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Kinetic analysis and chemical modification studies of nicotinate phosphoribosyltransferase from yeast

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KINETIC ANALYSIS AND CHEMICAL MODIFICATION STUDIES OF NICOTINATE PHOSPHORIBOSYLTRANSFERASE FROM YEAST

BY

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Abstract

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by

Susan Hess

Advisor: Professor Donald Sloan

Nicotinate phosphoribosyltransferase (NaPRTase) from Baker's yeast catalyzes the formation of nicotinate mononucleotide and pyrophosphate from phosphoribosyl α-1-pyrophosphate (PRibPP) and nicotinate, concomitant with ATP hydrolysis. Using purified NaPRTase, initial velocity measurements varying one substrate concentration at different fixed levels of the second substrate, and maintaining the third substrate constant, helped to define a sequential mechanism for the enzyme which contained a single ping-pong step. Furthermore, an exchange of label was observed between ATP and [^{14}C]-ADP, which reinforced the previous hypothesis from initial velocity data, that the binding of ATP to the enzyme involved a single ping-pong step. The inhibition of this rate of exchange by PRibPP, was attributed to substrate inhibition, whereas the inhibition exerted by pyrophosphate, was interpreted as being due to the stimulation of the ATPase activity of the enzyme. These results combined with other kinetic, isotope exchange, product inhibition, and flow dialysis binding studies previously accomplished in our laboratory, suggested that the enzyme proceeds through an ordered Uni Uni Bi Ter Ping Pong kinetic mechanism in which ATP binds to the enzyme first to form ADP and a phosphorylated enzyme. Subsequently, PRibPP and nicotinate bind to the enzyme in order, which is then followed by the random release of the products, nicotinic acid mononucleotide and pyrophosphate, and lastly phosphate.

Chemical modification and pH dependency studies of the enzyme were performed to gain insight into the chemical mechanism occurring at the active site. Incubations of NaPRTase with pyridoxal 5'-phosphate followed by sodium borohydride reduction led to inactivation of the enzyme. Pyridoxal was a less effective inhibitor than pyridoxal 5'-phosphate. The inactivation of the enzyme by pyridoxal 5'-phosphate was reversible upon flow dialysis, whereas reduction of the enzyme-pyridoxal complex rendered the inhibition irreversible. The presence of ATP or PRibPP, with or without Mg^{2+}, provided protection against this inactivation, while a kinetic analysis revealed the inhibition to be competitive, and noncompetitive, respectively. One mole of [^{3}H]-pyridoxal 5'-phosphate per mole of enzyme was required to completely inactivate the enzyme. The amount of pyridoxal 5'-phosphate incorporated into the enzyme was reduced in the presence of MgATP and MgPRibPP to 0.2 and 0.6, respectively, whereas in the combination of both of the two substrates there was no incorporation of pyridoxal 5'-phosphate. Taken together, these results suggest the presence of a lysine residue at the active site of the enzyme which can form a Schiff base intermediate with pyridoxal 5'-phosphate.

pH stability studies indicated the enzyme was stable over the pH range 4.1-9.6. Subsequently, plots of log Vmax and log Vmax/Km *vs.* pH for ATP, yielded pKa values of 5.3 and 5.6, respectively. The profile of log Vmax for PRibPP yielded a pKa value of 5.8 and a pKb value of 8.6, whereas a plot of log Vmax/Km yielded a pKa value of 5.5. These findings suggest the presence of a carboxyl or imidazole group of an amino acid residue , in addition to a lysine residue, at the catalytic site of NaPRTase.

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INTRODUCTION

The coenzyme, nicotinamide adenine dinucleotide (NAD), has a multitude of functions pervading different aspects of cellular metabolism. One of the major well known roles of NAD is to function as a hydride and electron carrier in the oxidation-reduction reactions of numerous dehydrogenases (Weiner and Van Eys,1983). The nucleus of the cell houses a secondary function of NAD that involves its use as a substrate for the synthesis and turnover of polymers of ADP-ribose (Hayaishi and Ueda, 1977). Recently, a third role has surfaced in which NAD has been implicated as another route by which Ca^{2+} is released from intracellular storage (Clapper *et al.*, 1987).

The biosynthesis of NAD from nicotinic acid was first established in mammalian tissue, yeast, and *E. coli* as proceeding through the following steps (Preiss and Handler, 1958a, 1958b; Imsande, 1961) which are known collectively as the Preiss-Handler pathway (the abbreviations in these equations are defined later in the narrative).

NaPRTase

Nicotinic acid + PRibPP <===> Nicotinic acid mononucleotide (NaMN) + pyrophosphate (PPi) (1)

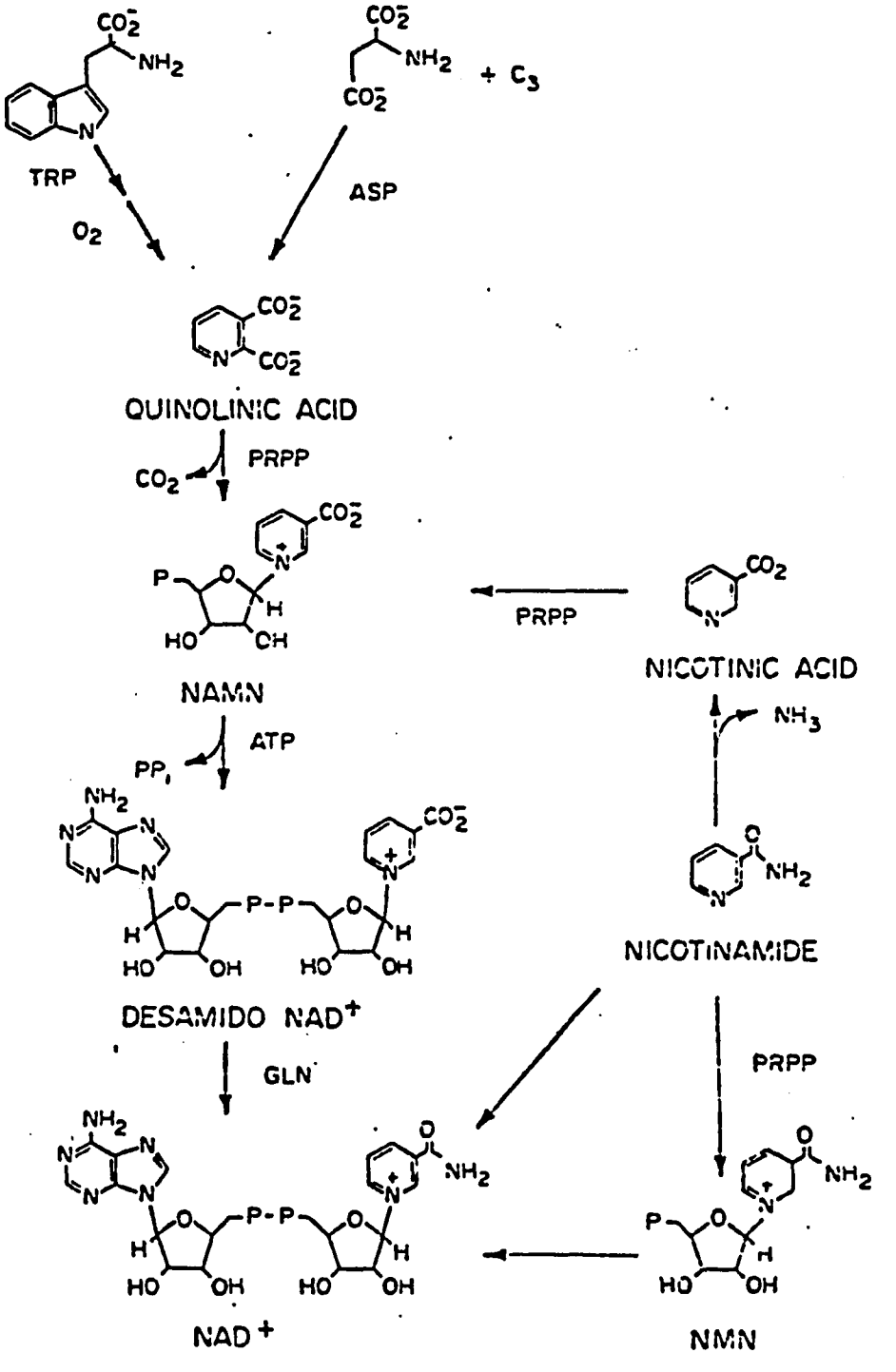
NaMN Adenyltransferase

NaMN + ATP <===> Nicotinic acid adenine dinucleotide (NaAD) + PPi (2)

NAD Synthase

NaAD + ATP + glutamine <===> Nicotinamide adenine dinucleotide (NAD) + glutamate + AMP + PPi (3)

FIGURE 1. Interrelationship of Biosynthetic Pathways of NAD.


TRP
O_2
ASP
+ C_3
QUINOLINIC ACID
PRPP
CO_2
NAMN
ATP
PP_i
DESAMIDO NAD+
GLN
NAD+
PRPP
NICOTINIC ACID
NH_3
NICOTINAMIDE
PRPP
NMN

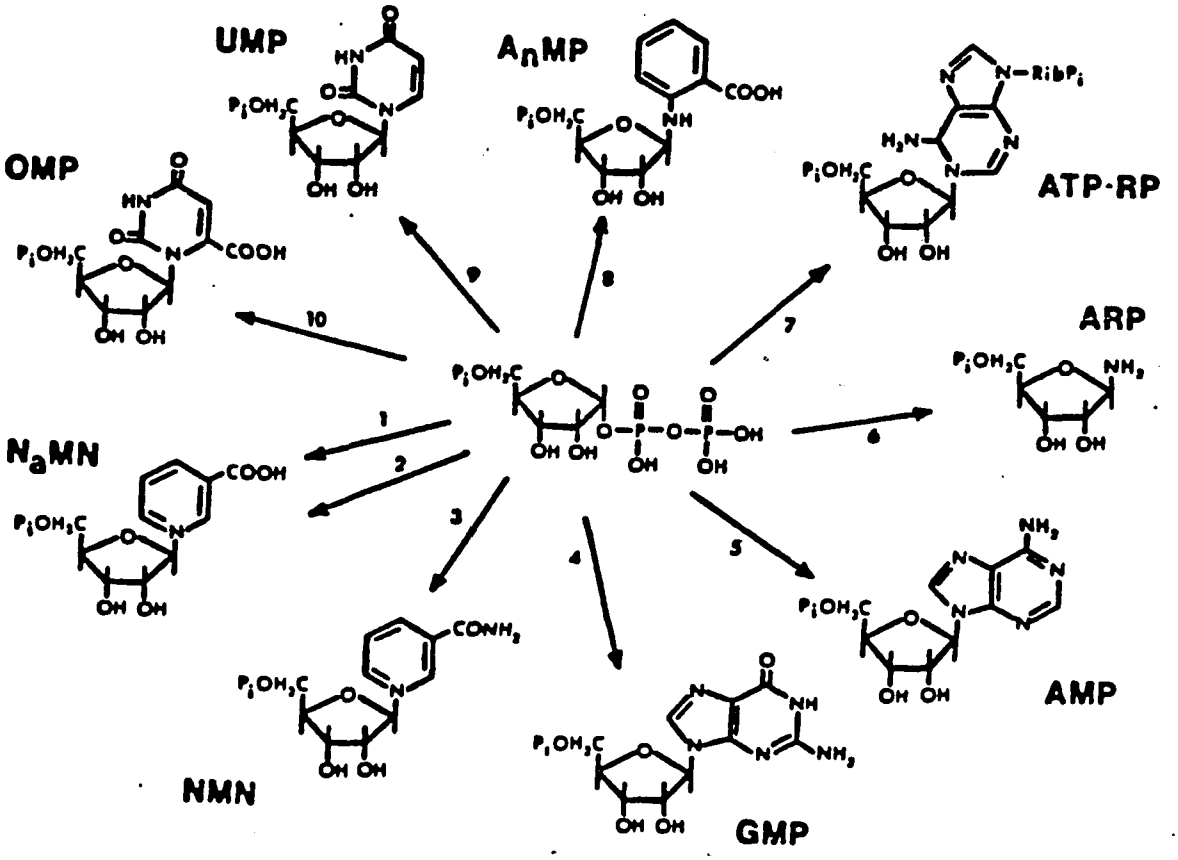
The first enzyme in the pathway, nicotinate phosphoribosyltransferase (NaPRTase) [EC 2.4.2.11] which was originally known as nicotinate mononucleotide pyrophosphorylase (Imsande and Handler, 1961), utilizes either exogenous, endogenous, or recycled nicotinate formed from NAD degradation.

The biosynthesis of NAD also proceeds through an alternate second route in which the enzyme nicotinamide phosphoribosyltransferase (NAmPRTase) [EC 2.4.2.12] (Dietrich *et al.*, 1966) converts exogenously obtained nicotinamide, in the presence of PRibPP (phosphribosyl α-1-pyrophosphate) to NMN, which subsequently reacts with ATP to form NAD (Preiss and Handler, 1958a). Because nicotinate and nicotinamide are obtained exogenously, NaPRTase and NAmPRTase are involved in the salvage pathway for NAD biosynthesis (Gholson, 1966; Musick, 1981).

In addition to these two salvage routes, quinolinate (an intermediate from tryptophan degradation, or the condensation of aspartic acid and glyceraldehyde 3-phosphate) can be converted to NaMN by the enzyme quinolinate phosphoribosyltransferase (QPRTase) [EC 2.4.2.19], which is involved in the denovo pathway for NAD biosynthesis (Nishizuka and Hayaishi, 1963). De novo signifies that the synthesis of the pyridine ring of NAD is derived from simpler metabolites (Mann and Byerrum, 1974). Fig. 1 summarizes the interrelationship of the three biosynthetic pathways involved in NAD synthesis.

The three enzymes mentioned previously are not only involved in pyridine nucleotide metabolism, but also comprise a group of ten enzymes known as the phosphoribosyltransferases (PRTases), which are part of the biosynthetic pathway of purines, pyrimidines, as well as the aromatic amino acids, tryptophan and histidine (Musick, 1981) (Fig.2). Each of these enzymes has an absolute requirement for a divalent cation, and catalyzes the cleavage of the pyrophosphate moiety from PRibPP concomitant with an anomeric inversion of the ribofuranose ring. The resulting ribose

FIGURE 2. Phosphoribosyltransferase (PRTase)-Catalyzed Reactions and the Metabolic Fate of Phosphoribosyl α-1-pyrophosphate. The Following Enzymes are Represented: 1) Nicotinate PRTase, 2) Quinolinate PRTase, 3) Nicotinamide PRTase, 4) Hypoxanthine-Guanine PRTase, 5) Adenine PRTase, 6) Glutamine-Dependent Aminotransferase, 7) ATP-PRTase, 8) Anthranilate PRTase, 9) Uracil PRTase, 10) Orotate PRTase.


UMP
AnMP
OMP
ATP-RP
ARP
NaMN
AMP
NMN
GMP
1
2
3
4
5
6
7
8
9
10

phosphate group is transferred to one of a group of specific aromatic nitrogenous bases which include orotate, hypoxanthine, adenine, guanine, quinolinate, and anthranilate.

My work in the laboratory has been concerned with the purification of yeast NaPRTase, and the kinetic and chemical mechanism by which it operates. NaPRTase has been partially and completely isolated from human erythrocytes (Niedel and Dietrich, 1973), bovine liver (Imsande and Handler, 1961; Smith and Gholson, 1969), hog liver (Hayakawa *et al.*, 1984a), *Bacillus subtilis* (Imsande, 1964), and other microorganisms (Baeker*et al.*, 1976; Dudley and Willett, 1965; Sundaram *et al.*, 1960), Ehrlich ascites cells (Seifert *et al.*, 1966), *Astasia longa* (Kahn and Blum, 1967), and yeast (Kosaka *et al.*, 1971; Kosaka *et al.*, 1977, Hanna *et al.*, 1983).

All of these enzymes from different sources can be categorized into three classes according to their response to ATP. The enzyme from yeast (Honjo *et al.*, 1966; Kosaka *et al.*, 1971; Hanna and Sloan, 1980), and *B. subtilis* (Imsande, 1964) and *E.coli* (Imsande, 1961) have an absolute requirement for ATP. Evidence from these sources has demonstrated that one mole of ATP is consumed for one mole of NaMN produced. In contrast, the enzyme from the protozoan *Astasia longa* (Kahn and Blum, 1967) is ATP-independent. NaPRTases isolated from bovine liver (Smith and Gholson, 1969), hog liver (Hayakawa *et al.*, 1984b), and human erythrocytes (Niedel and Dietrich, 1973) comprise a class in which ATP acts as an allosteric modifier, by lowering the Km values for both nicotinate and PRibPP. Although ATP is not an obligatory substrate of these enzymes, in the presence of the compound, a 1:1 stoichiometry between ATP cleavage and NaMN formation has been observed (Smith and Gholson, 1969; Niedel and Dietrich, 1973). NaPRTase purified from yeast (Kosaka *et al.*, 1971; Kosaka *et al.*, 1977) exhibited maximum activity at pH 8.0, while a broad pH optimum between 6.5 and 8.0 was observed in human erythrocytes (Niedel and Dietrich, 1973). Bovine liver (Imsande and Handler, 1961) and hog liver

NaPRTase (Hayakawa *et al* ., 1984) had similar pH optima at pH 7.2, and 7.3-7.4, respectively.

The molecular weight of the hog liver (Hayakawa *et al.*, 1984a) and human erythrocyte enzyme (Niedel and Dietrich, 1973) in the presence and absence of ATP, were estimated to be 120,000 (subunit 63,000), and 86,000, respectively. With regard to the bovine liver enzyme (Smith and Gholson, 1969), duplicate runs were used to determine the molecular weight, in the absence of ATP to be 74,400 and 71,600, respectively, while in the presence of ATP the molecular weights were determined to be 81,300 and 82,200. In contrast, the molecular weight estimated for the yeast enzyme was 43,000 (Kosaka *et al.*, 1971; Hanna *et al.*, 1983) indicating that the mammalian NaPRTase may exist as a dimer.

NaPRTase from bovine liver (Imsande and Handler, 1961), and yeast (Kosaka *et al.*, 1971) demonstrate a requirement for Mg^{2+}, whereas the enzyme from hog liver shows a requirement for a divalent cation, in which Mn^{2+} and Co^{2+} were found to be more effective than Mg^{2+}. The activity of these three enzymes in the presence of a divalent cation was enhanced by ATP. Maximal activity in both bovine liver and yeast was observed using equimolar concentrations of Mg^{2+} and ATP suggesting that a Mg-ATP complex was the true substrate of the reaction, and that free ATP inhibits the reaction, or that excess ATP bind to free Mg^{2+} which is needed to catalyze the reaction. Orthophosphate was shown to stimulate NaPRTase activity in hog liver (Hayakawa *et al.*, 1984b), bovine liver (Imsande and Handler, 1961) and yeast (Kosaka *et al* ., 1971).

Other purine nucleotides in place of ATP were screened for their ability to stimulate NaPRTase activity. GTP and ITP could substitute for ATP on the yeast enzyme, while the hog liver and human erythrocyte enzyme were very specific for ATP. The enzyme is also highly specific for nicotinate (Honjo *et al.*, 1966; Hayakawa *et al.*, 1984a). Nicotinamide, quinolinic acid and other purines and pyrimidines were

ineffective as substrates.

In examining the yeast enzyme for stability against heat inactivation, ATP, and other analogs as well as nicotinate and PRibPP, provided considerable protection (Kosaka *et al.*, 1971; Honjo *et al.*, 1966). The addition of Mg^{2+} to the solutions enhanced this protective effect (Kosaka *et al.*, 1971).

Analysis of the kinetic mechanism through which the enzyme proceeds involved initial velocity measurements, isotope exchange and product inhibition studies, which have been carried out only for the enzyme isolated from yeast. Kosaka *et al.* (1971) first demonstrated that NaPRTase catalyzed ATP-ADP exchange in the presence of Mg^{2+}, and the absence of other substrates, at a rate eight times faster than the overall reaction. This indicated that the enzyme can be phosphorylated in the absence of other substrates, and that the phospho-enzyme may be a catalytic intermediate. Using [^{32}P]-ATP hydrolytic activity was not observed, unless both nicotinate and PRibPP were present suggesting that the phosphorylated enzyme was relatively stable. Subsequently, the ^{32}P-phosphoenzyme was chromatographically isolated, and was shown to release all the ^{32}P from the enzyme only in the presence of both nicotinate and PRPP (Kosaka *et al.*, 1977). In addition, the velocity of ATP-ADP exchange decreased with addition of PRibPP and/or nicotinate, indicating that PRibPP and nicotinic acid could add randomly only after ATP was bound to the enzyme. This data did not reveal whether ADP was released prior to addition of PRibPP and nicotinate (a ping-pong step) or after the addition of all of the substrates.

