the amount of PBG-deaminase cross-reacting immunologic material (CRIM) in erythrocyte lysates from 32 heterozygous individuals of 22 unrelated AIP families. Two classes of mutations were identified. In 21 of the AIP families, the amount of enzyme protein was directly proportional to the amount of enzymatic activity (i.e., the CRIM/activity ratio was $\simeq$1.0); these mutations were classified CRIM-negative. In seven affected members from a family of Basque ancestry, the PBG-deaminase mutation was CRIM-positive; the CRIM/activity ratio of $\simeq$1.65 indicated the presence of immunoreactive, non-catalytic enzyme produced by the mutant allele. Although only 1 of the 22 AIP families had a CRIM-positive mutation, this finding provided

evidence for heterogeneity of the genetic defect in AIP.

G. Genetic and Molecular Studies

In the past several years, chromosomal localization of the genes coding for a number of heme biosynthetic enzymes has provided insight into the organization of the genes of a biosynthetic pathway in the human genome.

In 1981, Wang et al. (63), used somatic cell hybridization techniques to localize the structural gene for human PBG-deaminase to the distal portion of the long arm of chromosome 11 (11q23→11qter). These studies used human-mouse hybrids derived from the fusion of 2S murine erythroleukemia (MEL) thymidine kinase deficient (TK^-) cells and human Lesch-Nyhan (HLN) $HGPRT^-$ fibroblasts to map the PBG-deaminase structural gene to chromosome 11. Further regional localization was achieved by characterization of 2S MEL ($HGPRT^-$) x human fibroblast hybrids containing an X/11 translocation.

More recently, the human gene for ALA-D has been assigned to chromosome 9. In 1983, Eiberg et al. (64) used linkage analysis to show that the gene for ALA-D was syntenic with markers on chromosome 9. Somatic cell hybridization studies similar to those used for

mapping PBG-deaminase further localized the gene to 9q13→ter (65). Finally, Beaumont et al. (66) also used somatic cell hybridization to confirm the chromosomal assignment of the gene.

Ultimately, the investigation of the molecular nature of the genetic defects in the porphyric disorders will depend on the isolation of full-length cDNA and genomic sequences encoding the heme biosynthetic enzymes. To date, four enzymes of the heme biosynthetic pathway have been cloned. Romeo et al. (67,68) used immunoprecipitation to clone DNA sequences complementary to the mRNAs of uroporphyrinogen decarboxylase and PBG-deaminase from rat. The cDNAs were identified by hybrid selected translation. An ALA-S clone has also been isolated from a chicken erythroid cDNA library prepared in the bacteriophage expression vector λgt11 and screened for expressed fusion protein with rabbit anti-chicken liver ALA-S (69). The only human cDNA clone isolated and sequenced thus far is a full length sequence (1200 bp) for ALA-D (70,71). An 827 bp clone was isolated from a pEX human liver expression library and used to screen a human pKT218 human liver library. The full length cDNA contained an open reading frame as well as 5′ and 3′ untranslated regions.

II. OBJECTIVES

PBG-deaminase and ALA-D are two of the enzymes essential for the biosynthesis of heme, a tetrapyrrole crucial to such biological functions as oxygen transport and oxidative phosphorylation. The activity of ALA-D is known to be inhibited by lead and other heavy metals (11-13,32) and, as such, it provides a unique opportunity for the study of the interaction of environmental and genetic factors in the pathogenesis of disease. Mutations in both ALA-D and PBG-deaminase have been identified but they have not been extensively characterized biochemically or immunologically. Therefore, the objective of the proposed research was to study the biochemical properties of normal and mutant ALA-D and PBG-deaminase. Efforts were also directed toward the isolation of a cDNA for human ALA-D. These studies concentrated on five specific areas:

1. Identification of the CRIM-positive and CRIM-negative mutations of PBG-deaminase and the characterization of their physical, kinetic, and immunologic properties.
2. Purification to homogeneity of human ALA-D to be used for the production of monoclonal antibodies and the determination of N-terminal amino acid sequence.
3. Use of monoclonal antibodies for the rapid

affinity purification of the ALA-D isozymes and as immunologic probes for their characterization.

4. Characterization of the ALA-D isozymes including the determination of the effect of lead and zinc on the physical and kinetic properties of each isozyme and the investigation of the possible pharmacogenetic susceptibility to metal poisoning for individuals with the ALA-D 2 allele.

5. Application of recombinant DNA techniques to isolate full-length cDNA and genomic sequences encoding ALA-D for characterization of the structure and organization of the ALA-D structural gene.

III. MATERIALS

Item	Company	Location
Zetabind nylon membranes	AMF Cuno Micro-filtration Products Div.	Meriden, CT
Amicon Ultrafilters Centricon Ultrafilters	Amicon Corp.	Lexington, MA
Bio-Dot™ Micro-filtration Apparatus HRP Color Development Reagent 2-Mercaptoethanol	Bio-Rad Laboratories	Richmond, CA
DL-dithiothreitol Mouse Immunoglobulin Subtype Identification Kit Ethidium Bromide	Boehringer Mannheim Biochemicals	Indianapolis, IN
Pipes Pronase B grade (Lot 200191)	Calbiochem-Behring Corp.	San Diego, CA
Peroxidase conjugated goat anti-rabbit antibody Peroxidase conjugated rabbit anti-mouse antibody Peroxidase conjugated sheep anti-rabbit antibody	Cappel Laboratories	Cochranville, PA
Complete Freund's Adjuvant Incomplete Freund's Adjuvant	Difco Laboratories	Detroit, MI
Immulon flat bottom ELISA plates	Dynatech Laboratories	Alexandria, VA
Potassium thiocyanate	Fisher Scientific Co.	Fair Lawn, NJ

High resolution Tris-barbital buffer	Gelman Instrument Co.	Ann Arbor, MI
Restriction Enzymes (BamHI, HindIII, PstI, RsaI)	International Biotechnologies, Inc.	New Haven, CT
Balb/c mice	Jackson Laboratories	Bar Harbor, ME
Sea Kem agarose	Marine Colloids Division, FMC Corp	Rockland, ME
Zinc acetate	Matheson, Coleman and Bell	East Rutherford, NJ
M13 Universal Primer Restriction Enzymes (BamHI, HindIII, PstI, RsaI)	New England Biolabs	Beverly, MA
Agarose IEF CNBr-activated Sepharose 4B Gelbond film Pharmalyte ampholytes Sepharose 4B	Pharmacia Fine Chemicals	Piscataway, NJ
Fluorescamine	Pierce Chemical Co.	Rockford, IL
Polaroid Type 58 film	Polaroid Corp.	Cambridge, MA
Uroporphyrin standards	Porphyrin Products	Logan, UT
Nitrocellulose	Schleicher and Schuell	Keene, NH
Ammonium sulfate (enzyme grade) Bovine serum albumin Ethylene diamine tetraacetic acid, tetra sodium salt	Schwarz/Mann Research Laboratories	Orangeburg, NY
ALA (hydrochloride) Alkaline phosphatase conjugated goat anti-mouse antibody Bovine serum albumin Diaminobenzidine tetrahydrochloride	Sigma Chemical Co.	St. Louis, MO

DL-dithioerythrytol DL-dithiothreitol Human serum albumin p-nitrophenyl phosphate, disodium		
Stractan	St. Regis Paper Co.	Tacoma, WA
DEAE-cellulose Whatman No. 3MM filter paper Whatman No. 17 filter paper	Whatman Laboratory Products	Clifton, NJ
Bovine pancreatic trypsin	Worthington Biochemical Corp.	Freehold, NJ

All other chemicals were of the highest grade available. Outdated packed red blood cells were obtained from the Greater New York Blood Center. Fresh whole blood was obtained from individual donors.

IV. ACUTE INTERMITTENT PORPHYRIA: CHARACTERIZATION OF A NOVEL MUTATION IN THE STRUCTURAL GENE FOR PORPHOBILINOGEN DEAMINASE. DEMONSTRATION OF NON-CATALYTIC ENZYME INTERMEDIATES STABILIZED BY BOUND SUBSTRATE.

A. Methods

1. Human Subjects and Preparation of Erythrocyte Lysates

Subjects included heterozygotes with AIP from unrelated families representing a variety of different ethnic backgrounds or countries of origin. The clinical diagnosis of AIP in each subject was documented by demonstration of half-normal PBG-deaminase activity in erythrocyte lysates and/or increased urinary excretion of PBG and ALA.

Heparinized blood was collected from each AIP heterozygote and 35 age- and sex-matched normal individuals, with informed consent. Following centrifugation at 2500 g, the erythrocytes were removed, washed twice with 0.9% NaCl, and then used immediately or stored at -20°C. For specific experiments, human erythrocytes were separated into age-fractions on discontinuous Stractan gradients according to the method of Corash et al. (72). Erythrocytes were lysed by the addition of 3 vol of 1.0 mM sodium phosphate buffer, pH 8.1, containing 1 mM DTT, 1 mM $MgCl_2$, and 0.05% Triton X-100 (lysis buffer). The lysates were centrifuged at 30,000 g for 20 min and the supernatants were removed and

used for assays of protein and PBG-deaminase activity. Samples were diluted and then reassayed to insure that all lysates contained equal enzymatic activities prior to kinetic, heat denaturation, rocket immunoelectrophoretic, and immunotitration studies. For protease digestion experiments, the protein concentrations of the lysates were equalized by dilution with lysis buffer.

2. Assays

a. PBG-Deaminase Assay.

Enzymatic activity was determined by the quantitation of uroporphyrin fluorescence as previously described (56). One unit of enzymatic activity equalled that amount of enzyme which formed 1 nmol of uroporphyrin per h at 37°C.

b. Protein Assay.

Protein concentrations were determined by the fluorescamine procedure as previously described (20).

3. Kinetic, Heat Denaturation, and Protease Stability Studies

For kinetic studies, 50 ul of erythrocyte lysate was incubated in the standard assay with the final substrate concentration ranging from 0 to 50 uM. The K_m values were determined from Lineweaver-Burk plots.

To determine the effect of heat denaturation on erythrocyte PBG-deaminase activity, 500 ul aliquots of the lysates, which were equalized for activity, were placed in screw-capped vials and incubated at 65° or 70°C for 2 h, and then cooled to 0°C. Following centrifugation at 10,000 g for 10 min to remove precipitated proteins, 50 ul of the supernatant was removed and immediately assayed for PBG-deaminase activity.

To study the effect of trypsin and pronase on PBG-deaminase activity, erythrocyte lysates were equalized for protein concentration (to 80 mg/ml by dilution with lysis buffer). Aliquots (40 ul) of each lysate were placed at 0°C, then 10 ul of lysis buffer containing 125 ug/ml of pronase or 0.0625 ug/ml of trypsin was added to give a final protease concentration of 25.0 ug/ml or 0.0125 ug/ml, respectively. The reaction mixtures were preincubated for 0 to 60 min at 37°C, after which time 500 ul of 0.1 M Tris-HCl buffer, pH 8.1, containing 0.1 mM DTT, and 200 ul of 0.5 mM PBG were added immediately and the PBG-deaminase activity determined as described above.

4. Immunologic Studies

a. Characterization of Anti-PBG-Deaminase.

Anti-human PBG-deaminase was raised in New Zealand rabbits immunized with the homogeneous A

intermediate and the IgG fraction was purified as previously described (49). This antibody preparation was shown to be specific for purified human PBG-deaminase (or the human enzyme in erythrocyte lysates or murine erythroleukemia-human fibroblast somatic cell hybrids) by Ouchterlony double-immunodiffusion and competitive immunoprecipitation studies (49,63). Ouchterlony immunodiffusion gels showed a single arc of identity between the homogeneous enzyme and that in human erythrocyte lysates or liver homogenate supernatants when stained for protein and activity using PBG as substrate (Desnick et al., unpublished results).

b. Immunotitration of Anti-Human PBG-Deaminase.

Homogeneous PBG-deaminase intermediate A (40 ng of enzyme diluted with BSA to 50 ul; final protein concentration of 40 mg/ml) and 50 ul of rabbit anti-PBG-deaminase IgG (in serial dilutions) were mixed and incubated at 37°C. After 30 min, 50 ul of goat anti-rabbit IgG was added and the mixture was incubated at 37°C for 30 min. To insure quantitative precipitation of the anti-PBG-deaminase antibodies, the samples were left at 4°C overnight. The mixtures were then centrifuged at 4,000 g for 15 min, and the activity in the supernatant was determined by the standard assay described above. For immunotitration of the immunoreactive PBG-deaminase in erythrocyte lysates from normal and AIP heterozygotes, the lysate activities were equalized and then either 50 ul or 25

ul aliquots were titrated against the antibody as described above.

5. Immunologic Quantitation and Characterization of PBG-Deaminase in Erythrocyte Lysates

a. Rocket Immunoelectrophoresis.

Rocket immunoelectrophoresis of human PBG-deaminase was performed as previously described (49). An agarose solution (1%) was prepared in 0.06 M Tris-barbital buffer, pH 8.8, and bridges containing 4 ml of agarose solution were poured into a 5 x 7.5 cm plate in which a double thickness glass microscope slide had been placed in the center. After the agarose had gelled, the slide was removed and 4.0 ml of the agarose solution containing 15 ul of rabbit anti-human PBG-deaminase IgG (11 ug protein) was poured into the center area. Wells were cut at the cathodal end of the antibody-containing gel and 5-10 ul samples were applied. Electrophoresis was carried out in a tank (Chemtron Model 200, Milan, Italy) containing 0.06 M Tris-barbital buffer, pH 8.8, at a constant current of 30 mA for 4 h at room temperature. Following electrophoresis, the gel was thoroughly washed in 0.9% NaCl for 10-12 h with several changes and then the antibody containing part of the gel was overlayed with 300 ul of peroxidase-conjugated goat anti-rabbit IgG which had been diluted 1:2 with 0.9% NaCl. The plate was incubated at room temperature in a moist

chamber to prevent drying. Following another overnight wash with 0.9% NaCl, the gel was stained for peroxidase with 50 ml of 0.1 M Tris-HCl buffer, pH 7.6, containing 25 mg diaminobenzidine tetrahydrochloride and 0.15 ml of 3% hydrogen peroxide. After staining for 10 to 20 min at room temperature, the rockets were visualized using an indirect light source and the gel was photographed and stored in 0.9% NaCl at 4°C.

b. Isoelectric Focusing and Crossed-Immunoelectrophoresis.

Isoelectric focusing of the PBG-deaminase intermediates was performed on horizontal agarose slab gels using the Pharmacia system according to the manufacturer's instructions. Gels were prepared by heating a mixture of 0.3 g agarose IEF, 3.6 g sorbitol and 27 ml distilled water in a boiling water bath. After cooling to 75°C, 1.9 ml of pH 5-8 Pharmalyte ampholines were added. The mixture was then poured into a horizontal casting frame (11.4 x 22.5 cm; with Gelbond film backing) which had been preheated to about 60°C using a portable hairdryer. Gels were allowed to harden at least 1 h at 4°C or stored overnight at 4°C in a moist chamber. Prior to isoelectric focusing, erythrocyte lysates were heated at 60°C for 1 h, centrifuged at 10,000 g for 20 min, and then aliquots of the supernatants (50-100 ul) were pipetted onto Whatman No. 17 paper strips and placed 1 cm from the cathode. The cathode contained 1.0 M

NaOH and the anode 0.05 M H_2SO_4. Focusing was carried out for 2.5 h at 10 W (constant power) at 4°C. The filter paper strips were removed after the hemoglobin had migrated about 1 cm from the origin. After focusing, the pH gradient was determined by removing a 0.5 cm gel strip, cutting it into 1 cm pieces which were soaked in distilled H_20, and then the pH values of the leached ampholytes were measured. For visualization of activity bands, the gel was overlaid with Whatman No. 3MM filter paper saturated with 0.5 mM PBG, covered with polyvinylchloride film, and incubated at 37°C for 90 min in the dark. The overlay was removed and the gel was exposed to ultraviolet light for 10 min to oxidize the uroporphyrinogen to uroporphyrin, and the red fluorescent bands of PBG-deaminase activity were rapidly photographed under UV light with Polaroid Type 58 film using a Wratten No. 4 filter.

