

"Research Note"

INVOLVEMENT OF ESSENTIAL LYSINE RESIDUES IN THE CATALYTIC ACTIVITY OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE PURIFIED FROM *STREPTOMYCES AUREOFACIENS**

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Abstract – Glucose 6- phosphate dehydrogenase from *streptomyces aureofaciens* was purified and inactivated by pyridoxal 5'-phosphate (PLP). The inactivation was a pseudo-first order and time-dependent reaction. Complete inactivation was achieved at 0.2mM PLP within 16 minutes. The type of inhibition was competitive with respect to Glucose 6- phosphate. Spectral characteristics of PLP-enzyme complex corresponded to the formation of a Schiff's base between PLP and lysine residue(s) of the enzyme. Intrinsic protein fluorescence sharply decreased upon PLP modification with about a 10 nm red shift. The presence of glucose 6-phosphate in the incubation mixture prevented the fluorescence change. Fluorescence studies revealed that NAD⁺ and NADP⁺ binding induces different conformational changes in pyridoxylated enzyme. The stoichiometry of PLP binding to the enzyme showed that 2 moles of lysine residues were modified per mole of enzyme. The data indicated that the modified lysine residues are involved in substrate binding and/or catalytic activity of this enzyme.

Keywords – Glucose 6-phosphate dehydrogenase, pyridoxal 5'-phosphate, essential lysine residue

1. INTRODUCTION

Glucose 6- phosphate dehydrogenase (G6PD), E.C.1.1.1.49, an important enzyme in the hexose monophosphate pathway, is NADP⁺ specific or NADP⁺ preferred in eukaryotes and many prokaryotes [1]. Some prokaryotes possess a single G6PD that can utilize both NAD⁺ and NADP⁺ as a coenzyme [2]. A well studied example of dual nucleotide specific G6PD is the enzyme from *leuconostoc mesenteroides* [2-4]. Another dual coenzyme specific enzyme is G6PD from *streptomyces aureofaciens* [5]. Primary studies have shown that most probably, two different forms of this enzyme are responsible for NAD⁺ and NADP⁺ binding [6]. Denaturation of *S. aureofaciens* G6PD by urea and reactivation in the presence of NAD⁺ or NADP⁺ revealed that these coenzymes pull the enzyme towards different conformational structures [7]. We have recently purified this enzyme and studied its interaction with NAD⁺ and NADP⁺ using different fluorescent probes [8]. This study showed that the G6PD undergoes different conformational changes upon NAD⁺ and NADP⁺ binding, and modification of the enzyme by pyridoxal 5'-phosphate, a competitive inhibitor with respect to glucose 6- phosphate, pulls its structure into a conformation suitable for NAD⁺ binding. The detailed structure function relationship of *streptomyces aureofaciens* G6PD, however, is not yet understood and amino acids involved in the substrate binding or catalytic activity are not known.

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In the present study G6PD from *Streptomyces aureofaciens* was purified and the presence of lysine residues essential for the catalytic activity was shown using enzyme modification by pyridoxal phosphate (PLP), a lysine modifying reagent. The kinetics of the enzyme inhibition by this reagent was also studied.

2. MATERIALS AND METHODS

Streptomyces aureofaciens (#1119) was obtained from the Iranian Scientific Research Organization (Tehran). Sephadex G100 and β -mercaptoethanol were purchased from Sigma Co. (U.S.A). Pyridoxal phosphate, EDTA, Tris, Tryptose and agar were from Merck Co. (Germany). All other chemicals were reagent grade.

a) Enzyme purification

The cells were grown and the enzyme purified as described previously [7]. The purification procedure involved ammonium sulfate fractionation and chromatography on DEAE-Cellulose and Sephadex G100 columns. The purified enzyme had the same specific activity as before.

b) Enzyme assay

Standard assays of G6PD for NAD^+ and NADP^+ -linked activities were performed according to Neuzil et al [5] in a Perkin-Elmer spectrophotometer model 551S at 25°C.

