Allosteric Properties of Nucleoside Diphosphatase. Activation by Pyridoxal 5′-Phosphate and Specific Modification of Effector Binding Sites†

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ABSTRACT: The enzyme activity of purified nucleoside diphosphatase from rat liver is enhanced by pyridoxal 5′-phosphate at low concentrations. The Michaelis constant of activation for pyridoxal 5′-phosphate was 5.2 μM. No activation was observed with pyridoxal itself or with pyridoxine 5′-phosphate or pyridoxamine 5′-phosphate. The activation was ascribed to the increase of affinity for substrate, with the maximal velocity being essentially unchanged. The activation by pyridoxal 5′-phosphate is due to Schiff base formation with ε-amino groups of lysine residues of the enzyme. This was established by treatment of the enzyme with pyridoxal 5′-phosphate, followed by reduction with $NaBH_4$, and by isolation of N^6-pyridoxyllysine. The reduced derivative of the enzyme was no longer sensitive to pyridoxal 5′-phosphate, but its specific activity was not significantly altered. The modified enzyme contained approximately 2 mol of pyridoxal 5′-phosphate/mol of the enzyme. It was more resistant to heat denaturation and showed a broader pH-stability curve than the native enzyme. Nucleoside diphosphatase is also activated by ATP and other nucleoside triphosphates (Yamazaki, M., and Hayaishi, O. (1968), *J. Biol. Chem. 243,* 2934). Maximal velocity obtained with pyridoxal 5′-phosphate was 70–80% of that with ATP. Pyridoxal 5′-phosphate did not further stimulate the reaction which was maximally activated by ATP, and ATP showed no stimulation of the reaction which was activated by pyridoxal 5′-phosphate. The pyridoxyl phosphate derivative of the enzyme was not sensitive to ATP. ATP protected the enzyme against the desensitization with pyridoxal 5′-phosphate. These results suggest that pyridoxal 5′-phosphate and ATP bind to the same site on the enzyme. IDP also protected the enzyme against the desensitization. This finding is consistent with the observation that plots of reaction rates against IDP concentrations are sigmoidal.

Nucleoside diphosphatase, a microsomal enzyme, catalyzes the hydrolysis of the terminal phosphate of IDP, UDP, GDP, and thiamine pyrophosphate (Plaut, 1955; Yamazaki and Hayaishi, 1968). Nucleoside diphosphatase is an allosteric enzyme which is activated by ATP and other nucleoside triphosphates (Yamazaki and Hayaishi, 1965, 1968). The effect of ATP was ascribed to a decrease in the apparent K_m for substrate. ATP also stabilized the enzyme against heat inactivation. Schramm and Morrison (1968, 1969) studied kinetic properties of the enzyme in detail. However, the ATP binding site has not yet been investigated by chemical modification of the enzyme.

Pyridoxal phosphate, an important cofactor for many enzymes, forms Schiff bases with lysine residues of many additional enzymes which have no obvious requirement for it as a cofactor (Rippa et al., 1967). Marcus and Hubert (1968) demonstrated an almost complete desensitization of kidney fructose 1,6-bisphosphatase toward AMP inhibition by modification with pyridoxal phosphate.

We describe here the effects of pyridoxal phosphate on rat liver nucleoside diphosphatase. Pyridoxal phosphate is bound to the enzyme at the ε-amino group of the lysine residue in the allosteric site and enhances the enzymatic activity. Reduction of the enzyme–pyridoxal phosphate complex with sodium borohydride leads to the formation of active pyridoxyl phosphate derivative of the enzyme, which is almost completely desensitized toward ATP activation. Properties of the desensitized enzyme are also described.

Materials and Methods

Materials. IDP was prepared from ADP by deamination with nitrous acid (Kleinzeller, 1942) and was purified by column chromatography on Dowex 1-formate with the use of ammonium formate as the eluting system (Hurlbert et al., 1954). Pyridoxal phosphate was purified by column chromatography on Amberlite IRC-50 (H^+) (Peterson and Sober, 1954). Pyridoxal phosphate and nucleotides were purchased from Sigma. N^6-Pyridoxyllysine was prepared from poly(L-lysine) (Sigma Type IB) as described by Schnackerz and Noltmann (1971). Other chemicals were of analytical grade.