Crossed-immunoelectrophoresis of the separated PBG-deaminase intermediates was performed by placing an unstained lane from the focusing gel at the cathodal end of a rocket immunoelectrophoresis plate. The remainder of the plate was filled with 8 ml of 1% agarose containing 40 ul of anti-PBG-deaminase IgG and rocket immunoelectrophoresis was carried out as described above. Crossed-immunoelectrophoretic gels were stained for protein with Coomassie Brilliant Blue R-250 as previously described (49).

B. Results

1. Immunologic Identification of PBG-Deaminase Mutant Classes

The PBG-deaminase CRIM status was determined in 165 AIP heterozygotes from 92 unrelated families representing at least 20 different ethnic or demographic backgrounds (Table 3). For these studies, equal amounts of erythrocyte PBG-deaminase activity from each AIP heterozygote and normal individuals were subjected to rocket immunoelectrophoresis. AIP heterozygotes whose rocket peak heights were essentially identical to those of normal individuals were designated CRIM-negative (CRIM/activity ratio $\simeq$ 1.0). In contrast, AIP heterozygotes whose rocket peak heights were greater than those of normal individuals were designated CRIM-positive (CRIM/activity ratio $>$ 1.0), i.e., the increased CRIM represented non-catalytic, immunoreactive PBG-deaminase expressed by the mutant allele. As shown in Figure 5, four major PBG-deaminase mutant classes were readily identified: 1) CRIM-negative Type 1 heterozygotes with half-normal PBG-deaminase activity, 2) CRIM-negative Type 2 heterozygotes with normal erythrocyte activity, 3) CRIM-positive Type 1 heterozygotes whose peak heights were slightly greater than those of normal individuals, and 4) CRIM-positive Type 2 heterozygotes whose peak heights formed "railroad tracks", indicating the presence of markedly more non-catalytic PBG-deaminase than

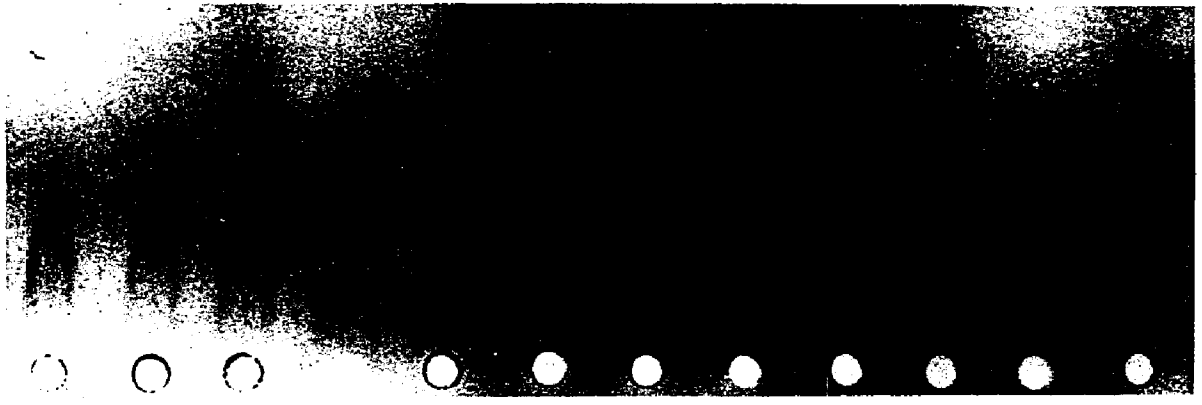
Table 3. Immunologic Characterization of the Defective PBG-Deaminase in AIP Heterozygotes from Unrelated Families

Family Ancestry*	Number of Families	Number of Patients	PBG-Deaminase Mutant Class			
			CRIM-Positive		CRIM-Negative	
			Type 1	Type 2	Type 1	Type 2
Basque	1	7	X			
Finnish	4	11	X			
Finnish	2	4		X		
Lapplander	1	9		X		
German	1	2		X		
Irish/Norwegian	1	3		X		
Scottish	1	2		X		
American Black	6	7			X	
Dutch	1	1			X	
English	2	2			X	
English/French	1	1			X	
English/German	2	3			X	
English/Polish	1	1			X	
Finnish	23	38			X	
Japanese	3	10			X	
Lapplander	1	4			X	
German	7	7			X	
German/Dutch	1	1			X	
Hungarian	1	1			X	
Irish	3	3			X	
Irish/English	1	1			X	
Irish/Scottish	1	2			X	
Italian	5	6			X	
Italian/French	1	2			X	
Norwegian	1	1			X	
Polish	3	4			X	
Spanish	8	14			X	
Swedish	5	8			X	
Swiss	1	1			X	
American Black**	1	2				X
Finnish**	1	5				X
Norwegian/Finnish**	1	2				X
TOTAL	92	165	5	6	78	3

* Both parents were from the same ethnic group or country unless otherwise indicated.

** AIP heterozygotes with normal erythrocyte activity.

Figure 5. Rocket immunoelectrophoresis of PBG-deaminase in erythrocyte lysates from unrelated AIP heterozygotes representing each of the four mutant classes. Equal enzymatic activities were applied to the gel, except in wells 9-11 in which the activity was diluted as indicated. Normal individuals, wells 1, 5, 7, 9 and 12; CRIM-negative Type 1 heterozygote (with half-normal erythrocyte activity), well 2; CRIM-negative Type 2 heterozygote (with normal erythrocyte activity), well 3; CRIM-positive Type 1 heterozygote, well 4; CRIM-positive Type 2 heterozygote, wells 6, 8, 10 and 11.


1 N
2 AIP NEG-1
3 AIP NEG-2
4 AIP POS-1
5 N
6 AIP POS-2
7 N
8 AIP POS-2
9 N (1/6)
10 AIP POS-2 (1/6)
11 AIP POS-2 (1/4)
12 N

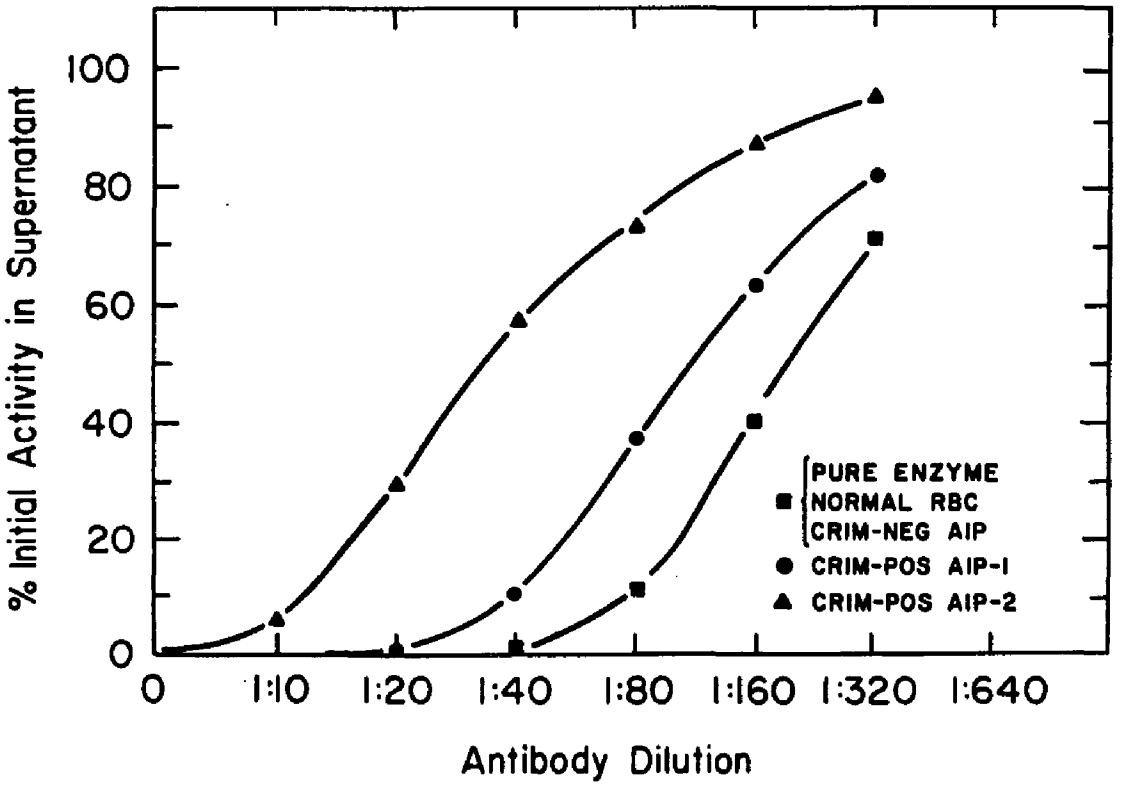
that observed in the CRIM-positive Type 1 heterozygotes. Review of the medical histories did not reveal notable differences in clinical disease expression in affected individuals among the four mutant classes.

2. Quantitation of PBG-Deaminase CRIM

The amount of immunoreactive PBG-deaminase protein in each AIP heterozygote was determined by immunotitration. When equal amounts of erythrocyte enzyme activity from CRIM-negative Type 1 and Type 2 heterozygotes and normal individuals were immunotitrated, the same antibody dilution (1:200) was required to precipitate 50% of the activity from each of these erythrocyte supernatants and their immunotitration profiles were essentially identical (Figure 6). The identity of their immunotitration curves confirmed the rocket immunoelectrophoretic studies which indicated the absence of non-catalytic enzyme protein in either type of CRIM-negative heterozygote. Since the amount of CRIM detected by either technique corresponded to the amount of erythrocyte activity used, the CRIM/activity ratios for the CRIM-negative Type 1 and Type 2 heterozygotes and normal individuals were 1.0.

In contrast, immunotitration of identical amounts of activity from the CRIM-positive Type 1 and Type 2 heterozygotes required antibody dilutions of 1:116 and 1:35 to precipitate 50% of the supernatant activities,

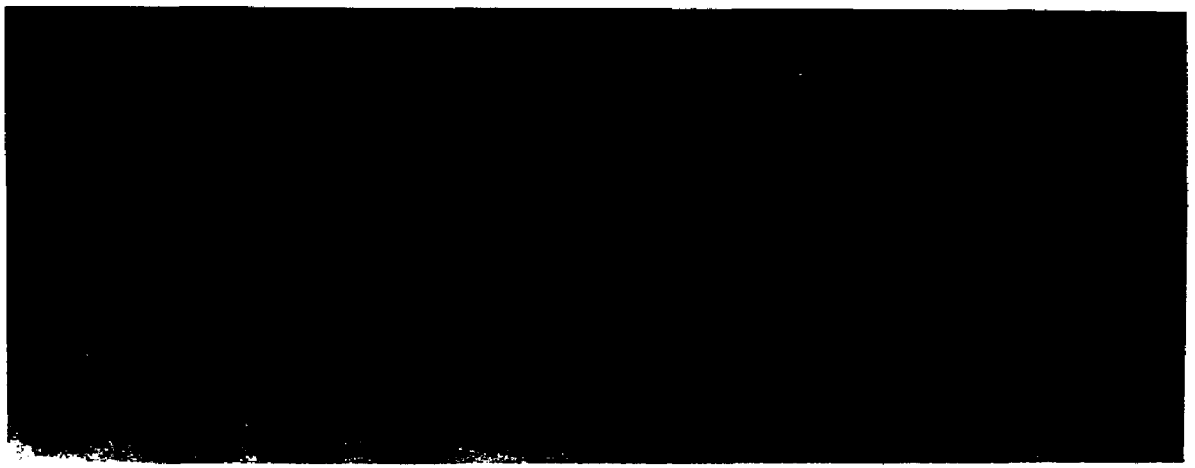
Figure 6. Immunotitration of PBG-deaminase with rabbit anti-human PBG-deaminase IgG. Immunotitration curves are shown for 25 ul aliquots (equalized for enzymatic activity and protein concentration with BSA) of homogeneous enzyme (■) and erythrocyte lysates of a normal individual (■), a CRIM-negative Type 1 heterozygote (■), a CRIM-positive Type 1 heterozygote (●), and a CRIM-positive Type 2 heterozygote (▲). Note that the curves for 25 ul of the homogeneous enzyme, normal lysate, and the CRIM-negative Type 1 lysate were essentially identical; the curve for 25 ul of the CRIM-negative Type 2 lysate also was identical (data not shown). Antibody dilutions of approximately 1:35, 1:116, and 1:200 were observed for 50% immunoprecipitation of enzyme from 25 ul of the CRIM-positive Type 2 lysate, the CRIM-positive Type 1 lysate, and the CRIM-negative Type 1 (or normal) lysates, respectively.


% Initial Activity in Supernatant
100
80
60
40
20
0
0
1:10
1:20
1:40
1:80
1:160
1:320
1:640
Antibody Dilution
PURE ENZYME
NORMAL RBC
CRIM-NEG AIP
CRIM-POS AIP-1
CRIM-POS AIP-2

respectively (Figure 6). The antibody dilutions required to precipitate 50% of the activity from 1:1 mixtures of normal and CRIM-positive Type 1 or Type 2 lysates were 1:146 and 1:60, respectively (data not shown). Based on these results, and assuming identical antibody avidities, the amounts of immunoreactive PBG-deaminase in these representative CRIM-positive Type 1 and Type 2 heterozygotes were about 1.7- and 5.7-fold that in normal lysates (i.e., CRIM/activity ratios of 1.7 and 5.7). Analogously, the CRIM/activity ratios determined by immunotitration for the 18 CRIM-positive Type 1 and the 20 CRIM-positive Type 2 heterozygotes ranged from 1.6 to 1.8 (mean ± 1 SD = 1.7 ± 0.07) and from 4.9 to 6.0 (mean ± 1 SD = 5.7 ± 0.3), respectively (data not shown).

The amount of immunoreactive PBG-deaminase in the CRIM-positive Type 2 heterozygotes also was determined by quantitative rocket immunoelectrophoresis. As shown in Figure 7, the rocket peak heights for 7 and 10 mU of PBG-deaminase activity in the CRIM-positive Type 2 erythrocytes were essentially equal to those obtained for 40 and 56 mU of normal lysate activity, respectively. Thus, the CRIM/activity ratio determined by rocket immuno-electrophoresis was about 5.7, consistent with the values for the CRIM-positive Type 2 heterozygotes obtained by immunotitration.

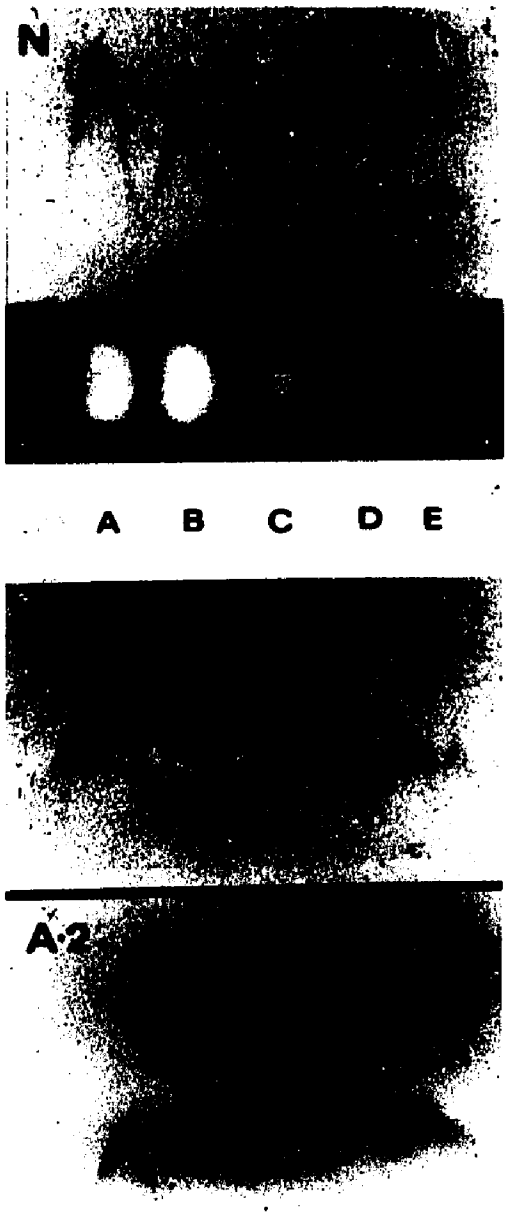
Figure 7. Estimation of the immunoreactive PBG-deaminase in a CRIM-positive Type 2 heterozygote. From left to right: wells 1-5 contained equal increments of normal erythrocyte PBG-deaminase activity (32 to 62 mU) and wells 6-9 contained equal increments of lysate activity (7 to 40 mU) from the CRIM-positive Type 2 heterozygote. Note that the peak heights for 40 and 56 mU of normal lysate activity were about equal to those obtained for 7 and 40 mU of the CRIM-positive Type 2 lysate. Thus, the ratio of immunoreactive PBG-deaminase to activity (CRIM/activity ratio) for the CRIM-positive Type 2 heterozygote was about 5.7. See text for details.


