INTERACTION OF NAD^+ AND $NADP^+$ WITH NATIVE AND PYRIDOXAL PHOSPHATE-MODIFIED GLUCOSE 6-PHOSPHATE DEHYDROGENASE PURIFIED FROM STREPTOMYCES AUREOFACIENS[*]

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Abstract - Interaction of glucose 6-phosphate dehydrogenase from *S. aureofaciens* with NAD^+, $NADP^+$ and glucose 6-phosphate were investigated using different fluorescent probes. Binding of NAD^+, $NADP^+$ and S-NADPH to the native enzyme quenched intrinsic protein fluorescence by 100%, 10% and 21%, respectively, from which K_d values of NAD^+ (6.5 mM), $NADP^+$ (92.0 μM) and S-NADPH (122.0 μM) were calculated. Binding of NAD^+, $NADP^+$ and S-NADPH to the pyridoxylated enzyme in which pyridoxal 5`-phosphate occupied a glucose 6-phosphate site, quenched the fluorescence of the pyridoxal group on the enzyme by 20%, 57% and 96%, respectively. K_d values for the pyridoxylated enzyme were also calculated for NAD^+ (1.0mM), $NADP^+$ (301.0μM) and S-NADPH (151.0μM). When NAD^+ was bound to the native enzyme-S-NADPH complex, to which S-NADPH was bound to only one subunit leaving the other free, the S-NADPH fluorescence was quenched with a 10 nm blue shift in its emission spectrum. $NADP^+$ binding, however, enhanced S-NADPH fluorescence. The fluorescence of S-NADPH bound to the pyridoxylated enzyme was enhanced upon NAD^+ binding with a 5 nm blue shift, while $NADP^+$ binding had no effect. A substrate analog, glucose 1-phosphate, inhibited the enzyme competitively with respect to glucose 6-phosphate and uncompetitively with respect to NAD^+. Binding of NAD^+ to enzyme-glucose 1-phosphate complex quenched protein fluorescence (44%) with decreasing K_d value from 6.5 mM in the absence of glucose 1-phosphate to 2.2 mM in its presence. $NADP^+$, however, showed opposite effects. The data demonstrated that *S.aureofaciens* glucose 6-phosphate dehydrogenase undergoes different conformational changes upon NAD^+ and $NADP^+$ binding, and modification of glucose 6-phosphate binding site by pyridoxal 5`-phosphate pulls the enzyme in a conformation suitable for NAD^+ binding.

Keywords – Glucose 6 phosphate dehydrogenase**,** streptomyces aureofaciens, pyridoxal phosphate

1. INTRODUCTION

Glucose 6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) catalyzed the first reaction of a hexose monophosphate pathway [1-2]. In prokaryotes and eukaryotes, G6PDs are either NAD^+ or $NADP^+$ specific generation NADH and NADPH, respectively [3-4]. In some organisms, however, a single enzyme is able to utilize both NAD^+ and $NADP^+$. The best known example of this kind is G6PD from *L. mesenteroides* [5-7].

Several investigations including kinetic [8-9], fluorescence [10-11], denaturation and renaturation [12], protection [13-14] and X-ray structure [15-16] studies revealed that *L.mesenteroides* G6PD undergoes different conformational isomers for binding to NAD^+ or $NADP^+$.

Among other dual nucleotide specific enzymes G6PD from   *S. aureofaciens* has been studied the least. Primary kinetic and inhibition studies have shown two different forms of *S. aureofaciens* G6PD for binding to NAD^+ or $NADP^+$ [17-18]. Recent inhibition studies (unpublished data) and kinetics of denaturation and renaturation [19] have demonstrated two distinct conformational forms of this enzyme for NAD^+ and $NADP^+$ binding. Structural studies have shown two lysine residues essential for the catalytic activity of *S. aureofaciens* G6PD [20]. However the mechanism by which coenzyme utilization in this enzyme is regulated is not yet understood.

In the present work kinetic and binding constants of several ligands were determined and conformational isomers suitable for NAD^+ or $NADP^+$ binding were extensively studied using three different fluorescent probes. The data suggested that glucose 6-phosphate binding pulls the enzyme to a conformational form favoring NAD^+ binding.