Assay of Enzyme Activity. The nucleoside diphosphatase activity was assayed by measuring inorganic phosphate liberated from IDP by the method of Fiske and Subbarow (1925). The standard incubation mixture contained, in a volume of 1.0 mL, 50 μmol of triethanolamine hydrochloride (pH 7.4), 4 μmol of $MgCl_2$, 0.25 μmol of IDP, 0.2 μmol of pyridoxal phosphate or 0.2 μmol of ATP where indicated, and the enzyme. The reaction mixture was incubated in the absence of enzyme at 37 °C for 5 min, and the reaction was then started by the addition of enzyme. After further incubation for 5 min at 37 °C, the reaction was stopped by the addition of 0.2 mL of 30% trichloroacetic acid. The enzyme activity with thiamine pyrophosphate as substrate was assayed by measuring the formation of thiamine monophosphate (Yamazaki and Hayaishi, 1968). Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Enzyme Purification. Purification was started from 850 g of rat livers. Solubilization of nucleoside diphosphatase from liver microsomes and fractionation with ammonium sulfate were performed as described by Kuriyama (1972). After di-

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alysis overnight against 5 L of 20 mM Tris-HCl[1] (pH 7.4) containing 0.15 M NaCl, the ammonium sulfate fraction was placed on a column (2.5 × 30 cm) of DEAE-Sephadex A-50 previously equilibrated with the same buffer. The column was washed with 20 mM Tris-HCl (pH 7.4) containing 0.2 M NaCl, and the enzyme was eluted from the column with a linear gradient consisting of 0.2 to 0.4 M NaCl in 20 mM Tris-HCl (pH 7.4) in a total volume of 600 mL. The active fractions were collected (190 mL) and concentrated to 6 mL by ultrafiltration using a Diaflo membrane UM 10 (Amicon, U.S.A.). The concentrated enzyme solution was applied to a Sephadex G-200 column (2.8 × 90 cm) which had been equilibrated with 20 mM Tris-HCl (pH 7.4) containing 0.4 M NaCl. The fractions eluted at 1.33–1.78 of V/V_0 were combined (70 mL) and dialyzed against 2 L of 30 mM potassium phosphate (pH 6.5). The enzyme solution was then placed on a hydroxylapatite column (2 × 25 cm) equilibrated with the same buffer, and the enzyme was eluted from the column with a linear gradient consisting of 30 (pH 6.5) to 180 mM potassium phosphate (pH 7.5) in a total volume of 450 mL. The active fractions were combined (50 mL) and dialyzed against 2 L of 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl. The enzyme solution was subjected to DEAE-Sephadex column chromatography. The elution conditions were the same as described above. A single protein peak was coincident with the enzyme activity. The purified enzyme was dialyzed against 2 L of 50 mM triethanolamine hydrochloride (pH 7.4) and was stored at −20 °C until use.

Discontinuous Gel Electrophoresis. The purity of the enzyme preparation was checked by discontinuous gel electrophoresis using 7.5% acrylamide gel, pH 8.5, as the separation gel (Davis, 1964). The electrophoresis was run at 5 °C.

Determinations of Molecular Weight. Molecular weights of the purified enzyme and its subunit were determined by gel filtration through Sephadex G-200 (Andrews, 1965) and by sodium dodecyl sulfate gel electrophoresis (Weber and Osborn, 1969), respectively, using several marker proteins as molecular weight standards.

Chemical Modification of Enzyme. The enzyme was incubated with 1 mM pyridoxal phosphate at 37 °C for 15 min in 1.0 mL of 50 mM triethanolamine hydrochloride (pH 7.4). The mixture was then cooled to 0 °C and 1 drop of octyl alcohol was added to prevent foaming. Then, 1 mg of solid $NaBH_4$ was added and the mixture was allowed to stand for 10 min at 0 °C, followed by exhaustive dialysis at 4 °C against 50 mM triethanolamine hydrochloride (pH 7.4).

In this experiment, pyridoxal phosphate at a concentration of 1 mM was used for the modification of the enzyme, since treatment with 0.1 or 0.25 mM pyridoxal phosphate produced a partially desensitized enzyme but not a completely desensitized one.