32
40
48
56
62
NORMAL (mU)
7
8
9
10
40
AIP CRIM-POS TYPE 2 (mU)

3. Characterization of the PBG-Deaminase Intermediates in the AIP Mutant Classes

Crossed-immunoelectrophoresis of erythrocyte PBG-deaminase provided further information concerning the nature of the enzymatic defect in the four AIP mutant classes. For these studies, PBG-deaminase in erythrocyte lysates was first subjected to isoelectric focusing in agarose gels to separate the enzyme-intermediates. Five major PBG-deaminase activity bands were observed in normal and CRIM-positive Type 1 and Type 2 lysates (Figure 8), as well as in CRIM-negative Type 1 and Type 2 lysates (data not shown). There were no significant differences in the pI values for each activity band in lysates from the different mutant classes. Following crossed-imunoelectrophoresis, a precipitin line of identity with peaks corresponding to each of the five intermediates was observed in lysates from normal individuals and AIP heterozygotes from each mutant class. The typical profiles obtained for erythrocyte PBG-deaminase from normal individuals and all CRIM-negative Type 1 and Type 2 heterozygotes are shown in Figure 8. In normal lysates, the A intermediate had the highest peak height, with B, C, D, and E intermediates having sequentially decreasing peak heights (upper gel). In contrast, lysates from CRIM-positive Type 1 heterozygotes (middle gel) had increased amounts of all five intermediates, the immunoreactive B intermediate being most

Figure 8. Isoelectric focusing and crossed-immuno-electrophoresis of PBG-deaminase in erythrocyte lysates from normal individuals (N; 40 mU) and CRIM-positive Type 1 (A-1; 40 mU) and Type 2 (A-2; 8mU) heterozygotes. Isoelectric focusing revealed five bands when stained for activity in normal, CRIM-positive Type 1 and Type 2, as well as CRIM-negative Type 1 and Type 2 lysates (not shown). No significant differences in the pI values for each band in the different lysates were observed. Following crossed-immunoelectrophoresis of the focused lysate proteins, only immunoreactive proteins corresponding to the activity bands were observed in the normal, CRIM-positive Type 1 and Type 2 (or CRIM-negative Type 1 or 2; not shown) heterozygotes. Note the distribution of immunoreactive enzyme intermediates in the CRIM-positive Type 1 and Type 2 heterozygotes compared to those in the normal erythrocyte lysate. See text for details.


N
A B C D E
A2

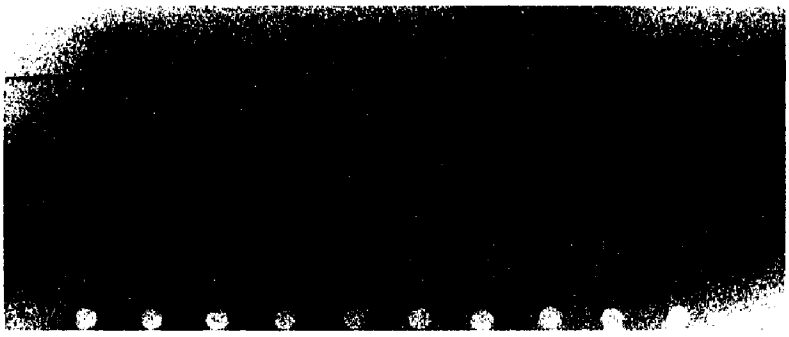
elevated (49). Lysates from the CRIM-positive Type 2 heterozygotes (lower gel) had a distinctly different profile: the A peak appeared decreased while the B, C, D, and E peaks were markedly increased. For comparison, the amount of Type 2 activity applied to this gel was one-fifth of that used for crossed-immunoelectrophoresis of the normal or CRIM-positive Type 1 enzyme.

To further characterize the amount of immunoreactive PBG-deaminase intermediates in CRIM-positive Type 2 heterozygotes, the erythrocyte enzyme intermediates from CRIM-positive Type 2 and normal individuals were isolated by DEAE-cellulose chromatography (49), and equal activities of the A, B, C, and D forms were subjected to rocket immuno-electrophoresis. As shown in Figure 9, each of the intermediates from the CRIM-positive Type 2 lysate had an increased amount of immunoreactive enzyme protein when compared with that obtained with the respective normal intermediate. Notably, the CRIM-positive B, C, and D intermediates were markedly increased whereas the A form, the free enzyme, was only slightly increased.

4. <u>Characterization of the Immunoreactive PBG-Deaminase in Age-Fractionated Erythrocytes from the CRIM-Positive Type 2 Heterozygotes</u>

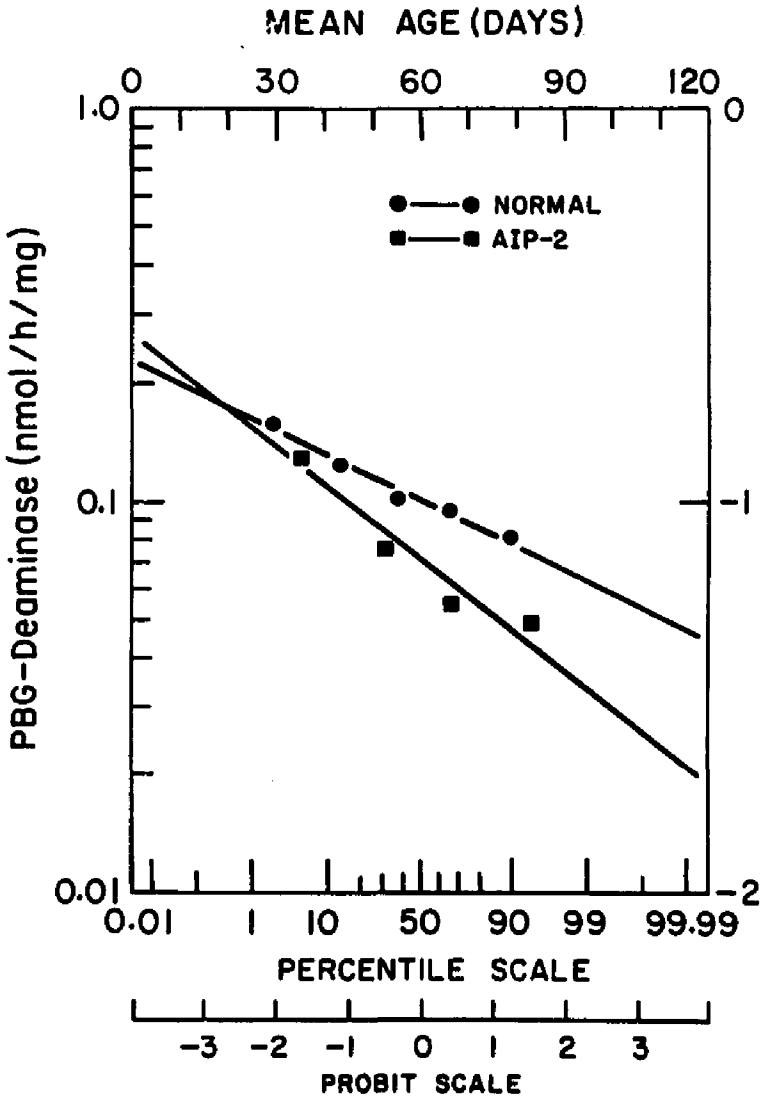
Erythrocytes from a CRIM-positive Type 2 heterozygote and a normal individual were fractionated into

Figure 9. Rocket immunoelectrophoresis of PBG-deaminase intermediates in erythrocytes from a CRIM-positive Type 2 heterozygote and a normal individual. Equal activities of each intermediate, isolated by DEAE-cellulose chromatography, were immunoelectrophoresed. From left to right: wells 1, 2, 4, 6, 8 and 10, normal erythrocyte PBG-deaminase intermediates A, A, B, C, D and A, respectively; wells 3, 5, 7 and 9, CRIM-positive Type 2 PBG-deaminase intermediates A, B, C and D, respectively. Note that each intermediate from the CRIM-positive Type 2 heterozygote had an increased amount of immunoreactive protein, the greatest amounts in the B, C, and D or substrate-bound intermediates.



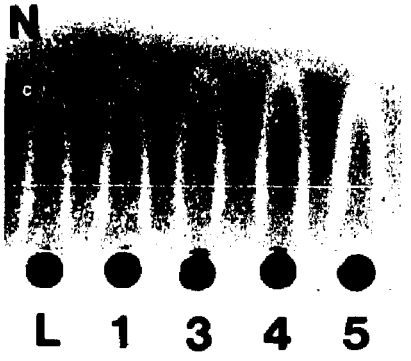
age-cohorts on discontinuous Stractan density gradients, according to the method of Corash et al. (72). As shown in Figure 10, four age-cohort fractions were obtained from a CRIM-positive heterozygote and five fractions were obtained from normal erythrocytes. These fractions ranged in mean age from 30 to about 90 days old. The lysates of the normal erythrocyte fractions (youngest to oldest) had PBG-deaminase activities of 0.159, 0.126, 0.102, 0.095, and 0.081 U/mg protein compared to 0.103 for the unfractionated lysate. The lysate activities of the fractionated CRIM-positive Type 2 heterozygote (youngest to oldest) were 0.136, 0.077, 0.054, and 0.050 U/mg protein, while the activity in the unfractionated lysate was 0.068 U/mg. As shown in Figure 11 (upper gel), rocket immunoelectrophoresis of equal amounts of activity from the unfractionated normal lysate and from the five age-fractionated lysates (youngest to oldest) revealed no difference in rocket peak height, indicating that the amount of activity corresponded to the amount of immunoreactive enzyme protein in each age-fraction. In contrast, rocket immunoelectrophoresis of 0.2 activity units of the unfractionated lysate (L), and the four age-fractionated lysates from a Type 2 heterozygote, demonstrated increasing amounts of cross-reactive enzyme protein with increasing erythrocyte age (Figure 11, lower gel).

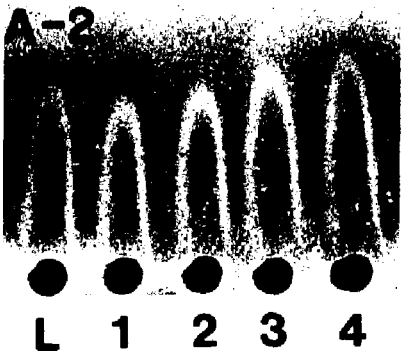
Figure 10. Age-fractionation of erythrocytes from a normal individual (●) and a CRIM-positive AIP Type 2 heterozygote (■). Erythrocytes were separated in discontinuous Stractan gradients by isopycnic centrifugation (16). Five fractions with mean ages of about 30, 43, 55, 66 and 80 days were separated from normal erythrocytes. Four fractions with mean ages of 35, 52, 66 and 84 days were separated from a CRIM-positive Type 2 heterozygote.



DISTANCE FROM TOP OF GRADIENT

Figure 11. Rocket immunoelectrophoresis of PBG-deaminase in age-fractionated erythrocyte lysates from a normal individual (N) and a CRIM-positive AIP Type 2 heterozygote (A-2). Equal amounts of enzymatic activity were applied in each well. No differences were observed in the rocket peak heights of immunoreactive enzyme in the normal lysate (L) or normal age-fractions (wells 1-5, youngest to oldest). In contrast, increasing amounts of immunoreactive enzyme were observed in the age-fractionated lysates (wells 1-4, youngest to oldest) from the CRIM-positive AIP Type 2 heterozygote.


N
L 1 3 4 5

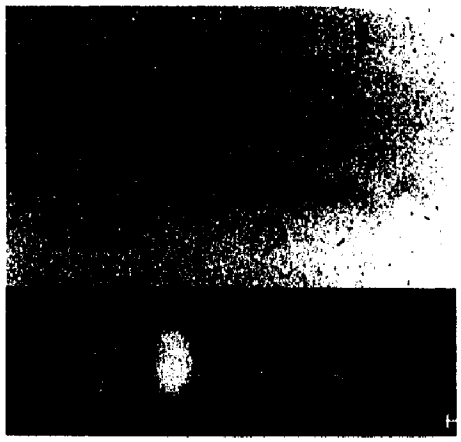

A-2
L 1 2 3 4

As shown in Figure 12, crossed-immunoelectrophoresis of a lysate from the youngest Type 2 age-fraction revealed a profile in which the B intermediate had the highest peak height (middle gel), whereas, crossed-immunoelectrophoresis of a lysate from the oldest Type 2 age-fraction (lower gel) revealed a profile in which the C intermediate was the highest peak. Presumably, the C or dipyrrole-enzyme intermediate represents the rate-limiting step in the step-wise conversion of the monopyrrole to the linear tetrapyrrole and/or the most stable enzyme-substrate complex (46,49).

5. Kinetic, Heat Denaturation, and Protease Stability Studies

The physicokinetic properties of the PBG-deaminase in erythrocyte lysates from normal individuals and each of the AIP mutant classes are summarized in Table 4. The K_m values calculated from Lineweaver-Burk plots were essentially the same ($\simeq$6 uM) from all sources. However, heat denaturation and protease digestion studies revealed differences among the AIP mutant classes. When the lysates were heat denatured at 65° and 70°C, the CRIM-positive Type 2 enzyme was most stable and was slightly activated at 65°C. The CRIM-positive Type 1 and CRIM-negative Type 1 activities had mean heat inactivation values which were similar to those of the normal enzyme. Interestingly, the

Figure 12. Crossed-immunoelectrophoresis of PBG-deaminase in normal erythrocytes (N) and in age-fractionated erythrocytes (A-2yng, youngest; A-2old, oldest) from a CRIM- positive AIP Type 2 heterozygote. Note the different profiles of the intermediates in the youngest (B>C>D>E>A) and oldest (C>B>D>E>A) age-fractions.



A B C D E

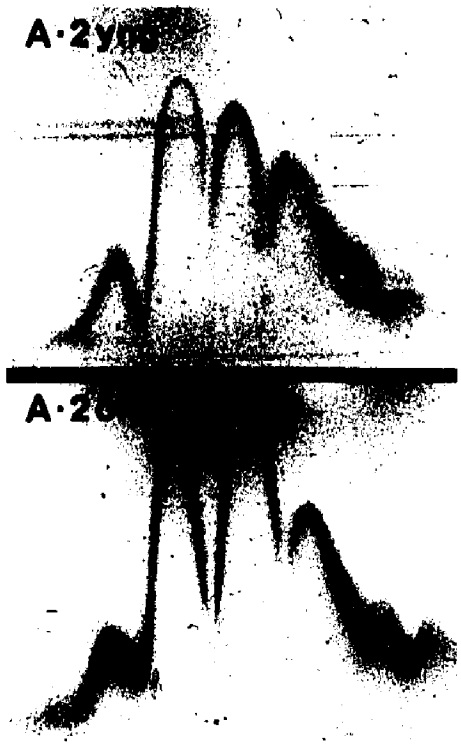


Table 4. Comparison of the PBG-Deaminase Mutant Classes

Property	Normal	Acute Intermittent Porphyria			
		CRIM-Negative		CRIM-Positive	
		Type 1	Type 2	Type 1	Type 2
Specific Activity (U/mg prot.):					
Mean	0.063	0.030	0.060	0.032	0.034
± 1 SD	0.008	0.004	0.008	0.003	0.004
(n)	(35)	(118)	(9)	(18)	(20)
CRIM/Activity Ratio:					
Mean	1.0	1.0	1.0	1.7	5.7
± 1 SD	0.06	0.05	0.06	0.09	0.23
(n)	(35)	(118)	(9)	(18)	(20)
Apparent Km (μM):	5.9	5.7	6.0	5.5	5.9
Heat Stability:* (% int. act.)					
65°C:					
Mean (n=6)	95	95	85	99	106
Range	(89-101)	(92-100)	(82-87)	(93-106)	(95-115)
70°C:					
Mean (n=6)	62	60	58	60	62
Range	(52-66)	(53-66)	(54-61)	(59-61)	(60-63)
Protease Stability: (% int. act.)					
Pronase:					
Mean (n=6)	39	35	39	41	56
Range	(31-48)	(26-51)	(23-50)	(31-56)	(42-67)
Trypsin:					
Mean (n=6)	72	67	71	69	74
Range	(63-79)	(60-74)	(62-79)	(51-81)	(63-85)
Isoelectric Focusing Profile:	N1**	N1	N1	N1	N1
Crossed-Immunoelectrophoresis Profile:	N1	N1	N1	B>A>>C>D>E	B>C>>D>E>>A

*Stability after 120 min at 65°C and 70°C expressed as percent of initial activity.