c) Enzyme modification by PLP

G6PD was modified with PLP as essentially described before [9]. *S. aureofaciens* G6PD (150 $\mu\text{g/ml}$) was incubated with different concentrations of PLP (0.1-0.8 mM) in 30 mM phosphate buffer pH 7.8 at 25°C in the dark. Aliquots were removed at different time intervals and assayed immediately for the enzyme activity. A control sample was subjected to the same procedure, but omitting G6PD. After 20 minutes incubation, acetic acid (0.5M) was added to the incubation mixture to bring the pH to 6. To reduce the Schiff's base formed, a fresh aqueous solution of 0.25 M NaBH_4 was added in portions of 20 μl until a concentration of 0.1 M NaBH_4 was achieved in the enzyme solution; n-octanol (20 μl) was added to prevent foaming. The reaction mixture was maintained at pH 6 during the reduction. The reduction was allowed to proceed for 10 min, after which the enzyme was dialyzed against the phosphate buffer with several changes.

d) Other methods

Fluorescence studies were performed in 30mM phosphate buffer pH 7.8 containing the indicated ligands (see Figure legends) using a Perkin-Elmer spectrophotofluorimeter Model LS-3P at room temperature. Absorbance studies were done in the above buffer using a Perkin-Elmer spectrophotometer Model 551S at room temperature. Protein was measured by the method of Lowry et. al. [10]. The lines of the double reciprocal plots were drawn using linear regression analysis and Excel 2000 program.

3. RESULTS

a) Inactivation of G6PD by PLP

Inactivation of the enzyme by different concentrations of PLP is shown in Fig. 1. Inactivation was pseudo-first order and a time-dependent reaction. With 0.2 mM PLP the activity loss was completed in 16 minutes. The activity of the control sample in the absence of PLP did not change up to the 16 minutes studied. The pseudo- first order constant, k' , were determined from the data in Fig. 1, and half – times ($t_{0.5}$)

were calculated using the equation $t_{0.5} = 0.693/k'$. Plotting $t_{0.5}$ values versus $\log \text{PLP}$ [11] gave a straight line with a slope of 0.78 (Fig. 2). The inhibition was competitive with respect to glucose 6-phosphate (Fig. 3).

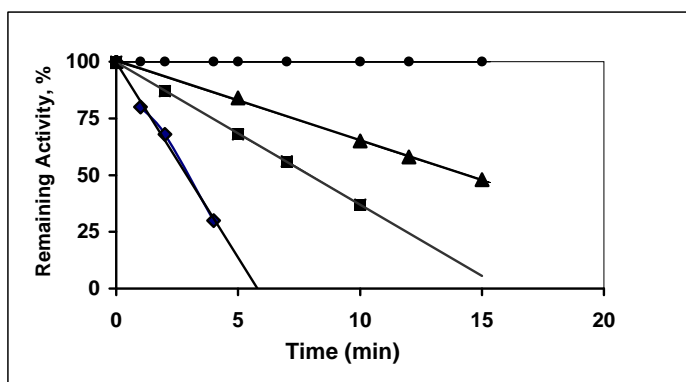


Fig. 1. Time – and concentration-dependent inactivation of G6PD by PLP. The enzyme (0.4 μM) was incubated in 30 mM phosphate buffer pH 7.8 with PLP at concentrations of zero (\bullet), 0.1 mM (\blacktriangle), 0.2 mM (\blacksquare) and 0.8 mM (\blacklozenge). Each point represents the average of 2 experiments. For details see text