Identification of N^6-Pyridoxyllysine in the Modified Enzyme. The reduced pyridoxal phosphate–enzyme complex was dialyzed for 24 h against several changes of distilled water and then hydrolyzed in sealed tubes in the presence of 6 N HCl for 22 h at 110 °C. The hydrolysate was concentrated under reduced pressure, dissolved in water, and lyophilized. This procedure was repeated several times to free the product completely from residual HCl. Aliquots of the hydrolysate were applied to sheets of Whatman 3MM paper and subjected to ascending paper chromatography in two solvent systems: (1) 2-propanol–pyridine–acetic acid–water (30:20:6:24) (Forrey et al., 1971); (2) water–methanol–ethanol–benzene–pyridine–dioxane (25:25:10:10:10:10) (Dempsey and Snell, 1963). After drying of the paper sheets, a fluorescent spot was visible under UV light with a mobility in each solvent system identical with that of the synthetic pyridoxyllysine.

[1] Abbreviations used are: Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; DEAE, diethylaminoethyl.

TABLE I: Purification of Nucleoside Diphosphatase.[a]

	protein (mg)	enz act.[b] total units	enz act.[b] units/mg of protein
microsome extract	4020	8820 (100)[c]	2.19
$(NH_4)_2SO_4$ (35–75%)	2060	7440 (84)	3.61
1st DEAE-Sephadex	205	7160 (81)	34.9
Sephadex G-200	58.5	5700 (65)	97.4
hydroxylapatite	12.3	3410 (39)	278.3
2nd DEAE-Sephadex	3.36	1200 (14)	358.1

[a] Purification was started from 850 g of rat livers. [b] The enzyme activity was determined under the standard assay conditions, except that 2 μmol of IDP was used as substrate in the absence of the activator. One unit of enzyme activity was defined as the amount which catalyzed the liberation of 1 μmol of P_i/min under the assay conditions. [c] Yield of nucleoside diphosphatase.

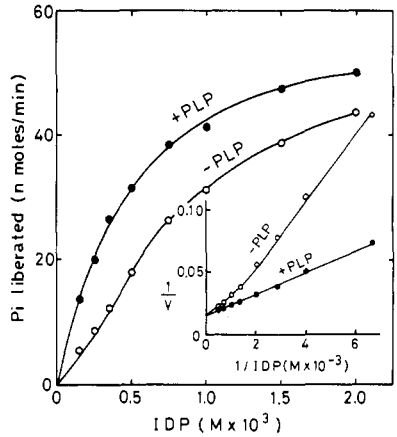

FIGURE 1: Effect of pyridoxal phosphate (PLP) on the nucleoside diphosphatase activity with varying concentrations of IDP. The reaction mixture (1.0 mL) contained 50 μmol of triethanolamine hydrochloride, pH 7.4, 4 μmol of $MgCl_2$, the enzyme, 0.2 μmol of pyridoxal phosphate where indicated, and varying amounts of IDP. Enzyme activities in the presence (●) and absence (○) of pyridoxal phosphate.

Results

Molecular Weights of Nucleoside Diphosphatase and Its Subunits. By the procedures described under Materials and Methods, nucleoside diphosphatase was purified about 160-fold from the microsome extract of rat liver with a yield of 14% (Table I). The purified enzyme preparation gave a single band in both disc gel electrophoresis and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Comparison of its mobility with those of several marker proteins in the sodium dodecyl sulfate–polyacrylamide gel electrophoresis indicated that the dissociated subunit had a molecular weight of 63 000. The molecular weight of purified nucleoside diphosphatase was estimated to be about 130 000 from column chromatography on Sephadex G-200. Therefore, nucleoside diphosphatase appears to be composed of at least two identical subunits.

Activation of Nucleoside Diphosphatase by Pyridoxal Phosphate. Nucleoside diphosphatase activity was found to be stimulated by pyridoxal phosphate. Stimulation was more pronounced at low substrate concentrations, and the effect disappeared as the substrate level increased (Figure 1). The Lineweaver–Burk plot gave a concave line in the absence of

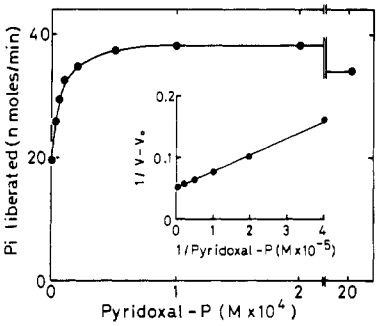

FIGURE 2: Effect of pyridoxal phosphate concentrations on the nucleoside diphosphatase activity. The standard assay conditions were used except that the concentration of pyridoxal phosphate was varied as indicated. V_0 refers to the reaction rate in the absence of pyridoxal phosphate.

