ADPGLUCOSE PYROPHOSPHORYLASE: EVIDENCE FOR A LYSINE RESIDUE AT THE ACTIVATOR SITE OF THE ESCHERICHIA COLI B ENZYME[1]

Thomas Haugen[2], Armana Ishaque, and Jack Preiss
Department of Biochemistry and Biophysics
University of California
Davis, California 95616

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SUMMARY

Pyridoxal-P can be covalently linked to E. coli B ADPglucose pyrophosphorylase by reduction with sodium borohydride. The modified enzyme is almost fully active when less than 1 mole of pyridoxal-P is incorporated per mole of enzyme subunit and is no longer dependent on the presence of allosteric activators in reaction mixtures for high activity. The allosteric activators, fructose-P_2 or hexanediol 1,6 bisphosphate, decrease the incorporation of pyridoxal-P into enzyme suggesting that the pyridoxal-P is linked at or near the allosteric activator binding site. Acid hydrolysis of the modified enzyme yields pyridoxyllysine suggesting that the epsilon amino group of lysine is functional in the binding of the allosteric activators of the enzyme.

Previous studies (1,2) showed that in vitro allosteric regulation of bacterial glycogen occurs at the level of ADPglucose pyrophosphorylase. The ADPglucose pyrophosphorylase from Escherichia coli B is regulated via activation by glycolytic intermediates, notably fructose-P_2, and via allosteric inhibition by 5'AMP, ADP, and P_i. Kinetic studies on ADPglucose pyrophosphorylases with altered allosteric properties and isolated from E. coli B glycogen deficient (2,3) and glycogen excess mutants (1-4) have strongly indicated that the allosteric phenomena observed in vitro are operative in vivo and that fructose-P_2 is the most important physiological activator (2,3). Positive correlations between cellular fructose-P_2 levels in E. coli strains and their glycogen synthetic rates have been observed also suggesting the importance of fructose-P_2 in the in vivo regulation of glycogen synthesis (5).

It was shown (2,4,6-8) that the activator site of the E. coli ADPglucose pyrophosphorylase is relatively non-specific. Many analogues of fructose-P_2 are capable of stimulating this enzyme. One of the most effective compounds

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[2]Predoctoral trainee of USPHS Training Grant GM 119.

is pyridoxal phosphate (4,6). We have now found that pyridoxal-P is covalently linked by reduction with sodium borohydride to a lysine residue probably situated at or near the activator site of ADPglucose pyrophosphorylase.

MATERIALS AND METHODS

Assay of Enzyme - Pyrophosphorolysis of ADPglucose. Formation of [^{32}P] ATP from $^{32}PP_i$ and ADPglucose was assayed as previously described (8). The reaction mixture contained 10 µmoles Tris-Cl buffer (pH 8.0), 100 µg of bovine serum albumin, 2.0 µmoles of $MgCl_2$, 0.2 µmole of ADPglucose, 0.5 µmole of $^{32}PP_i$ (5×10^5 - 5×10^6 cpm per µmole), 0.4 µmole of fructose-P_2 where indicated, and enzyme in a volume of 0.25 ml.

ADPglucose Pyrophosphorylase. Enzyme was prepared from Escherichia coli B strain AC70R1 cells as previously described (9). Protein concentrations were determined by the method of Lowry (10) using bovine serum albumin as standard, or by using the relation $A^{0.1\%}_{280nm} = 1.0$. This value was obtained by relating A_{280nm} values of the enzyme to nitrogen content (11) and amino acid composition (unpublished data). A molecular weight value of the subunit of the enzyme of 50,000 was used (9).

Reagents. Hexanediol 1,6 bis-phosphate (12), pyridoxyl-valine (13), and ε-pyridoxyl-lysine (14) were synthesized as described. All other reagents were the highest purity commercially obtainable.