**N1 = A>B>>C>D>E profile on IEF and crossed-immunoelectrophoresis.

CRIM-negative Type 2 activity was less stable than the normal enzyme following heat treatment at 65°C. When equal amounts of erythrocyte protein from each of the mutant classes were subjected to pronase or trypsin digestion, the activities of the CRIM-positive Type 1, both CRIM-negative Type 1 and 2 heterozygotes, and normal individuals were equally digested by either protease. In contrast, the CRIM-positive Type 2 activity was more stable to pronase digestion and appeared slightly more stable to trypsin degradation. Rocket immunoelectrophoresis of equal activities of each mutant class did not reveal any differences in the peak height of the enzyme before or after pronase or trypsin digestion (data not shown), suggesting that the partially degraded enzyme proteins lost activity, but fully retained their antigenic properties.

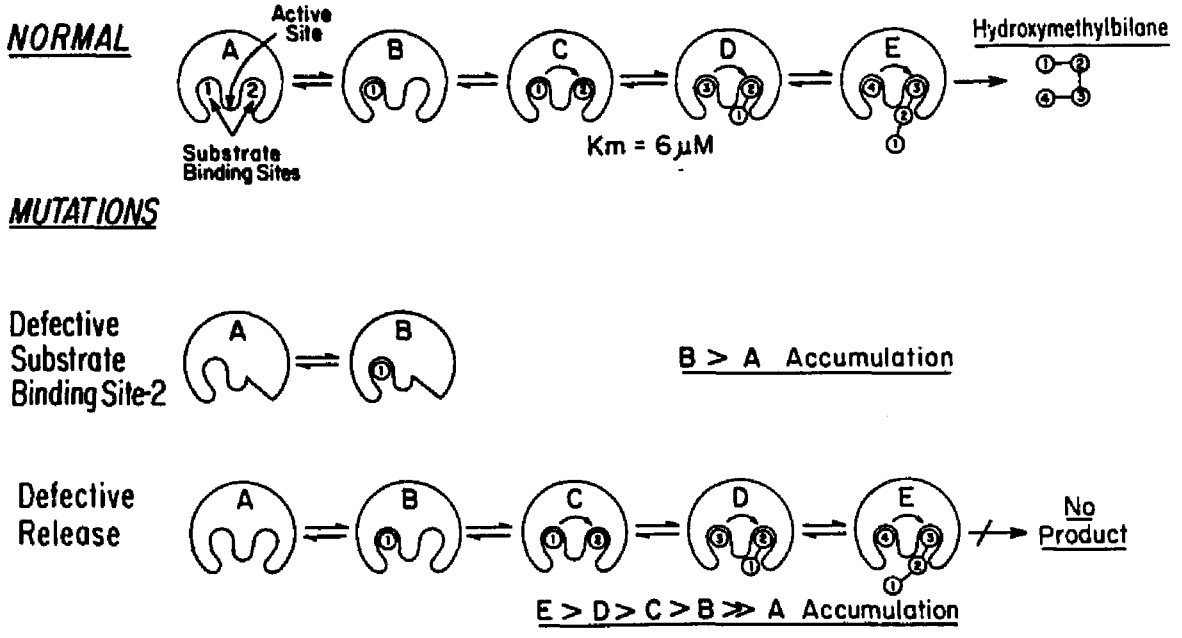
C. Discussion

Four major mutant classes were identified by immunologic and physicokinetic studies of PBG-deaminase in erythrocytes from unrelated AIP heterozygotes with this dominantly inherited, inborn error of metabolism. Two of these mutant classes had been defined previously by immunologic studies of heterozygotes from 22 unrelated AIP families (49). Using monospecific antibodies to quantitate immunoreactive enzyme protein, the heterozygotes from all but one of these families were CRIM-negative (CRIM/activity

ratio ≃ 1.0), i.e., immunoreactive, non-catalytic enzyme protein encoded by the mutant allele was not detectable in erythrocyte lysates. In only one of 22 AIP families, the mutation was CRIM-positive (CRIM/activity ratio ≃ 1.7), consistent with a structural gene mutation in the allele encoding PBG-deaminase. In this communication, two additional PBG-deaminase mutant classes, designated CRIM-negative Type 2 and CRIM/positive Type 2, were identified and characterized. Each of these mutations was novel and provided further insight into the nature of the defects underlying the PBG-deaminase deficiency in AIP.

Characterization of the CRIM-positive mutations was permitted by the recognition that PBG-deaminase catalyzed the formation of hydroxymethylbilane via five stable enzyme intermediates, designated A-E (46-48). Previously, a model was proposed (Figure 13) which predicted the different types of mutations which might be revealed by immunologic characterization of the PBG-deaminase intermediates in CRIM-positive heterozygotes (49). This model assumes that the normal enzyme has two binding sites for PBG and one active site (73), with the normal reaction mechanism involving the step-wise formation of the mono-, di-, tri-, and tetrapyrroles by the enzyme-substrate intermediates, A through E. Mutations which render substrate binding site-1 defective would not permit PBG binding, and would result in the accumulation of the free enzyme. A mutation in the

Figure 13. Possible alterations in the CRIM-positive Type 1 and Type 2 non-catalytic enzyme proteins resulting from different mutations in the PBG-deaminase structural gene. The normal enzyme is assumed to have one active site and two substrate binding sites; the stepwise formation of the mono-, di-, tri-, and tetrapyrrole-enzyme intermediates (B, C, D, and E, respectively) is shown. Mutations which alter substrate binding site-2 and product release as well as the expected formation and/or accumulation of the enzyme intermediates for each are depicted. See Discussion for details.


NORMAL
Active Site
A
B
C
D
E
Hydroxymethylbilane
Substrate Binding Sites
Km = 6 μM
MUTATIONS
Defective Substrate Binding Site-2
B > A Accumulation
Defective Release
No Product
E > D > C > B ≫ A Accumulation

second substrate binding site would lead to the accumulation of both the B and A intermediates, presumably with more B than A. If a mutation resulted in altered binding and/or catalysis, a K_m mutation would result and all of the enzyme-substrate forms would accumulate, perhaps the B or C form in greater amounts, depending on whether the substrate binding or active site was more defective. If a mutation resulted in increased binding and/or defective release of the product, only the substrate-bound forms would accumulate and, presumably, the A form would not be increased.

Immunologic characterization of the CRIM-positive Type 1 mutation revealed that each of the enzyme intermediates had elevated levels of cross-reactive material and the non-catalytic B enzyme-substrate intermediate was present in the greatest amount as previously described (49). These findings best fit the model for a K_m mutation with a markedly altered, but functional, active site and an altered substrate binding site-2 (Figure 13). In addition, the immunoreactive gene product produced by the mutant allele was somewhat unstable, since the CRIM/activity ratio was 1.7, whereas, a totally stable mutant protein would have had a CRIM/activity ratio of 2.0 in the heterozygous lysate.

In marked contrast, the CRIM-positive Type 2 mutants had a CRIM/activity ratio of about 5.7 (range from 4.9 to 6.0), an unexpected finding, and a crossed-immunoelectrophoretic profile in which the immunoreactive B, C, D, and E

enzyme-substrate intermediates were markedly elevated, while the A intermediate, the free enzyme, was only slightly increased. These findings best fit the model which predicted increased binding or defective release of the bound substrate molecules (Figure 13). It appears that the substrate-bound intermediates are particularly resistant to degradation in the erythrocyte, as evidenced by the increasing CRIM-activity ratio in the older age-fractionated erythrocytes (Figures 11 and 12). Thus, the Type 2 structural gene mutation represents a unique type of stability defect, one in which the non-functional mutant protein is more stable to in vivo proteolysis than the normal, catalytically active erythrocyte enzyme. Of the numerous human protein mutations characterized to date, only two hyperstable variants have been identified, hemoglobins Hacettepe and Agenogi (74-76). In each of these mutant hemoglobins, a β-chain amino acid substitution resulted in the formation of new ionic bonds in the molecule which rendered the mutant proteins more stable to heat denaturation than the normal protein. By analogy, an amino acid substitution in the mutant PBG-deaminase monomer could markedly alter substrate binding and/or product release rendering the mutant substrate-enzyme complexes more stable. Of particular interest was the observation that the presence of the immunoreactive, non-catalytic enzyme in these heterozygotes altered the heat and protease stabilities of

the activity expressed by the normal allele. The mechanism by which the accumulated mutant enzyme stabilized the normal erythrocyte activity remains unclear. Since PBG-deaminase is an extremely hydrophobic protein, one possible explanation might be that the normal and mutant proteins aggregate, thereby permitting the hyperstable mutant protein to increase the stability of the normal enzyme.

The CRIM-negative Type 2 mutation was differentiated from the CRIM-negative Type 1 mutations by the fact that the level of erythrocyte PBG-deaminase activity in these individuals was within the normal range. The normal erythrocyte PBG-deaminase levels in these individuals did not result from elevated reticulocyte counts, nor were they due to the biologic range in the expression of a single normal allele (e.g., high heterozygote level), since the activity ratios of erythrocyte PBG-deaminase to ALA-D and uroporphyrinogen III cosynthase were normal (Desnick, unpublished observations). Several hypotheses have been advanced to account for the normal erythrocyte PBG-deaminase activities in these symptomatic patients. It has been suggested that these AIP heterozygotes have half-normal hepatic PBG-deaminase activities and normal erythrocyte levels (61). This hypothesis requires the presence of two structural genes encoding hematopoietic (e.g., erythrocytic) and non-hematopoietic (e.g., hepatic) isozymes or a regulatory gene locus which controls either the tissue

specific expression of a single structural gene or the differential expression of hematopoietic and non-hematopoietic isozymes. Support for this concept will await determination of the hepatic activity in these patients. However, the fact that human PBG-deaminase has been assigned to a single, narrow, chromosomal location (63) and the finding that monospecific antibodies against the purified erythrocyte enzyme form a single line of identity with partially purified hepatic and erythrocytic PBG-deaminase activities, argue against this hypothesis (Ostasiewicz and Desnick, unpublished results). Nevertheless, it is possible that two or more structural genes are present as a gene family in the same chromosomal region, analogous to the β-globin gene complex (77), and that the anti-erythrocyte PBG-deaminase antibodies did not recognize unique determinants of a hepatic isozyme. Another explanation which also may depend on the occurrence of separate genes encoding hepatic and erythrocytic enzymes, is the suggestion that the defect in AIP results from a regulatory gene mutation (12). Such a mutation could result in normal hematopoietic, but half-normal hepatic gene expression.

Finally, one could account for the rare individuals with normal erythrocyte activity by the segregation of a variant allele with high normal activity (i.e., a hypermorph). It is well known that there is a large range

(about three-fold) in normal erythrocytic PBG-deaminase levels and that this variation is genetically determined (38,78). The occurrence of two codominant PBG-deaminase alleles was hypothesized recently to account for the distribution of the erythrocyte activities in over 200 normal individuals (79). For the two allele model, the mean activities for the trimodal distributions were about 130, 170, and 220 pmol/30 min/mg protein, respectively. If an individual inherited such a hypermorphic allele and an AIP mutant allele, the level of erythrocyte PBG-deaminase activity may be within the normal range (but the non-hematopoietic activities could be half-normal). Symptomatic individuals in such a family have been described (80); normal individuals with the hypermorphic allele had high normal activities of erythrocyte PBG-deaminase, whereas symptomatic heterozygous individuals, who had the hypermorphic allele and a mutant AIP allele, had erythrocyte activities in the normal range. However, characterization of the erythrocyte enzyme in six asymptomatic, apparently normal members of the large Finnish CRIM-negative Type 2 family did not reveal activity levels consistent with the presence of a hypermorphic allele. Therefore, it is likely that insight into the precise nature of the defect in these CRIM-negative Type 2 heterozygotes will be revealed by molecular studies of their PBG-deaminase genes.

In summary, immunologic and biochemical characteri-

zation of the enzymatic defect in a large series of unrelated AIP families identified four classes of PBG-deaminase mutations. At the molecular level, the two CRIM-positive mutations most likely result from single base substitutions in the exons of the structural gene. The CRIM-negative mutations may represent mutations which 1) markedly alter enzyme stability or antigenicity, 2) result in early chain termination, or 3) alter mRNA transcription or processing. The classification of these mutations should facilitate the initial selection of appropriate candidates for future molecular genetic analyses, analogous to the characterization of the genetic defects in the human thalassemias (77).

V. IMMUNOAFFINITY PURIFICATION AND CHARACTERIZATION OF δ-AMINOLEVULINIC ACID DEHYDRATASE ISOZYMES FROM HUMAN ERYTHROCYTES

A. Methods

1. Production and Purification of Monoclonal Antibodies to ALA-D

Human ALA-D was purified to homogeneity from erythrocytes by the method of Anderson and Desnick (20). Female Balb/c mice (6-16 week old) were given primary subcutaneous immunizations of 20 ug of purified ALA-D in complete Freund's adjuvant in the inguinal and axillary regions and in the hind foot pads. Subcutaneous booster injections of 10 ug in incomplete Freund's adjuvant were given in the inguinal and axillary regions 7 and 14 days later. After 5 weeks, a final subcutaneous boost of 10 ug into the inguinal, axillary, and tail regions, as well as the hind foot pads was given, and three days later the spleen was removed, macerated, and fused to the SP2/0 mouse myeloma cell line by the method of Kennett (81). Hybridomas were selected by growth in HAT medium (81). Anti ALA-D secreting hybridomas were titered by RIA (82) and ELISA (83), and subcloned into microtiter plates by limiting dilution at an average density of 1 cell/well as described by McKearn (84).

Antibody-containing ascites fluid was obtained by injecting $1x10^6$ to $1x10^7$ hybridoma cells into mice which had been primed intraperitoneally with 0.5 cc of pristane one week beforehand. IgG from ascites fluid was partially purified by 50% ammonium sulfate precipitation (85) followed by DEAE-cellulose chromatography using 0.04 M sodium phosphate buffer, pH 8.0 (86).

2. Isotype Analysis of Monoclonal Antibodies

Monoclonal antibodies were isotyped according to the manufacturer's suggestions using the Boehringer-Mannheim isotyping kit.

3. Antigenic Specificity of Monoclonal Antibodies

A Bio-Dot™ microfiltration apparatus was used according to manufacturer's suggestions to determine the monoclonal antibody concentrations which would saturate 0.06 ug of ALA-D purified through the ammonium sulfate fractionation stage or 3 ng of immunoaffinity purified enzyme bound to nitrocellulose. The antibodies were then used at saturating concentrations in the microfiltration assay singly or in combination. The nitrocellulose membranes were dried overnight and the intensity of each dot was quantitated using a Vis-UV-2 Chromatogram Analyzer (Farrand Optical Co. Inc., New York, NY) in the reflectance mode. A significant increase in the intensity of the dots

resulting from incubation of a mixture of antibodies compared to single antibodies incubated under the same conditions was used as an indication that the monoclonal antibodies recognized different epitopes on the enzyme molecule.