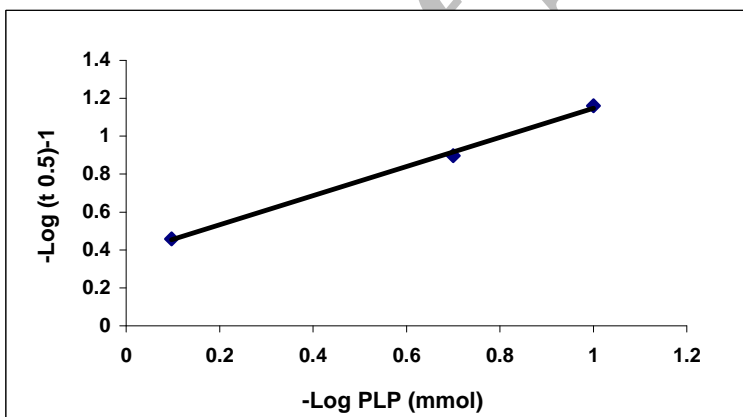


Fig. 2. Determination of the order of PLP inhibition. The data were taken from Fig. 1

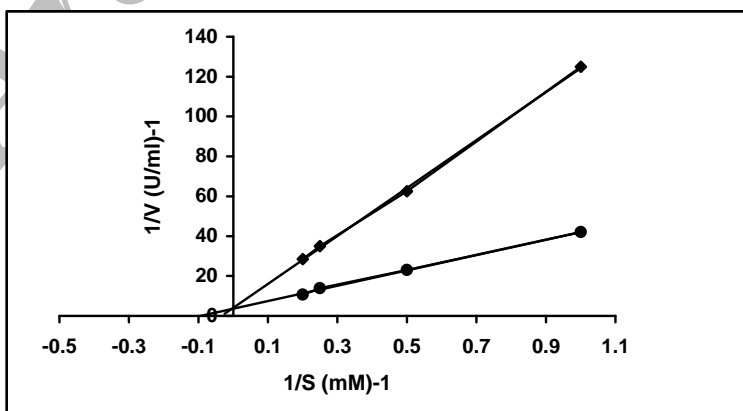


Fig. 3. Lineweaver-Burk plot of G6PD inhibition by PLP. The enzyme (0.4 μM) activity was measured in the absence (\bullet) and presence (\blacklozenge) of PLP (0.8 mM) at different concentrations of glucose 6- phosphate. Each point is the average of duplicate tests. For details see text

b) Spectral characterization of PLP-modified G6PD

Absorption spectra of normal and reduced PLP-modified enzyme showed an increase in the absorption of the latter between 325 nm and 345 nm corresponding to the formation of a secondary amine (reduced Schiff's base) (Fig. 4). The difference absorption spectrum of unreduced PLP-modified enzyme and normal enzyme showed a maximum at 430 nm (Fig. 5), suggesting again Schiff's base formation. When reduced, pyridoxylated enzyme was excited at 280 nm, a decrease in the fluorescence at 340 nm and an increase in fluorescence at about 390 nm was observed (Fig. 6).

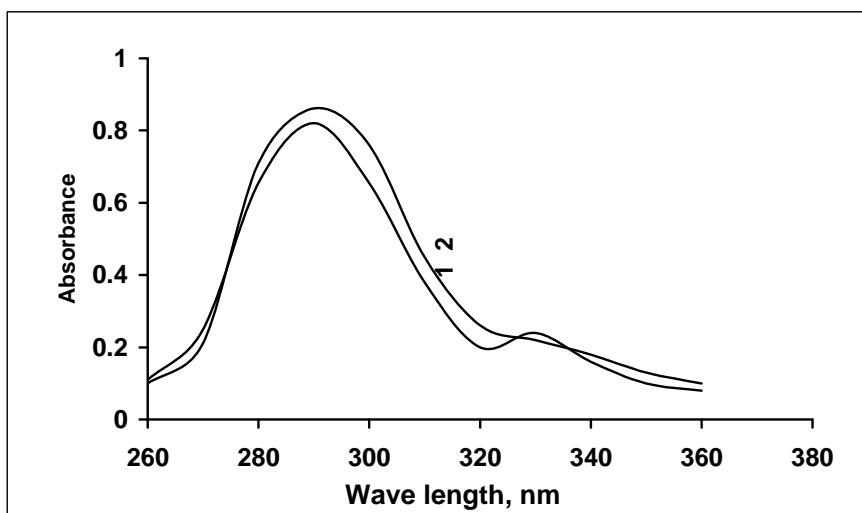


Fig. 4. Absorption spectra of normal and reduced PLP-modified G6PD. The enzyme ($1.5 \mu\text{M}$) was inactivated with PLP (0.6 mM) in 30 mM phosphate buffer pH 7.8 and reduced with NaBH_4 as described in methods. A control enzyme was taken in which PLP was omitted. PLP-modified enzyme (1); normal enzyme (2)