The initial velocites of a reaction can be determined by varying one substrate concentration at different concentrations of the second substrate maintaining the third substrate constant (Cleland, 1977). The data can then be plotted by several methods in order to determine the kinetic mechanism and parameters governing the reaction. Perhaps, the most common and advantageous procedure is to linearize the data by using the reciprocal form of the Michaelis-Menton equation.

$$1/v = 1/V + Ka/V\ (1/A)$$

where v = Initial velocity

V = Maximal velocity

Ka = Michelis constant for A

A = Substrate

The data is then plotted as a double reciprocal plot (Lineweaver-Burk plot) of 1/v vs. 1/[A].

Enzyme mechanisms can be categorized into two main classes, sequential and ping-pong, which can be distinguished from one another by the type of pattern visualized on a double reciprocal plot. A sequential mechanism is defined as one in which all the substrates bind to the enzyme before any products are released. However, it does not indicate the order of substrate addition. This pathway usually exhibits a group of lines (at different fixed substrate concentrations) on a double reciprocal plot that intersect at a common point, either on the y-axis (also termed as competitive), or to the left of the y-axis (also termed noncompetitive). On the other hand, in a ping-pong mechanism, one of the substrates interacts with the enzyme to form a modified enzyme which releases product before the next substrate can bind. This mechanism usually exhibits a group of parallel lines on a double reciprocal plot, which is also termed "uncompetitive".

Initial velocity measurements performed by Kosaka *et al.*, (1977) gave intersecting patterns on double reciprocal plots which provided a better fit to the sequential mechanism, although parallel patterns, which were indicative of a ping-pong mechanism, were observed when either PRibPP or nicotinic acid were fixed at high

concentrations. Although the exchange data revealed the possibility of a ping-pong step, the inital velocity data indicated that this reaction proceeded predominately through a random sequential mechanism in which ATP bound first to the enzyme, followed by the random addition of PRibPP and nicotinate. However, an alternate ping-pong pathway also operates in which subsequent formation of a phosphorylated enzyme by ATP, is followed by the random order binding of the other substrates.

Although the kinetic mechanism was characterized, discrepancy between the data from initial velocity and isotope exchange studies raised some doubts as to whether the enzyme followed both an alternate ping-pong and sequential pathway. In addition the order of product release was not clearly defined. When analyzing the kinetic mechanism, Kosaka *et al.*, (1971, 1977) monitored enzyme activity by a sensitive radioactive assay which measured the production of [^{14}C]-NaMN from [^{14}C]-nicotinate. However, to accomplish an even more detailed kinetic analysis than done previously using this assay method would be quite time consuming. Subsequently, Hanna and Sloan (1981) developed a high pressure liquid chromatography (HPLC) assay which was able to follow the disappearance of substrates and the appearance of products, simultaneously. In addition, the stoichiometry between ATP cleavage and NaMN formation could be determined with ease. Thus, this assay offered advantages over the radioactive assay in that one could monitor the enzyme's activity over a fairly short period of time without the use of radioactive materials. A detailed description of this radioactive and hplc assay is presented in the Methods and Results section.

Using this HPLC assay and partially purified NaPRTase, Hanna and Sloan (1980) were able to investigate the kinetic mechanism in greater detail. Double reciprocal plots of the inital velocity *vs.* PRibPP concentration at different concentrations of ATP, demonstrated inhibition of the enzyme at high concentrations of PRibPP and at all concentrations of ATP. The greatest extent of inhibition occurred with low

concentrations of ATP, suggesting that PRibPP competes with ATP for the ATP binding site. Furthermore, at low concentrations of PRibPP, the lines formed a parallel pattern , which indicated the possibility of a ping-pong step, which provided additional evidence for the formation of a phosphorylated enzyme intermediate from ATP. These preliminary studies were subsequently repeated and extended using purified NaPRTase from a new and improved procedure (Hanna *et al.*, 1983). Initial velocity studies of the rate of NaMN formation over a series of concentrations of one substrate, at fixed values of the second substrate, and maintaining the third substrate constant, gave four intersecting and two parallel patterns on double reciprocal plots. Two of the patterns were similar to a couple of the patterns obtained by Kosaka *et al.*, (1977), which again suggested a sequential mechanism. However, in order to fully characterize the kinetic mechanism of a three-substrate reaction, and allow simplification of the calculation of the kinetic constants, initial velocity measurements should be performed over a series of concentrations of one substrate over fixed concentration ratios of the other two substrates (Rudolph and Fromm, 1979). These studies were carried out, and are discussed in the Methods and Results section.

Product inhibition studies with pyrophosphate revealed that pyrophosphate inhibited ATP action, noncompetitively, while towards nicotinate and PRibPP, PPi acts as an uncompetitive and competitive inhibitor, respectively. These patterns were similar to the product inhibition patterns observed for the kinetic mechanism of chicken liver pyruvate decarboxylase (Barden *et al.*, 1972), and suggested first that ATP bound to the enzyme at a different site from nicotinate and PRibPP, and second, that the binding of nicotinate and PRibPP was ordered.

Isotope exchange studies were performed in order to determine whether the mechanism was sequential or ping-pong, and the order of substrate addition. Exchange experiments between the substrate/product pairs [^{14}C]-nicotinate/NaMN and [^{32}P]-PPi/PRibPP was not observed in the absence of the other substrates which also

suggested that nicotinate and PRibPP bind sequentially to the enzyme. An exchange of label between [^{14}C]-ADP and ATP was determined, and these studies are described in the Methods and Results section. In addition, flow dialysis binding studies demonstrated that enzyme preincubated with ATP and Mg^{2+}, did not bind [^{14}C]-nicotinate, suggesting that PRibPP binds first to the phosphorylated enzyme followed by the addition of nicotinate.

An interesting feature of this enzyme is its ability to hydrolyze ATP, independently of its capacity to function as a phosphoribosyltransferase. When enzyme solution containing ATP and Mg^{2+}, was incubated with PRibPP, production of ADP was not observed. However, incubation of the enzyme solution with NaMN alone, NaMN and nicotinate, or PPi alone promoted ATP hydrolysis. These results indicated that NaMN or PPi could bind to the phosphorylated enzyme, thereby allowing release of phosphate from the enzyme. The addition of PRibPP to the enzyme incubation mixtures containing either product inhibited the ATPase activity of the enzyme. Nicotinate had no effect on the product-induced ATPase activity. This study demonstrated that PRibPP, but not nicotinate, could bind to the phosphorylated enzyme, which strengthened our previous hypothesis deduced from the initial velocity studies, that binding of PRibPP and nicotinate was sequential. In addition, these results indicate that release of the products NaMN and PPi is random, which is then followed by phosphate. Furthermore, complexes of Cr(III)-PPi, but not Cr(III)-ATP (which was found to be a competitive inhibitor of the reaction with respect to ATP), had the ability to promote ATP hydrolysis.

The key role of ATP in the catalytic mechanism of yeast, *E. coli*, and *B. subtilis* NaPRTase has been open to various discussions. Imsande and Prestige (1964) proposed that the function of ATP may be to serve as a control point by which NAD synthesis can be linked to energy metabolism. Interestingly, studies conducted by Micheli *et al.*, (1986) in human erthrocytes (in which ATP acts as an allosteric

modifier) have demonstrated that inhibition of the production of nicotinic acid nucleotides by adenine was due to its conversion into ATP, which in turn (depending on the nicotinate concentration) inhibited NaPRTase. These results have suggested that ATP plays an important role in the synthesis of NaMN. This observation together with the finding that PRibPP exerted a noncompetitive inhibition on NaPRTase (Micheli *et al.*, 1985) inferred that the synthesis of pyridine nucleotides may be controlled by the cellular energy level (Micheli *et al.*, 1986).

In comparison to the NaPRTase from *Astasia longa* (Kahn and Blum, 1968) in which the reaction is independent of ATP, the obligatory hydrolysis of ATP renders the yeast NaPRTase reaction essentially irreversible which shifts the equilibrium of the reaction in the direction of pyridine nucleotide synthesis (Kosaka *et al.*, 1971, 1977). A possible consequence of this effect is an increased role in the utilization of the salvage pathway of which NaPRTase constitutes a part (Kosaka *et al.*, 1971).

On a thermodynamic level, Honjo *et al.*, (1966) speculated that the energy derived from ATP hydrolysis could be utilized to form the high energy pyridine-nitrogen ribose bond of NaMN. However, this hypothesis may be disputed on the grounds that ATP is not a required substrate for *Astasia longa* (Kahn and Blum, 1967), or bovine liver (Smith and Gholson, 1969).

Other phosphoribosyltransferases that utilize ATP, in addition to yeast NaPRTase are the salvage pyridine nucleotide enzyme mentioned earlier, nicotinamide phosphoribosyltransferase (NAmPRTase), and adenosine triphosphate phosphoribosyltransferase (ATP-PRTase, EC 2.4.2.17). As stated previously, NAmPRTase catalyzes a reaction similar to NaPRTase using nicotinamide as the pyridine base. Earlier work on the enzyme demonstrated that ATP was required for activity in erythrocytes (Dietrich *et al.*, 1966) and Ehrlich ascites cells (Dietrich *et al.*, 1965). However, in rat liver enzyme, ATP can be replaced by sufficiently high concentrations of MgPRibPP (Powanda *et al.*, 1969). Studies by Sloan *et al.*, (1984)

have detected NAmPRTase activity in yeast, which appears to be ATP-independent. In contrast, ATP-PRTase utilizes ATP to form a component of the main product, phosphoribosyl-ATP. Interestingly, in comparison to NaPRTase, this enzyme was once thought to form a phosphoribosyl-enzyme covalent intermediate (Bell and Koshland, 1970). However, studies by Morton and Parsons (1976) demonstrated that the enzyme forms a tightly bound E-PRib-ATP complex.

ATP-utilizing enzymes encompass a vast group which can be divided into two main categories: the kinases which catalyze the transfer of a phosphate group from a donor to an acceptor molecule, and ATPases, in which the free energy derived from ATP hydrolysis is linked to a metabolic function. Some metabolic processes driven at the expense of ATP are: oxidative phosphorylation in the mitochondria catalyzed by F1 ATPase (Senior and Wise, 1983; Vignais and Satre, 1984), the maintenance of specific ion gradients such as those found in $(Na^+ + K^+)$-ATPase (Dahl and Hokin, 1974) and the $(Ca^{2+} + Mg^{2+})$-ATPase of sarcoplasmic reticulum (Hasselbach, 1978), and rough endoplasmic reticulum (Imamura and Schulz, 1985). In addition, two rather unique functions: DNA degradation by the double-stranded DNA exonuclease V enzyme (Telander Muskavitch and Linn, 1981), and the supercoiling of relaxed -duplex DNA by the topoisomerase DNA gyrase (Gellert, 1981), are both coupled to ATP hydrolysis.

The unique capacity of NaPRTase to catalyze an enzymatic reaction at the expense of ATP, is shared by only a few other enzymes. Three representative enzymes that possess this capability are ATP citrate lyase [EC 4.1.3.8], succinyl - CoA synthetase [EC 6.2.1.6], and pyruvate carboxylase [EC 6.4.1.1]. ATP citrate lyase, as it's name implies belongs to a class of enzymes known as lyases which cleave C-C, C-O, C-N, and other bonds in a way that does not require hydrolysis or oxidation (Spector, 1982a). This enzyme catalyzes the first step of lipogenesis in the cytosol of mammalian tissues (Houston and Nimmo, 1984):

$$\text{ATP + citrate + CoA} \xleftrightarrow{Mg^{2+}} \text{acetyl CoA + oxaloacetate + ADP + PI}$$

Kinetic analysis of the enzyme first suggested a sequential pathway in which MgATP bound to the enzyme first, followed by the random order addition of Mg-citrate and CoA (Plowman and Cleland, 1967). However, isotope-exchange studies between [^{14}C]-ADP and ATP in the absence of citrate and CoA, together with the isolation of a phosphorylated enzyme (after incubation of the enzyme with [γ-^{32} P]-ATP) indicated the presence of an alternate ping-pong pathway (Plowman and Cleland, 1967). These results were reminiscent of the kinetic mechanism proposed for yeast NaPRTase by Kosaka *et al.* (1971,1977). These experiments were performed on ATP citrate lyase before it was known that the enzyme was prone to proteolytic degradation during the purification procedure (Singh *et al.*, 1976).

In order to resolve the discrepancy between the results of the kinetic and isotope-exchange studies, subsequent investigations were carried out on undegraded ATP citrate lyase (Houston and Nimmo, 1984). The data from these studies indicated that the enzyme followed a double displacement mechanism, in which ATP bound first to form a phosphorylated enzyme and the release of ADP, prior to random addition of Mg-citrate and CoA. This enzyme can be phosphorylated in response to insulin and glucagon (Alexander *et al.*, 1979). A comparison of the kinetic properties of phosphorylated and unphosphorylated ATP citrate lyase revealed that the phosphorylated form had a considerably higher Km for ATP than the unphosphorylated form, although no other kinetic differences were evident between the two forms (Houston and Nimmo, 1985).

Succinyl-CoA synthetase and pyruvate carboxylase constitute a class of enzymes known as ligases (or synthetases) the function of which is to make covalent

bonds at the expense of ATP (Spector, 1982b). Therefore, the enzymatic reactions in this class usually involve three substrates. Succinyl-CoA synthetase catalyzes the following reaction:

$$\text{ATP + succinate + CoA} \xrightleftharpoons{Mg^{2+}} \text{ADP + PI + succinyl-CoA}$$

Kinetic studies revealed that succinyl-CoA synthetase acts through a sequential mechanism, in which ATP is bound to the enzyme first followed by random binding of coenzyme A and succinate to form a quaternary complex, prior to the release of products (Moffet and Bridger, 1970). Although this enzyme follows sequential kinetics a phosphorylated enzyme was isolated (Kreil and Boyer, 1964), along with succinyl phosphate which was noncovalently bound to the enzyme (Nishimura and Meister, 1965). However, these forms of the enzyme do not exist independently. In addition, there is suggestive evidence for the formation of a succinyl-enzyme (Benson *et al.*, 1969). ATP-ADP exchange is observed with this enzyme, however, this partial reaction is relatively slow, suggesting that this reaction is not evidence of a catalytic intermediate (Bridger *et al.*, 1986). In the presence of substrates expecially succinyl-CoA, the exchange rate increases. This phenomenon in which the rate of a partial reaction is accelerated by the addition of other substrates is known as substrate synergism. This effect demonstrated in succinyl-CoA synthetase is in contrast to the decrease of ATP-ADP exchange detected in yeast NaPRTase, in the presence of PRibPP and/or nicotinate described previously (Kosaka *et al.*, 1977, Hanna *et al.*, 1983).

The third enzyme to be discussed is pyruvate carboxylase which catalyzes the conversion of pyruvate and HCO_3^- into oxaloacetate which is coupled to the hydrolysis

of ATP (McClure *et al.*, 1971; Spector, 1982). The reaction can be divided into two partial reactions:

$$\text{E-biotin} + \text{ATP} + HCO_3^- \xrightarrow[\text{acetyl CoA}]{Mg^{2+}} \text{E-biotin-}CO_2 + \text{ADP} + \text{Pi} \quad (1)$$

$$\text{E-biotin-}CO_2 + \text{pyruvate} \xrightarrow[\text{acetyl CoA}]{Mn^{2+}} \text{E-biotin} + \text{oxaloacetate.} \quad (2)$$

Net:

$$\text{MgATP} + HCO_3^- + \text{pyruvate} \quad <===> \quad \text{MgADP} + \text{Pi} + \text{oxaloacetate}$$

Biotin, which is covalently linked to a lysine residue is situated between two subsites, and functions as a spacer arm shuttling a carboxyl group between the two flanking subsites (Spector, 1982b). Isotope exchange was observed between ATP and ADP, and ATP and Pi, and these partial reactions depended on the presence of acetyl CoA which functions as an allosteric activator, and Mg^{2+}, HCO_3^- , and K^+. The exchange of label between pyruvate and oxaloacetate only partially depended on acetyl CoA and cations. A net exchange of label was also observed betwen HCO_3- and oxaloacetate, but only at chemical equilibrium.

As mentioned earlier, a phosphorylated enzyme intermediate was detected for both succinyl-CoA synthetase and ATP citrate lyase. Although a phosphoenzyme intermediate was not detected in pyruvate carboxylase, the observed ADP-ATP exchange in the absence of all other substrates except Mg^{2+}, suggested the possibility of its existence. In the case of succinyl-CoA synthetase, a phosphohistidine residue

was isolated from the phosphorylated intermediate (Kreil and Boyer, 1964), Studies with ATP citrate lyase have demonstrated that the phosphate group is bound to a γ-carboxyl group of a specific glutamyl residue to form enzyme-glutamyl phosphate (Suzuki *et al*, 1969). Subsequent studies revealed that citrate also binds to the γ-carboxyl group of a glutamyl residue as in the phosphorylated enzyme (Suzuki,1971). This evidence taken together suggested that the high energy produced by ATP hydrolysis is retained in the linkage between the phosphate group (derived from ATP) and the γ-carboxyl group of the glutamyl residue, thereby producing the high energy enzyme-glutamyl-phosphate intermediate. Thereafter, citrate becomes bound to the enzyme through the same γ-carboxyl group of the glutamyl residue, to form an acid anhydride linkage, which yields a second intermediate, enzyme-glutamyl-citrate, prior to release of phosphate. Finally, experiments suggest that oxaloacetate is split off the second intermediate to form E-acetyl CoA (Suzuki *et al.*, 1969). Therefore, the high energy derived from ATP is reserved and passed down through the bonds between phosphate and the enzyme, citrate and the enzyme, and acetate and the enzyme, where this energy is finally transferred to acetyl CoA.

Similarly, the chemical reactions of both succinyl-CoA synthetase, and pyruvate carboxylase, which result in the activation of succinate to form E-succinyl-phosphate, and the carboxyl group to form E-CO_2-phosphate, respectively, both proceed through the formation of a phosphorylated enzyme derived from the hydrolysis of ATP (Spector, 1982b). Thus, as hypothesized for the ATP citrate enzyme, these enzymes conserve the energy of the terminal pyrophosphate bond of ATP by transferring it through linkages of successive reactive intermediates. Fig.3A,B, and C are the proposed chemical mechanisms for ATP citrate lyase, succinyl-CoA synthetase, and pyruvate carboxylase, respectively.

In order to elucidate the mechanism of an enzymatic reaction at the molecular level, it is necessary to first establish the kinetic mechanism, and then to ascertain

FIGURE 3. Proposed Chemical Mechanism for ATP-Citrate Lyase (A), Succinyl-CoA Synthetase (B), and Pyruvate Carboxylase (C).

$$E + ATP \xleftrightarrow{Mg^{2+}} E\sim P + ADP$$

$$E\sim P + citrate \longleftrightarrow E\cdots citryl\sim P$$

$$E\cdots citryl\sim P \longleftrightarrow E\sim citryl + P_i$$

$$E\sim citryl \longleftrightarrow E\sim acetyl + oxaloacetate$$

$$E\sim acetyl + CoA \longleftrightarrow E + acetyl\sim CoA$$

$$ATP + E \xleftrightarrow{Mg^{2+}} E\sim P + ADP$$ } Phosphotransferase

$$E\sim P + succinate \longleftrightarrow E\cdots succinyl\sim P$$ } Phosphotransferase

$$E\cdots succinyl\sim P \longleftrightarrow E\sim succinyl + P_i$$ } Succinyltransferase

$$E\sim succinyl + CoA \longleftrightarrow E + succinyl\sim CoA$$ } Succinyltransferase

$$\text{Sum: } ATP + succinate + CoA \longleftrightarrow ADP + P_i + succinyl\sim CoA$$

$$ATP + E \xleftrightarrow[acetyl\text{-}CoA]{Mg^{2+}} E\sim P + ADP$$ } Phosphoryltransferase

$$E\sim P + bicarbonate \xleftrightarrow[acetyl\text{-}CoA]{Mg^{2+}} E\cdots carboxyl\sim P$$ } Phosphoryltransferase

$$E\cdots carboxyl\sim P \xleftrightarrow[acetyl\text{-}CoA]{Mg^{2+}} E\sim CO_2 + P_i$$ } Carboxyltransferase

$$E\sim CO_2 + pyruvate \xleftrightarrow[acetyl\text{-}CoA]{Mn^{2+}} E + oxaloacetate$$ } Carboxyltransferase

$$\text{Sum: } ATP + bicarbonate + pyruvate \longleftrightarrow ADP + P_i + oxaloacetate$$

the role that various amino acids would play in binding and/or catalysis at the active site.