4. Immunotitration of Monoclonal Antibodies

A noncompetitive indirect ELISA was used to titer the anti-ALA-D monoclonal antibodies. Decreasing amounts of DEAE-cellulose purified antibodies were added to the wells of 96 well microtiter plates containing 0.6 ug of homogeneous ALA-D per well. The antigen-antibody complexes were incubated with alkaline phosphatase conjugated anti-mouse IgG and quantitated photometrically after reaction with p-nitrophenyl phosphate.

5. Monoclonal Affinity Purification of ALA-D

a. Coupling of Antibody to Sepharose.

Monoclonal antibody, which had been purified as described above, was coupled to CNBr-activated Sepharose 4B as suggested by the manufacturer for 2 h at room temperature in a ratio of 5 mg protein to 1 g dry Sepharose. The beads were blocked for 2 h at room temperature with 1 M ethanolamine and the antibody-coupled Sepharose was washed five times in alternating high and low pH buffers. Following the final wash, antibody-coupled Sepharose was

equilibrated in 7 mM potassium phosphate buffer, pH 6.8, containing 0.1 mM DTT and 0.02% sodium azide.

b. Preparation of Lysate for Affinity Chromatography.

ALA-D was partially purified from human erythrocyte lysates using DEAE-cellulose chromatography and ammonium sulfate fractionation as described previously (20).

c. Affinity Purification of ALA-D.

Affinity chromatography was carried out at 4°C. Ammonium sulfate precipitated ALA-D was applied at 0.2 ml/min to a 0.9 x 15 cm monoclonal antibody 6-1-7 affinity column which had previously been equilibrated with 7 mM potassium phosphate buffer, pH 6.8, containing 0.1 mM DTT and 0.02% sodium azide. After washing with phosphate buffer until the effluent was free of protein, ALA-D was eluted from the column by the addition of 3 M KSCN to the wash buffer. The eluted protein was dialyzed against 0.05 M potassium phosphate buffer, pH 6.8, containing 0.25 M KCl and 1 mM DTT, assayed for activity and protein, and concentrated in an Amicon ultrafiltration cell using a PM 10 filter followed by an Amicon Centricon 10 concentrator.

After chromatography, the immunoaffinity column was routinely regenerated with 0.1 N HCl followed by equilibration in starting buffer.

6. SDS Polyacrylamide Gel Electrophoresis

Electrophoresis was performed according to the method of Laemmli (87). Denatured protein (1-3 ug) was applied to a 5 mm 6% stacking gel, pH 6.8, atop a 100 x 60 x 0.50 mm 10% separating gel, pH 8.8. Electrophoresis was carried out at 200 V, constant voltage, at 4°C, until the bromophenol blue dye front was within 2 mm of the bottom edge. The gel was stained for 15 min in 0.25% Coomassie Brilliant Blue G-250 dissolved in 45% methanol and 9% glacial acetic acid and destained in a solution of 7.5% glacial acetic acid and 5% methanol.

7. Assays

a. ALA-D Assays.

Enzymatic activity during protein purification was monitored by the standard assay as previously described (20). For characterization studies and assays of purified enzyme, the semimicro method of Sassa (35) was employed. This assay was modified for studies involving metals in which 0.05 M sodium Pipes, pH 6.5, was substituted for 0.05 M sodium phosphate buffer, pH 6.5. Enzyme activity for both assays is expressed as umol of PBG formed per hour at 37°C.

b. Protein Assay.

Protein concentrations were routinely determined by an adaptation of the fluorescamine procedure by Bishop et al (88).

8. Starch Gel Electrophoresis

Electrophoresis was performed according to the method of Battistuzzi et al. (89) with the single modification that the substrate buffer was 0.1 M potassium phosphate, pH 6.8, containing 0.05 mM zinc.

9. Isoelectric Focusing

Agarose gel isoelectric focusing was performed according to the method suggested by the manufacturer (Pharmacia Fine Chemicals). The gradient contained pH 4.0-6.5 Pharmalytes™ in a 1% Agarose IEF gel. The cathode consisted of 1 M NaOH and the anode of 0.05 M H_2SO_4. Immunoaffinity-purified ALA-D isozymes were dialyzed against 7 mM potassium phosphate buffer, pH 6.8, containing 0.1 mM DTT and 0.02% sodium azide, spotted onto Whatman number 17 filter paper strips and placed 1 cm from the cathode. Focusing was carried out at 10 W (constant power) at 4°C for 1.5 h after which time the voltage was adjusted so as not to exceed 1000 V and focusing was continued an additional 1.5 h. Each lane was cut into 0.5 cm sections and the sections crushed in 1.0 x 10 cm test tubes containing 150 ul of 0.25

M potassium phosphate buffer, pH 6.8. After an overnight soak at 4°C, the leached enzymes were assayed by the addition of 50 ul of 20 mM DTT, 50 ul of 0.5 mM zinc acetate, and 250 ul of 5 mM ALA as described previously. The pH gradient was determined by soaking gel strips in 1 ml of water overnight at 4°C and then measuring the pH.

10. Native Polyacrylamide Gel Electrophoresis

Native polyacrylamide gels were run by a modification of the method of Cawley (90) using a 7% polyacrylamide separating gel and a 2.5% polyacrylamide stacking gel. Gels (76 mm x 60 mm x 0.75 mm) were run at 100 V constant voltage at 4°C for 3 h. The running buffer was 50 mM Tris 4 mM glycine, pH 8.3. Gels were stained for protein by the method of Blakesley and Boezi (91).

11. DEAE-Cellulose Profiles of ALA-D Isozymes

Whole blood was obtained from identified 1-1, 1-2, and 2-2 individuals, and washed and lysed as previously described. Following dialysis in 7 mM potassium Pipes, pH 6.8, containing 0.05 mM zinc acetate, a Gilson Minipuls 2 multi channel peristaltic pump (Gilson Medical Electronics, Inc., Middleton, WI) was used to apply 10-15 ml of lysate at 0.4 ml/min simultaneously to each of three 0.9 x 15 cm columns of DEAE-cellulose previously equilibrated in the above buffer. The columns were washed in the same buffer

and then a single gradient of 0.1-0.5 M KCl in 7 mM potassium Pipes, pH 6.8, was used to elute all three enzyme forms. The five chamber gradient was constructed with equal volumes (50 ml) of 0.1, 0.2, 0.3, 0.4, and M 0.5 KCl. Fractions (0.8 ml) were collected and immediately assayed for activity as described previously.

B. Results

1. Production and Characterization of Anti-Human ALA-D Monoclonal Antibodies

Three anti-human ALA-D antibody-producing clones were obtained from two fusions. The first fusion resulted in 7 hybridomas, of which 1 was positive for antibody as determined by RIA and ELISA; the second fusion yielded 2 antibody-producing clones out of 102 hybridomas. Two subclonings of each of the positive hybridomas into 96 well microtiter plates by limiting dilution at a cell density of 1 cell/well resulted in greater than 70% of the subcloned hybridomas producing antibodies to ALA-D. This was taken as evidence of the monoclonal nature of the three antibody producing cell lines and the three clones were designated as 6-1-7, 2-51-1, and 2-102-5.

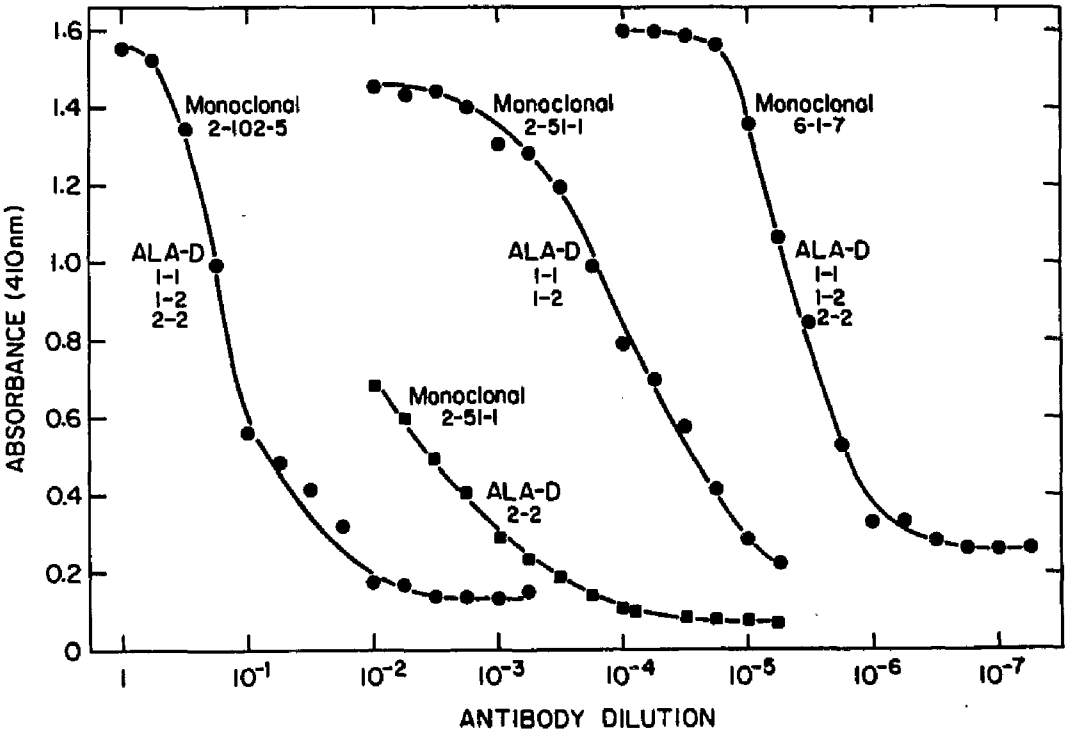
Isotype analysis of crude ascitic fluids demonstrated clearly that each of the three monoclonal antibodies was of the IgG½2B isotype. None of the antibodies were found to inhibit the enzyme activity when ALA-D was assayed in the

presence of monoclonal antibody, however, immunotitration assays revealed significant differences in the affinities of the three antibodies for the enzyme. Whereas the 6-1-7 and 2-51-1 monoclonal antibodies had titres of 5×10^{-5} and 1×10^{-4}, respectively, the titre of monoclonal antibody 2-102-5 was significantly lower at 1×10^{-1} (Figure 14). Immunotitration analysis also showed that although monoclonal antibodies 6-1-7 and 2-102-5 recognized all three ALA-D isotypes equally, antibody 2-51-1 exhibited markedly greater affinity for the 1-1 and the 1-2 isozymes than for the 2-2 isozyme.

Dot blot analysis was unable to show that the antibodies competed for different epitopes on the enzyme molecule. When antigen was incubated with mixtures of antibodies, each at saturating concentrations, the intensity of the dots as quantitated using a reflectance densitometer was only marginally increased compared to the intensity of the dots obtained when the antibodies were used singly.

ELISA indicated that all of the monoclonal antibodies were able to recognize ALA-D which had been denatured by heating at 95°C for 15 min in the presence of 0.2% SDS. The antibodies also recognized human liver ALA-D making them useful for isolation of enzyme from this source (unpublished data).

Figure 14. Titration of ALA-D isozymes 1-1, 1-2, and 2-2 with monoclonal anti-ALA-D antibodies by indirect ELISA. Decreasing amounts of DEAE-cellulose purified monoclonal antibodies 2-102-5, 2-51-1, or 6-1-7 were added to 0.6 ug of homogeneous ALA-D 1-1, 1-2, or 2-2. The antigen-antibody complexes were then incubated with alkaline phosphatase conjugated anti-mouse IgG and quantitated photometrically at 410 nm after reaction with p-nitrophenyl phosphate. Antibodies 2-102-5 and 6-1-7 reacted similarly with all three isozymes, however, this was not the case for monoclonal 2-51-1 which exhibited markedly greater affinity for the 1-1 and 1-2 than for the 2-2 isozyme. Absorbance in negative control wells containing 0.6 ug BSA and monoclonal antibody was less than 0.01. Similarly, anti-mouse IgG did not cross-react with any of the isozymes.


ABSORBANCE (410nm)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1
10-1
10-2
10-3
10-4
10-5
10-6
10-7
ANTIBODY DILUTION
Monoclonal 2-102-5
ALA-D 1-1 1-2 2-2
Monoclonal 2-51-1
ALA-D 1-1 1-2
Monoclonal 6-1-7
ALA-D 1-1 1-2 2-2
Monoclonal 2-51-1
ALA-D 2-2

2. Monoclonal Affinity Purification of ALA-D

Each of the three monoclonal antibodies was purified from ascites fluid by ammonium sulfate precipitation and DEAE-cellulose chromatography and coupled to CNBr-activated Sepharose 4B as described in Methods. Partially purified ALA-D (ammonium sulfate fractionated) was bound to each of the immunoaffinity columns and several elution conditions were evaluated. These included the use of KSCN at concentrations of 1 to 4 M; 3 M KCl; 0.1 M Tris-HCl, pH 9.0; glycine HCl, pH 3.5; 4 M urea; 0.1 M ALA; glycine-10% dioxane, and 5% ethylamine. Only KSCN was able to elute the enzyme from the columns and KSCN at a concentration of 3 M in 7 mM potassium phosphate buffer, pH 6.8, provided the optimal condition for elution of ALA-D with maximum recovery of activity in all three cases. The presence of KSCN in the eluant did not interfere with the fluorescamine protein determinations, however, fractions had to be dialyzed against potassium phosphate buffer, pH 6.8, in order to monitor activity accurately. SDS-PAGE indicated that enzyme of comparable purity could be obtained using any of the three antibody columns, however, since antibody 6-1-7 showed the greatest binding capacity for the enzyme, this antibody was chosen for routine immunoaffinity purification of ALA-D.

Under the described conditions, the capacity of the 6-1-7 affinity column was 100 ug of ALA-D bound per ml of

affinity resin. Approximately 60% of the activity applied to the immunoaffinity column could be recovered after dialysis of the KSCN-treated sample into 7 mM potassium phosphate buffer, pH 6.8. The elution profile is shown in Figure 15 and a typical purification scheme is presented in Table 5. The enzyme was purified approximately 19,500-fold with a 26% overall yield. Enzyme obtained by this method was greater than 95% pure as estimated by SDS-PAGE (Figure 16). Reapplication of purified enzyme to the same or a different affinity column did not improve purity significantly. The same monoclonal affinity column has been reused on numerous occasions with little loss of binding capacity. To assure consistent column starting conditions, it is washed extensively with 0.1 M HCl followed by 7 mM potassium phosphate buffer, pH 6.8, before each purification.

3. Characterization of ALA-D Isozymes

a. Starch Gel Eletrophoresis.

Figure 17 shows the migration of the three ALA-D isozymes from lysates on starch gel electrophoresis after staining for activity. In agreement with previously reported data (44), the 2-2 isozyme was found to be the most electronegative, the 1-1 the most electropositive, and the 1-2 isozyme intermediate in charge.

Figure 15. Affinity chromatography of human ALA-D on anti-ALA-D monoclonal IgG-Sepharose. ALA-D which had been purified through DEAE-cellulose chromatography and ammonium sulfate precipitation was applied to a column (0.9 x 15 cm) of monoclonal antibody coupled to CNBr-activated Sepharose 4B. The column was washed with 7 mm potassium phosphate buffer, pH 6.8 and the enzyme eluted with 3 M KSCN as described in Methods. ●-● , ALA-D activity; ■-■ , protein concentration.