2. MATERIALS AND METHODS

a) Materials

S. aureofaciens (strain 1119) obtained from the Iranian Scientific Research Organization (Tehran). NAD^+, $NADP^+$, S- $NADP^+$, glucose 6-phosphate, glucose 1-phosphate, pyridoxal 5`-phosphate (PLP), DEAE-cellulose and Sephadex G-100 were prepared from Sigma Chemical Co. (USA). Yeast G6PD was obtained from the Merck Co. (Germany). All other chemicals were reagent grade. All solutions except the enzyme solution used in the fluorescence studies were filtered through a Millipore membrane ($0.45\mu m$) 2 times before use.

b) Enzyme purification

S. aureofaciens was grown according to Behal et al. [21]. The cells were suspended in 0.2 M Tris-HCl buffer pH=7.4 containing 15% (v/v) glycerol, 1 mM β-mercaptoethanol, 2 mM EDTA and 0.05 mM PMSF and sonicated at 22 KHz for 30 seconds. Sonication repeated 13 cycles to disrupt the cells (90%). The broken cells were then centrifuged at 20,000 g for 30 minutes. The supernatant (crude extract) was kept at -20ºC for the enzyme purification.The enzyme was purified as described before [18, 19] by chromatography on DEAE-cellulose and Sephadex G-100.

c) Enzyme assay

G6PD activity was measured in 50 mM imidazole-HCl buffer pH=6.6 containing 2 mM NAD^+, 5mM glucose 6-phosphate and 10 mM $MgCl_2$ as reported previously [17,18].

d) Determination of protein

Protein concentration was measured by Lowry's method, [22] using bovine serum albumin as a standard.

e) SDS-gel electrophoresis

SDS-gel electrophoresis was done according to Hames and Rickwood's method [23].

f) PLP-modification

G6PD was modified with PLP using the method described by Milhausen and Levy [24].An enzyme containing 1.15 mole of PLP group/mole of enzyme was prepared.

g) Preparation of S-NADPH

S-NADPH was prepared by reducing S-NADP$^+$ using yeast G6PD in the 89 mM triethanolamine buffer pH=7.4 containing 10 mM S-NADP$^+$, 40 mM glucose 6-phosphate, 6.9 mM MgCl$_2$ and yeast G6PD (32 μg/ml) [25]. S-NADPH was then purified on DEAE-cellulose column by the procedure first described by Winer [26]. Fractions that display absorbance at 260 and 399 nm were pooled and the concentration of S-NADPH was determined using an extinction coefficient of 11700 M^{-1} cm^{-1} at 399 nm [11]. Because of photosensitivity of S- NADPH, the reaction was done at dark and all experiments with S-NADPH were conducted with minimum exposure to light [27, 28].

h) Equilibrium dialysis

Binding of NADP$^+$ to G6PD was measured in 0.03 M phosphate buffer pH=7.6 at room temperature by equilibrium dialysis. Each 1.0 ml of enzyme solution was dialyzed against 10 ml of buffer containing NADP$^+$ (20-400 μM). The protein concentration was (4.4 μM).

A control experiment showed that equilibrium was completed in less than 10 hrs. The dialysis was done for about 20 hrs to reach complete equilibration. The NADP$^+$ concentration determined inside and outside of dialysis tubing by yeast G6PD [25].

i) Fluorescence studies

All fluorescence studies were done in 0.03 M phosphate buffer, pH=7.6 at 25ºC using a Perkin-Elmer L5-3B fluorimeter. The native and PLP-modified enzymes were titrated with different concentrations of ligands. The fluorescence values were then corrected for dilution [10, 11]. For the titration of native G6PD with NAD$^+$, NADP$^+$ and S-NADPH, intrinsic protein fluorescence was measured at 340 nm when excited at 290 nm [10]. When the fluorescence of the PLP group in a pyridoxylated enzyme was monitored, the protein was excited at 325 nm and the fluorescence was measured at 392 nm. In the experiments, where the fluorescence of S-NADPH was measured, the excitation wavelength was 399 nm and the emission wavelength was 490 nm.

K_d values for the ligands binding to native and pyridoxylated enzymes were measured from the fluorescence quenching and equilibrium dialysis data using Scatchard plots [29]. Double reciprocal plots of fluorescence quenching (ΔF %) vs. ligand concentration yielded a straight line from which ΔF_{max}, the maximal fluorescence change corresponding to complete saturation by ligands, and K_d values were determined [10, 30]. The concentration of bound ligands were calculated on the assumption that is directly proportional to the fractional fluorescence change, therefore [ligand bound] = n. [E_t]. $\Delta F/\Delta F_{max}$, where [E_t] = enzyme concentration, ΔF = fluorescence change in each ligand concentration and n=the number of ligand binding sites per molecule of enzyme, which according to equilibrium dialysis experiments is assumed to be two. The lines of double reciprocal plots and Scatchard plots were drawn using linear regression analysis and Excel 2000 program.

The linearity of Scatchard and double reciprocal plots and the accuracy of ΔF_{max}, which is reflected in the abscissa intercept of Scatchard lines, was near one (one ligand bound per enzyme subunit); all supported the correct evaluation of the binding constants [11].

3. RESULTS

a) Enzyme purification

The purified enzyme had a specific activity of 3.2 U/mg. protein corresponding to that reported before [18, 19]. SDS-gel electrophoresis of the purified enzyme showed a single band corresponding to a molecular weight of 51.5 kDa .The molecular weight of the native enzyme was also determined using activity staining and Ferguson plots (data not published yet) and was found to be 100 kDa.