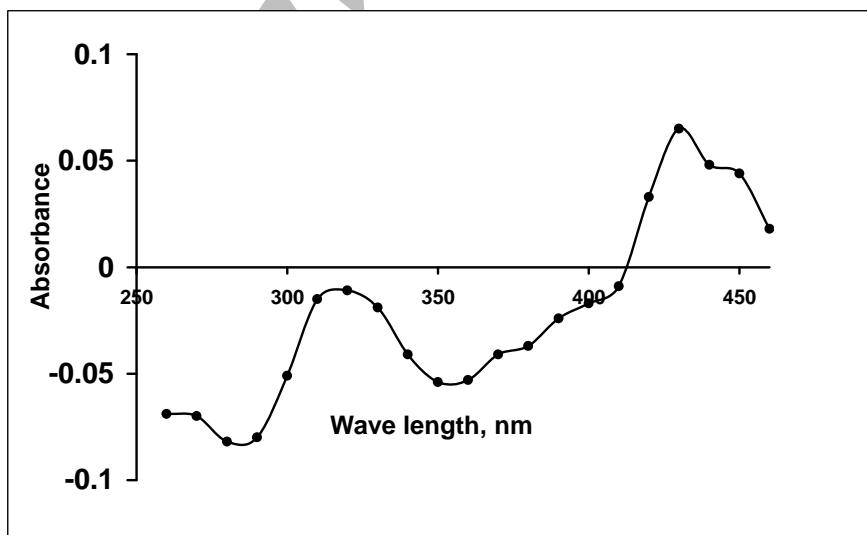


Fig. 5. Difference spectrum of PLP-G6PD reversible adduct. The enzyme ($0.4 \mu\text{M}$) was inactivated with PLP (0.4 mM) as described in the methods. The absorption spectra for the test and control samples measured from which the difference spectrum was calculated

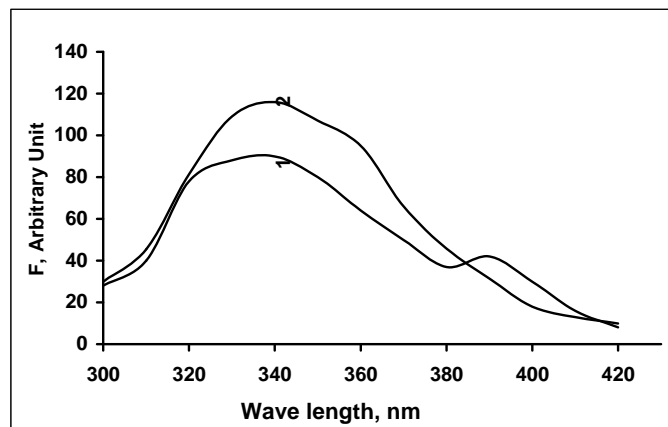


Fig. 6. Emission spectra of normal enzyme (2) and reduced pyridoxyl-G6PD (1). The enzyme and PLP concentrations and the procedure are the same as in Fig. 4. The excitation wave length was 280 nm

c) Effects of coenzyme binding on PLP and protein fluorescence

The maximum fluorescence of the PLP group bound to G6PD decreased when excited at 330 nm upon NAD^+ and NADP^+ binding (Fig. 7). The decrease in the fluorescence by NADP^+ , however, was more pronounced than that of NAD^+ . The data shown in Fig. 8 demonstrated that protein fluorescence of G6PD (excited at 280 nm) sharply decreased upon PLP modification with about a 10 nm red shift in the emission spectrum. The presence of glucose 6- phosphate in the incubation mixture during modification significantly prevented the protein fluorescence change.

d) Stoichiometry of PLP binding

The stoichiometry of PLP binding was determined following reduction of Schiff's base with NaBH_4 , using a molar extinction coefficient of $8600 \text{ M}^{-1}\text{cm}^{-1}$ at 316 nm [12] and M.W. of 107 KD_a for dimeric enzyme [13]. The results showed that 2 moles of lysine residues were modified per mole of dimer enzyme for complete activity loss (Fig. 9).