Modification of the Enzyme by Pyridoxal-phosphate and $NABH_4$. ADPglucose pyrophosphorylase preparations were dialysed against 0.1 M Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid-NaOH), pH 7.0, buffer containing 0.5 mM dithioerythritol. The reaction mixtures contained enzyme, 0.1 M Hepes (pH 7.0), and pyridoxal-P. Protein concentrations were adjusted so that the molarity of pyridoxal-P was always at least four fold higher than the subunit concentration. The mixtures were incubated at 25° for 30 minutes and then $NaBH_4$ was added in amounts that were at least four reducing equivalents excess over the pyridoxal-P. Reduction was allowed to proceed for an additional 30 minutes. When necessary, protein was concentrated by precipitation with $(NH_4)_2SO_4$ at a final concentration of 70% saturation. Precipitated protein was collected by centrifugation at 20,000 x g for 20 minutes. Resuspended pellets or protein solutions were dialysed against 0.05 M Tris-Cl (pH 7.2) containing 0.5 mM dithioerythritol. After dialysis, incorporation of pyridoxyl-P groups into the protein was measured by the absorbance at 325 nm after subtracting the end absorbance of the unmodified protein (15). Alternatively, the increased phosphate content of the enzyme was measured by the method of Ames and Dubin (16). The molar absorbance of the pyridoxyl-phosphate group attached to the enzyme was measured by plotting the P content against the increased absorbance at 325 nm and found to be 4700 at pH 7.2. This compares well with the value of 5350 reported by Greenwell et al. (15) for pyridoxyl-phosphate groups on aspartate transcarbamylase.

RESULTS

Reduction of the Pyridoxal Phosphate-Enzyme Complex. Figure 1 shows the effect of reduction on the pyridoxal-P enzyme mixture as a function of the pyridoxal phosphate concentration. The untreated enzyme has a fructose-P_2

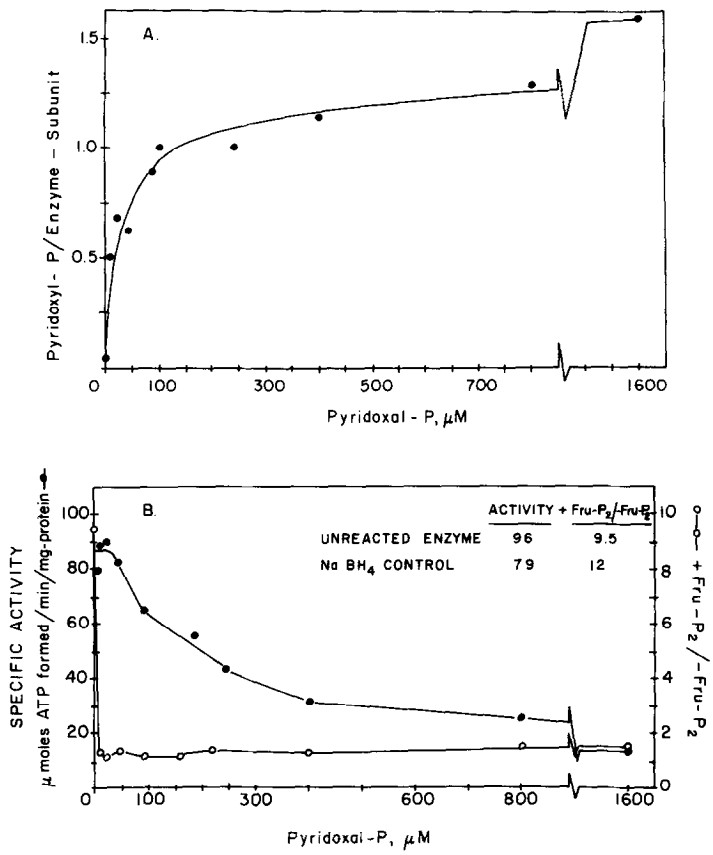

Figure 1. Modification of enzyme by pyridoxal-P and $NaBH_4$. Conditions are described in Materials and Methods. The reaction mixture contained 2 mg of enzyme in a volume of 5 ml. (A) Incorporation of pyridoxal-P into enzyme as a function of pyridoxal-P concentration. The pyridoxyl-P content was measured by determining phosphate bound to enzyme. (B) Effect of modification on kinetic properties of enzyme. Enzyme from part A was assayed for activity. The specific activity is measured in the presence of fructose-P_2.

activated specific activity of 90-100 μmoles $min^{-1}mg^{-1}$ in the pyrophosphorolysis direction and is stimulated about ten fold by fructose-P_2. When one pyridoxal-P has been covalently linked per subunit of enzyme, stimulation by fructose-P_2 has been reduced to 1.5-fold or less. The fructose-P_2 stimulated specific activity is less than 33% decreased. It is likely that this decrease in activity is due to addition of pyridoxal-P to sites other than the activator site because at concentrations of pyridoxal-P where more than one pyridoxal-P per subunit is covalently bound the enzyme is progressively inactivated. The

stimulation by fructose-P_2, however, remains minimal.