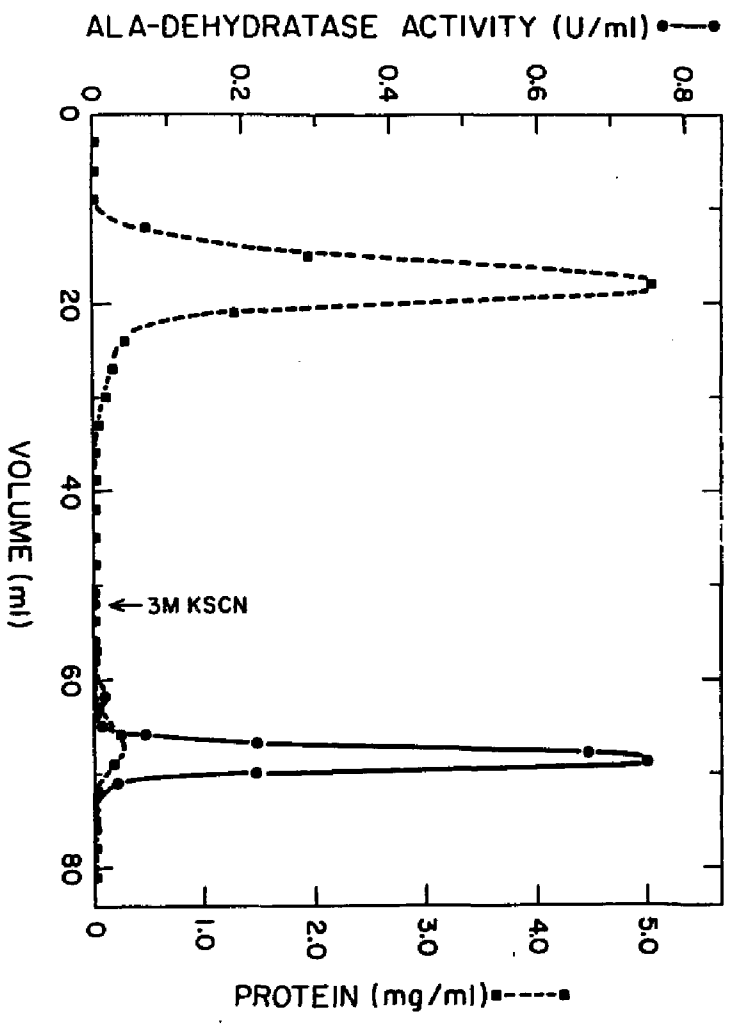
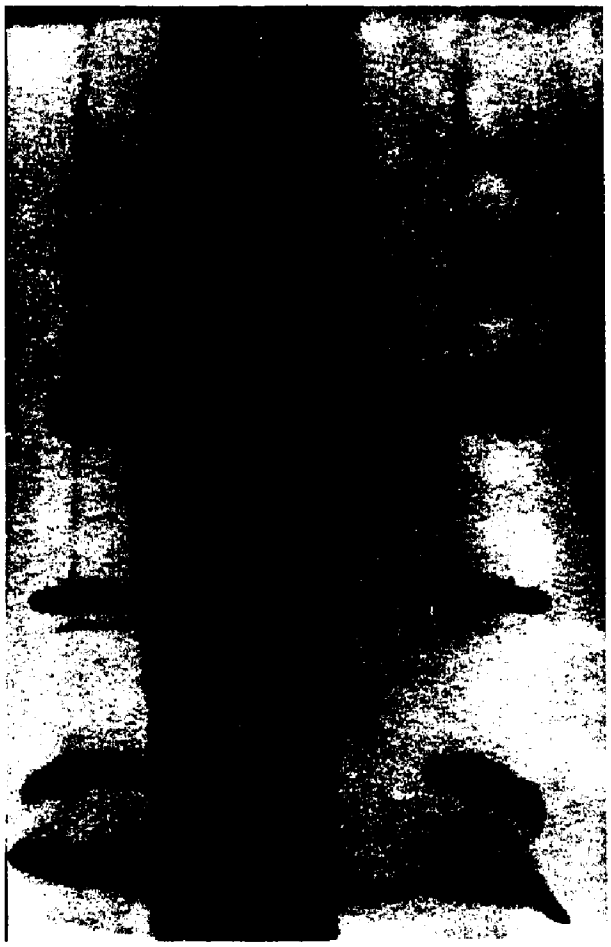

ALA-DEHYDRATASE ACTIVITY (U/ml)
0
0.2
0.4
0.6
0.8
VOLUME (ml)
0
20
40
60
80
3M KSCN
0
1.0
2.0
3.0
4.0
5.0
PROTEIN (mg/ml)

Table 5. Purification of ALA-D from human erythrocytes

Step	Specific Activity	Yield	Purification
	units/mg.	%	-fold
Erythrocyte lysate	0.00068	100	---
DEAE-cellulose	0.75	65	1103
45% $(NH_4)_2SO_4$ ppt.	0.87	65	1279

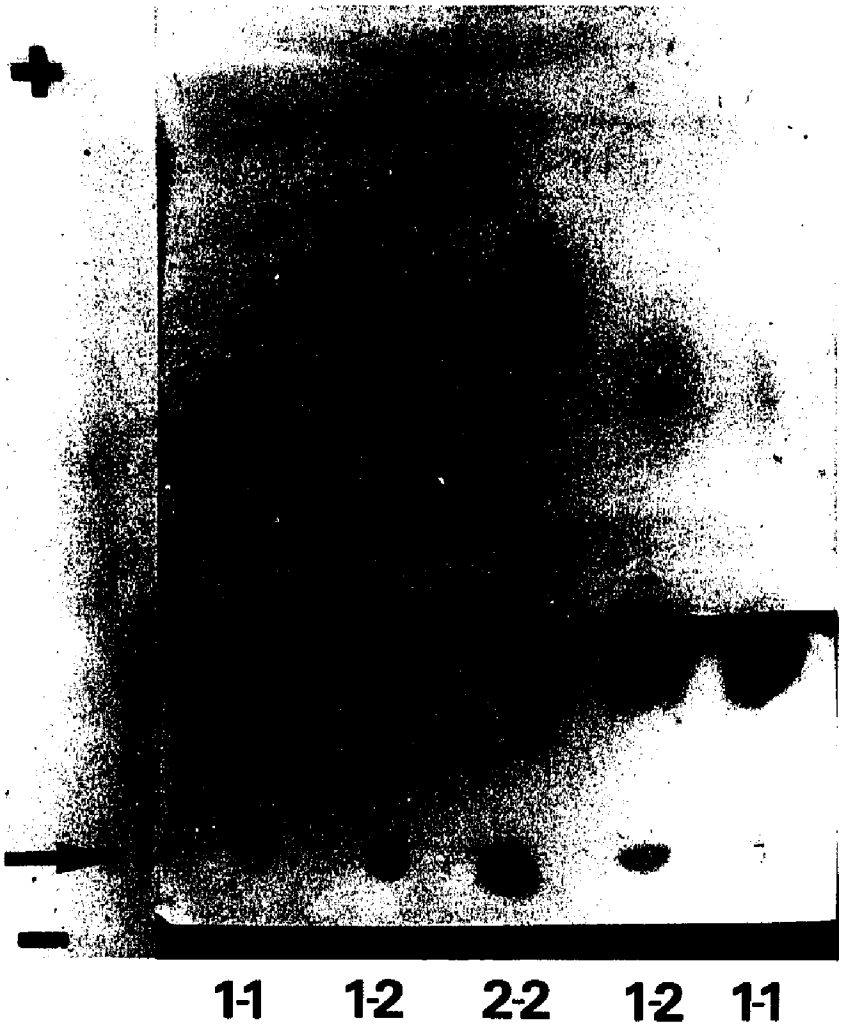
Conventional Purification				Immunoaffinity Purification			
	units/mg	%	-fold		units/mg	%	-fold
Octyl-Sepharose	2.7	46	3,970	Anti-ALAD	13.3	26	19,559
Phenyl-Sepharose	9.7	28	14,118				
Sephadex G-200	12.5	26	18,382				

Figure 16. SDS-polyacrylamide gel electrophoresis of immunoaffinity purified ALA-D from human erythrocytes. Lanes: 2, 10 ug of DEAE-cellulose purified enzyme; 3, 30 ug of ammonium sulfate precipitated enzyme; 4, 1.5 ug of affinity purified ALA-D; 1 and 5, protein standards (phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, δ -lactalbumin, 11 ug total).



1 2 3 4 5

Figure 17. Starch gel electrophoresis of ALA-D isozymes.


1-1
1-2
2-2
1-2
1-1

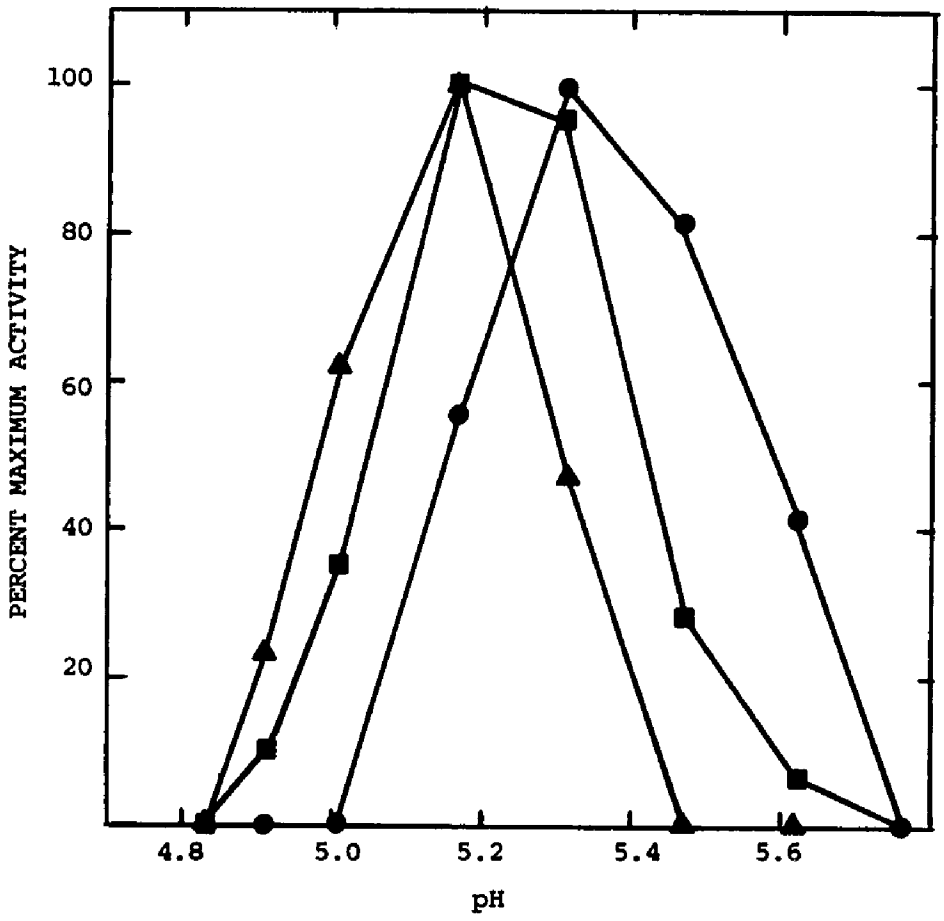
b. Isoelectric Focusing.

When the three immunoaffinity-purified isozymes were subjected to isoelectric focusing in agarose gels, different isoelectric points were observed (Figure 18). Enzyme activity for the 1-1 protein was detected in the pH range 5.1-5.6 with maximum activity at pH 5.3 while the more electronegative 2-2 protein exhibited activity in the lower pH range of 4.9-5.3 with maximum activity at pH 5.2. Activity for the 1-2 isozyme was detected in the broader pH range of 4.9-5.6, overlapping the isoelectric points of the other two isozymes.

c. Native Polyacrylamide Gel Electrophoresis.

In accordance with starch gel electrophoresis and isoelectric focusing data, when immunoaffinity purified ALA-D isozymes were subjected to native polyacrylamide gel electrophoresis, the 2-2 isozyme migrated the furthest (most anodal), the 1-1 isozyme the least (most cathodal) and the 1-2 isozyme to an intermediate position (data not shown). Electrophoresis time was increased to 3 h due to the small gel size (76 mm x 60 mm x 0.75 mm). The lengthened run time resulted in slightly decreased resolution of the bands, but was necessary in order to fully separate the three enzyme forms.

Figure 18. Isoelectric focusing profiles of ALA-D isozymes. Agarose isoelectric focusing was performed as described in Methods. The lanes were cut into 0.5 x 1.0 cm pieces which were crushed and soaked in phosphate buffer and then assayed for activity. ●–● , 1-1; ■–■, 1-2; ▲–▲ , 2-2.


PERCENT MAXIMUM ACTIVITY
100
80
60
40
20
4.8
5.0
5.2
5.4
5.6
pH

d. DEAE-Cellulose Chromatography.

Charge differences in the enzyme forms were also detectable using DEAE-cellulose chromatography. As would be predicted from starch gel electrophoresis data, when lysates or partially purified (ammonium sulfate fractionated) enzyme preparations were chromatographed on DEAE-cellulose, the more negatively charged 2-2 isozyme bound most avidly to DEAE-cellulose. At saturating, but not inhibitory, zinc concentrations, a salt concentration of 0.230 M KCl was needed to elute the 2-2 protein while 1-2 and 1-1 enzyme forms were eluted from the column at concentrations of 0.205 M KCl and 0.180 M KCl, respectively (Figure 19).

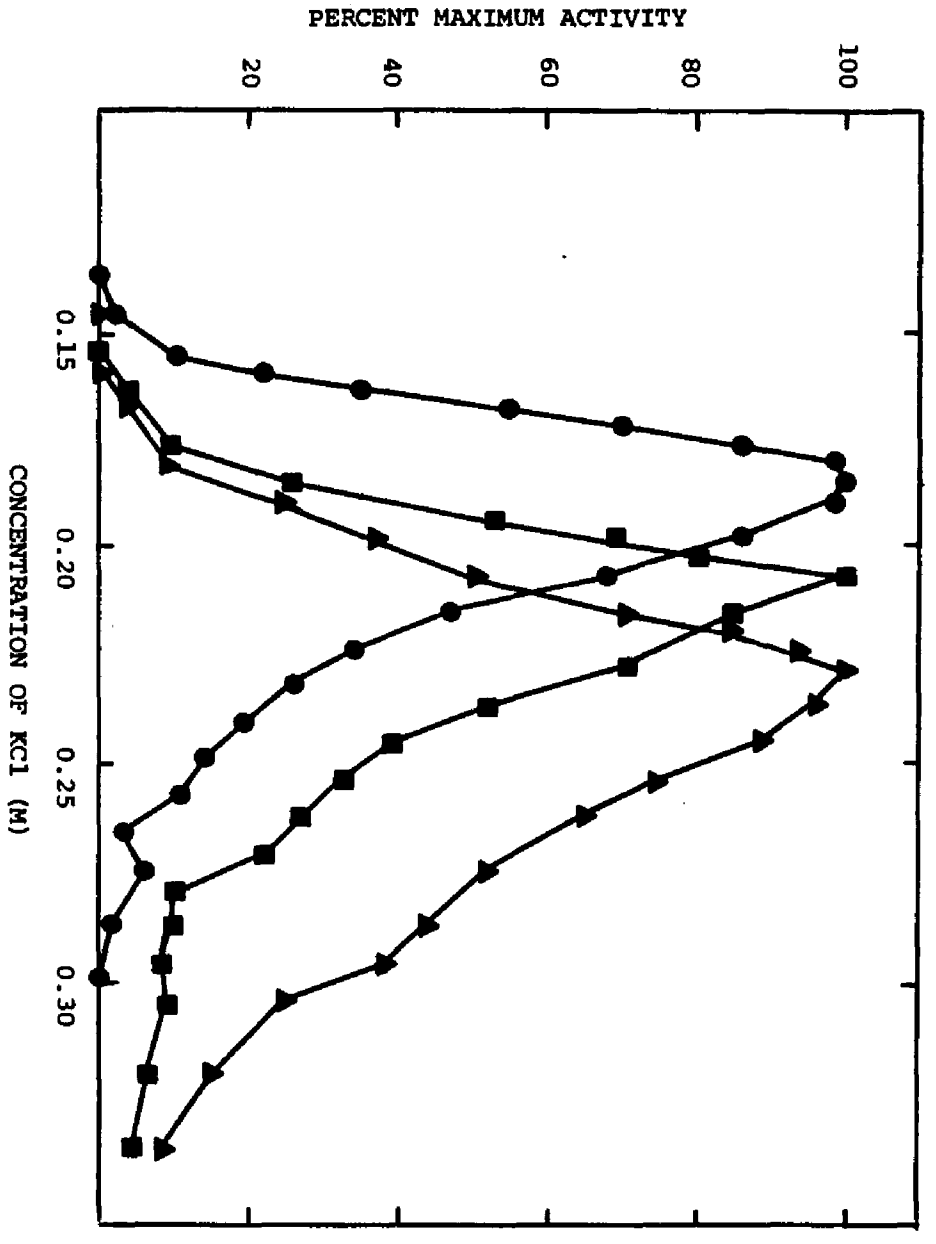
If partially purified (ammonium sulfate fractionated) enzyme preparations were chromatographed on DEAE-cellulose without prior treatment with zinc, multiple peaks were observed for the three enzyme forms. These peaks may represent the different mobilities of the homooctameric enzyme molecules which are not saturated with zinc, but which contain varying amounts of the metal.

e. Activators and Inhibitors.