For this thesis research, initial velocity measurements and ATP-ADP exchange studies were accomplished, and combined with data from other kinetic and isotope exchange studies previously done in our laboratory, in order to clearly define the kinetic mechanism of NaPRTase. Thereafter, substrate analogs of nicotinic acid, 3-pyridylacetate and nicotinaldehyde were investigated in order to determine their effect on NaPRTase activity. Finally, chemical modification with PLP (a modifier of lysine residues) and pH dependency studies were initiated to determine specific amino acids that are essential for the catalytic mechanism of the enzyme.

MATERIALS AND METHODS

Materials

Pressed Baker's yeast (Budweiser brand) was purchased from Valente Yeast, Inc., Flushing, NY. ATP (disodium salt), ADP (sodium salt), nicotinic acid, NaMN (acid form), PRibPP (sodium salt), nicotinaldehyde, orotic acid, pyridoxal 5'-phosphate, 3 pyridylacetate, POPSO, Trizma base, ammonium phosphate, and ammonium sulfate were purchased from Sigma Chemical Co., St. Louis, MO. Sodium pyrophosphate and magnesium chloride were obtained from Baker Chemical Co., Phillipsburg, NJ. CHES and PIPES were supplied by Calbiochem, Inc., La Jolla, CA. Potassium phosphate (mono-and dibasic salts), and ammonium acetate were supplied by Fisher ScientificCo., Fairlawn, NJ. Sodium borohydride was obtained from Aldrich Chemical Co., Milwaukee, WI. Phosphocellulose was purchased from Brown Co., Berlin, NH. Hydroxylapatite, protein assay kit, and reagents for SDS gel electrophoresis (excluding acrylamide and bis-acrylamide) including molecular weight standards were supplied by Biorad Laboratories, Richmond, CA. AcrylaGel, Bis-AcrylaGel, and Hydrofluor were obtained from National Diagnostics, Somerville, NJ. [4-^{3}H]-PLP was a generous gift from Dr. Jack Preiss, Dept. of Biochemistry, Michigan State University, East Lansing, MI. [7-^{14}C]-nicotinic acid (free acid), [^{32}P]-ADP, Protosol and Econofluor were products of New England Nuclear, Boston, MA. [^{3}H]-sodium borohydride was purchased from Amersham, Arlington Heights, NJ. All other reagents were analytical grade.

Enzyme Purification

NaPRTase was partially purified according to the procedure of Hanna and Sloan (1983) with some modifications.

Batch Procedure. Baker's yeast (20 lbs) was suspended in 6 liters of .3 M phosphate buffer at pH 8.0 and 1 liter toluene at 30°C, and stirred for 3 1/2 hours. The pH was periodically adjusted to pH 8.0 with 5 N KOH, and then allowed to stand overnight at 4°C. The suspension was centrifuged at 12,000 rpm for 15 min (thereby removing cellular debris and lipids), and the supernatant (9.2 liters) was collected. The autolysate was adjusted to pH 5.0 with 8 N acetic acid (.760 liter), followed by the addition of a few drops of octanol (antifoaming agent). The acidified autolysate was brought to 50% saturation with ammonium sulfate, and the suspension was allowed to stand overnight at 4°C. The protein precipitate collected from the 0-50% ammonium sulfate cut, was dissolved in a minimum volume of 10 mM phosphate buffer at pH 8.0 and dialyzed against 20 liters of the same buffer overnight. In order to remove nucleic acid materials from this preparation, a final concentration of 50 mM $MgCl_2$ (.086 liter) was added to the dialyzed supernatant (1.625 liters), stirred for 30 min and centrifuged. The pH of the supernatant collected was adjusted to pH 6.0 with 2 M sodium acetate buffer at pH 6.0, followed by the addition of orotic acid (.66 gram, to protect OPRTase). Each liter of supernatant (1.927 liters) was brought to 50% saturation with 1 liter of 95% ethanol (precooled to -26°C), and centrifuged. The precipitate collected was dissolved in a minimum volume of 5 mM phosphate buffer at pH 6.0, and dialyzed against 20 liters of the same buffer overnight at 4°C. Thereafter, this dialyzed suspension was centrifuged and the supernatant collected (1.3 liters).

Phosphocellulose Column.The extract was applied to a phosphocellulose column equilibrated with 5 mM phosphate buffer, and washed with the same buffer to remove any unbound protein from the column. NaPRTase, but not OPRTase was retained by the

column. Subsequently, NaPRTase was eluted with 40 mM phosphate buffer at pH 6.7, followed by a 1M KCl wash. Active fractions were pooled (.270 liter) and brought to 70% saturation with ammonium sulfate. The precipitate was collected and dissolved in a minimum volume of 5 mM phosphate buffer at pH 7.5 and dialyzed against 12 liters of the same buffer overnight.

Hydroxylapatite Column I and II

At this stage of the purification, some modifications were made because previous attempts at purifying the enzyme according to the established procedure provided unsatisfactory yields of the enzyme. The dialyzed solution (.035 liter) was incubated with 2.0 mM PRibPP, 1.0 mM $MgCl_2$, and 5.0 mM nicotinate at 25°C for 15 min (to convert any phosphorylated enzyme back to its native form), and then applied to a hydroxylapatite column (25 x 3 cm), equilibrated with 5 mM phosphate buffer at pH 7.5. After washing the column with the same buffer, NaPRTase was eluted with .5 M phosphate buffer at ph 7.5. Active fractions were pooled and dialyzed against 4 liters of 5 mM phosphate buffer at pH 7.5. The dialyzed enzyme solution (.047 liter) was incubated again with PRibPP, $MgCl_2$, and nicotinate and reapplied to the hydroxylapatite column equilibrated with 5 mM phosphate buffer at pH 7.5. Subsequently, NaPRTase was eluted with a 5-100 mM phosphate linear gradient (pH 7.5). Active fractions were pooled, and dialyzed in 20 l of 5 mM phosphate buffer at pH 7.5, and stored at -76°C. The volume of the dialyzed enzyme was 20 ml.

Protein Determination

Solution protein concentrations were determined using the Bio-Rad protein assay developed by Bradford (1976).

Radioactive Assay

Enzyme activities for the initial stages of the purification procedure were

determined by measuring the production of [^{14}C]-nicotinic acid mononucleotide (NaMN) from [^{14}C]-nicotinic acid according to the method of Kosaka *et al.*, (1971) with some modifications. The standard reaction mixture (.2 ml at 37°C) contained 5 mM $MgCl_2$, 1 mM ATP, 1 mM PRibPP, 1 mM [^{14}C]-nicotinic acid (.5mCi/mmole), and enzyme in 50 mM Tris-phosphate buffer at pH 8.0. The reaction was terminated in a boiling water bath after 15 min.

Using a capillary tube, aliquots were spotted 15 times at the origin of 1.5 x 13 inch strips of Whatman No.1 filter paper and developed by descending chromatography in 1 M ammonium acetate buffer at pH 5.0, 95 % ethanol (3:7 v/v) for approximately 4 hours. [^{14}C]-NaMN remained close to the origin, whereas [^{14}C]-nicotinic acid migrated further to the front (approximately 6-7 cm). Each strip was cut into 8 pieces (1 cm each), which were placed in a vial with 4 ml of Hydrofluor, and then counted with a Tracor 6800 Analytical Scintillation Counter. The activity was determined as the % counts of NaMN/total counts which was then expressed in units of µmoles NaMN formed/min.

HPLC Assay

The later stages of purification of NaPRTase and the following studies employed a standard HPLC assay developed by Hanna and Sloan (1980). This assay required the use of a Waters HPLC instrument supplied with a model 6000A solvent delivery system, model U6K sample injector, model 440 absorbance detector, and a Houston Omniscribe recorder. A 4 mm x 30 cm Waters µBondapak C_{18} column was connected between the solvent system and the detector. When not in use, the column was stored in 10% methanol.

The assay mixture contained 5.0 $MgCl_2$, 1.0 mM ATP, 1.0 mM PRibPP, and 1.0 mM nicotinic acid in 50 mM Tris-phosphate buffer at pH 8.0. The reaction was initiated with the addition of enzyme (volume and specific activity is included for each

experiment), followed by incubation at 37°C. At varying time intervals, aliquots of the incubation mixture were withdrawn and placed in a boiling water bath for 2 min, in order to terminate the reaction. Thereafter, each sample was filtered through a .45-µm HA-type Millipore filter. Sample volumes of 5-20 µl were injected (using a Hamilton 801 microliter syringe) onto the C_{18} column, equilibrated with 50 mM ammonium phosphate buffer at pH 6.0. The base nicotinate and the nucleotides NaMN, ATP, ADP were eluted from the column at a flow rate of 1.0 ml/min, and were detected at 254 nm (0.02-0.10 sensitivity setting). The authors determined that a direct relationship exists between peak height and concentrations of reactants and products. Therefore, values for the peak height (cm) of NaMN were converted to NaMN concentrations, from a graph plotting peak height *vs.* known concentrations of this nucleotide. The enzyme activity was then determined by calculating the change in concentration of NaMN (µM) with time. The units of activity are expressed either as µmolarity of the solution of NaMN formed/min or µmoles NaMN formed/min. A typical analysis is shown in Fig. 4 and Fig. 5.

Initial Velocity Studies

Initial velocities were determined for a series of concentrations of one substrate over a fixed concentration ratio of the other two substrates. The incubation mixture (4.94 ml) contained 50 mM Tris-phosphate buffer, pH 8.0, 5 mM $MgCl_2$, and10-60 µM ATP, at fixed micromolar Na-to-PRibPP ratios. The reaction (37°C) was initiated with the addition of 60 µl of enzyme (specific activity 4.4 µmoles/min/mg), and 1 ml aliquots were removed at time intervals of 1, 3, 5, and 10 min. The above experiment was repeated with PRibPP concentrations ranging from 4-30 µM at fixed micromolar ATP-to-Na concentration ratios, and with Na concentrations ranging from 12-40 µM at fixed micromolar ATP-to-PRibPP ratios. All of the fixed concentrations ratios are described in the legend to Fig. 6. The HPLC

FIGURE 4. Elutions of a Stock Solution of ATP, ADP, Nicotinate, and NaMN Through a μBondapak C_{18} Column Using 25 mM Ammonium Phosphate Buffer over a Range of pH Values. Stock solutions of each reactant were used to assign the peaks. The HPLC elution conditions were: 5 μl sample injection volumes, 0.7 ml/min flow rate.

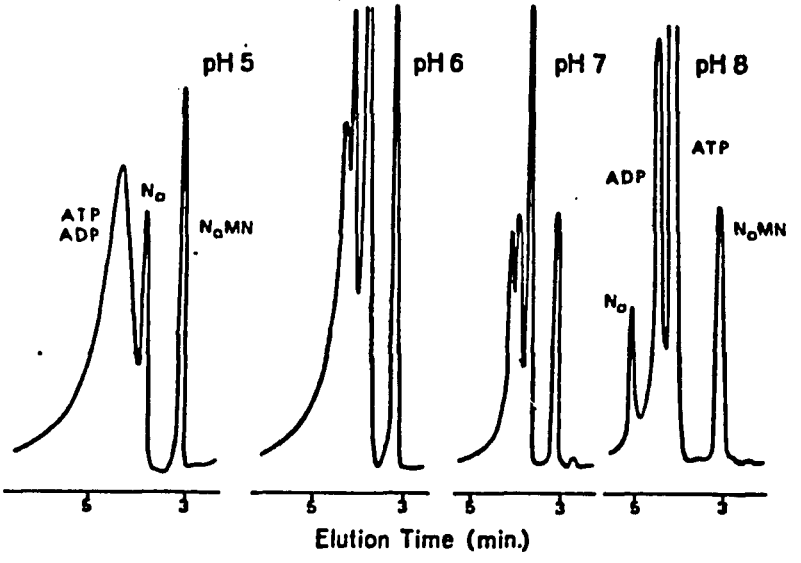

pH 5
pH 6
pH 7
pH 8
ATP
ADP
N_a
N_aMN
5
3
Elution Time (min.)

FIGURE 5. HPLC Elution Profile of Nicotinate Phosphoribosyltransferase Assay Solution. NaPRTase (25 µl, 4 mg/ml) was incubated with assay mixture containing 5 mM $MgCl_2$, 100 µM nicotinate, 75 µM ATP, 30 µM PRibPP, in 50 mM Tris-HCl (pH 8.0). At appropriate time intervals (0-15 min) aliquots of this mixture were injected onto a Waters μC_{18} reversed phase column using an isocratic 25 mM ammonium phosphate elution buffer (pH 8.0).

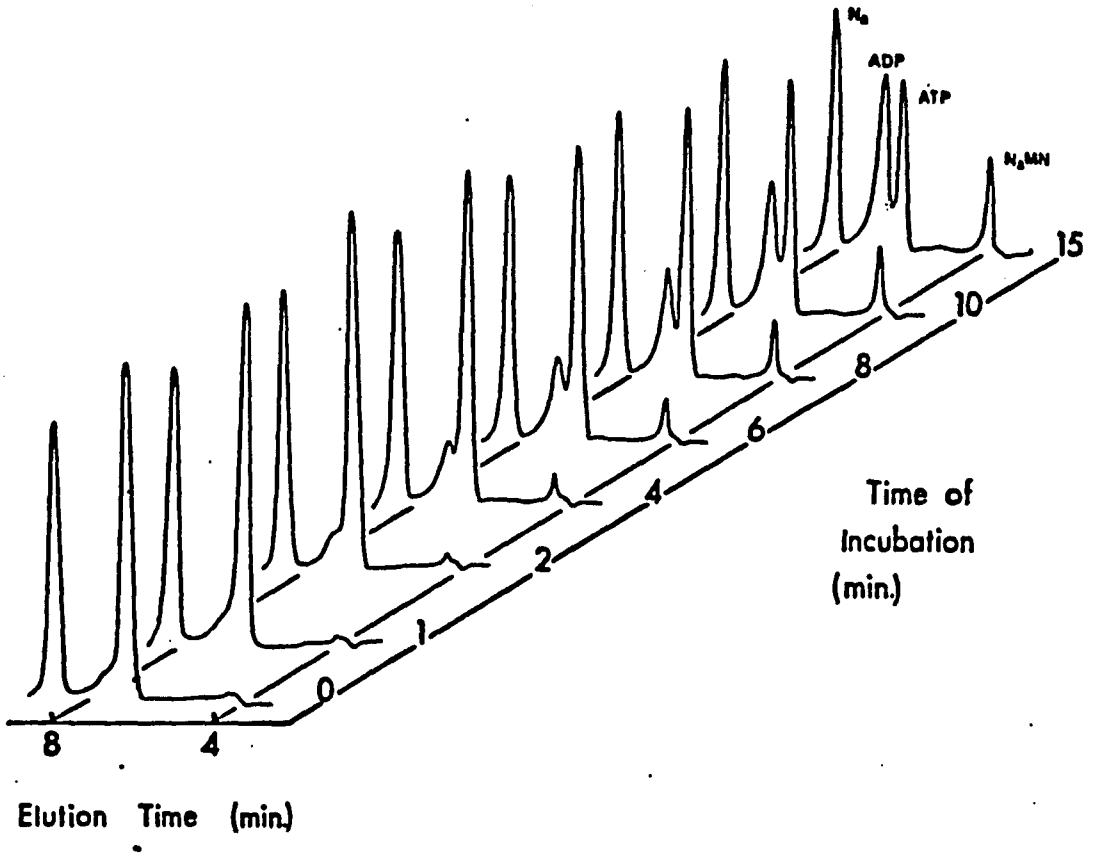
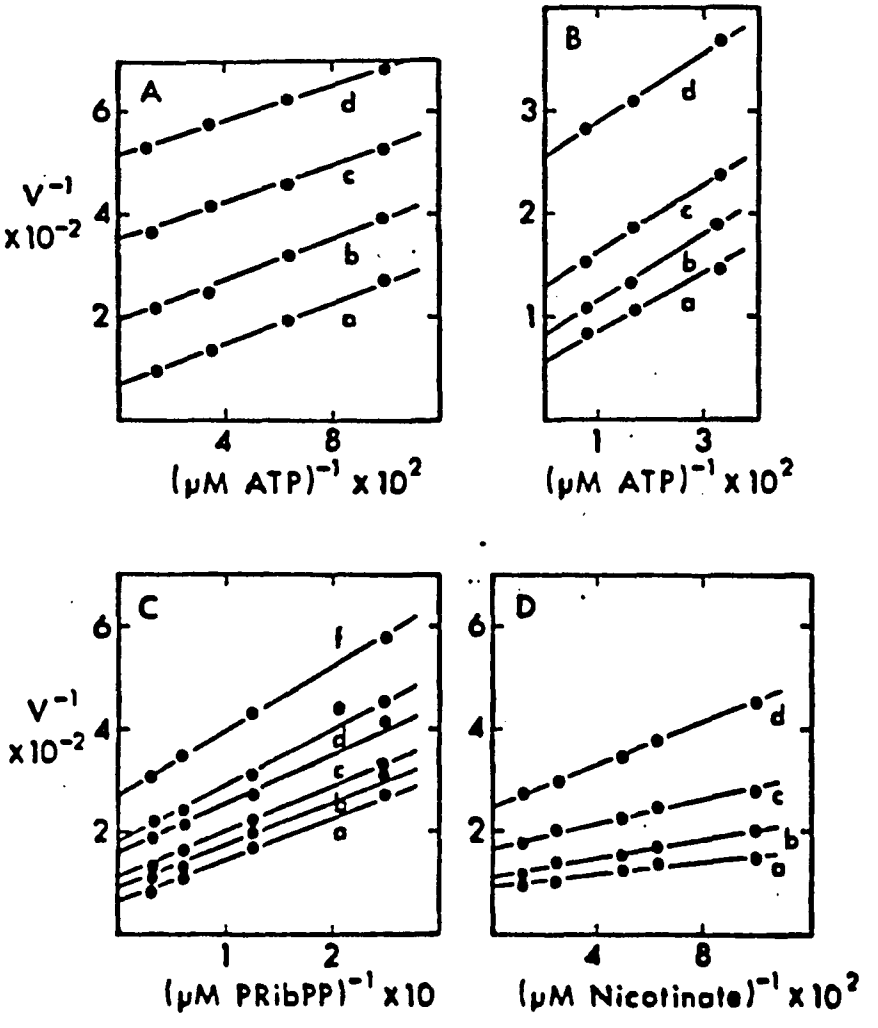

N_a
ADP
ATP
N_aMN
15
10
8
6
4
2
1
0
Time of
Incubation
(min.)
8
4
Elution Time (min.)

FIGURE 6. Initial Velocity (V) Double Reciprocal Plots of the Rate of NaMN Formation *versus* One Substrate Concentration in the Presence of Constant Ratio Concentrations of the Other Substrates. A, V^{-1} *versus* $[ATP]^{-1}$. The micromolar nicotinic acid-to PRibPP ratios employed were 120:24 (a), 60:12 (b), 24:4 (c), and 10:2 (d). B, V^{-1} *versus* $[ATP]^{-1}$. The micromolar nicotinate-to-PRibPP ratios employed were 160:32 (a), 80:16 (b), 40:8 (c), and 24:4 (d). C, V^{-1} *versus* $[PRibPP]^{-1}$. The micromolar ATP-to-nicotinate ratios employed were 240:16 (a), 120:80 (b), 60:40 (c), 30:20 (d), 24:16 (c), and 18:12 (f). D, V^{-1} *versus* $[nicotinate]^{-1}$. The micromolar ATP-to-PRibPP ratios employed were 240:32 (a), 120:16 (b), 60:8 (c), and 30:5 (d).


A
B
C
D
V^{-1} x 10^{-2}
(μM ATP)$^{-1}$ x 10^2
(μM ATP)$^{-1}$ x 10^2
(μM PRibPP)$^{-1}$ x 10
(μM Nicotinate)$^{-1}$ x 10^2
a
b
c
d
f

elution conditions were: 25 mM ammonium phosphate buffer, pH 6.0,1 ml/min flow rate, and 25 µl sample injection volume.

Exchange of [^{32}P]-ADP into ATP

The assay mixture (4.94 ml) contained 50 mM Tris-phosphate buffer, pH 8.0, 5 mM $MgCl_2$, 100 µM [^{32}P]-ADP (42.6 mCi/mmole), and 500, 100, or 20 µM ATP, in the absence or presence of either 100 µM PRibPP or 100 µM PPi. The reaction was initiated with the addition of 60 µl of enzyme (specific activity 4.4 µmoles/min/mg), and 1 ml aliquots were withdrawn at time intervals of 3, 5, 10, and 15 min. Thereafter, each sample was injected onto an Analytical Sepralyte quaternary amine ion exchange column which yielded a complete separation of ATP and ADP using a 0.2 M ammonium phosphate buffer, pH 2.7. Fractions eluting from the column containing ATP and ADP were collected, dissolved in 4 ml Hydrofluor, and the radioactivity was determined with a Tracor 6800 Analytical Scintillation Counter.