When enzyme was incubated with pyridoxal-P without $NaBH_4$ or when enzyme was incubated with 1 m$\underline{M}$ $NaBH_4$ without pyridoxal-P and then dialyzed, no effect on the ability of fructose-P_2 to stimulate the enzyme activity was observed. At higher concentrations of $NaBH_4$ (5 m$\underline{M}$) inactivation to an extent less than 30% was observed. Most experiments involved concentrations of $NaBH_4$ less than 1 m$\underline{M}$ where no inactivation occurred due solely to this reagent.

Protection by Fructose-P_2 and Hexanediol 1,6 bisphosphate. Pyridoxal phosphate, 40 $\mu\underline{M}$, and enzyme subunits, 20 $\mu\underline{M}$, in one ml of 0.1 $\underline{M}$ Hepes (pH 7.0) buffer was incubated alone or with the addition of 1.5 m$\underline{M}$ fructose-P_2 or 0.5 m$\underline{M}$ 1,6 hexanediol bisphosphate. After 30 minutes at room temperature, the mixture containing only pyridoxal-P, enzyme and buffer was noticeably more yellow than the others, indicating formation of a Schiff's base. These mixtures were then made about 2 m$\underline{M}$ in $NaBH_4$ by adding 10 μl of a 0.2 $\underline{M}$ $NaBH_4$ solution and then dialyzed to remove unreacted low molecular weight material. The addition of the activators fructose-P_2 or 1,6 hexanediol bisphosphate in the reaction mixture had markedly decreased the incorporation of pyridoxal phosphate groups (Table 1). The stimulation by fructose-P_2 (determined by the ratio, activity in presence of fructose-P_2/activity in absence of fructose-P_2) is greater with the enzyme fractions protected by the allosteric activators than with the enzyme incubated with pyridoxal-P in the absence of allosteric activators (Table 1).

Identification of a Pyridoxyllysine Residue. Enzyme was incubated as described in Methods with 160 $\mu\underline{M}$ pyridoxal phosphate and then reduced with $NaBH_4$. This resulted in the incorporation of 1.2 pyridoxyl phosphate groups per subunit. A portion of the modified enzyme (60 nmoles of subunits) was hydrolyzed in 6 $\underline{M}$ HCl at 109° for 22 hours. The hydrolysate was evaporated using a stream of N_2 and dissolved in a small amount of H_2O. This solution was passed onto a Dowex 50 (NH_4^+ form) column (0.5 x 20 cm) and eluted with 0.1 $\underline{M}$ citrate-NH_4OH (pH 5.0), followed by elution with H_2O, and then with 0.03

Table 1. Effect of hexanediol 1,6 bisphosphate and fructose-P_2 on the covalent binding of pyridoxal-P to ADPglucose pyrophosphorylase. See text for procedure.

	P-pyridoxyl / enzyme subunit	μmoles ATP formed / min-mg		+ fru-P_2 / - fru-P_2
		+ fru-P_2	- fru-P_2	
Unreacted enzyme	--	90	11.3	8
Unprotected	0.75	90	68.0	1.3
+ Hexanediol 1,6 P_2, 0.5 mM	0.23	79	14.8	5.3
+ Fructose-P_2, 1.5 mM	0.36	67	18.2	3.7