The three ALA-D isozymes were inhibited similarly by the substrate analogs levulinic acid and succinylacetone and by the metal chelator EDTA. In addition, no differences were observed with lead which was found to inhibit the enzymes at concentrations as low as 0.3

Figure 19. DEAE-cellulose elution profiles of ALA-D isozymes. DEAE-cellulose chromatography was performed as described in Methods in the presence of 0.05 mM zinc.

●-●, 1-1; ■-■, 1-2; ▲-▲, 2-2.


PERCENT MAXIMUM ACTIVITY
20
40
60
80
100
0.15
0.20
0.25
0.30
CONCENTRATION OF KCl (M)

uM. When the affinity purified enzymes were assayed in the presence of 80 uM lead, only 20% of the initial activity remained after a 10 min preincubation followed by a 60 min assay.

Zinc activated all three isozymes equally up to a concentration of 0.1 mM and acted as an inhibitor at greater concentrations. However, when enzymes were treated with inhibitory concentrations of lead (400 uM), excess zinc at as much as 10 mM could fully restore activity.

Interestingly, lead and zinc had different effects on purified and crude enzyme preparations. Whereas purified enzyme was susceptible to zinc inhibition at concentrations greater than 0.1 mM, activity in the lysate was preserved at 0.3 mM zinc. On the other hand, crude enzyme was more susceptible to lead inhibition than purified enzyme, with only 10% of activity remaining after incubation with 10 uM lead while 100 uM lead was needed to inhibit purified enzyme to the same extent.

f. Thermal Stability.

The stabilities of post affinity enzyme preparations as determined at 37°, 50°, and 60°C revealed no differences among the enzyme forms. At 50°C, the isozymes had a $t_{1/2}$ of 50 minutes. The $t_{1/2}$ was increased to 90 minutes by the addition of 2 mg/ml BSA. No thermostability differences were detected in either lysates or packed red blood cells incubated at 37°C.

g. pH Optima.

Measurement of ALA-D activity over a pH range of 5.25 to 7.8 in 75 mM sodium phosphate buffer revealed no difference in the pH optima of the three isozymes. Maximum activity for all three enzyme forms was observed at pH 6.50. The pH optima remained unchanged when the isozymes were assayed in the presence of lead at 50% inhibitory concentrations.

h. Kinetic Analyses.

For kinetic studies, enzyme preparations were saturated with zinc by preincubation with 0.1 mM zinc acetate for 30 min at 37°C followed by assay in 0.05 M sodium Pipes, pH 6.8. Both partially purified and affinity-purified enzymes had similar apparent K_m values of 0.3 mM.

C. Discussion

This is the first report of the immunoaffinity purification of human ALA-D and of the biochemical characterization of the three isozymes 1-1, 1-2, and 2-2, of this enzyme. Three monoclonal antibodies, 6-1-7, 2-51-1, and 2-102-5 were raised to human enzyme purified by conventional means (20). None of the antibodies recognized the enzyme active site as evidenced by the finding that they had no effect on enzyme activity. Immunotitration curves revealed that the affinities of the antibodies for ALA-D

differed greatly. Antibodies 6-1-7 and 2-102-5 showed similar affinities for the three enzyme forms and exhibited titres of 5×10^{-5} and 1×10^{-1}, respectively. Interestingly, antibody 2-51-1, with an intermediate titre, showed a markedly greater affinity for the 1-1 and the 1-2 isozymes than for the 2-2 isozyme, indicating that this monoclonal antibody distinguishes between the antigenic sites which characterize the two enzyme forms. From these data, it would be expected that the 1-2 isozyme should have a titre falling between those of the homoproteins, however, this was found not to be the case (Figure 14). These results can be explained in terms of the numbers and identities of epitopes found on the surface of the ALA-D molecule. Although the native enzyme is a homooctomer, the number of epitopes which appear on the surface of the molecule may be limited, with the majority of sites buried within the enzyme. The homomeric 1-1 or 2-2 isozymes which are characterized by dissimilar epitopes would show different titration curves. However, the titration curve of the 1-2 enzyme would be dependent on whether or not its surface antigenic site(s) was of the 1 or 2 variety. In this case, it appears that this particular heterozygous protein exhibited the surface epitope of the 1 subunit and was, therefore, recognized as a 1-1. Alternatively, because of its markedly higher affinity for the 1 epitope, monoclonal antibody 2-51-1 may bind preferentially to this

site. If the ALA-D protein cannot physically accommodate the binding of a second antibody molecule to the 2 epitope, the titration curve of the hybrid protein would once again resemble that of the 1-1.

Because the monoclonal antibodies recognized the three isozymes differently, it would appear that they recognize at least two distinct antigenic sites on the enzyme. Experiments using dot blot analysis with mixtures of antibodies were unable to confirm this. Combinations of antibodies, each at saturating concentrations, resulted in only slightly higher reflectance readings as compared to those obtained when the antibodies were used singly. It is likely that the antibodies recognize different epitopes, but are precluded from binding to the molecule simultaneously by steric hindrance or the possibility that the epitopes overlap.

Using the monoclonal antibodies, three different immunoaffinity columns were made and tested for the purification of ALA-D. Bound enzyme was eluted from each of the columns using 3 M KSCN resulting in comparable recoveries and purifications. Because of its greater binding capacity and the fact that it recognized all enzyme forms equally, the immunoaffinity column made with antibody 6-1-7 was the column of choice for routine purification work. Typically, the column bound 100 ug of ALA-D per ml of affinity resin. Approximately 60% of the activity applied

to the immunoaffinity column could be recovered after dialysis of the KSCN-eluted sample into potassium phosphate buffer, pH 6.8, resulting in an overall yield of 26%. By this method, ALA-D was purified from fresh human erythrocytes approximately 19,500-fold and was shown to be greater than 95% pure as estimated by SDS-PAGE. The specific activity of the immunoaffinity purified enzyme was 13.3 units/mg. This is lower than previously reported values for the human enzyme purified by conventional means (20,92) although higher than for other sources including, guinea pig erythrocytes, 3.6 units/mg (93) and murine liver, 12 units/mg (17). This relatively low specific activity may be due to denaturation of ALA-D by 3 M KSCN which inhibits the enzyme activity by as much as 60% at that concentration (unpublished data). Indeed, loss of activity associated with the harsh elution conditions often necessary for the disassociation of the antigen-antibody complex is a major difficulty of immunoaffinity purifications (94). However, this disadvantage is offset by shortened purification times and by the purity of the enzyme obtained, making it an ideal method, not only for routine purification, but also for isolation of protein for tryptic digestion and sequence analysis.

This new methodology represents a significant improvement over the conventional purification procedures for ALA-D in that it allows for a rapid (3-4 days) isolation

of enzyme of comparable purity to that obtained by the previously used method which required 3 to 4 weeks of work (20). Enzyme can be purified in bulk through the initial stages and stored at -20°C as a stabilized ammonium sulfate precipitate which can then be used as needed for immunoaffinity purification. This procedure also allows for the rapid concentration of ALA-D from sources with low or small amounts of activity, i.e., human liver.

The 6-1-7 immunoaffinity column was used in the purification of three human ALA-D isozymes for their characterization. The frequencies of the two alleles responsible for this polymorphism have been well documented (45,89), however, until this report, no comparison had been made of their physical and kinetic properties. The three enzyme forms were distinguishable by starch gel electrophoresis in agreement with previously reported data (45,89) and by isoelectric focusing (pI 1-1 = 5.31; pI 1-2 = 5.17 - 5.31; pI 2.2 = 5.31). Slightly lower pIs (4.9,5.1) for human erythrocyte ALA-D have been reported by other investigators (20,95); differences in methodology may be responsible for these variations. Charge differences were also identifiable by DEAE-cellulose chromatography, with the isozymes eluting from the columns at different salt concentrations (1-1 = 0.180 M KCl; 1-2 = 0.205 M KCl; 2-2 = 0.230 M KCl). These results support the earlier finding of the 2-2 protein being the most electronegative, the 1-1 the

most electropositive, and the 1-2 intermediate in charge. Differences in the stabilities of the three enzyme forms equalized for protein and activity were not found at either 37°, 50°, or 60°C; the pH optima and apparent K_m values were all similar and in close agreement with the previously documented values of pH = 6.5 and K_m = 0.3 mM (20).

Since ALA-D is a metalloenzyme, efforts were directed towards the study of the effects of metals on the isozyme forms. The enzyme is activated by zinc at concentrations of up to 0.1 mM and inhibited by higher concentrations of zinc (20,92) and it is generally agreed that the native octameric protein contains 8 Zn^{2+} atoms, although reports of the number of zinc atoms necessary for full catalytic activity vary (18,31,96). Lead is a potent reversible inhibitor of ALA-D and inhibition of enzyme activity is used as a diagnostic indicator of lead poisoning (40). It is not clear whether lead inactivates the enzyme by displacing essential zinc molecules (18) or whether it has its own distinct binding site (29).

The studies reported here did not reveal any differencies in the effects of either of these metals on the activities of the three isozymes. This does not rule out the possibility that this polymorphism may have a biological significance in terms of the metal binding capabilities of the enzyme forms. The difficulty in characterizing a difference with respect to metal binding may lie in the high

affinity of this enzyme for these metals. The ubiquitous nature of zinc as a laboratory contaminant present at relatively high levels makes it extremely difficult to fully inactivate the enzyme in the absence of chemical chelators, although inactivations of up to 88% have been reported (92). If the association of metals with ALA-D is a cooperative one, with the reaction of the initial "slow binding" ion(s) occuring at concentrations too low to allow for accurate quantitation of enzyme activity, differences in metal binding among the three isozymes would not be detected. Alternatively, if the binding of metal to the enzyme is very rapid, lengthy assay times may preclude discrimination of metal binding differences between the isozymes.

Clearly, the question of metal binding with respect to the different isozymes of ALA-D warrants further investigation. The identification of the amino acid difference between the two allelic forms may provide an answer, however, ultimately, the sequencing and characterization of the enzyme active and metal binding sites and their relationship to each other in the different isozyme forms will be necessary. To this end, the immunoaffinity methodology described in this communication can be used to rapidly purify enzyme from individual 1-1, 1-2, and 2-2 donors for tryptic digestion and amino acid sequencing.

VI. MOLECULAR CLONING OF A cDNA FOR HUMAN δ-AMINOLEVULINATE DEHYDRATASE

A. Methods

1. Production of Rabbit Anti-Human ALA-D Antibodies

Female New Zealand rabbits were inoculated with 150 ug of homogeneous ALA-D [isolated from human erythrocytes, (20)] in Freund's adjuvant and boosted with 150 ug and 75 ug ALA-D at one month intervals. The polyclonal rabbit anti-human ALA-D IgG was partially purified (97) and tested for antigenic specificity (49). Prior to screening the expression vector cDNA library, the antibody IgG preparation was absorbed against an E. coli lysate that was immobilized on Sepharose 4B (98).

2. Antibody Screening of a pEX1 Human Liver cDNA Expression Library

The construction of an adult human liver cDNA expression library from the cDNA inserts in a library of 240,000 independent clones (99) and the pEX1 expression vector have been described previously (100). Optimal anti-ALA-D antibody concentrations for screening were determined by dot blot analysis with 1 ul aliquots of homogeneous ALA-D from human erythrocytes (diluted with 1 mg/ml human serum albumin in 25 mM sodium phosphate buffer, pH 6.8) applied to nitrocellulose strips (101).

The library was plated at 5000 colony-forming units per 137 mm Petri dish. Bacteria were replica plated onto nitrocellulose filters and screened by the colony blot procedure of Stanley (102). Nonfat dry milk (2.5%) was substituted for fetal calf serum for all blocking and antibody incubation steps (103). Horseradish peroxidase-conjugated goat anti-rabbit IgG and HRP Color Development Reagent were used as described by the manufacturer (Bio-Rad). This same procedure also was used to screen purified colonies. To determine the specificity of antigen expression by the antibody-positive colonies, duplicate experiments were conducted with purified human ALA-D added to saturate the anti-ALA-D IgG preparation (11 mg antigen/mg antibody). Anti-human serum albumin was used to isolate human serum albumin cDNA colonies from the pEX1 library. These clones were used as controls for antibody screening.

3. Amino Acid Sequences of ALA-D

The N-terminal amino acid sequence of ALA-D purified from human erythrocytes was determined by Dr. D. Martin Watterson, Vanderbilt University. Partial amino acid sequences for two chymotryptic peptides, as well as a tryptic and a CNBr peptide from bovine ALA-D were kindly provided by Dr. David Shemin of Northwestern University.

4. Construction of Mixed Oligonucleotide Probes and Oligonucleotide Primers

Mixed and unique oligonucleotides were synthesized on a Sam One Synthesizer (Biosearch, San Rafael, CA) using β-cyanoethyldiisopropyl-phosphoramidite chemistry. Two sets of oligonucleotide mixtures (probes 1 and 2), each containing all possible amino acid coding sequences, were prepared to correspond to two non-overlapping regions of the N-terminal sequence of human ALA-D (Table 6). Two overlapping sets of oligonucleotide mixtures, one containing all possible amino acid coding sequences (probe 3) and a longer one allowing guanosine-thymidine mispairing (probe 4), were prepared to correspond to part of the CNBr active site peptide from bovine ALA-D.

After partial determination of the nt sequence of the human ALA-D clone described in Results, section 3, six evenly spaced unique oligonucleotides were synthesized for use as internal primers to facilitate further DNA sequence analyses. All synthetic oligonucleotides were 5'-end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol; New England Nuclear) using T4 polynucleotide kinase (104). Additional purification of the oligonucleotides proved to be unnecessary for use either as probes or as primers.

Table 6. Construction of mixed oligonucleotide probes corresponding to bovine and human ALA-D peptide amino acid sequences.

Amino-terminal sequence and codons for human ALA-D*

			1				5					10				
Amino acids:		NH_2-	Met-	Gln-	Pro-	Gln-	Ser-	Val-	Leu-	His-	Ser-	Gly-	Tyr-	Lys-	His-	
Codons:			ATG	CA(A/G)	CCN	CA(A/G)	(TC/AG)N	GTN	(C/T)TN	CA(C/T)	(TC/AG)N	GGN	TA(C/T)	TG(C/T)	CA(C/T)	
Probe 1:	5'		ATG	CA(A/G)	CCN	CA(A/G)										3'
Anticodons:			TAC	GT(C/T)	GG(C/T)	GT(C/T)	(AG/TC)N	CAN	(A/G)AN	GT(A/G)	(AG/TC)N	CCN	AT(A/G)	AC(A/G)	GT(A/G)	
Probe 2:	3'											CCN	AT(A/G)	AT(A/G)	GT(A/G)	5'

Active site CNBr fragment sequence and codons for bovine ALA-D (92)

		1				5				
Amino acids:		-Met-	Val-	Lys-	Pro-	Gly-	Arg-	Pro-	Tyr-	
Codons:		ATG	GTN	AA(A/G)	CCN	GGN	(C/A)GN	CCN	TA(C/T)	
Probe 4:	5'	ATG	GT(G/T)	AAG	CC(G/T)	GG(G/T)	(C/A)G(G/T)	CC(G/T)	TA	3'
Anticodons:		TAC	CAN	TT(C/T)	GGN	CCN	(G/T)CN	GGN	AT(A/G)	
Probe 3:	3'	TAC	CAN	TT(C/T)	GGN	CC				5'

*N = A, T, G or C.