The above experiment was repeated in the absence of [^{14}C]-ADP in order to determine the overall rate of the reaction. The activity was determined using the standard HPLC assay procedure. HPLC elution conditions were: 35 mM ammonium phosphate buffer, pH 4.9,1 ml/min flow rate, and sample injection volume of 25 µl.

Studies with Pyridylacetate

Preliminary studies were initiated to examine the effect of this nicotinic acid analog 3-pyridylacetate on the enzymatic reaction. To test for substrate specificity, 10 µl of NaPRTase (specific activity, 1.7 µmoles/min/mg) was incubated with assay mixture (1.109 ml) containing 50 mM Tris-phosphate buffer, pH 8.0, 5 mM $MgCl_2$, 500 µM ATP, 500 µM PRibPP, and 6 mM pyridylacetate at 37°C. At time intervals of 1, 2, 3, 5, and 10 min, 200 µl aliquots were removed, and the enzyme activity was measured. The same experimental conditions as described above were employed to

test for inhibition of the enzyme by pyridylacetate, by including 40 μM nicotinate in the assay mixture. The presence of nicotinate without pyridyl acetate in the assay mixture served as a control. The HPLC elution conditions were: 50 mM ammonium phosphate buffer, pH 6.0; 1ml/min flow rate, and 5 μl sample injection volume.

Kinetic Analysis with Nicotinaldehyde

To test for substrate specificity, 10 μl of NaPRTase (specific activity, 1.2 μmoles/min/mg) was incubated with 0.19 ml standard assay mixture containing either 1 mM nicotinaldehyde or 1 mM nicotinate at 37°C for 50 min. The standard assay mixture containing 1 mM nicotinaldehyde in the absence of enzyme served as a control. Aliquots of 5 μl were injected onto the C_{18} column equilibrated with 50 mM ammonium phosphate buffer, pH 6.0 with 5% acetonitrile (gradient 20 min-program 10).

To test the possibility of nicotinaldehyde irreversibly inhibiting the reaction, 100 μl of enzyme (specific activity 1.7 μmoles/min/mg) was incubated with 10 μl of 5 mM phosphate buffer, pH 6.0, prior to removing a 20 μl aliquot at zero time. The remaining enzyme mixture (90 μl) was reacted with 1 μl of a final concentration of 10 mM nicotinaldehyde, at 25°C. At time intervals of 5, 10, 20, and 60 min, 20 μl aliquots were withdrawn and added to 480 μl of standard assay mixture, and incubated at 37°C for 60 min. The above experiment was repeated twice with the addition of 1 mM ATP to enzyme (in place of phosphate buffer), prior to the addition of 1 and 10 mM nicotinaldehyde. Sample volumes of 25 μl were then injected onto the μBondapak column equilibrated with 25 mM ammonium phosphate buffer, pH 6.0.

To test for reversible inhibition, 100 μl of NaPRTase (specific activity 1.7μmoles/min/mg) was incubated (37°C) with 2.5 ml of standard assay mixture (1.2 mM in place of 1 mM nicotinate) in the absence and presence of 4.2 mM nicotinaldehyde. Assay mixture in the absence of enzyme served as a control. At

time intervals of 5, 10, 15, 30, and 60 min, 0.5 ml aliquots were removed, and the enzyme activity was determined. HPLC conditions were the same as stated for the irreversible inhibition experiments.

Time Dependent Inactivation by PLP

A 50 µl volume of NaPRTase (specific activity 1.7 µmoles/min/mg) in 5 mM phosphate buffer at pH 6.0, was incubated with 0- 0.5 mM PLP at 25°C, in a final volume of 100 µl. In order to minimize photodegradation of PLP and pyridoxyl-P derivatives, the room lights were shut off, and the incubation, reduction, and assay mixtures were stored in metal foil. At time intervals ranging from 5-60 min, a 10 µl aliquot was withdrawn from the mixture, and reacted with 190 µl of standard assay mixture at 37°C for 60 min. The experiment was repeated with 5 mM PLP, followed by $NaBH_4$ reduction. The residual enzyme activity was determined by HPLC as described previously. Sample volumes of 5 µl were injected onto a C_{18} µBondapak column equilibrated with 50 mM ammonium phosphate buffer, pH 6.0 at a flow rate of 1 ml/min.

Concentration Dependence Inactivation by PLP

A 10 µl aliquot of NaPRTase (specific activity 1.6 µmoles/min/mg) was incubated with 30 µl of 0.025-2.5 mM PLP at room temperature in the presence of 50 mM phosphate buffer at pH 8.0. After 30 min, a 10 µl aliquot of a final concentration of 40 mM $NaBH_4$ in 50 mM phosphate buffer (pH 8.0)was added to the reaction mixture, and then maintained on ice for 15 min. The final pH of the pyridoxyl-P-enzyme (reduced inactivated enzyme) was 8.5, which falls in the pH range where the enzyme is stable. Following reduction, an assay mixture (final volume, 0.2 ml) containing 5 mM $MgCl_2$, 500 µM ATP, 500 µM PRibPP, and 500 µM nicotinate in 50 mM Tris-phosphate buffer at pH 8.0 was incubated with the reduced sample at 37°C

for 15 min. The residual enzyme activities were monitored using HPLC. The HPLC conditions are the same as described in the previous experiment.

Reactivation of PLP-Treated NaPRTase using Flow Dialysis

NaPRTase (100 µl, specific activity 1.6 µmoles/min/mg) in 5.0 mM phosphate buffer at pH 7.0 was incubated with 5 mM PLP at room temperature for 60 min. The above experiment was repeated, followed by reduction of the pyridoxal-P-enzyme with 8 mM $NaBH_4$, which was maintained on ice for 15 min. A10 µl aliquot was removed, and reacted with standard assay mixture at 37°C for 60 min. Subsequently, the residual enzyme activity was determined.

Thereafter, a Technilab (Fisher Scientific) flow dialysis apparatus first described by Colowick and Womack (1969), was employed to determine the extent of reactivation of the pyridoxal-P-enzyme and its reduced form. The inactivated enzyme (90 µl) was dialyzed against 50 mM phosphate buffer at pH 8.0. Aliquots of 10 µl were withdrawn at different time intervals, and assayed for residual activity as described earlier.

In addition, a preliminary study of the enzyme in the presence of PLP without $NaBH_4$ was performed as described above, except that flow dialysis took place in the light. The HPLC elution conditions were the same as described in the previous experiment.

pH Dependence of Inactivation by PLP

A 10 µl volume of NaPRTase was incubated with 10 µl of .1M buffer at pH 5.1-9.1, in the absence and presence of 25 µM PLP, at room temperature for 30 min (buffers used throughout this pH range are described in the pH stability study in "Methods"). A 1 µl volume of a final concentration of 5 mM $NaBH_4$ was added to the incubation mixture, and maintained on ice for 15 min. Subsequently, a 10 µl aliquot

was withdrawn and incubated with reaction mixture containing 50 mM Tris-phosphate buffer at pH 8.0, 5 mM $MgCl_2$, 300 µM ATP, 300 µM PRibPP, and 300 µM nicotinate at 37°C in a final volume of 0.6 ml. The HPLC elution conditions were: 50 mM ammonium phosphate buffer, pH 6.5, 1 ml/min flow rate and 20 µl sample injection volume.

Protection of NaPRTase by Substrates

The enzyme (10 µl, specific activity 1.6 µmoles/min/mg) was incubated with 125 µM PLP in the absence and presence of each of the substrates (5 mM ATP, 5 mM PRibPP, 5 mM nicotinate, and 25 mM $MgCl_2$) separately or in combination with each other, at room temperature for 30 min, in a final volume of 40 µl. This was followed by reduction with 10 µl of a final concentration of 40 mM $NaBH_4$ as described earlier. In order to maintain the same concentration of substrates in the final assay mixture for all samples, the substrate incubated with enzyme and PLP was omitted from the assay mixture. For example, in the sample containing enzyme, PLP, and ATP, no additional ATP was added to the assay mixture. The final concentrations of each substrate in the assay mixture was 1 mM ATP, 1 mM PRibPP, 1 mM nicotinate, and 5 mM $MgCl_2$ in 50 mM Tris-phosphate buffer, pH 8.0. A volume of 150 µl of this mixture was added to each reduced incubation mixture, and incubated at 37°C for 15 min. The residual enzyme activity was measured by the HPLC procedure. The HPLC elution conditions were the same as described for the reactivation experiment.

Kinetic Studies of Substrate Protection by ATP

Reaction mixtures (1.0 ml) containing 50 mM Tris-phosphate buffer at pH 8.0, 5 mM $MgCl_2$, 60-540 µM ATP, in the presence and absence of 250 and 500 µM PLP, and enzyme (5 µl, specific activity 1.6 µmoles/mg) were incubated at room temperature for 5 min. Subsequently, 5 µl of 500 µM PRibPP-nicotinate was added to

the mixture to initiate the enzyme reaction, and incubated at 37°C. At time intervals of 3 and 5 min, 200 µl aliquots were withdrawn from the sample and the initial velocity of the reaction was determined. The HPLC elution conditions were: 50 mM ammonium phosphate buffer, pH 6.0, 1 ml/min flow rate and 7.5 µl sample injection volume.

Kinetic Studies of Substrate Protection by PRibPP

Reaction mixture (1.0 ml) containing 50 mM Tris-phosphate buffer at pH 8.0, 5.0 mM $MgCl_2$, varying concentrations of PRibPP (15-140 µM), , and enzyme (5 µl) were incubated in the absence and presence of 60 and 120 µM PLP at room temperature for 5 min. A final concentration of 500 µM ATP-nicotinate was added to initiate the reaction, which was then assayed following the conditions described above for ATP. The HPLC elution conditions were: 50 mM ammonium phosphate buffer, pH 6.0, 1 ml/min flow rate, and 75 µl sample injection volume.

Study of the Comparison of Inactivation by Pyridoxal 5'-Phosphate and Pyridoxal.

A 10 µl volume of NaPRTase (specific activity 1.7 µmoles/min/mg) was incubated with 100 and 500 µM of pyridoxal 5'-phosphate and pyridoxal at room temperature for 30 min, followed by reduction with 1 µl of a final concentration of 20 mM $NaBH_4$. A 10 µl aliquot was withdrawn from the pyridoxyl-enzyme, and incubated at 37°C with standard assay mixture containing 5 mM $MgCl_2$, 200 µMATP, 200 µM PRibPP, 200 µM nicotinate, in 50 mM Tris-phosphate at pH 8.0 in a final volume of 0.5 ml. Aliquots of 0.1 ml were withdrawn at 3 and 5 min. and the initial velocity of the reaction was determined. The HPLC elution conditions were: 50 mM ammonium phosphate buffer, pH 6.5, 1 ml/min flow rate, and 20 µl sample injection volume.

Stoichiometry of PLP inactivation using [^{3}H]-Sodium Borohydride

In order to determine the number of moles of PLP bound to the enzyme the procedure of Uyeda *et al.*, (1985) was followed with some modifications. A 10 µl aliquot of the enzyme (specific activity 1.6 µmoles/min/mg) was reacted with 30 µl of PLP ranging from 0.023 mM-2.44 mM in the absence and presence of substrates at room temperature for 30 min. Subsequently, the Schiff base was reduced with 40 µl of a final concentration of 20 mM [^{3}H]- sodium borohydride (3.93 x 10^4 dpm/nmole), and placed on ice for 15 min. The enzyme reduced with $NaBH_4$ in the absence of PLP served as the control. Thereafter, the ^{3}H-labeled protein was precipitated with 1 ml of cold 10% TCA, prior to the addition of 50 µl of BSA (1 mg/ml, added for nonspecific binding to filter), and each sample was left to stand under the hood for 1 1/2 hours. Each sample was transferred onto glass filters (#31, 25 mm-Schleicher and Schuell) to which a glass chimney was attached to a filter flask. In order to make a quantitative transfer, each sample tube was rinsed with 1 ml of cold 10% TCA 4 times, and each wash was transferred to the glass filter. In order to eliminate a high background count, each filter was then washed 13 times with 10 ml of cold 10% TCA, followed by 10 ml of 95% ethanol. Each filter was transferred to a paper towel, air dried, and incubated in 0.5 ml of Protosol at 37°C overnight, to allow solubilization of protein from the filter. Scintillation fluid (5 ml-Econofluor) was added to each solubilized filter, followed by the addition of 50 ul of glacial acetic acid (to prevent chemiluminescence). The radioactivity was determined with a Tracor Analytic Scintillation Counter.

In order to correlate the number of moles of PLP bound/mole enzyme with the amount of enzyme activity remaining in each sample, the experiment was repeated under the same conditions except that the Schiff base was reduced with cold $NaBH_4$. Each reduced sample was incubated with 120 ul of assay mixture containing 5 mM $MgCl_2$, 1 mM ATP, 1 mM PRibPP, and 1 mM nicotinate in 50 mM Tris-phosphate

buffer (pH 8.0), at 37°C for 15 min and the residual enzyme activity was determined. The HPLC elution conditions were: 50 mM ammonium phosphate, pH 6.5, 1 ml/min flow rate, and 5 µl sample injection volume.

[^{3}H]-PLP Labeling of NaPRTase

The enzyme (15 µl, specific activity 1.2 µmoles/min /mg) in 5 mM phosphate buffer at pH 7.8 was incubated with 10 µl of [^{3}H]- (PLP) (2.2 mM, specific activity 3.24 x 10^5 dpm/nmole) for 1 hour, in the dark, at room temperature, in the absence and presence of either 22.2 mM MgATP and/or 22.2 mM MgPRibPP. Subsequently, the [^{3}H]-PLP-enzyme complex was reduced with 50 mM $NaBH_4$ and placed on ice in the dark for 15 min. Control samples containing only enzyme were also treated with and without $NaBH_4$.

Incorporation of [^{3}H]-PLP in NaPRTase, using the procedure described by Morell *et al.*, (1986) was measured by subjecting the reduced [^{3}H]-PLP enzyme to sodium dodecyl sulfate polyacrylamide disc gel electrophoresis (SDS PAGE) according to the method of Laemmli (1970), with the modifications of a 7.5% separating gel, and boiling of each prepared sample for 3 min. The gels were then fixed in 50% TCA overnight, stained for 1 hour at 37°C with .1% Coomassie blue in 50% TCA, and destained in 7% acetic acid. Thereafter, the gels were scanned at 600 nm using a VICON Linear Gel Scanner (Brinkmann Instrument , Inc., Westbury, New York), and then cut into 2 mm wide slices using a Mickle Gel Slicer (Brinkmann Instrument, Inc.). Each slice was dissolved in 6 ml of Econofluor (New England Nuclear) containing 5% Protosol at 37°C overnight, and the radioactivity in each sample was counted as described previously.

As stated previously in the purification procedure, NaPRTase could not be purified to homogeneity, therefore other protein bands were present in the sample gels in addition to the NaPRTase protein band. In order to determine the identity of the

NaPRTase band and quantitate the protein present exclusively in that band, SDS PAGE molecular weight zebra standards (containing the proteins lyzozyme-14,400, soybean trypsin inhibitor-21,500, carbonic anhydrase-31,000, ovalbumin-42,699, bovine serum albumin-66,200, and phosphorylase-97,400) were run at 4 different concentrations (2-15 µg) on SDS PAGE along with the radioactive samples as described above. A plot of log molecular weight vs. mobility for the molecular weight standards at each concentration yielded a straight line. By determining the mobility of each protein band present in each radioactive sample the molecular weight of each band could be extrapolated from this graph.

Using this method I determined that NaPRTase (M.W. 44,000) was represented by protein peak 1 (*vida infra*) although there was a slight uncertainty that peak 2 may be NaPRTase. Protein bands 1 and 2 were quantitated in relationship to known concentrations of a standard protein according to a modified method of Hochberg and Wolfson (1979). For example, the peak representing the molecular weight protein standard ovalbumin at 4 concentrations , and protein peak 1 and 2 (of the gel scan) from each radioactive sample were cut out, weighed, and the ovalbumin peak weights were plotted *vs.* concentrations of ovalbumin. Because the zebra molecular weight standards containing equal concentrations of protein do not always absorb Coomassie blue with the same intensity, there is variation in the absorbance recorded for each protein peak at 600 nm. To minimize the error in quantitating protein peaks 1 and 2, this procedure was also applied to 3 of the other molecular weight standards. Therefore 4 protein concentration values were estimated for both peaks 1 and 2.

The slight discrepancy present in assigning either peak 1 or 2 to NaPRTase was resolved by evidence presented below. As shown in in "Results", there was a marked decrease in [^{3}H]-PLP labeling in protein peak 1 when PRPP was present, which was not evident in protein peak 2. Moreover, when quantitating the number of moles of PLP bound to each protein peak in the presence and absence of substrate, the results

obtained with peak 1 strongly supported data derived from the previous protection studies. For these reasons the final calculations of the number of moles of PLP bound to NaPRTase are represented only for peak 1.

pH Stability Studies

Incubations of 10 µl NaPRTase (specific activity, 1.7 µmoles/min/mg) in 5 mM phosphate buffer, pH 7.8, with 10 µl of .1 M buffer were performed through the pH range 4.1-9.6., on ice for 30 min. The buffers were selected on the basis of having a pKa value approximately within 1 pK unit from the pH being studied, displaying minimal interference with the HPLC elution profile, and exerting no inhibition on enzymatic activity. These buffers were: potassium acetate, pKa 4.75(pH 4.0-6.2), PIPES, pKa 6.8 (pH 6.2-7.7), POPSO, pKa 7.8 (pH 7.6-8.3), and CHES, pKa 9.5 (pH 8.3-9.6). Thereafter, the reaction (37°C) was initiated with the addition of a 10 µl aliquot of the incubation mixture to assay mix (final volume 0.5 ml) containing 50 mM Tris-phosphate, pH 8.0, 5 mM $MgCl_2$, 500 µM ATP, 500 µM nicotinate, and 100 µM PRibPP. At time intervals of 3 and 5 min, 100 µl aliquots were withdrawn and the initial enzyme activity was determined. The HPLC elution conditions were: 50 mM ammonium phosphate buffer, pH 6.5,1 ml/min flow rate, and 20 µl sample injection volume.

Kinetic Analysis of NaPRTase pH Dependence- ATP Concentration Variation

The pH dependence of the enzyme was investigated to assess the pKa values of enzyme groups that may be involved in catalysis and/or binding of ATP. The reaction (37°C) was initiated with the addition of 10 µl enzyme (specific activity, 1.7 µmoles/min/mg) to assay mixture (final volume 0.5 ml) containing 50 mM buffer (pH 4.9-8.5), 5 mM $MgCl_2$, 40 µM PRibPP, 240 µM nicotinate and 60-240 µM ATP. The buffers used were: potassium acetate (pH 4.9, 5.2, and 5.4), PIPES (pH 6.2, and 7.1),

POPSO (pH 7.6), and CHES (pH 8.2, and 8.5). It should be noted that above pH 8.5, precipitation in the assay mixture occurred possibly due to Mg^{2+} complexing with phosphate at a higher pH. Therefore, the alkaline pH range above 8.5 could not be examined. At time intervals of 3 and 5 min, 100 µl aliquots were removed, and the initial enzyme activity was determined. The HPLC elution conditions were the same as described in the above experiment.

Kinetic Analysis of NaPRTase pH Dependence-PRibPP Concentration Variation

The same experimental conditions as described above for ATP were employed, except the assay mixture contained 50 mM buffer (pH 4.8-8.6), 5 mM $MgCl_2$, 240 µM ATP, 240 µM nicotinate and 15-75 µM PRibPP.

RESULTS

Enzyme Purification

Table 1 summarizes the total protein, total activity, and specific activity for the column chromatography steps involved in the purification procedure. The results obtained for the autolysis, ammonium sulfate and ethanol fractionation are the same as illustrated in Hanna *et al.*, (1983). The enzyme eluted off the second hydroxylapatite column had a specific activity value of 1.6 units/mg, which is lower than that achieved previously by Kosaka *et al.*, (1971), and in this laboratory (4.4 units/mg). In view of the time constraint, I elected to use the enzyme preparation at this stage of the purification for the chemical modification and pH dependency studies. The initial velocity measurements and isotope exchange studies were performed using a previously purified enzyme preparation (specific activity 4.4 units/mg.) from this laboratory.

HPLC Elution Profile Varying pH and Enzyme Incubation Time

Fig. 4 illustrates an HPLC elution profile over a range of pH values. Maximum separation of reactants and products occured at pH 8.0, although a complete separation of NaMN from ATP, ADP, and Na was achieved at pH 6.0. The column material is fairly unstable at pH 8.0, therefore, enzyme activity was usually monitored at pH 6.0. The HPLC elution profile is also represented at varying enzyme incubation times (Fig. 5). As the time of incubation of the enzyme increases, the height of the NaMN and ADP peaks increase concomitant with a decrease in the ATP and nicotinate peak heights.