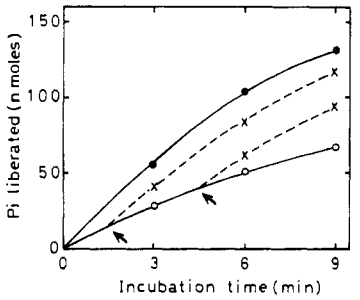

FIGURE 3: Effect of addition of pyridoxal phosphate during the course of the reaction. The reactions were performed in the presence (●-●) and absence (○-○) of pyridoxal phosphate under the standard assay conditions. At the times indicated by the *arrow*, 0.2 μmol of pyridoxal phosphate was added (x—x).

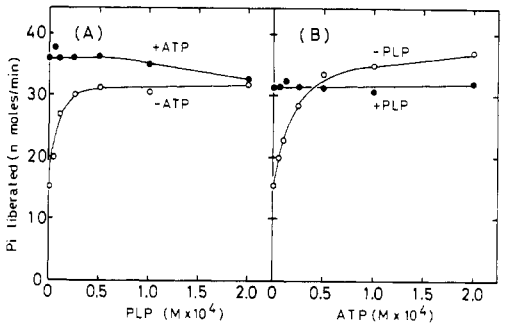

FIGURE 4: Competition between pyridoxal phosphate (PLP) and ATP on activation of nucleoside diphosphatase. (A) The standard assay conditions were used except that the concentration of pyridoxal phosphate was varied and 0.2 μmol of ATP was added where indicated. Enzyme activities in the presence (●) and absence (○) of ATP. (B) The standard assay conditions were used except that the concentration of ATP was varied and 0.2 μmol of pyridoxal phosphate was added where indicated. Enzyme activities in the presence (●) and absence (○) of pyridoxal phosphate.

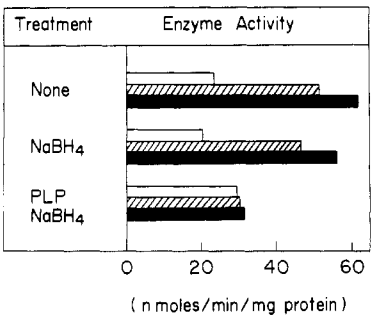

FIGURE 5: Modification of nucleoside diphosphatase by pyridoxal phosphate and $NaBH_4$. Nucleoside diphosphatase (0.2 mg) was treated with 1 mM pyridoxal phosphate (PLP) followed by reduction with $NaBH_4$ as described under Materials and Methods. In the control experiment the enzyme was treated with $NaBH_4$ alone. After dialysis the enzyme activity was determined under the standard assay conditions in the presence and absence of pyridoxal phosphate or ATP: (open bars) activities in the absence of the activator; (hatched bars) activities in the presence of pyridoxal phosphate; (solid bars) activities in the presence of ATP.

pyridoxal phosphate, but was normalized in its presence. The apparent K_m values for IDP in the presence and absence of pyridoxal phosphate were calculated to be 0.57 and 2.94 mM, respectively; V_{max} was essentially unchanged.

The effect of pyridoxal phosphate concentration on the rate of reaction is shown in Figure 2. Maximal velocity was obtained with 50 μM pyridoxal phosphate, and the Michaelis constant of activation (K_a) for pyridoxal phosphate was 5.2 μM under the conditions employed. Pyridoxal phosphate could not be replaced by other vitamin B_6 compounds. Pyridoxine phosphate and pyridoxamine phosphate at 0.2 mM and pyridoxal, pyridoxine, and pyridoxamine at 2 mM showed no stimulatory effect. The stimulatory effect of pyridoxal phosphate occurred without a measurable time lag when pyridoxal phosphate was added to an otherwise complete reaction mixture during incubation (Figure 3).

Pyridoxal phosphate also stimulated the enzyme reaction with thiamine pyrophosphate as substrate; the reaction rate with 2 mM thiamine pyrophosphate was increased to 2.3-fold by the presence of 0.2 mM pyridoxal phosphate.