The PLP modified enzyme (1.15 mole PLP/mole of enzyme) showed the spectral characteristic of reduced pyridoxyllysine (24) having maximum absorbance at 325 nm and maximum fluorescence at 392 nm when exciting at 325 nm (Fig. 1).
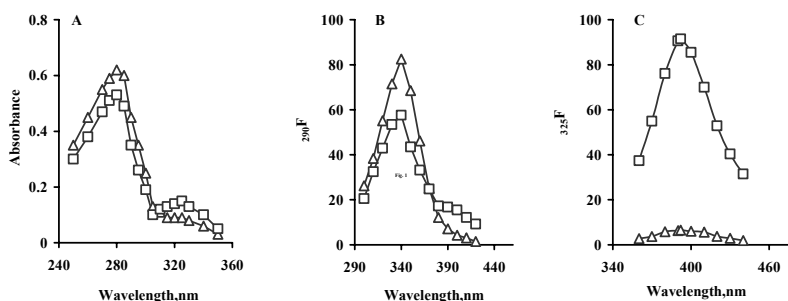

Fig.1. Spectral characteristics of reduced PLP-modified G6PD. The enzyme (1.1 mg/ml) was modified with PLP and absorption and fluorescence measurements were done in 30 mM phosphate buffer pH=7.6 as described in the methods. Absorbance (A) and fluorescence spectra exciting at 290 nm (B) and 325 nm (C) were measured at 25 ^{o}C . Each point represents the average of two independent experiments.
Δ, native enzyme; □, PLP- modified enzyme

b) Equilibrium dialysis

The results of equilibrium dialysis experiments are shown as Scatchard plot (Fig. 2), from which a stoichiometry of 1.8 binding sites per dimer enzyme and a K_d of 95.0 μM for NADP$^+$ binding were calculated.
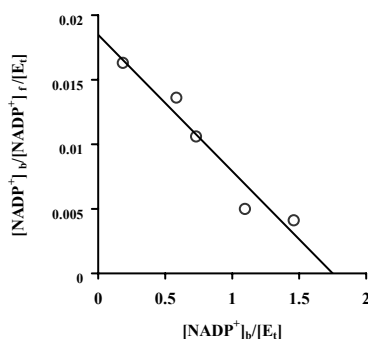

Fig.2. Scatchard Plot of equilibrium binding of NADP$^+$ to G6PD. The enzyme (0.44 mg/ml) in the presence of (20-400μM) NADP$^+$ was dialyzed and NADP$^+$ concentration measured as described in the methods. Each point represents the average of two independent experiments. Subscripts b and f indicate bound and free coenzyme Concentration and [E_t] represents total enzyme concentration

c) Fluorescence studies

Binding of NAD^+ and $NADP^+$ to the native G6PD was demonstrated by monitoring the intrinsic protein fluorescence (Fig. 3). Maximum fluorescence quenching upon NAD^+ binding was 100% and K_d value of 6.5 mM was calculated. For $NADP^+$ binding, ΔF_{max} was only 10% and K_d value was 92.0 µM.

NAD^+ and $NADP^+$ Binding to the pyridoxylated enzyme was monitored by measuring the fluorescence quenching of the PLP group bound to the enzyme (Fig. 4). Maximum fluorescence quenching for NAD^+ and $NADP^+$ was 20% and 57%, respectively. K_d values were 1.0 mM for NAD^+ and 301.0 µM for $NADP^+$. Binding of S-NADPH to the native and pyridoxylated enzyme was also measured using similar experiments (data not shown) and K_d value of 122.0 µM and 151.0µM, were obtained, respectively.