Kinetic Analysis of NaPRTase

In order to clearly define the kinetic mechanism for a three substrate reaction, the procedure described by Segal (1975) and Ruldolph and Fromm (1979) was employed , which involved measuring the initial velocities of the reaction for a series

Table 1. Purification of NaPRTase from Baker's Yeast. [a]

	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)
Phosphocellulose Chromatography	399	98	.25
Hydroxylapatite I	263	122	.46
Hydroxylapatite II	68	106	1.56

[a] Typical yield after purification was approximately 5%. One unit of activity is equal to 1 µmol NaMN formed/min.

of concentrations of each substrate, at a fixed concentration ratio of the other two substrates.

Fig. 6, A and B, represent double reciprocal plots of the concentration of ATP *vs.* the rate of formation of NaMN over fixed concentration ratios of Na-to-PRibPP. These two plots represent kinetic studies performed with two different enzyme preparations, over two ATP concentration ranges. Computer analysis of the data was carried out using an adaptation of the HYPER program described by Cleland (1979) for an Apple II computer. Dr. Rosalyn Strauss of the Biomedical Department of the City College of New York accomplished the conversion of FORTRAN to BASIC language. Lines a-d of Fig. 6A are parallel (uncompetitive pattern) with slopes equaling (x 10^{-3}) 1.94 ± 0.1, 2.0 ± 0.6, 2.09 ± 0.4, and 1.97 ± 0.3. The same slope (3.5 x 10^3 + 0.6 x 10^3) was established for lines b-d of Fig. 6B. Secondary plots (data not shown) of the y-intercepts of Fig. 6, A and B vs. PRibPP or nicotinate were linear within experimental error.

An analysis of variance was performed on the data, which was described by Cleland (1979) as the square of the differences between the experimentally determined velocities, and the theoretical velocities determined from a specific rate equation. This value is then divided by the number of degrees of freedom. The units of σ^2 are the velocity measurements squared, and the units of σ are equal to the velocity measurements and to Vmax (umoles of NaMN formed/min/mg). The values of σ^2 , and σ determined for the data represented in Fig.6, A and B were 2.3 x 10^{-2} $(units/mg)^2$ and 0.15 unit/mg (4.4 % of Vmax), respectively. These results suggested that ATP hydrolysis involved a ping pong step.

Fig. 6C shows a double reciprocal plot of the concentrations of PRibPP vs. the rate of formation of NaMN over fixed concentration ratios of ATP-to-nicotinate. Lines a-f yielded consecutively increasing slopes (x 10^{-3}) with values of 0.85 ± 0.3, 0.92 ± 0.04, 0.93 ± 0.06, 1.00 ± 0.07, 1.08 ± 0.04, and 1.27 ± 0.07, which intersect far

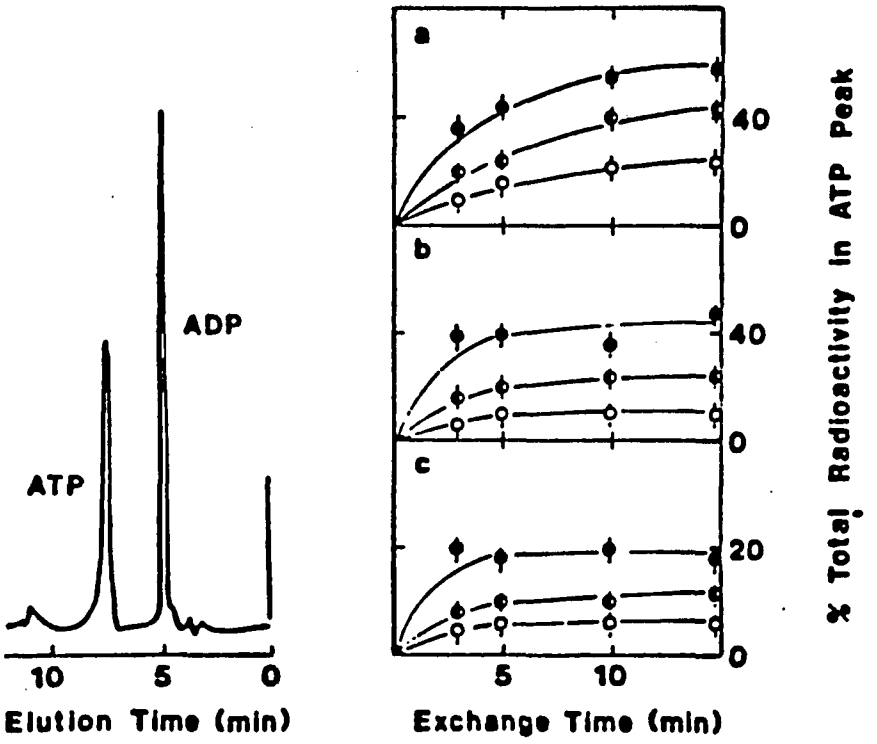
the left of the y axis (noncompetitive pattern). σ^2 and σ values were determined to be equal to 1.4 x 10^{-2} (units/mg)^2 and 0.12 units/mg (3.5 % of Vmax), respectively.

Fig. 6D illustrates a double reciprocal plot of the concentrations of nicotinate *vs.* the reaction rate over fixed concentration ratios of ATP-to PRibPP. The family of lines a-d intersect with slopes (x 10^{-3}) equal to 0.63 ± 0.02, 0.73 ± 0.11, 1.17 ± 0.05, and 1.98 ± 0.20. The σ^2 and σ values were determined to be 7 x 10^{-3} (units/mg)^2 and 0.083 units/mg (2.1 % of Vmax), respectively. Taken together, the results in Fig. 6 suggest a sequential mechanism which involves a single ping-pong step.

Isotope Exchange Studies

Isotope exchange between [^{14}C]-ATP and ADP in NaPRTase was previously observed by Kosaka *et al.*, (1971). These exchange studies were repeated using [^{14}C]-ADP and in addition, the effect of PPi on the exchange rate was determined. Fig. 7 A illustrates the HPLC elution profile for the complete separation of ATP and ADP using a Sepralyte quaternary amine ion exchange column. As shown in Fig. 7 B, the total radioactivity present in the ATP peak at different exchange times was decreased in the presence of PRibPP, and PPi. The data in Table 2 , shows that the initial rates of exchange of label were approximately three times faster than the initial rate of the overall reaction. This result, together with the initial velocity studies, supported the idea that the binding of ATP to the enzyme involved a ping-pong step. The presence of either PRibPP or PPi in the incubation mixture inhibited the rate of exchange. This inhibition by PRibPP was interpreted as being due to substrate inhibition. Previous studies by Hanna and Sloan (1980) demonstrated that inhibition of the reaction occurred at relatively high concentrations of PRibPP at all concentrations of ATP. This indicated that PRibPP competes with ATP for the ATP binding site, in addition to binding to its own site on the enzyme. Cleland (1977) suggested that the

FIGURE 7. (A) Elution Profile of a Solution of 100 μM ATP and 100 μM ADP. Stationary phase: Analytichem Sepralyte quaternary amine ion-exchange column. Mobile phase: 50 mM ammonium phosphate (pH 2.7). HPLC conditions: 10 μl sample injection volume, 1 ml/min flow rate. (B) Time Course for the NaPRTase-Catalyzed Exchange of [^{14} C]-ADP (100 μM) Label into ATP in the Absence (a) and Presence of PPi (b) and PRibPP (c). Concentrations of ATP used were 20 μM (open circles), 100 μM (half-closed circles), and 500 μM (closed circles).


ATP
ADP
10
5
0
Elution Time (min)
A
a
b
c
40
0
40
0
20
0
5
10
Exchange Time (min)
B
% Total Radioactivity in ATP Peak

rate of exchange between a substrate/product pair could be inhibited or activated by other substrates in a two site enzymatic mechanism. The inhibition of the exchange rate in the presence of PPi could be explained in terms of ATPase activity of the enzyme, which is stimulated by the addition of PPi. A decrease in the amplitude of the ATP peak concomitant with an increase in the amplitude of the ADP peak was also observed. Thus new equilibrium concentrations of the reactants were achieved, the values of which could alter the exchange-rate of the reaction (Cleland, 1977).

Effect of 3-pyridylacetate on NaPRTase Activity

When the nicotinic acid analog 3-pyridylacetate (6 mM) was examined for its ability to catalyze the NaPRTase reaction, the formation of product was not observed, indicating that this compound was not phosphoribosylated by the enzyme. Subsequent examination of the analog's inhibitory capability, revealed that this compound exerted no effect on the enzyme's activity, and thus may not have been bound.

Effect of Nicotinaldehyde on NaPRTase Activity

When the enzyme was incubated in an assay mixture containing nicotinaldehyde in place of nicotinic acid (in order to test for this compound's specifcity in the reaction), no enzyme activity was observed. This analog also showed no inhibitory effect when the same assay conditions were employed in the presence of nicotinic acid. These results again suggested that the presence of an ionizable carboxyl group on nicotinic acid may be important for binding, by interacting with nearby amino acid residues at the active site of the enzyme. Furthermore, no inhibitory effect was observed when nicotinaldehyde was preincubated with enzyme, in the presence and absence of ATP, prior to addition to the assay mixture. Although the preliminary results were negative, a more detailed analysis should be carried out of the reversible inhibition of NaPRTase in the presence of lower concentrations of nicotinate (closer to

TABLE 2. Calculated Nicotinate Phosphoribosyltransferase-Catalyzed Rates of Exchange (V*) of Radioactivity between [^{14} C]ADP and ATP. Incubation mixtures contained 5 mM $MgCl_2$, 0.1 μM enzyme, 50 mM Tris-Cl (pH 8), 100 μM [^{14} C]ADP, and one of the below-described ATP concentrations.

Conditions	V*		
	500 μM ATP	100 μM ATP	20 μM ATP
	μM [^{14} C]ATP/min		
No additions	14.5[a]	11.5	8.0
+ 100 μM PPi	9.0	6.0	4.2
+ 100 μM PRibPP	5.0	2.6	2.0

[a] Initial rates of the nicotinate phosphoribosyltransferase-catalyzed formation of NaMN (micromolar product/min) using 100 μM PRibPP, 125 μM nicotinate, 5 mM MgCl2, 0.1 μM enzyme, and the concentrations of ATP described above (500, 100, and 20 μM) were equal to 5.2, 3.8, and 3.0, respectively.

the K_m value). In addition,an irreversible inhibition experiment should be performed, which would involve preincubation of the enzyme with ATP, PRibPP, and Mg^{2+}, prior to the addition to assay mixture (containing nicotinic acid and buffer).

PLP Inactivation of NaPRTase as a Function of Time

Incubation of NaPRTase with concentrations of PLP ranging from 0.5-5mM resulted in a concentration-dependent decrease in the catalytic activity. In each incubation, the enzyme activity rapidly decreased within 2 min, and then approached an equilibrium value which exhibited some fluctuation at prolonged incubation time (2-60 min). This biphasic effect of PLP on an enzyme was previously studied by Chen and Engel (1975), who proposed that PLP inactivation proceeded through the following mechanism, in which the initial, reversible, noncovalent binding is rapid (E • PLP), followed by a slower, reversible, covalent binding (Schiff base- E- PLP).

$$E + PLP \underset{K_{-1}}{\overset{K_1}{<===>}} E \text{ pyridoxal 5'-phosphate } (E \bullet PLP) \underset{K_{-2}}{\overset{K_2}{<===>}} E'\text{-PLP}$$

Fluctuations in the equilibrium value attained during PLP inactivation of NaPRTase can be explained in the following manner. Because of the prolonged assay time, reversibility of the Schiff base could be significant and could lead to reactivation of the enzyme upon dilution with the assay solution. Because of this significant reversibility of the Schiff base formation, $NaBH_4$ was added to enzyme incubated with PLP. Anderson *et al.*, (1966), first demonstrated that $NaBH_4$ reduced the Schiff base, thereby rendering the inactivation irreversible. The addition of $NaBH_4$ abolished the fluctuations in the equilibrium value. Therefore, subsequent PLP studies (except kinetic studies) were performed in the presence of $NaBH_4$.

A plot of the log % activity vs. time (0-20 min) of the varying concentrations

of PLP was attempted in order to determine the rate constant k_2 (limiting rate constant) and the k_i value (dissociation constant) for PLP. However, the lines of the graph were nonlinear.

PLP Inactivation followed by $NaBH_4$ Reduction

When NaPRTase was incubated with various concentrations of PLP followed by reduction with $NaBH_4$, there was a concentration dependent decrease in enzyme activity, and almost 100% inactivation was attained with 2.5 mM PLP (Fig.8). The enzyme treated with $NaBH_4$ in the absence of PLP caused no appreciable loss in activity (80% residual activity compared with enzyme alone). This concentration-dependent inactivation with PLP suggested that a lysine residue may be essential for activity at the active site of the enzyme. The reaction representing formation of a Schiff base, followed by its reduction with $NaBH_4$ is shown below.

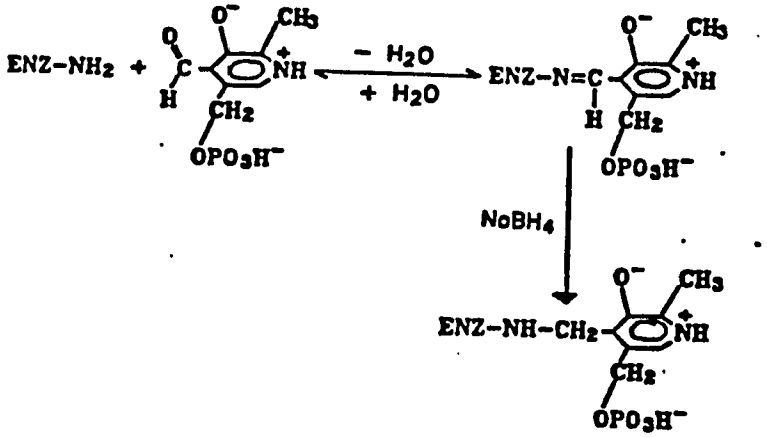
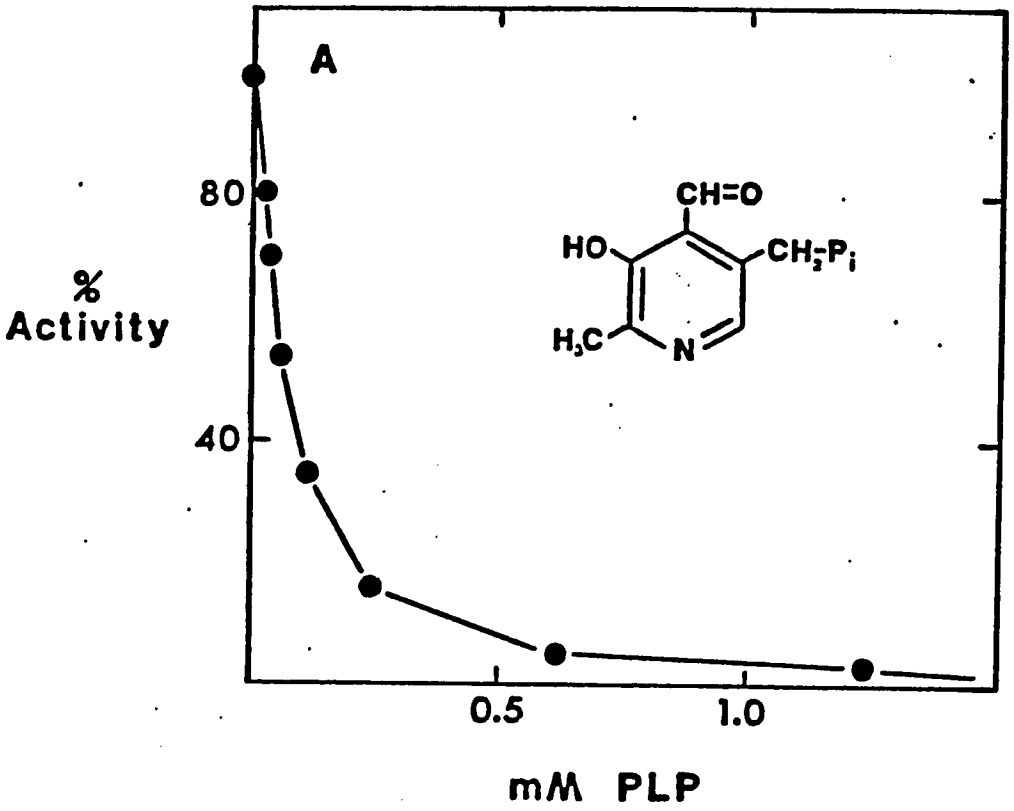


FIGURE 8. NaPRTase Activity as a Function of Pyridoxal Phosphate Concentration. NaPRTase was treated with varying concentrations of PLP for 30 min at room temperature, followed by reduction with $NaBH_4$. The residual activities were then determined as described in "Methods". The activity of the enzyme treated with $NaBH_4$ but without PLP treatment is defined as 100%.


A
80
40
% Activity
0.5
1.0
mM PLP
CH=O
HO
CH₂-Pi
H₃C
N

Reactivation of NaPRTase

The reversibility of PLP inactivation of NaPRTase in the presence and absence of $NaBH_4$ was examined, and the results are presented in Table 3. In the presence of PLP and the absence of $NaBH_4$, the inactivation is completely reversible, and upon flow dialysis there is full recovery of activity after 90 min. In the presence of $NaBH_4$, PLP inactivation of the enzyme was irreversible. This data may be interpreted as the formation of a reversible Schiff base between the aldehylic group of PLP and the ε-amino group of a lysine residue of the enzyme, which upon reduction with $NaBH_4$ becomes irreversible.

When an experiment was performed under the same conditions, with the exception that flow dialysis was carried out in the light, the inactivation by PLP in the absence of $NaBH_4$ was irreversible. This preliminary result suggested the possible involvement of a histidine residue at the active site of NaPRTase. Previous studies (Hathaway and Noltmann, 1977; Rippa *et al.*,1969; Sund *et al* .,1973; Stark *et al.*,1973; Kumaga and Miles,1974), revealed that PLP can act as a photosensitizing agent, rendering the inactivation irreversible. When protein inactivated with PLP is irradiated, the Schiff base between the modifier and the lysine residue undergoes nucleophilic attack by a nearby base, which has been identified as the imidazole ring of a histidine (Hathaway and Noltmann 1977, Hucho *et al.*, 1973). More detailed experiments of the photoinactivation by PLP of NaPRTase should be carried out by future researchers in order to establish if an essential histidine residue is involved in catalysis of this enzyme.

pH Dependence of PLP Inactivation of NaPRTase

The effect of pH on PLP inactivation of NaPRTase was studied, and the results are illustrated in Fig. 9 . In the absence of PLP, the enzyme activity slightly increased as the pH became more alkaline. Analogous results were obtained in the pH stability

TABLE 3. Reactivation of NaPRTase by 5 mM Pyridoxal Phosphate in the Presence and Absence of 8 mM $NaBH_4$.

Conditions	Time of Flow Dialysis (min)	% Residual Activity
Enzyme alone	-----	100
Enzyme + 5 mM PLP	30	59
	90	100
Enzyme + 5 mM PLP + 8 mM $NaBH_4$	30	35
	60	28

NaPRTase was reacted with 5 mM PLP at room temperature for 60 min, followed by reduction with 8 mM $NaBH_4$. The residual activities were determined as described in "Methods". Thereafter, using flow dialysis with 50 mM phosphate buffer, pH 8.0, samples were removed at varying time intervals and the residual activities were once again determined. The activities are expressed as a percentage of the activity of a control sample treated in the same manner but with the omission of PLP.

study of the enzyme (*vida infra*). In the presence of PLP, the degree of inactivation increased with the pH of the incubation mixture. Previously, Rippa *et al.*, (1967) observed that the extent and rate of inactivation of 6-phosphogluconic dehydrogenase by PLP also increased as the pH of the incubation mixture became more alkaline. Means and Feeney (1968) observed that when protein solutions were reacted with aliphatic aldehydes or ketones, followed by $NaBH_4$ reduction, the amino groups were converted into mono-or dialkyl products. This reaction depended on the pH, and at pH 9.0 at 0°C, only modification of amino groups occurred. The major product, formed from reduction of the protein solutions incubated with formaldehyde, was identified as ε-N,N-dimethyl lysine. They concluded from these studies that the pH dependency of formation of alkylated compound was attributed to the stability of $NaBH_4$, and also to the increasing formation of Schiff base. At pH 7.0 this reaction occurred very slowly, while optimal conditions for the reaction were achieved close to pH 10.0. The results obtained with NaPRTase could also be interpreted in a similar manner, in that with increasing pH, the inactivation exerted by PLP increases, which is a reflection of Schiff base formation between a lysine group and PLP, and the stability of $NaBH_4$. The equation below illustrates the above reaction described by these authors.