Relationship to ATP Activation. Since the nucleoside diphosphatase reaction is also stimulated by ATP, the effect of pyridoxal phosphate on the reaction in the presence of ATP was investigated. Pyridoxal phosphate did not further stimulate the reaction which was maximally activated with ATP, and higher concentrations of pyridoxal phosphate reduced the reaction rate to the level obtained with pyridoxal phosphate alone (Figure 4A). Maximal velocity obtained with pyridoxal phosphate was 70–80% of that with ATP. Likewise, ATP did not further stimulate the reaction which was activated by pyridoxal phosphate; in other words, in the presence of 0.2 mM pyridoxal phosphate the enzyme lost sensitivity toward ATP activation (Figure 4B). These results suggest that pyridoxal phosphate and ATP compete for the same site on the enzyme and that pyridoxal phosphate has a higher affinity for the site than ATP. In fact, the K_a for ATP was 24 μM which is about five times that for pyridoxal phosphate.

Modification with Pyridoxal Phosphate and $NaBH_4$. In order to know whether the pyridoxal phosphate activation is due to Schiff base formation with its aldehyde group, the enzyme-pyridoxal phosphate complex was reduced by $NaBH_4$. As shown in Figure 5, treatment of nucleoside diphosphatase with pyridoxal phosphate followed by reduction with $NaBH_4$ resulted in marked changes of the properties of the enzyme; namely, the enzyme was desensitized toward the activation by pyridoxal phosphate as well as ATP. No change in the response to pyridoxal phosphate or ATP was found in the control experiment where the enzyme was treated with $NaBH_4$ alone. Although the modified enzyme with pyridoxal phosphate and $NaBH_4$ was no longer sensitive to pyridoxal phosphate or ATP, its activity in the absence of the activator was higher than that in the control experiment. This result can be explained by the decreased K_m of the modified enzyme for substrate as described below.

Experiments were done to analyze the effects of ATP, IDP, and $MgCl_2$ on the modification of the enzyme. As shown in Table II, the presence of 5 mM ATP or 10 mM IDP provided appreciable protection against the desensitization by 1 mM

TABLE II: Protection of Nucleoside Diphosphatase against Desensitization.[a]

	rel enz act.[b]		
	none	+Pyr *P*	+ATP
native	1.00 (23.4)	2.10	2.78
modified	1.00 (27.8)	1.15	1.22
modified in the presence of 5 mM ATP	1.00 (23.9)	1.81	2.42
modified in the presence of 10 mM IDP	1.00 (21.1)	1.72	2.26
modified in the presence of 10 mM $MgCl_2$	1.00 (28.3)	1.16	1.23

[a] Pyridoxal phosphate treatment and reduction with $NaBH_4$ were carried out as described in Figure 5. [b] Relative reaction rates under the standard assay conditions in the presence and absence of pyridoxal phosphate or ATP. The enzyme activities in the absence of the activator, expressed as nmol min^{-1} (mg of protein)$^{-1}$, are shown in parentheses.

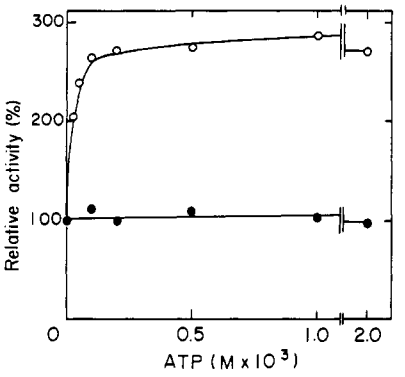

FIGURE 6: Effect of ATP concentration on the activities of native (○) and modified (●) nucleoside diphosphatase. The modified enzyme was prepared as described in Figure 5. Standard assay conditions were used except that the concentration of ATP was varied as indicated.

pyridoxal phosphate. On the other hand, 10 mM $MgCl_2$ showed no protection.

The Modified Enzyme: Absorption Spectrum and Isolation of N^6-Pyridoxyllysine. The absorption spectrum of the reduced enzyme-pyridoxal phosphate complex showed an absorption band with a maximum at 325–330 nm that was not present in the original enzyme. The absorption maximum at 325 nm is characteristic of the reduced pyridoxal phosphate Schiff base (Fischer et al., 1963). Further evidence in favor of the formation of a pyridoxyl phosphate derivative of the enzyme was obtained by means of fluorescence spectroscopy. As expected, excitation of the modified enzyme at 327 nm gave the emission band at 390 nm characteristic of pyridoxamine phosphate residues (Churchich, 1965). The number of phosphopyridoxyl amino groups present in the reduced pyridoxal phosphate–nucleoside diphosphatase can be estimated from the absorbance at 325 nm by using the molar extinction coefficient of 10 150 for pyridoxyl phosphate–lysine (Fischer et al., 1963). It was calculated that approximately 2 equiv of pyridoxal phosphate was incorporated per mol of enzyme (molecular weight of 130 000).