M NH_4OH. Under these conditions, the neutral and acidic amino acids are eluted by the citrate buffer, and histidine and pyridoxyllysine by the NH_4OH solution. The fractions were neutralized by bubbling CO_2 gas into them. Fluorescent material (measured by excitation at 325 and emission at 398 nm) was eluted only by the NH_4OH wash (Figure 2) and was estimated to contain 70 nmoles pyridoxyllysine. After concentration, the material was subjected to electrophoresis in 1.6 M HCOOH on Whatman #3 paper (System A), followed by paper chromatography on Whatman #1 using butanol:pyridine:HOAc:H_2O (30:20:6:24). The material eluted from the fluorescent spot was estimated to contain 40 nmoles of pyridoxyllysine using the published spectral data (14). This material was then subjected to paper electrophoresis on Whatman #1 using pyridine:HOAc:H_2O (10:0.4:90) (pH 6.5) as solvent (System B). In each chromatographic or electrophoretic system the material co-migrated with standard pyridoxyllysine (Table 2). Unpublished data indicate that valine is the N-terminal amino acid of the E. coli B ADPglucose pyrophosphorylase. It has been observed by Benesch et al. (17) that in hemoglobin an N-terminal amino acid residue may also react with pyridoxal phosphate. Pyridoxylvaline was, therefore, run as a standard in these chromatography systems and was well resolved from pyridoxyllysine in each case (Table 2).

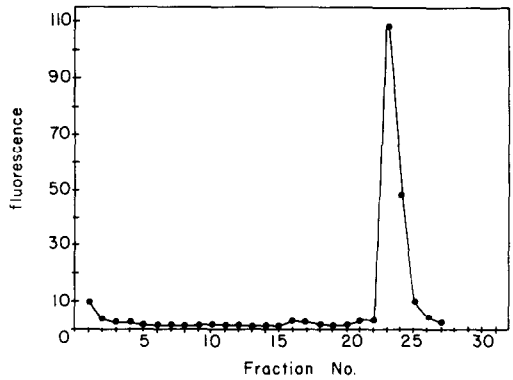

Figure 2. Chromatography of acid hydrolysate of 60 nmoles of enzyme subunits previously reacted with pyridoxal-P and $NaBH_4$. The material was applied to a column of Dowex 50 x 8, NH_4^+ form (20 x 0.5 cm), eluted with 0.1 M citrate-NH_4OH (pH 5.0) buffer, followed by H_2O at fraction #10 and then by 0.03 M NH_4OH at fraction #21. Synthetic pyridoxylvaline elutes at fractions 3-5, and synthetic pyridoxyllysine at fraction 22-26. The fraction volumes were 2.3 ml. Fluorescence was measured at 395 nm upon excitation at 325 nm.

Table 2. Electrophoresis and paper chromatography of pyridoxyllysine. See text for conditions. In electrophoresis system A, the mobility of unknowns and standards were compared to lysine. In system B the comparison was with pyridoxyllysine.

	electrophoresis system A relative mobility	paper chromatography Rf	electrophoresis system B relative mobility
lysine	1.00	--	--
synthetic pyridoxyl-lysine	0.93	0.24	1.00
synthetic pyridoxyl-valine	0.61	0.51	0.41
pyridoxyllysine from protein hydrolysate	0.88	0.22	0.96

DISCUSSION

It is likely that the lysine residue modified by the reduction of the pyridoxal phosphate-ADPglucose pyrophosphorylase complex is a residue at or near the activator site. The activity of the enzyme containing covalently

linked pyridoxyl-P is greatly increased when measured in the absence of fructose-P_2 and is not appreciably increased by addition of fructose-P_2 to the assay mixtures. The conditions required to form the reversible complex as measured in kinetic assays (concentration of pyridoxal-P required to give 50% of maximal stimulation = 12 μM; reference 4) compare well with the concentrations needed to obtain covalently attached pyridoxyl phosphate groups. The protection against modification by the activators fructose-P_2 and hexanediol bisphosphate can be viewed as a displacement of the pyridoxal phosphate by competition and is consistent with the concept that the site of modification is the activator site.

Many metabolites and a number of fructose-P_2 analogues have been shown to stimulate ADPglucose pyrophosphorylase (2,4,6-8) and, therefore, the activator site is relatively nonspecific. These compounds which compete for and activate by binding to a unique site on the enzyme contain certain common features. They are either bisphosphates (e.g. fructose-P_2), or compounds containing an aldehyde and a phosphate group (e.g. pyridoxal-P, erythrose-P) or compounds containing a carboxylic acid and a phosphate group (e.g. pyridoxic acid-5-P, P-enol pyruvate). It may be that the requirements for binding are satisfied by one phosphate group plus an additional anionic or aldehydic component (2,7). It is quite likely that these activators would bind to basic residues in the protein such as the epsilon amino group of lysine. The results presented suggest that pyridoxal-P binds via formation of a Schiff's base with the ε-amino of a lysine residue.

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