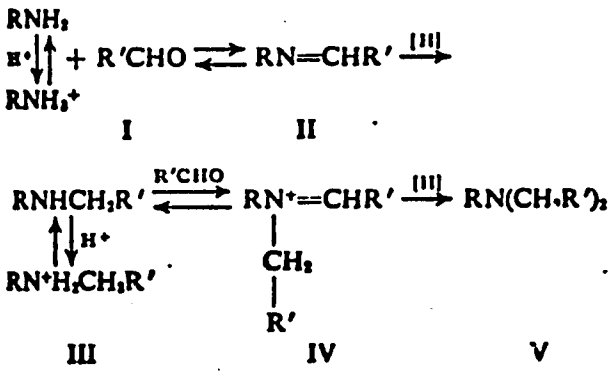
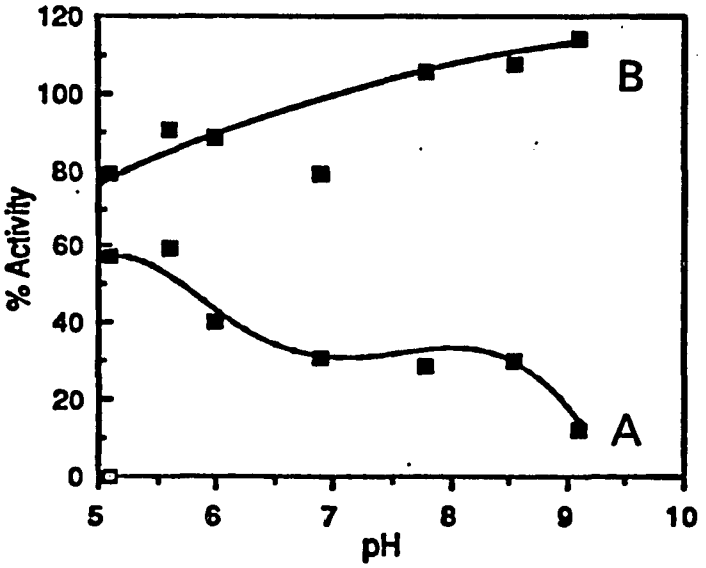


FIGURE 9. PLP Inactivation of NaPRTase as a Function of pH. NaPRTase was incubated in buffer at a pH range from 5.1-9.1 for 30 min, in the presence (A) and, absence (B) of 25 μM PLP, followed by reduction with 5 mM $NaBH_4$. The residual enzyme activities were determined as described in "Methods".


120
100
80
60
40
20
0
% Activity
5
6
7
8
9
10
pH
B
A

Protection of PLP Inactivation by Various Substrates

The effect of substrate protection on NaPRTase inactivation by PLP was investigated, and the results are summarized in Table 4 . ATP, and PRibPP provided some protection against inactivation by 125 μM PLP. Several combinations of reactants (PRibPP + Mg^{2+}, nicotinate + PRibPP + ATP), and a combination of all the substrates provided complete protection. Nicotinic acid was ineffective in preventing inactivation by PLP. Although $MgCl_2$ offered no protection against PLP inactivation, it enhanced the protection afforded by PRibPP, and ATP.

Reversible Inhibition Studies with PLP

Since MgATP and MgPRibPP protect the enzyme against PLP inactivation, the mode of inhibition with respect to both of these substrates was investigated in more detail. Fig.10 shows a double reciprocal plot of the velocity vs. concentration of ATP-Mg^{2+}, in the presence of 0, 250, and 500 μM PLP. The kinetic data was analyzed by a linear regression program using a Texas Instruments TI-53 calculator. The line representing the initial velocity at 500 μM PLP was nonlinear at low ATP concentrations. Therefore, the "best-fit" line was calculated using only the 3 data points at 540, 110, and 80 μM ATP. The intercepts (min^{-1}) of the lines at 0, 250, and 500 μM PLP were equal to 0.05 , 0.06 , and 0.02, respectively, which suggested that within experimental error, this group of lines intersect at a common point on the ordinate axis. This pattern of lines indicated that the inhibition was competitive with respect to ATP.

Fig. 11 represents a double reciprocal plot of the initial velocity vs. concentration of MgPRibPP, in the presence of 0, 60, and 120 μM PLP. This family of lines was analyzed as stated above for ATP. Intercepts (min -1) of 0.19, 0.23, and 0.32 were calculated for the data at 0, 60, and 120 μM PLP, respectively, and suggested that these group of lines intersect far to the left of the y-axis. These

TABLE 4. Substrate Protection of NaPRTase Against PLP Inactivation. [a]

Substrates	% Residual Activity
Control (No PLP)	100
No Substrate Additions	33
5 mM ATP	78 ± 2
25 mM Mg(II)	30 ± 3
5 mM ATP + 25 mM Mg(II)	88 ± 2
5 mM PRibPP	65 ± 5
5 mM PRibPP + 25 mM Mg(II)	102 ± 5
5 mM ATP + 5 mM PRibPP	90 ± 5
5 mM ATP + 5 mM PRibPP + 25 mM Mg(II)	103 ± 3
5 mM Nicotinate	37
5 mM Nicotinate + 25 mM Mg(II)	38 ± 2
5 mM ATP + 5 mM Nicotinate	88 ± 2
5 mM ATP + 5 mM Nicotinate + 25 mM Mg(II)	92 ± 2
5 mM Nicotinate + 5 mM PRibPP	67 ± 3
5 mM Nicotinate + 5 mM PRibPP + 25 mM Mg(II)	60
5 mM Nicotinate + 5 mM PRibPP + 5 mM ATP	102 ± 2
5 mM Nicotinate + 5 mM PRibPP + 5 mM ATP + 25 mM Mg(II)	118 ±12

[a] NaPRTase was preincubated with 125 μM PLP in the presence and absence of various substrates for 30 min at room temperature, followed by reduction with 40 mM $NaBH_4$. The residual activities were then determined as described in "Methods". The activity of the enzyme treated with 40 mM $NaBH_4$ but without PLP is defined as 100%.

FIGURE 10. Initial Velocity (V) Double Reciprocal Plot of the Inhibition of NaPRTase by PLP with ATP as the Variable Substrate. NaPRTase was preincubated for 5 min at room temperature, with varying concentrations of ATP in the presence of the following concentrations (μM) of PLP: 0 (A), 250 (B) and, 500 C). Thereafter the reaction was initiated with the addition of PRibPP and nicotinate. The initial velocities were then determined as described in "Methods".

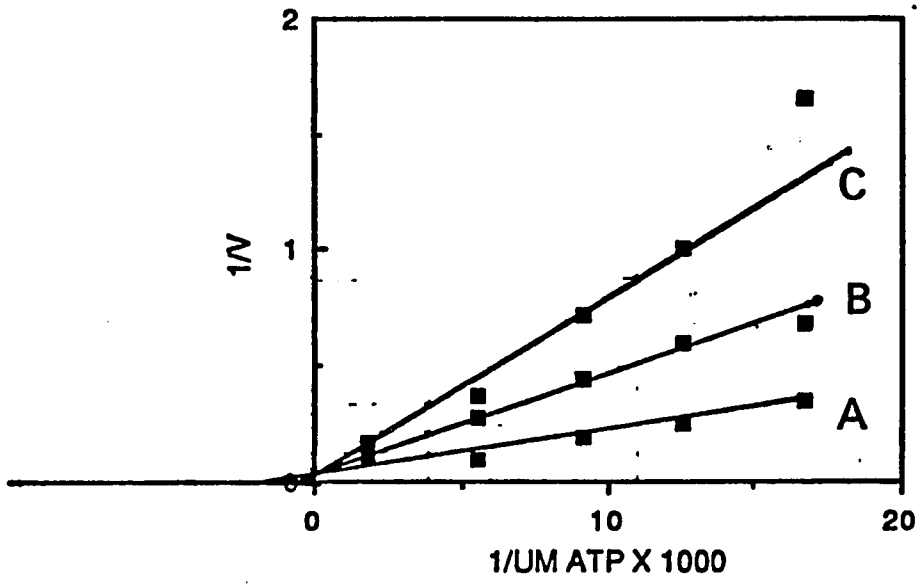
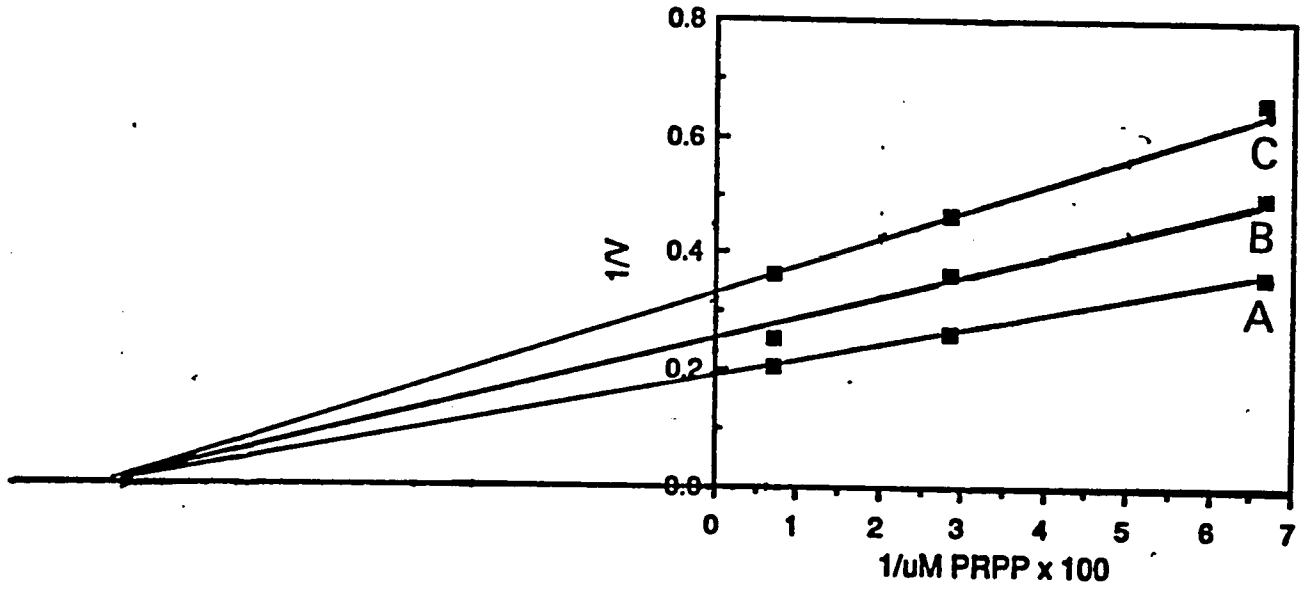

2
1
0
1/V
0
10
20
1/UM ATP X 1000
C
B
A

FIGURE 11. Initial Velocity (V) Double Reciprocal Plot of the Inhibition of NaPRTase by PLP with PRibPP as the Variable Substrate. The enzyme was preincubated for 5 min at room temperature, with varying concentrations of PRibPP, in the presence of the following concentrations (μM) of PLP: 0 (A), 60 (B) and, 120 (C). Thereafter, the reaction was initiated with the addition of ATP and nicotinate. The initial velocities were then determined as described in "Methods".


0.8
0.6
0.4
0.2
0.0
1/V
C
B
A
0
1
2
3
4
5
6
7
1/uM PRPP x 100

results indicate that PLP is noncompetitive with PRibPP.

Comparison of Inactivation by Pyridoxal Phosphate and Pyridoxal(PL)

As seen in Table 5, PLP is a more potent inhibitor of NaPRTase than PL. A concentration of 100 μM PLP significantly inactivated the enzyme, whereas the same concentration of PL did not show any effect. At a higher concentration (500 μM), PL was effective (61% remaining activity) though to a lesser extent than PLP (8% remaining activity). These findings suggested that the phosphate group of PLP may be important in the binding to an essential lysine residue at the enzyme active site.

Stoichiometry of PLP inactivation using [^{3}H]-$NaBH_4$.

The results of studies showing the relation between the loss of activity of the enzyme by PLP and the number of lysine residues modified is presented in Table 6. In this experiment, and prior experiments employing [^{3}H]-$NaBH_4$, the background count of the control sample (enzyme + $NaBH_4$ in the absence of PLP) was higher than some of the PLP-inactivated samples. This was attributed to insufficient washing of each filter (although precautions were taken to minimize this error), and to the possibility of some of the protein passing through the filter. The data presented here was obtained by subtracting the dpm of the control experiment from each of the pyridoxyl- enzyme samples. This yielded a negative value for the moles PLP incorporated/moles enzyme (represented by a dashed line) for some of the samples. In order to attempt a correction of this negative value, a positive value of the the dpm for the sample protected with ATP ($-.259 \times 10^5$) was added to each sample and a corrected value was obtained for moles PLP/moles enzyme. From both sets of data it can been seen that an increased incorporation of [^{3}H]-PLP into the enzyme led to a decreased enzyme activity. In the corrected values, full protection was observed with ATP-Mg^{2+}, which was reflected by 0 moles of PLP incorporated into the protein, and full

TABLE 5. Comparison of Inactivation by Pyridoxal 5'-Phosphate and Pyridoxal. [a]

Addition	Concentration (μM)	% Residual Activity
None	-----	100
PLP	100	30
PL	100	120
PLP	500	8
PL	500	61

[a]NaPRTase was incubated with the above concentrations of Pyridoxal 5'-Phosphate or Pyridoxal for 30 min, followed by reduction with 20 mM $NaBH_4$. The residual activities were determined as described in "Methods".

recovery of enzyme activity. PRPP-Mg^{2+} provided almost full protection, while a combination of the three substrates offered full protection.

The results from both sets of data were plotted, and are depicted in Fig. 12A and 12B. Each plot shows a biphasic curve indicating that approximately 4 lysine residues are modified with PLP. Extrapolation of the initial portion of the inactivation curve to zero activity, indicated that the modification of 1 essential lysine residue caused complete inactivation. A plot of % Activity *vs.* mM PLP (Fig. 13) showed a nonlinear curve similar to the inactivation curve obtained above. Because of the presence of other proteins in this enzyme extract that may bind to PLP, and the error introduced by the conditions of the experiment, these results were interpreted only as a rough estimation of the stoichiometry of PLP inactivation.

[^{3}H]-PLP labeling of NaPRTase

In order to determine the stoichiometry of PLP inactivation with greater accuracy than in the previous experiment using tritiated $NaBH_4$, [^{3}H]-PLP was used to label the protein , and the reduced derivatized samples were subjected to SDS disc gel electophoresis. This method offered a more direct measurement of PLP incorporation,and provided almost complete separation of NaPRTase from other contaminant proteins. Furthermore, the background count obtained in the control experiment (PLP + $NaBH_4$ in the absence of enzyme) was extremely low (data not shown). Fig. 14A and 14B represents plots of radioactivity (dpm) superimposed on the record of the gel scans (600 nm) *vs.* gel slice. As can be seen in the gel scan this procedure successfully separated NaPRTase (peak 1) from the other proteins present in the extract. A record of the gel scan of the enzyme extract in the absence of PLP and $NaBH_4$ gave a similar absorbance pattern of the proteins (data not shown). When enzyme was reacted with [^{3}H]-PLP followed by reduction with $NaBH_4$, there was a substantial amount of incorporation of [^{3}H]-PLP into the proteins (Fig. 14A). The

Table 6. Stoichiometry of PLP Inactivation of NaPRTase in the Presence and Absence of Substrates using [^{3}H]-$NaBH_4$. [a]

Residual PLP (mM)	Additions Substrates	PLP/enzyme (mol/mol)	Corrected PLP/enzyme (mol/mol)	% Activity
0	None	0	0	100
0.05	None	----	0.24	66
0.10	None	0.09	0.74	60
0.20	None	0.97	1.12	40
0.61	None	2.09	2.75	19
1.22	None	4.06	4.72	4
1.22	5 mM ATP + 25 mM Mg^{2+}	----	0	106
1.22	5 mM PRibPP + 25 mM Mg^{2+}	0.25	0.90	146
1.22	5 mM ATP + 5mM PRibPP + 25 mM Mg^{2+}	----	0.43	138

[a] NaPRTase was incubated with varying concentrations of PLP for 30 min, followed by reduction with [^{3}H]-$NaBH_4$. Subsequently, the radioactivity in each derivatized enzyme sample was counted. The experiment was repeated except that the enzyme-pyridoxal complex was treated with cold NaBH4 and the residual activities were determined as described in "Methods".

FIGURE 12. Residual Activity *vs.* Stoichiometry of PLP Inactivation using [^{3}H]-$NaBH_4$. NaPRTase was incubated with varying concentrations of PLP for 30 min at room temperature, followed by reduction using [^{3}H]-$NaBH_4$. Thereafter, the radioactivity of each PLP-$NaBH_4$ treated sample was counted. The experiment was repeated in the presence of cold $NaBH_4$, and the residual activity was determined as described in "Methods". The data was plotted as: Uncorrected moles PLP/mole enzyme (A), and Corrected moles PLP/mole enzyme (B).

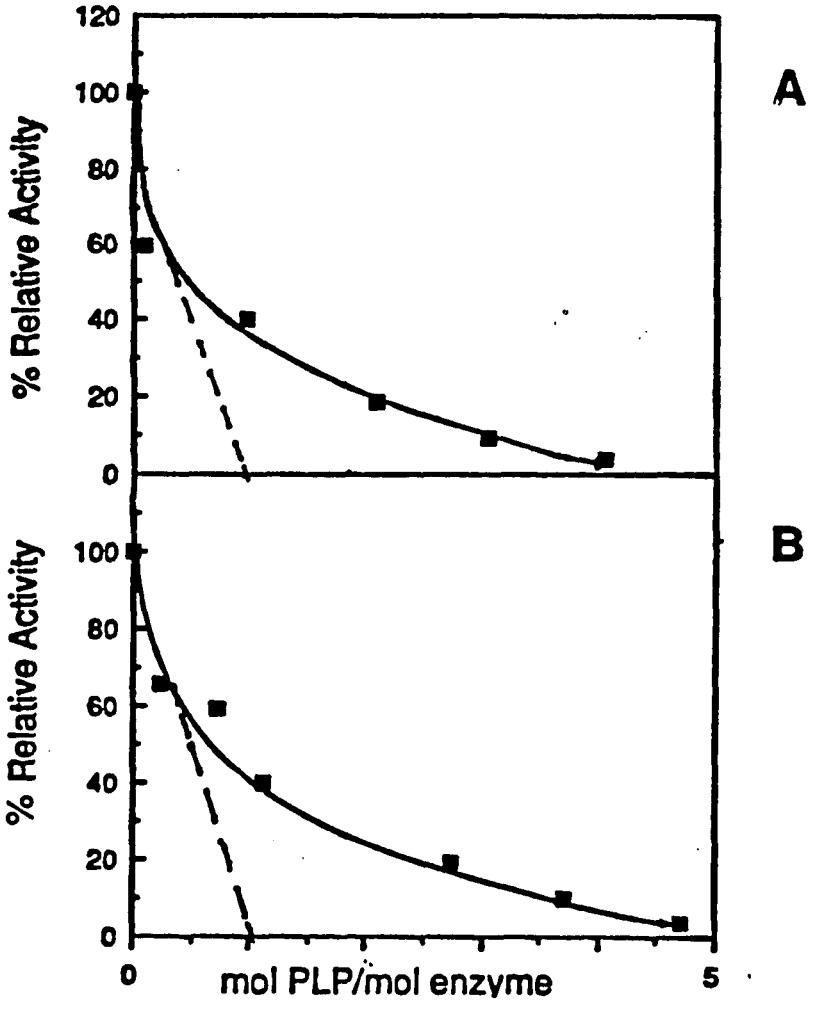
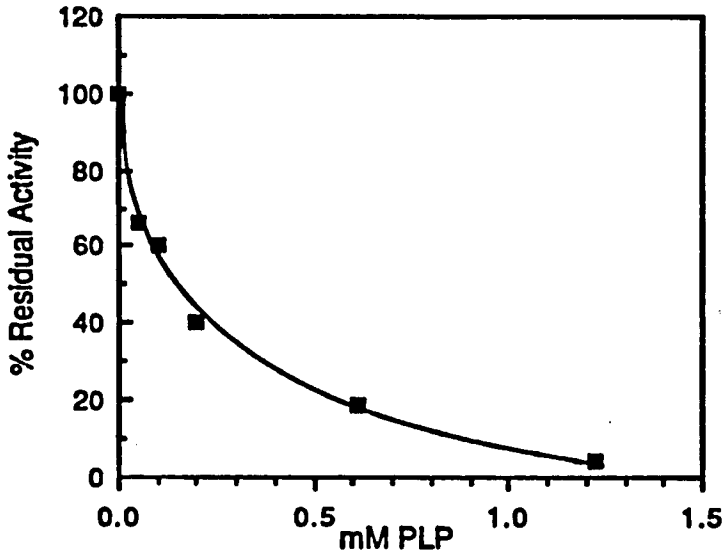

120
100
80
60
40
20
0
% Relative Activity
A
100
80
60
40
20
0
% Relative Activity
B
0
mol PLP/mol enzyme
5

FIGURE 13. Residual Activity of NaPRTase as a Function of PLP Concentration. NaPRTase was subjected to the conditions as stated previously, but reduction was in the presence of cold $NaBH_4$.