The modified enzyme was dialyzed against distilled water and then hydrolyzed with HCl, as described under Materials and Methods. N^6-Pyridoxyllysine was then identified in the acid hydrolysate by means of paper chromatography. In two different solvent systems, a fluorescent spot was observed with a mobility identical with that of synthetic N^6-pyridoxyllysine.

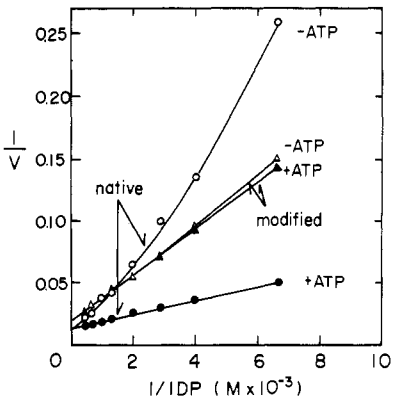

FIGURE 7: Lineweaver–Burk plots of the native and modified nucleoside diphosphatase in the presence and absence of ATP. The reaction system was the same as in Figure 1, except that 0.2 μmol of ATP was added in place of pyridoxal phosphate. The native enzyme activities in the presence (●) and absence (○) of ATP. The modified enzyme activities in the presence (▲) and absence (△) of ATP.

Kinetic Properties of the Modified Enzyme. The activity of the modified enzyme was not stimulated even by high concentrations of ATP (Figure 6). The modified enzyme gave a linear curve of Lineweaver–Burk plots in contrast to the concave line of the native enzyme in the absence of ATP (Figure 7). The K_m values of modified enzyme for IDP were 0.95 mM, irrespective of the presence or absence of ATP, while the K_m values of the native enzyme in the presence and absence of ATP were 0.48 and 2.94 mM, respectively. The decreased K_m value of the modified enzyme may be due to the covalent binding of pyridoxal phosphate at the regulatory site. Maximal velocity was slightly decreased in the modified enzyme.

The alteration of the regulatory properties of nucleoside diphosphatase after modification with pyridoxal phosphate was not due to dissociation of the enzyme to subunits, as studied by gel filtration with Sephadex G-200 column chromatography.

Stability of Native and Modified Enzymes. Pyridoxal phosphate protected nucleoside diphosphatase against heat inactivation. At 45 °C in 50 mM triethanolamine hydrochloride (pH 7.4), about 80% of the enzyme activity was destroyed in 16 min. This inactivation was decreased by 2 mM pyridoxal phosphate (Figure 8). In the presence of 0.2 mM pyridoxal phosphate, there was no protection of the activity against heat inactivation. The fixation of pyridoxal phosphate resulted in a profound increase in stability against heat denaturation. As shown in Figure 9A, the modified enzyme was more resistant to heat denaturation than the native enzyme. The pH stability of the modified enzyme was compared with that of the native enzyme. The modified enzyme showed a higher stability than the native enzyme, particularly in the alkaline pH range (Figure 9B).

Discussion

The present study has shown that pyridoxal phosphate stimulates the nucleoside diphosphatase reaction. Pyridoxal phosphate is active at very low concentrations and the K_a for pyridoxal phosphate is 5.2 μM, which is almost the same order of magnitude as the K_m values of several enzymes that require this compound as a coenzyme for catalytic activity (Barman, 1969). It seems possible that pyridoxal phosphate stimulates the nucleoside diphosphatase activity under intracellular conditions.

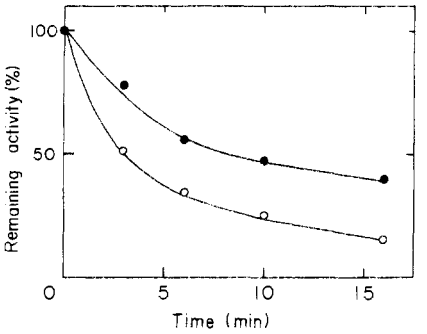

FIGURE 8: Effect of pyridoxal phosphate on heat inactivation of nucleoside diphosphatase. The enzyme was incubated at 45 °C in 50 mM triethanolamine hydrochloride (pH 7.4) with (●-●) or without (○-○) 2 mM pyridoxal phosphate. At the indicated times aliquots were withdrawn and assayed immediately for the activity of nucleoside diphosphatase under the standard assay conditions, except that 2 μmol of IDP was used as substrate in the presence of pyridoxal phosphate.