5. Screening Clones with Synthetic Mixed Oligonucleotides

Antibody-positive pEX1 clones, containing potential cDNA inserts for human ALA-D, were spotted and grown on Zetabind nylon membranes. The filters were treated for 15 min each with (i) 1.5 M NaCl, 0.5 M NaOH, (ii) 1 M Tris buffer, pH 7.8, (iii) 0.36 M NaCl, 20 mM phosphate buffer, pH 7.4, 1 mM EDTA and then baked in an 80°C vacuum oven for 2 h. A hybridization solution was prepared to contain 3.0 M $(CH_3)_4NCl$, 0.1 M Tris buffer, pH, 7.8, 10 mM EDTA, 10 ug/ml sonicated and denatured calf thymus DNA, 0.05% Ficoll, 0.05% dextran sulfate, 0.05% polyvinylpyrolidone and 0.1% SDS. The filters were rehydrated with hybridization solution, mechanically freed of residual bacterial debris, and prehybridized by shaking at 65°C for 2 h with two changes of solution. Hybridiation was carried out overnight in the same solvent with 5 ng/ml of a 5'-^{32}P-labeled mixed oligonucleotide probe at the appropriate temperature. After hybridization, the solvent was exchanged and the incubation continued for 1 h. Binding of probes to specific colonies was detected autoradiographically.

6. Characterization of Positive Clones

Plasmid DNA was purified (104) from the antibody-positive clones which also bound the mixed oligonucleotide

probes. These DNAs were digested with PstI, BamHI, RsaI, PstI+RsaI, and BamHI+RsaI, were electrophoresed in agarose gels containing 0.5 ug/ml EtdBr and then were visualized fluorographically. In some cases the DNAs were transferred to nylon membranes (Zetabind) by the method of Southern (105). The filters were baked and hybridized with radiolabeled mixed oligonucleotide probes using the method described above for colony blot hybridization.

The probe-binding cDNA insert from a pEX1 clone was recloned into the PstI site of the plasmid pUC9 (106) and subjected to further restriction analysis using various restriction endonucleases with 6-bp recognition sequences. The insert was removed from pUC9 by cleavage with HindIII+BamHI and recloned into bacteriophage M13mp18 and M13mp19 (107) replicative form DNA which had been cleaved with the same two restriction endonucleases. All DNA sequencing was carried out by primer extension (108) on the viral DNAs from the M13mp18 and M13mp19 clones using either the M13 universal primer or the synthetic oligonucleotide primers previously described.

B. Results and Discussion

1. Screening of a pEX1 Human Liver cDNA Expression Library

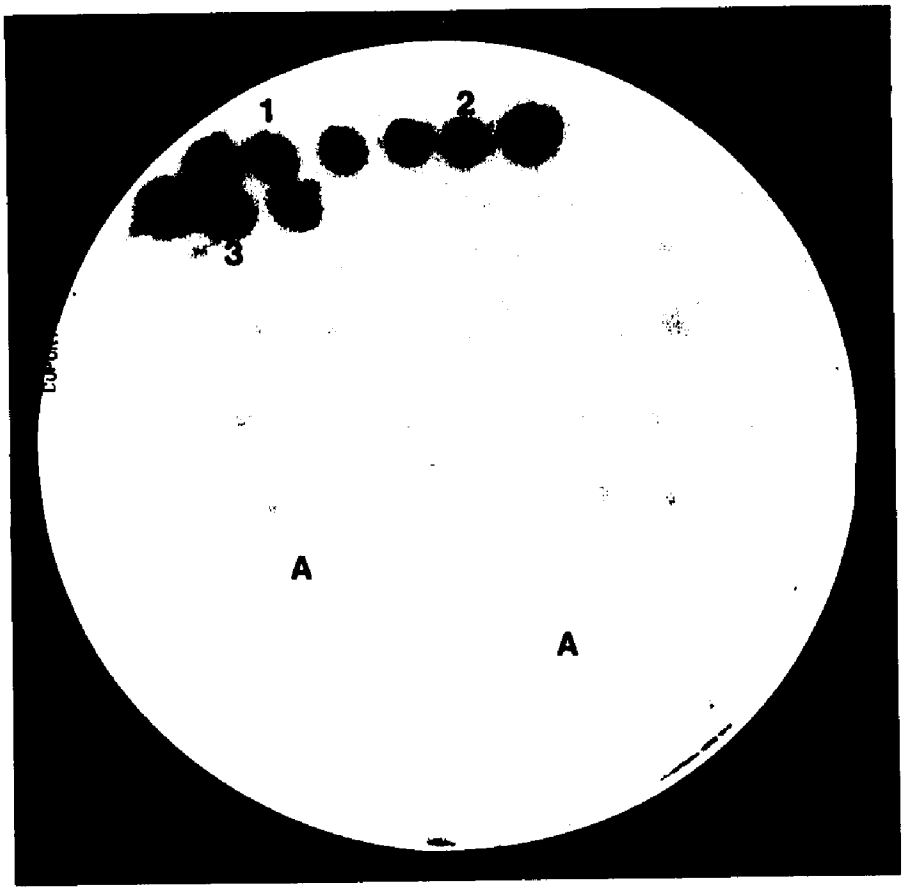
The pEX1 human liver cDNA expression library of Stanley and Luzio (100) was screened for human ALA-D by the

colony blot method of Stanley (102). Seventeen antibody-positive human ALA-D clones were identified. Each of these colonies, as well as pEX1 colonies containing human serum albumin cDNA inserts (102), were grown in triplicate arrays on two nitrocellulose filters for colony blot testing (e.g., Figure 20). One filter was screened with anti-human ALA-D IgG while the second was screened with anti-human ALA-D IgG which had been preabsorbed with purified human ALA-D. Of the original 17 antibody-positive colonies tested, seven remained positive after purification. Although easily detectable, the intensity of the horseradish peroxidase signal in the colony blot assay for each of these clones was less than the signal observed when the human serum albumin clones were screened with anti-human serum albumin. Of these, four (clones 1,2,3, and 5) showed significant inhibition of anti-human ALA-D IgG binding after preabsorption with the purified antigen. All 17 of the originally selected, antibody-positive human ALA-D cDNA clones were also tested using the mixed oligonucleotide probes (see below).

2. Mixed Oligo Hybridization to Antibody-Selected cDNA Clones

Table 6 shows the N-terminal amino acid sequence of human ALA-D, the partial amino acid sequence of the CNBr peptide containing the active site lysine of the bovine

Figure 20. Hybridization of probe 4 to the DNA from an array of the original 17 anti-body clones selected from the pEX1 library.


1
2
3
A
A

enzyme, and the mixed oligonucleotide probes which were synthesized to these amino acid sequences. Because of the codon redundancy of the serine residues at positions 5 and 9 of the N-terminal amino acid sequence, oligonucleotide mixtures were synthesized to correspond to all possible codons for amino acid 1-4 (probe 1) and 10 to 13 (probe 2). Two sets of mixed oligonucleotides were constructed to correspond to the amino acid sequence of the active site CNBr peptide of bovine ALA-D. Probe 3 was a complete oligonucleotide mixture based on amino acid residues 1 to 5. A second, longer (23-nt) set of mixed oligonucleotides (probe 4) was synthesized using guanine or thymine when either purine or either pyrimidine was possible, respectively.

The original 17 antibody-positive ALA-D clones, grown in triplicate arrays on four nylon (Zetabind) filters as described above, were processed to attach the denatured DNA to the filters. Hybridization was carried out in a solution containing 3 M $(CH_3)_4NCl$, a DNA reassociation solvent (110) known to remove the compositional dependence of DNA melting (111). A similar solvent has been used to wash filters after hybridization with mixed oligonucleotides (112). The enthalpy of bp formation and the reassociation rate constant in the base composition independent solvent were determined (Wetmur et al., unpublished results), thereby permitting the prediction of the melting temperature and reassociation rate

for any mixture of oligonucleotides at any oligonucleotide concentration. All hybridization and wash steps were carried out at the predicted melting temperature (when known). Neither of the N-terminal probes (1 and 2) bound to the filter. However, both probes 2 and 4 bound strongly to three triplicate sets of antibody-positive clones. Figure 20 shows the binding of probe 4 to the DNA of three antibody-positive clones, whereas the remainder of the putative ALA-D clones, as well as those for human serum albumin, did not bind any of the four mixed oligonucleotide probes.

3. Characterization of Antibody- and Probe-Positive Clones

The three antibody- and oligonucleotide-positive clones (1, 2, and 3) were further characterized. Plasmid DNAs were isolated, digested with the restriction endonuclease PstI, and analyzed by agarose gel electrophoresis. The cDNA insert removed from all three clones was approximately 800 bp. Double digestion with RsaI+PstI, HindIII or BamHI revealed identical restriction maps for the three clones. One of the cDNA inserts was recloned into the PstI cleavage site of the plasmid pUC9. The entire cDNA insert was removed from pUC9 using HindIII+BamHI and cloned into replicative form DNAs of bacteriophages M13mp18 and M13mp19 cut with the same two

restriction endonucleases. The inserts in the viral single-stranded DNAs of these bacteriophages were sequenced by primer extension. After partial sequence data were obtained using the universal M13 primer, additional oligonucleotide primers were synthesized for further sequence analysis.

Three regions of nearly complete sequence homology were found between the amino acid sequence predicted from the DNA sequence of the human ALA-D cDNA clone and the three peptide sequences determined from bovine ALA-D. The sequence data are summarized in Table 7. The sequence showed that both probes 3 and 4 contained sequences which precisely matched the cDNA of these clones for the first 14 nt (Tables 6 and 7). Neither the N-terminal sequence nor a methionine initiation codon was found in the predicted amino acid sequence 5′ to the first sequence identified. There was no 3′-poly d(A) sequence in the cDNA. The failure of these probes to bind to clone 5 cDNA could be due either to truncation of an ALA-D cDNA with loss of the binding site or, more likely, to a cDNA fortuitously coding for a strongly cross-reacting antigenic determinant.

C. Conclusions

1. A cDNA encoding the heme biosynthetic enzyme, ALA-D, was isolated from a human liver cDNA library cloned

Table 7. Human ALA-D cDNA and predicted amino acid sequences compared with bovine ALA-D peptide amino acid sequences*

Open reading frame amino acid 47:	
Human cDNA sequence:	GTG GCC TGT GAT GTC TGC CTG TGT CCC TAC
Predicted amino acids:	-Val-Ala-Cys-Asp-Val-Cys-Leu-Cys-Pro-Tyr-
Bovine sequence:**	-Val-Ala-Pro-Asp-Val-Cys-Leu-Cys-----Tyr-
Open reading frame amino acid 149:	
Human cDNA sequence:	GGG GAC CGC CGC TGC TAC CAG CTG CCC CCT GGA GCA CGA
Predicted amino acids:	-Gly-Asp-Arg-Arg-Cys-Tyr-Gln-Leu-Pro-Pro-Gly-Ala-Arg-
Bovine chymotryptic** peptide:	-Gly-Asn-Arg-Arg-Cys-Tyr-
Bovine tryptic peptide:**	-Cys-Tyr-Gln-Leu-Pro-----Gly-Ala-Arg-
Open reading frame amino acid 180:	
Human cDNA sequence:	ATG GTG AAG CCG GGA ATG CCC TAC CTG GAC ATC GTG CGG GAG
Predicted amino acids:	-Met-Val-Lys-Pro-Gly-Met-Pro-Tyr-Leu-Asp-Ile-Val-Arg-Glu-
Bovine CNBr peptide:(92)	-Met-Val-Lys-Pro-Gly-Arg-Pro-Tyr-Leu-Asp-Leu-Val-Arg-Glu-

*Amino acid differences are underlined.

**L.H. Oh, G. Hohberger, G.F. Barnard, and D. Shemin, unpublished results.

into the pEX1 expression vector. Only by the use of both monospecific antibody and mixed oligonucleotide probes could the identity of clones 1, 2, and 3 as human ALA-D clones be established. These three clones were found to contain identical cDNA inserts, suggesting that the library contained no other human ALA-D cDNA clones in which the cDNA was in the proper phase and orientation to lead to the formation of a fusion protein which could be detected by the colony blot procedure.

2. Colinearity of the four bovine peptide sequences and the predicted corresponding human amino acid sequences provided definitive identification that human ALA-D cDNA had been cloned (Table 7). First, the ten amino acid residues in the bovine chymotryptic peptide were found to differ from the predicted human sequence by two residues. In the predicted human sequence, a cysteine replaced proline at position 3 and a proline was inserted at position 9. The cysteine codon differs from the proline codon by two nt. Second, the predicted human amino acid sequence permitted the recognition of a 13 amino acid bovine sequence which was derived from the overlapping sequences of two bovine peptides: a chymotryptic sequence beginning at the first amino acid and a tryptic sequence beginning with the cysteine at position 5. Two differences were found between the predicted human and bovine sequences, an aspartic acid replacing asparagine at position 2 and an inserted proline

at position 10. The aspartic acid and asparagine codons differ by 1 nt. Third, the 14 amino acid bovine CNBr peptide had two differences between the predicted human and bovine ALA-D amino acid sequences. Both the substitution of methionine for arginine at position 6 and the conservative substitution of isoleucine for leucine at position 11 could result from as few as five nt substitutions, in 35 amino acids, excluding the predicted prolines which were in the human ALA-D cDNA but which were not detected during amino acid sequencing of two bovine polypeptides. This degree of amino acid sequence variation in different mammalian species is not unusual (113). Although the use of synthetic mixed oligonucleotides based upon amino acid sequence data for a different mammalian species may be insufficient for isolation of a cDNA clone, their use in conjunction with screening an expression library with an immunologic probe proved to be successful. The amino acid sequence data from a different mammalian species was sufficient to confirm the identification of a cDNA clone following nt sequence analysis.

3. The molecular cloning of a cDNA encoding human ALA-D represents the first report of a cDNA clone for a human heme biosynthetic enzyme. Of the seven other enzymes in the heme biosynthetic pathway, cDNA clones have been described for chick embryo ALA-synthase (69), rat porphobilinogen deaminase (68) and rat uroporphyrinogen

decarboxylase (67). The availability of the human ALA-D cDNA should facilitate the characterization of the structure, organization, and expression of the gene for ALA-D. In addition, this cDNA should permit investigations of the molecular lesion in the enzyme defect in the human porphyria due to ALA-D deficiency and of the molecular nature of the human ALA-D polymorphism.

VII. CONCLUDING REMARKS

The study of four types of mutant PBG-deaminase revealed the presence of different allelic mutations in the structural gene for PBG-deaminase and documented genetic heterogeneity in AIP. Two types of mutations proved particularly interesting. The CRIM-positive Type 2 mutation, with an unexpectedly high CRIM/activity ratio of about 5.7, exhibited increased resistance to degradation in the erythrocyte representing a unique type of stability defect in which the non-functional mutant protein is more stable to *in vivo* proteolysis than the normal, catalytically active erythrocyte enzyme. The mechanism by which the accumulated mutant enzyme stabilizes the normal erythrocyte activity is an area which deserves further investigation. The CRIM-negative Type 2 mutation was characterized by normal levels of erythrocyte PBG-deaminase activity in affected individuals; the basis for this finding has not been explored. In light of the recent discovery that the PBG-deaminase gene codes for two enzymes, hepatic and erythrocytic (Nordmann *et al.*, unpublished data), the comparison of the hepatic and erythrocyte enzymes in the CRIM-negative Type 2 mutant may help to explain the biochemical nature of this mutation. Ultimately, however, cDNA and genomic probes will be necessary in order to reveal the precise nature of the molecular genetic defects in this and the other PBG-deaminase mutations.

A new immunoaffinity purification for ALA-D which allows for rapid isolation of nearly homogenous enzyme was developed and used to purify the three human ALA-D isozymes 1-1, 1-2, and 2-2. Biochemical characterization of the enzyme forms revealed that they were distinguishable only on the basis of charge differences. Studies aimed at distinguishing the two allelic forms on the amino acid level are ongoing and currently directed towards the analysis of tryptic digests of the 1-1 and 2-2 proteins. The eventual isolation of cDNAs specific for the ALA-D 1 and 2 subunits would permit the examination and comparison of the active and metal binding sites for the isozymes and would elucidate the significance of this polymorphism. The isolation of the cDNA encoding human ALA-D will permit the study of the structure, organization, and expression of the ALA-D gene and will facilitate the investigation of the precise molecular lesions in the enzyme defect due to ALA-D deficiency.

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