120
100
80
60
40
20
0
% Residual Activity
0.0
0.5
1.0
1.5
mM PLP

presence of ATP-Mg^{2+} alone, or in combination with PRibPP, led to a decrease in [^{3}H]-PLP labeling of all the proteins in the gel (data not shown). In the presence of MgPRibPP alone, a decrease in incorporation of [^{3}H]-PLP was observed in peak 1, while an increased labeling of [^{3}H]-PLP was observed in peak 2 (Fig. 14B). This was an important observation, which with the results of the molecular weight calculations determined for each peak helped to establish the identity of NaPRTase as peak 1 (a complete explanation of this conclusion is discussed in "Methods").

The stoichiometry of [^{3}H]-PLP incorporation into NaPRTase in the presence and absence of substrates is presented in Table 7 .The slight overlap occurring between the NaPRTase peak and peak 2, introduces error in quantitating the protein and radioactivity present in the NaPRTase peak. Therefore, the results were expressed as the average value calculated between the maximum moles PLP/minimum moles protein and the minimum moles PLP/maximum moles protein. In the absence of substrates, 1 mole of PLP was incorporated in one mole of the protein, indicating the presence of 1 essential lysine residue at the active site. ATP-Mg^{2+} provided almost complete protection, which was reflected in the reduction in the amount of PLP incorporated (0.15 mol/mol). PRibPP-Mg^{2+} was less effective than ATP-Mg^{2+}, while the combination of ATP, PRibPP, and Mg^{2+} afforded complete protection against PLP inactivation. These results suggested that PLP forms a Schiff base with a lysine residue at or near the ATP and PRibPP binding site of NaPRTase.

Effect of pH on the Stability of NaPRTase

Enzymes can be irreversibly destroyed at either side of their pH optimum, and unless this effect is taken into consideration, it may be misunderstood as being caused by the ionization of a particular group on the enzyme. Therefore, a study of the pH stability of an enzyme is prerequisite for performing experiments on the pH dependence of the activity of an enzyme. The pH stability curve is determined by

FIGURE 14. Disc SDS Polyacrylamide Gel Electophoresis of NaPRTase. The enzyme was incubated with [^{3}H]-PLP in the absence (A) and presence (B) of MgPRibPP, for 1 hour to allow complete inactivation of the enzyme, followed by $NaBH_4$ reduction. The derivatized enzyme samples were then subjected to SDS PAGE. The solid line represents the absorbance of the protein at 600 nm, while the dotted line represents the radioactivity (dpm) present in the gel.

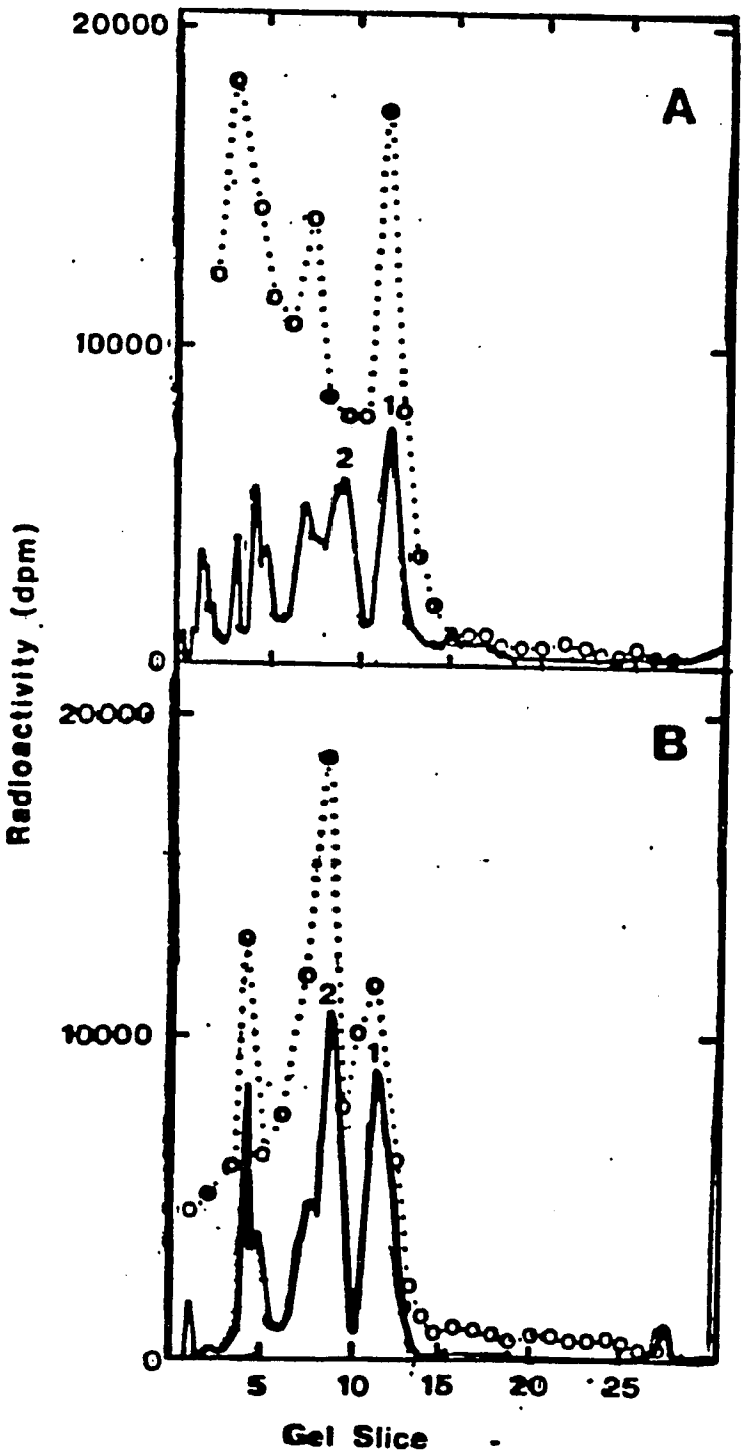

20000
10000
0
A
1
2
20000
10000
0
B
2
1
Radioactivity (dpm)
5
10
15
20
25
Gel Slice

Table 7. Stoichiometry of PLP inactivation of NaPRTase Utilizing [^{3}H]-PLP in the Presence and Absence of Substrates.[a]

Substrate	mol PLP/mol enzyme
None	1.0 + .4
22.2 mM ATP-Mg^{2+}	0.15 + .07
22.2 mM PRPP-Mg^{2+}	0.5 + .2
22.2 mM ATP-Mg^{2+} + 22.2 mM PRPP	0.09 + .03

[a] NaPRTase was reacted with 2.2 mM [^{3}H]-PLP in the presence and absence of either 22.2 mM MgATP or 22.2 mM MgPRibPP for 1 hour, to allow for total inactivation of the enzyme. Subsequently, the enzyme-PLP complex was reduced with 50 mM $NaBH_4$, and subjected to SDS PAGE. The gels were sliced and the radioactivity was determined. The protein in the NaPRTase peak was determined as described in "Methods".

preincubating the enzyme with buffer of varying pH values for at least as long as the assay time. Subsequently, the enzyme is assayed at its optimum pH. These studies were carried out for NaPRTase from pH 4.1-9.6, and are represented by Fig. 15. At pH 4.1, 82% of the activity remained. The enzyme remained stable over a broad pH range, although an increased stability was evident at a more alkaline pH value.

pH Dependency of NaPRTase Activity Varying ATP and PRibPP

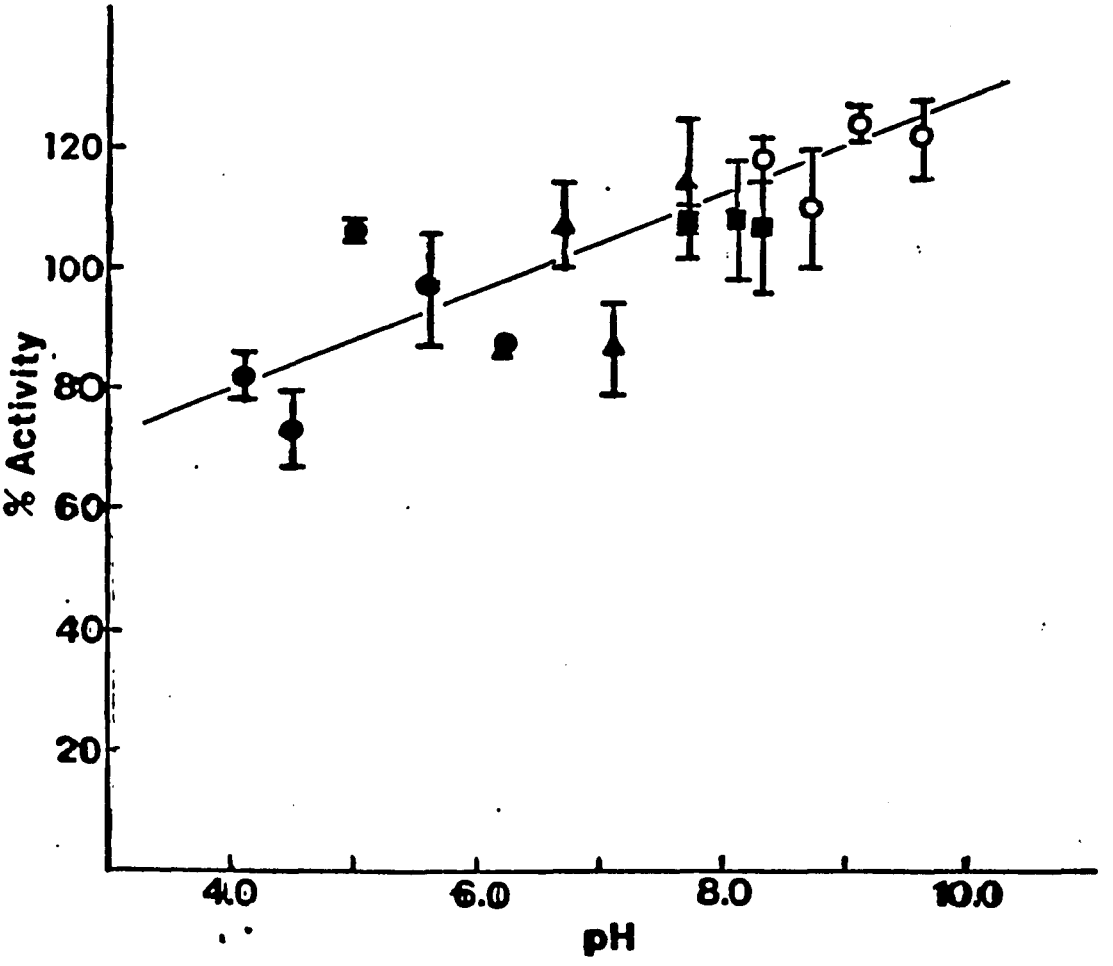
Since chemical modification with PLP and protection studies with MgATP and MgPRibPP suggested the presence of a lysine residue at the active site of the enzyme, pH dependency studies were initiated to provide further information about the pKa values of other amino acids, in addition to lysine, that are involved in this enzyme's function. The pH dependence of the kinetic parameters V and V/Km for MgATP and MgPRibPP was calculated from initial velocity data varying one of these substrates at saturating concentrations of the other two substrates. Analysis of the initial velocity data was performed using the HYPER program described by Cleland (1979) on an Apple II computer, in which FORTRAN was converted into BASIC language by Dr. Rosalyn Strauss. This program fits initial velocity data to the equation:

$$v = VA/(K + A)$$

where v= initial velocity
V= maximal velocity
A= varied substrate concentration
K= Michaelis constant for A

Thereafter, the kinetic constants V and V/K obtained from the HYPER program for each varied substrate, were fit to the following equation using the Bell program developed

FIGURE 15. pH Dependence of the Stability of NaPRTase. The enzyme was incubated with buffer at a range of pH values (4.1-9.6), on ice for 30 min. The buffers used were: potassium acetate (closed circles), PIPES (closed trianges), POPSO (closed squares), and CHES (open circles). Thereafter, an aliquot from the incubation solution was added to the assay mixture, and the initial velocities were determined as described in "Methods".


120
100
80
60
40
20
% Activity
4.0
6.0
8.0
10.0
pH

by Cleland (1979) which was also translated from FORTRAN to BASIC language by Dr. Rosalyn Strauss.

$$\log y = \log (C/\ 1 + H/Ka + Kb/H)$$

where y= V or V/K

C= pH independent value of y

Ka= dissociation constant for an acid

.Kb= dissociation constant for a base

The data was then plotted as the log V, log V/Km, and log Km *vs.* pH for MgATP and MgPRibPP, which is illustrated in Fig. 16 and 17, respectively. Fig. 16A shows a half-bell shaped curve in which V decreases at low pH giving a pKa value of 5.3 ± .04. As stated in the methods section, the pH values above 8.5 could not be examined, therefore a pKb value for the alkaline portion of the curve could not be determined. The log Vmax/Km profile (Fig. 16B) is essentially the same shape as the log V plot, giving a pKa value of 5.8 ± 0.1. Values of log Km for ATP vs. pH (Fig. 16C) did not significantly change indicating that the ionizable group most likely resided on the enzyme, and not on the substrate. These findings suggest that the group affecting catalysis and/or binding is likely to be a carboxyl group of aspartic acid or glutamate, or the imidazole moiety of a histidine residue.

As seen in Fig. 17A, the log V for PRibPP which is bell shaped decreases at low and high pH giving a pKa values of 5.8 ± 0.1 and a pKb value of 8.6 ± 0.1. The pKb value must be interpreted with caution in view of the fact that only one data point was obtained for this parameter on the alkaline side of the curve, and that some microprecipitation may have occurred which would also yield a decrease in the log V value (see Methods). A similar profile was obtained for log Vmax/Km for PRibPP

(Fig. 17B) giving a pKa value of 5.5 ± 0.1, although the alkaline portion of the curve displayed less of a decline than the log Vmax plot. As stated above, the kinetic parameters for the higher pH values above 8.6 could not be determined. The pKa value of ~ 5.6 indicates the possible involvement, as seen in the pH dependence study varying ATP, of an essential carboxyl or imidazole group. The pKb value of 8.6 determined from the log V profile for PRibPP is suggestive of a lysine residue involved in the binding and/or catalysis of the enzyme. This idea is also supported by the evidence from the chemical modification studies with PLP.

FIGURE 16. pH Dependence of NaPRTase Activity Varying ATP. The reaction was initiated with the addition of NaPRTase to assay mixture containing 50 mM buffer (pH 4.9-8.5), 5 mM $MgCl_2$, 40 µM PRibPP, and 60-240 µM ATP. The buffers used for this range of pH values were: potassium acetate (pH 4.0-6.2), PIPES (pH 6.2-7.7), POPSO (pH 7.6-8.3), and CHES (pH 8.3-9.6). The initial velocities were then determined as described in "Methods". The data was analyzed as described in "Results", and plotted as: log Vmax (A), log Vmax/Km (B)and, log Km (C).

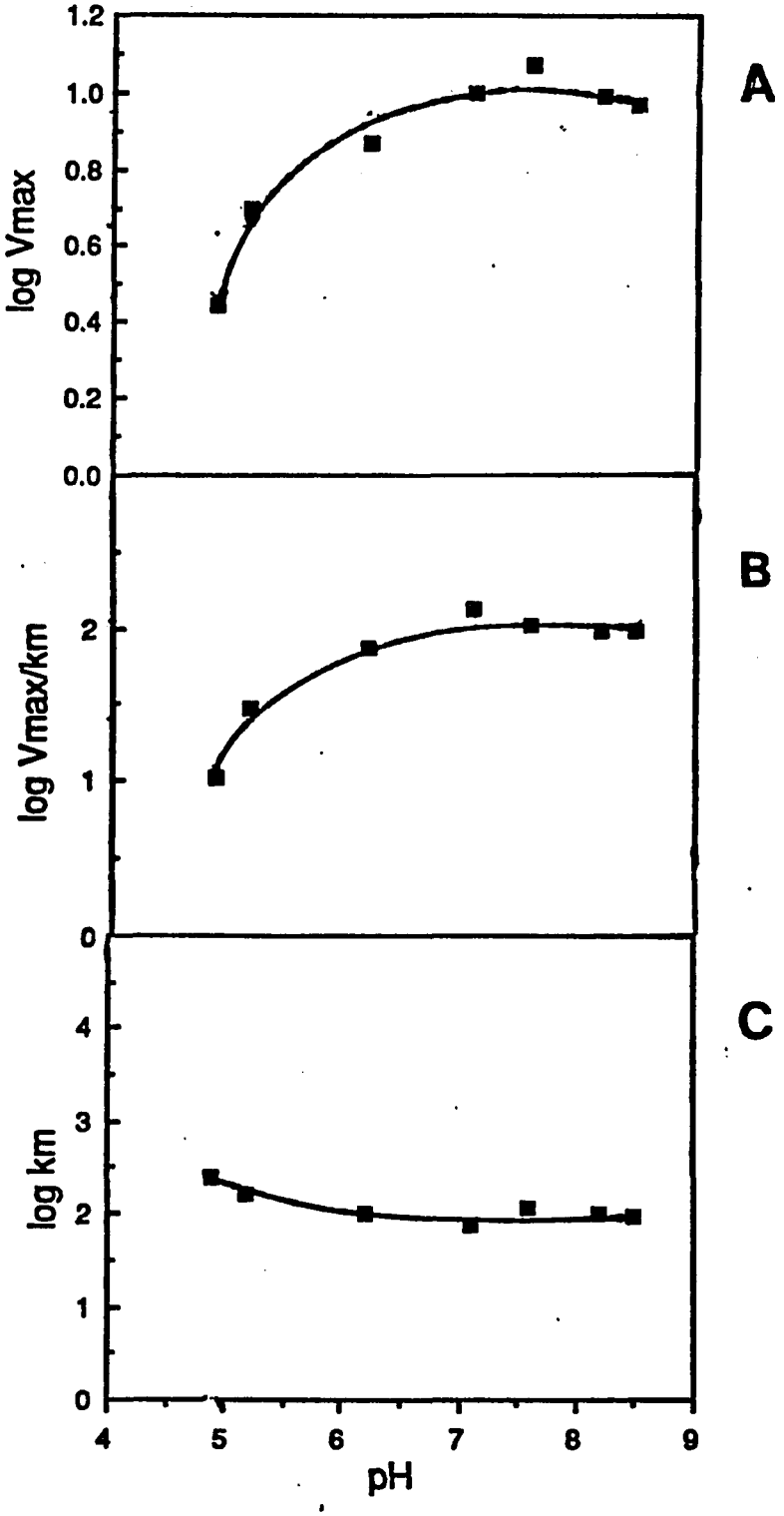
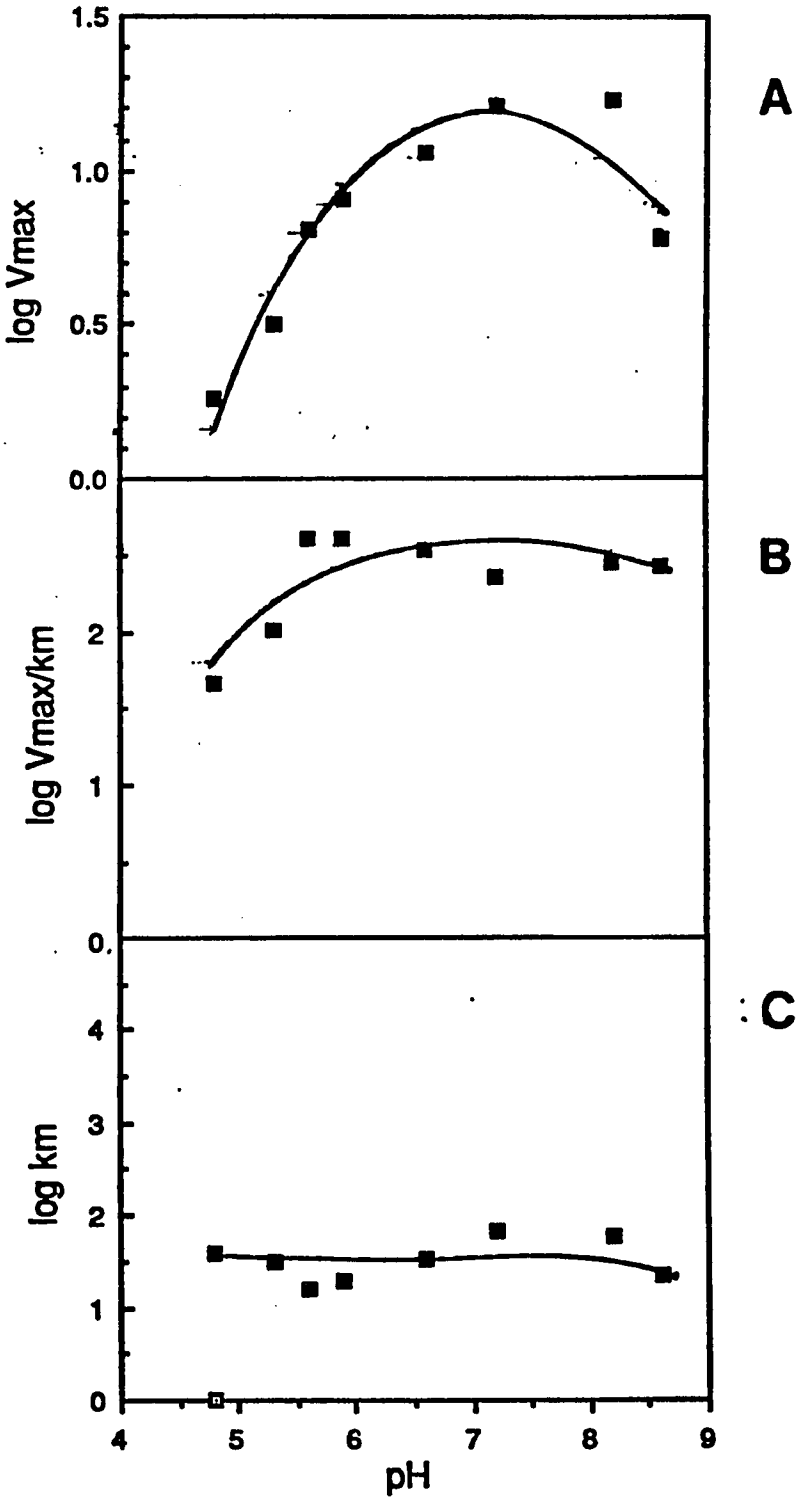

A
B
C
log Vmax
log Vmax/km
log km
pH
1.2
1.0
0.8
0.6
0.4
0.2
0.0
2
1
0
4
3
2
1
0
4
5
6
7
8
9

FIGURE 17. pH Dependence of NaPRTase Activity Varying PRibPP. The same conditions as stated previously for ATP were employed, except the assay mixture contained 50 mM buffer (pH 4.8-8.6), 5 mM $MgCl_2$, 240 μM ATP, and 15-75 μM PRibPP. The data was analyzed as described in "Results" and was plotted as A) log Vmax, B) log Vmax/Km, C) log Km.