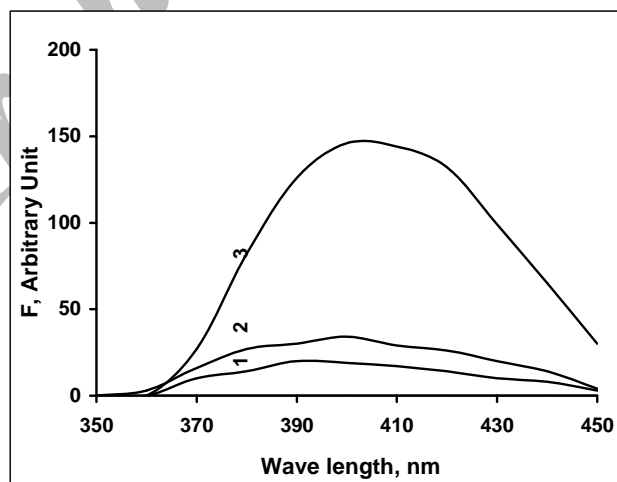


Fig. 7. The effects of NAD^+ and NADP^+ on the emission spectrum of reduced PLP-G6PD complex. The conditions for enzyme modification and reduction are the same as in Fig. 4. The fluorescence of PLP group was measured using excitation wave length at 330 nm in the presence of NAD^+ (2 mM) or NADP^+ (2 mM) in 30 mM phosphate buffer pH 7.8 at 25°C. (3), no coenzyme; (2), Plus NAD^+ ; (1), plus NADP^+

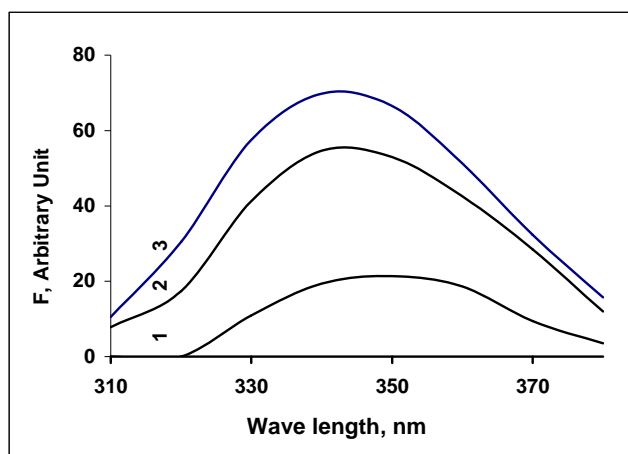


Fig. 8. The effect of glucose 6-phosphate on the intrinsic protein fluorescence of PLP-G6PD complex. The enzyme ($1.5 \mu\text{M}$) was inactivated with PLP (0.4 mM) in the absence and presence of glucose 6-phosphate as described in the text. Excitation wave length was 280 nm . (3), normal enzyme; (1), modified in the absence of glucose 6-phosphate, (2), modified in the presence of glucose 6-phosphate