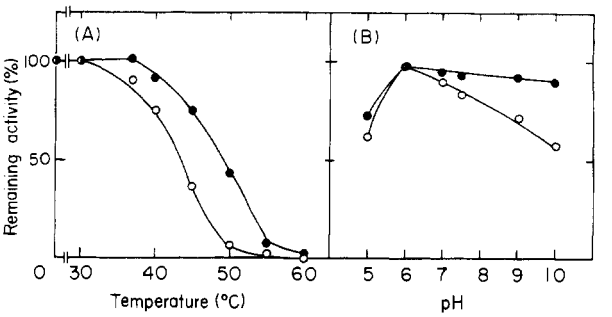

FIGURE 9: (A) Heat stability of the native (○) and modified (●) nucleoside diphosphatase. The enzymes were heated in 50 mM triethanolamine hydrochloride (pH 7.4) at the indicated temperatures for 5 min, and then the enzyme activity was measured under the standard assay conditions except that 2 μmol of IDP was used as substrate in the absence of activator. (B) pH stability of the native (○) and modified (●) nucleoside diphosphatase. The enzymes were incubated at 37 °C for 10 min at the indicated pH, and then the enzyme activity was measured under the standard assay conditions except that 2 μmol of IDP was used as substrate in the absence of activator. The following buffers were used: 50 mM sodium acetate (pH 5-6), 50 mM triethanolamine hydrochloride (pH 6-9), and 50 mM cyclohexylaminopropanesulfonic acid (pH 9-10).

The activation mechanism of nucleoside diphosphatase by pyridoxal phosphate is similar to that by ATP (Yamazaki and Hayaishi, 1968): (1) both activators decreased the K_m for substrate without affecting the V_{max}; (2) double-reciprocal plots gave linear curves in the presence of activators, whereas concave curves were obtained in their absence; (3) the stimulatory effects were exerted without a time lag; and (4) both ATP and pyridoxal phosphate protected the enzyme against heat denaturation. Three lines of evidence suggest that pyridoxal phosphate is bound to the enzyme at the same site as ATP: (1) reduction of the enzyme-pyridoxal phosphate complex with $NaBH_4$ yielded a modified enzyme which lost the sensitivity to either pyridoxal phosphate or ATP, (2) ATP protected the enzyme against the desensitization with pyridoxal phosphate and $NaBH_4$, and (3) pyridoxal phosphate and ATP appeared to compete for the same site on the enzyme in the stimulation of the reaction.

The results presented here suggest that the regulatory site of nucleoside diphosphatase contains a reactive lysine residue which forms a Schiff base with pyridoxal phosphate. This lysine residue appeared to participate in allosteric regulation of nucleoside diphosphatase activity, since modification of this lysine residue yielded a desensitized enzyme toward either ATP or pyridoxal phosphate activation. The lysine residue may be somehow involved in binding of the triphosphate moiety of ATP and other nucleoside triphosphates. Nucleoside diphosphatase is composed of two identical subunits, and about 2 equiv of pyridoxal phosphate was bound per mol of the enzyme. Therefore, the changes in the regulatory properties of nucleoside diphosphatase are thought to be the result of the modification of a single lysyl residue per subunit. Similar desensitization with pyridoxal phosphate was reported with fructose 1,6-bisphosphatase which was allosterically regulated by AMP (Marcus and Hubert, 1968; Krulwich et al., 1969; Colombo et al., 1972). It should be noted that our and their enzymes, both allosterically regulated by nucleotide, are desensitized by fixation of pyridoxal phosphate to the lysine residues in the regulatory sites.

Schramm and Morrison (1968, 1969) concluded that IDP is bound to the enzyme at two sites, one "catalytic" and the other "allosteric", since plots of reaction rates against IDP concentrations were sigmoidal in the absence of ATP but became hyperbolic in its presence. The present findings that (1) the plots became hyperbolic in the presence of pyridoxal phosphate, (2) the plots became also hyperbolic with the enzyme modified by pyridoxal phosphate and $NaBH_4$, and (3) the presence of IDP provided significant protection against the modification of the enzyme lend further support to the interpretation.