1.5
1.0
0.5
0.0
log Vmax
A
2
1
0
log Vmax/km
B
4
3
2
1
0
log km
C
4
5
6
7
8
9
pH

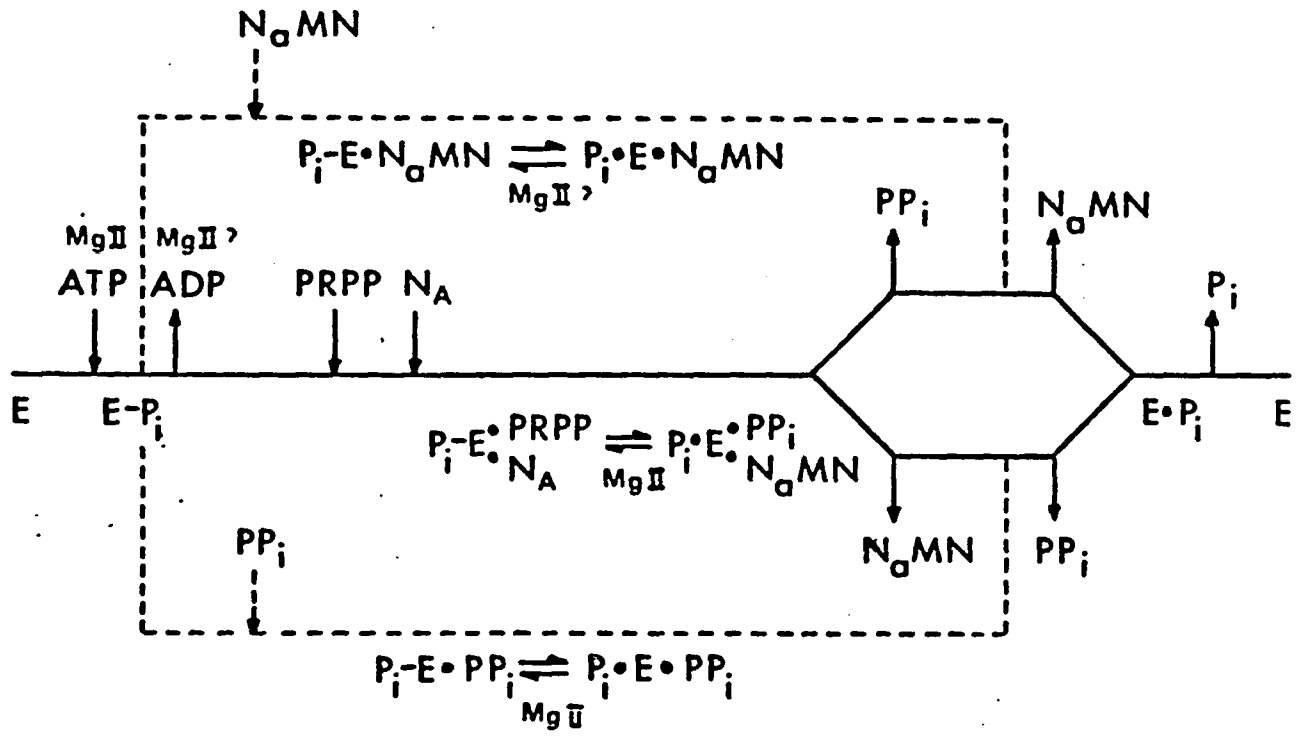
DISCUSSION

Initial velocity measurements varying the concentration of one substrate at a fixed concentration ratio of the other two substrates, helped to define a sequential mechanism with a single ping-pong step. ATP-ADP exchange studies suggested that ATP was the first substrate to bind to the enzyme, and also indicated the involvement of a ping-pong step. These results together with the other kinetic, isotope exchange, product inhibition, and flow dialysis binding experiments described earlier in the introductory section, indicated that NaPRTase follows an ordered Uni Uni Bi Ter Ping Pong Mechanism in which ATP binds to the enzyme first to form a phosphorylated enzyme prior to release of ADP. Subsequently, PRibPP and nicotinate bind in order to the phosphoenzyme, which is then followed by the random release of PPi and NaMN, and lastly Pi. Fig. 18 illustrates the postulated mechanism for NaPRTase.

In comparison to other PRTases studied in our laboratory, hypoxanthine-guanine phosphoribosyltransferase which catalyzes the formation of a purine nucleotide (either GMP or IMP) and PPi from PRibPP and a purine base (either guanine or hypoxanthine), proceeds through an ordered Bi Bi kinetic mechanism, in which PRibPP binds to the enzyme first forming a phosphoribosyl-enzyme intermediate, followed by the addition of the purine base (Ali and Sloan, 1982). Orotate phosphoribosyltransferase catalyzes the conversion of orotic acid and PRibPP to orotidine 5'-phosphate (OMP) and PPi, and follows a Bi Bi Ping Pong kinetic mechanism, in which an enzyme-phosphoribosyl intermediate is formed (Victor *et al.*, 1979).

Following the kinetic analysis, the effect of 3-pyridylacetate on NaPRTase was examined. This analog exerted no inhibitory effect, nor did it act as a substrate. This finding infers that the distance between the carboxyl group and the pyridine ring is critical for the binding of nicotinic acid. Similarly, in human erthryocyte NaPRTase,

FIGURE 18. The Proposed Ordered Uni Uni Bi Ter Ping Pong Kinetic Mechanism for Nicotinate Phosphoribosyltransferase, in which the Second and Third Products can Dissociate at Random. Dotted lines represent the sequences for the nicotinate phosphoribosyltransferase-catalyzed ATPase activity.


N_aMN
P_i-E•N_aMN ⇌ P_i•E•N_aMN
Mg II
Mg II
Mg II
ATP
ADP
PRPP
N_A
PP_i
N_aMN
P_i
E
E-P_i
P_i-E•PRPP / N_A ⇌ P_i•E•PP_i / N_aMN
Mg II
E•P_i
E
PP_i
N_aMN
PP_i
P_i-E•PP_i ⇌ P_i•E•PP_i
Mg II

3-pyridylacetate was found to be an extremely weak inhibitor (Gaut and Soloman, 1971).

PLP is an important cofactor for enzymes such as aminotransferases, deaminases, and other enzymes which catalyze reactions involving amino acids (Fasella, 1967). Studies by Fisher and colleagues (1958, 1963), first demonstrated that PLP is an essential constituent of muscle phosphorylase, and is bound to the enzyme through a Schiff base linkage. These studies also revealed that upon reduction of the Schiff base with $NaBH_4$, PLP was covalently bound to the ε-amino group of a lysine residue on the protein.

Investigations by Rippa *et al.*, (1967) revealed that PLP could be used as a chemical modifying reagent for identifying critical lysine residues of other enzymes which have no requirement for this cofactor. PLP reduced with $NaBH_4$ was also found to act selectively as an affinity label for enzymes possessing phosphate binding sites (Means and Feeney, 1971). Thereafter, this reagent was shown to be a useful diagnostic tool for the identification of lysine residues in or near phosphate binding sites of numerous proteins including several dehydrogenases (Anderson *et al.*, 1966; Rippa *et al.*, 1967; Stepp and Reed, 1985), glycolytic enzymes (Reuter *et al.*, 1983; Colombo and Marcus, 1974; Shapiro *et al.*, 1968), ATPases (Sugiyama and Mukohata, 1979; Maeda *et al.*, 1988), ribosomal proteins (Ohsawa and Gualerzi, 1983), and aminoacyl-tRNA synthetases (Piszkiewicz *et al.*, 1977). In addition, PLP has been used as a specific active site photosensitizer for the modification of essential histidine residues (Hathaway and Noltmann, 1977; Rippa and Pontremoli, 1969), and as an activator, specifically of ADP-glucose pyrophosphorylase (Morell *et al.*, 1988).

The experiments presented in this work suggested that the active site of NaPRTase contains an essential lysine residue which forms an inactive Schiff base complex with PLP. This conclusion is supported by the following experimental evidence:

1. The extent of inactivation of NaPRTase by the combined PLP-$NaBH_4$ treatment, during a fixed 30 min incubation period, increased as the PLP concentration increased. Adequate controls indicated that the loss of activity was not due to the addition of $NaBH_4$.

2. Inhibition of the enzyme by PLP was reversible upon dialysis, whereas this inactivation was made irreversible following reduction of the enzyme-PLP complex with $NaBH_4$. In addition, the degree of inactivation of NaPRTase by PLP increased as the pH became more alkaline. These results are indicative of Schiff base formation between the aldehydic group of PLP and the ε-amino group of a lysine residue. Recently, this reversibility of inhibition by PLP was also demonstrated in orotate PRTase (Ashton *et al.*, manuscript in preparation). In contrast, the PLP-$NaBH_4$ induced inhibition of thymidylate synthase remained reversible, indicating that in this case, PLP acts as a substrate analog rather than forming a Schiff base with an essential lysine residue (Chen *et al.*, 1988).

3. Stoichiometry experiments with [^{3}H]-PLP indicate that upon complete loss of activity, one mole of PLP is incorporated per mole of enzyme. The fact that the stoichiometry determined is a small integral number, and that NaPRTase is a monomer provides strong evidence for the specificity of the reaction.

4. Significant protection against PLP inactivation was observed with MgATP, which was reflected in the decreased stoichiometry of PLP binding (Table 7), and in the recovery of enzyme activity (Table 4). Activity was fully recovered with MgPRibPP (Table 4), while the data from the stoichiometry experiment showed that this substrate provided partial protection (Table 7). A combination of the two substrates afforded complete protection against inactivation. These results also inferred that modification occurred at or near the active site. The fact that PRibPP partially protected against inactivation, while ATP gave almost full protection is also an indication that PRibPP may partially bind to the nonphosphorylated form of

NaPRTase, but the geometry of the active site may not be conducive to the correct positioning of this substrate. Phosphorylation of the enzyme by ATP, may induce a conformational change in the enzyme, allowing PRibPP to bind in the proper orientation. Furthermore, the reversible inhibition data demonstrating that PLP is competitive with MgATP, but noncompetitive with MgPRibPP, is consistent with the ordered ping-pong kinetic mechanism proposed for this enzyme.

A closer look at the protection offered by ATP and PRibPP revealed that Mg^{2+} enhanced the protective effect provided by each of these substrates. Previous studies with the enzyme have demonstrated that Mg^{2+} is required for activity, and that maximal activity is obtained in the presence of equimolar concentrations of ATP and Mg^{2+} (Kosaka *et al.*, 1971). In addition, the Mg form of ATP and PRibPP has been shown to have a strongly marked protective effect against heat denaturation. These findings support the hypothesis that MgATP and MgPRibPP are the true substrates of the reaction. In chemical modification studies of both orotate PRTase (Ashton *et al.*, manuscript in preparation) and anthranilate PRTase (Grove and Levy, 1979), the Mg^{2+} combined with PRibPP also offered the best protection against inactivation by PLP. Interestingly, Mg^{2+} alone offered no protection against PLP inactivation in NaPRTase, whereas in anthranilate PRTase, Mg^{2+} slightly promoted the inhibition exerted by PLP (Grove and Levy, 1979).

A comparion of the inhibitory effect exerted by PLP and the nonphosphorylated aldehydes, nicotinaldehyde and pyridoxal, suggests that the specificity of the reaction may be due to a direct effect of the phosphate group. Inhibition of NaPRTase was not observed with nicotinaldehyde. In contrast, inhibition of nicotinamide deamidase activity by nicotinaldehyde was demonstrated , and was attributed to the presence of a reactive lysine residue (Schiff base formation), or a cysteine residue at the active site (Yan and Sloan, 1987). The combined pyridoxal-$NaBH_4$ treatement inactivated NaPRTase, however, much higher concentrations of pyridoxal were required (Table 5).

In enzymes utilizing organophosphate substrates, the formation of a Schiff base between PLP and a lysine residue is hypothesized to occur first through the formation of a noncovalent intermediate (Chen and Engel, 1975), which was suggested for NaPRTase (see Results section). The noncovalent intermediate is supposedly formed from ionic interactions between the phosphoryl group of PLP and a cationic group on the enzyme. On the other hand, inactivation of glutamate dehydrogenase by pyridoxal has been demonstrated, however, the formation of a noncovalent complex was not detected (Piszkiewicz and Smith, 1971a).

Further evidence that the phosphate group of PLP may be involved in directing this substance to a specific site, is the fact that each of the substrates, ATP and PRibPP, protected the enzyme against inactivation by PLP, while nicotinic acid had no effect.

The specificity of PLP for the lysine residue at the active site is dependent on the local tertiary stucture of the enzyme. Anomalous pK values have been found for the lysine residues that preferentially bind to PLP as compared with the pK values of model compounds (pK value of 10.2). For instance, a pK value of 5.9 was reported for an essential lysine residue in acetoacetate decarboxylase (Schmidt and Westheimer, 1971). Presumably, the chemical effects of nearby amino acids may account for the high reactivity of a particular lysine group (Ivanov and Karpeisky, 1969).

The exact role that this putative lysine residue plays in NaPRTase catalysis remains to be clarified. The possibility exists that this lysine residue when protonated can interact with the negatively charged phosphate moiety of the substrate. Recent studies of the modification of adenylate kinase by the affinity label adenosine diphosphopyridoxal have suggested that the ε-amino group of a specific lysine is located at or close to the subsite for the γ-phosphate moiety of ATP(Tagaya *et al.*, 1987). Studies using an ATP affinity label (Mg-oATP) to modify the MgATP binding site of pyruvate carboxylase also suggested that the lysine residue may be involved in

binding the terminal phosphate group of MgATP (Easterbrook-Smith *et al.*, 1976). These authors proposed that the electron withdrawing ability of the ε-NH_3^+ group would render the γ-phosphorus atom of MgATP more susceptible to nucleophilic attack by a carboxyl group, with subsequent formation of a carbonyl-phosphate intermediate. This role for the lysine residue has also been hypothesized for the active site of creatine kinase (James and Cohn, 1974).

Since the kinetic mechanism follows an ordered ping-pong pathway, the form of the enzyme present as V/K_{MgATP} is free enzyme, while $V/K_{MgPRibPP}$ represents the phosphorylated enzyme intermediate. The V/KMgATP profile shows an ionizable group having a pK value of 5.8. Similarly, the V/KMgPRibPP plot gave a pK value of 5.6. These two pK values may represent the involvement of a carboxyl or imidazole group in catalysis. The involvement of an essential imidazole group is also slightly suggested by the preliminary photoinactivation experiment performed with PLP.

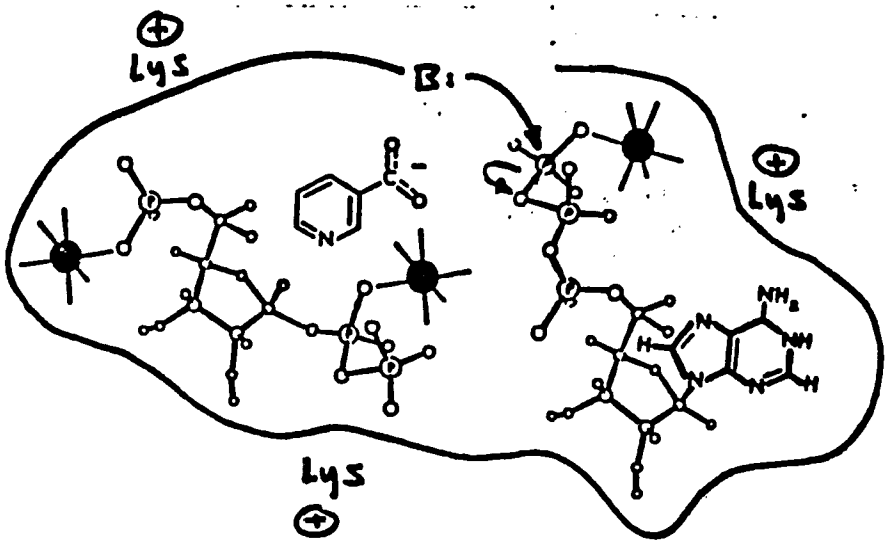
As mentioned earlier in the introductory section, phosphoryl groups of phosphoenzyme intermediates can be attached to the enzyme through different linkages including a phosphoramidate linkage to N-1 or N-3 of histidine, as in succinyl CoA synthetase (Kreil and Boyer, 1964; Hultquist et al., 1966), or through an acid anhydride linkage to a γ-carboxyl group of glutamic acid, as is the case with ATP citrate lyase. In addition, an enzyme bound phosphoryl group identified as an acyl phosphate was found in gastric H-K-ATPase (Walderhaug et al., 1985), and recently in phosphoglycolate phosphatase (Seal and Rose, 1987).

Based on the investigations of Easterbrook-Smith *et al.*, (1976) , and James and Cohn (1974), a similar chemical mechanism can be speculated for the first partial reaction of NaPRTase, which is consistent with the above kinetic, chemical modification, and pH dependency studies performed on NaPRTase. It is conceivable that an essential protonated lysyl residue forms a salt bridge with the terminal phosphoryl group of MgATP. In this ionic interaction, the ε-NH_3^+ group can make this terminal

phosphate moiety prone to nucleophilic attack by a nearby base, which could possibly be an essential imidazole or carboxyl group on the active site of the enzyme. Subsequently, a phosphorylated intermediate is formed between an enzyme bound acyl or imidazole group and phosphate, followed by the release of ADP. Fig. 19 illustrates this postulated mechanism for the first partial reaction of NaPRTase involving ATP.

In conclusion, future experiments may be designed utilizing purified NaPRTase, to determine the amino acid involved in binding the phosphate group in the phosphoenzyme intermediate. It would also be interesting to determine the sequence of residues at the active site of ATP and compare the sequence homology to that of the ATP utilizing enzymes mentioned earlier.

FIGURE 19. The Proposed Chemical Mechanism for the First Partial Reaction for Nicotinate Phosphoribosyltransferase.


Lys
B1
Lys
Lys

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