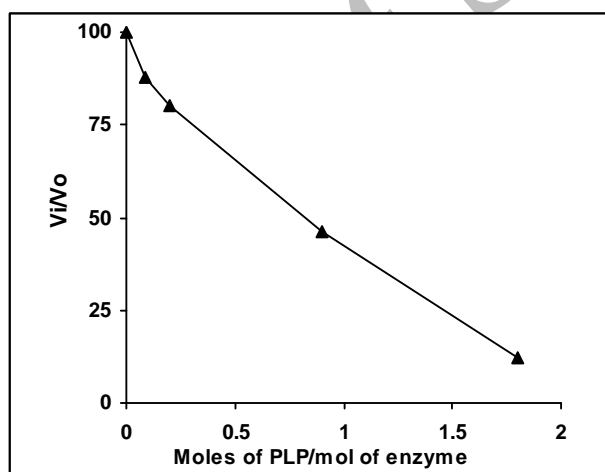


Fig. 9. Stoichiometry of PLP bound to G6PD. The enzyme ($1.5 \mu\text{M}$) was modified with different concentrations of PLP ($0\text{-}1 \text{ mM}$) and each time reduced with NaBH_4 as in the methods. The number of PLP bound per mole of enzyme calculated as described in the text. v_i and v_o are the initial velocities of the modified and normal enzyme, respectively

4. DISCUSSION

The involvement of essential lysine residues have been shown in the active sites of enzymes such as G6PD from *L. mesenteroides* [9], Ribulose 1,5-bisphosphate carboxylase [14], and rat liver phosphatidate phospho-hydrolase [15]. In the present study modification of G6PD from *S. aureofaciens* with PLP, a chemical modification reagent which reacts selectively with the ϵ -mino group of specific lysine residues [12], caused rapid inhibition of the enzyme. The difference spectrum (Fig. 5) of enzyme-PLP complex suggested that PLP was bound reversibly to primary amino groups as the keto-enamine form with characteristic absorption maximum at 430 nm [11]. When the reduced pyridoxyl enzyme was excited at 330 nm (Fig. 7), a maximum emission at 390 nm was observed.

These spectral characteristics are in agreement with those reported by Forry *et al.* [12] for phosphopyridoxyllysine. Similar absorption characteristics were found when PLP was reversibly bound to *L. mesenteroides* G6PD [16, 17], human erythrocyte G6PD [18], and phosphatidate phosphohydrolase [15].

The close similarity between the spectra of these enzymes in which PLP was shown to react with ϵ -amino group of lysine as well as that of PLP-modified *S. aureofaciens* G6PD, provides evidence that the essential residue is a lysine residue. Stoichiometry data, which showed only 2 moles of PLP bound per mole of dimmer enzyme, further support this conclusion. In Fig. 2 the average order of the reaction is equal to the slope of the line [11]. The slope of 0.78 obtained suggested that one mole of PLP is bound per active unit of G6PD.

The finding that the fluorescence of pyridoxyl enzyme, activated at 280 nm, increased at 390 nm and decreased at 330 nm (Fig. 6), is a result of the overlap of the absorption spectrum of the pyridoxyl group and the emission spectrum of tryptophan residues [12]. It is, therefore, concluded that the appearance of a new peak at 390 nm arises by energy transfer from excited aromatic amino acid residues (mainly tryptophan) on the protein to the bound pyridoxyl group.

Different effects of NAD^+ and NADP^+ on the maximum emission of the PLP group in the reduced PLP-enzyme complex (Fig. 7) revealed that NADP^+ binding induces larger conformational change in the enzyme than NAD^+ . Inhibition studies demonstrated that PLP is a competitive inhibitor with respect to glucose 6-phosphate (Fig. 3). It is, therefore, possible that PLP binds to the substrate binding site on the enzyme. The conformational change induced upon PLP binding, as indicated by intrinsic protein fluorescence, was protected by glucose 6-phosphate (Fig. 8) suggesting again that lysine residue modified by PLP is possibly the substrate binding site. These findings led us to the conclusion that glucose 6-phosphate pulls the enzyme to a conformation that is more suitable for NAD^+ binding. This has also been reported by the kinetics of denaturation and renaturation of this enzyme [7-8]. Similar conclusions have been reported for other dual nucleotide enzymes such as *L. mesenteroides* G6PD [18, 19].

NOMENCLATURE

G6PD	glucose 6-phosphate dehydrogenase
PLP	pyridoxal 5'-phosphate
NAD^+	nicotinamide adenine dinucleotide
NADP^+	nicotinamide adenine dinucleotide phosphate

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