The properties of the modified enzyme, pyridoxyl phosphate-enzyme, resembled those of the native enzyme in the activated state. The K_m of the modified enzyme for IDP was 0.95 mM, irrespective of presence or absence of pyridoxal phosphate, while those of the native enzyme were 0.57 and 2.97 mM in the presence and absence of pyridoxal phosphate, respectively. The modified enzyme was more resistant to heat denaturation, which is in agreement with the observations that pyridoxal phosphate protected the native enzyme against heat denaturation. These results suggest that the modified enzyme has similar conformations of the protein molecule to those in the activated state of the native enzyme. This result was comparable to phosphorylase b which contains pyridoxal phosphate as an essential constituent. Reduced phosphorylase b has 50-60% of the specific activity of the native enzyme (Strausbauch et al., 1967).

Acknowledgments

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Uridine Diphosphate Galactose 4-Epimerase: Nucleotide and 8-Anilino-1-naphthalenesulfonate Binding Properties of the Substrate Binding Site†

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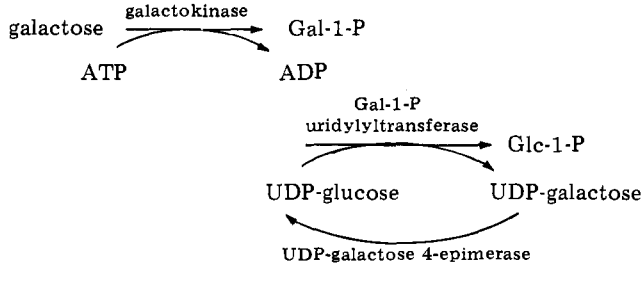
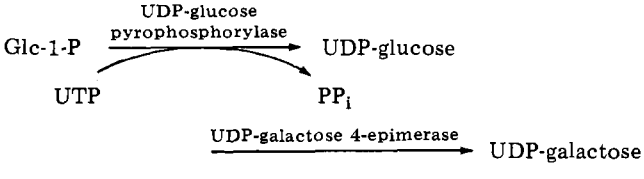
ABSTRACT: *Escherichia coli* UDP-galactose 4-epimerase in its native form (epimerase·NAD) binds 8-anilino-1-naphthalenesulfonate (ANS) at one tight binding site per dimer with a dissociation constant of 25.9 ± 2.1 μM at pH 8.5 and 27 °C. This appears to be the substrate binding site, as indicated by the fact that ANS is a kinetically competitive reversible inhibitor with a K_i of 27.5 μM and by the fact that ANS competes with UMP for binding to the enzyme. Upon binding at this site the fluorescence quantum yield of ANS is enhanced 185-fold, and its emission spectrum is blue shifted from a λ_{max} of 515 to 470 nm, which suggests that the binding site is shielded from water and probably hydrophobic. Competitive binding experiments with nucleosides and nucleotides indicate that nucleotide binding at this site involves coupled hydrophobic and electrostatic interactions. The reduced form of the enzyme (epimerase·NADH) has no detectable binding affinity for ANS. The marked difference in the affinities of the native and reduced enzymes for ANS is interpreted to be a manifestation of a conformational difference between these enzyme forms.

UDP[1]-galactose 4-epimerase, which catalyzes the interconversion of UDP-galactose and UDP-glucose, is one of the three enzymes of the Leloir pathway for the conversion of galactose to Glc-1-P. This pathway is the major route by which nutrient galactose enters the energy-producing metabolism of most organisms. The other two enzymes of the pathway are galactokinase and galactose-1-phosphate uridylyltransferase, which by coupled action convert galactose to UDP-galactose in preparation for the action of the epimerase. The Leloir pathway is illustrated by Scheme I. Another important function of UDP-galactose 4-epimerase is the net production of UDP-galactose from UDP-glucose under conditions in which galactose is not available as a nutrient but galactosyl units are needed for the biosynthesis of cellular constituents such as glycoproteins. UDP-glucose is produced under these conditions

SCHEME I.



SCHEME II



primarily by the action of UDP-glucose pyrophosphorylase on Glc-1-P and UTP. This is illustrated by Scheme II.

The *E. coli* UDP-galactose 4-epimerase is a dimer of identical subunits which contains one molecule of very tightly

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[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonate; NAD, nicotinamide adenine dinucleotide; UMP, UDP, and UTP, uridine 5′-mono-, di-, and triphosphates; AMP, ADP, and ATP, adenosine 5′-mono-, di-, and triphosphates.

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