The comparison of the fluorescence titration for the native and PLP-modified enzyme is shown in Table 1. Binding of glucose 6-phosphate to native or PLP-modified enzyme did not affect the fluorescence of protein or PLP-group.
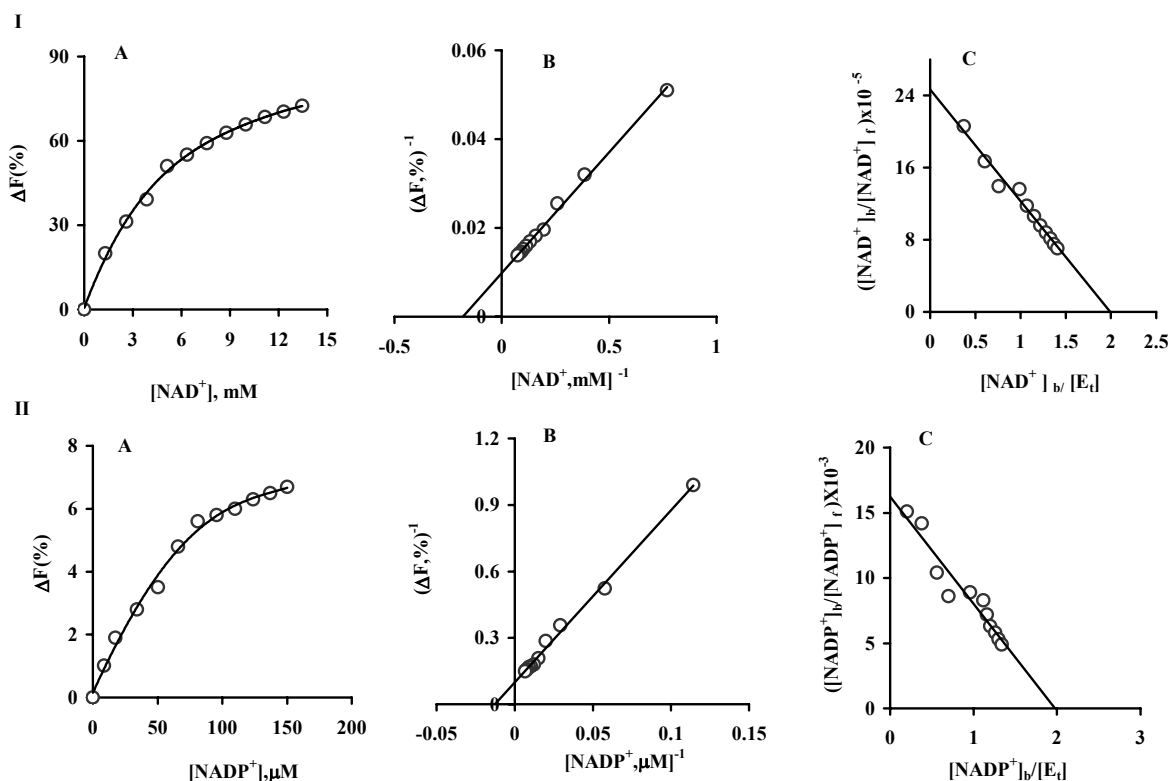

Fig.3. Fluorescence quenching of the native G6PD by NAD^+ (I) and $NADP^+$ (II). Intrinsic protein fluorescence was measured in 30 mM phosphate buffer pH=7.6 containing enzyme (72.0 µg/ml) and different concentration of each coenzyme as described in the methods. The data in B and C were taken from A. Subscripts b, f and $[E_t]$ as in Fig. 2. Each experiment was done in duplicate
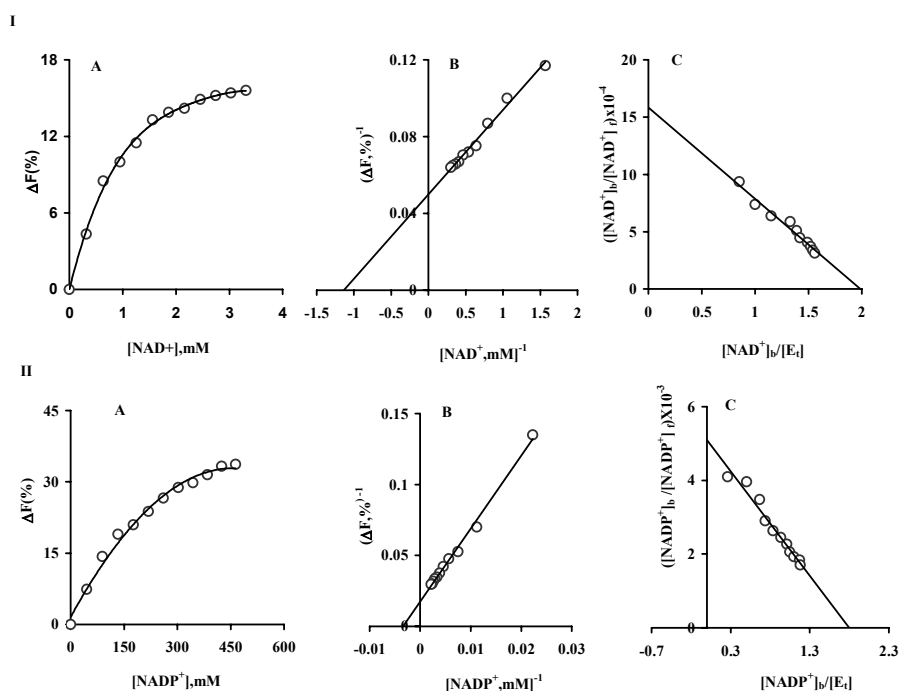
Fig.4. Fluorescence quenching of the PLP-modified G6PD by NAD$^+$ (I) and NADP$^+$ (II).
Fluorescence quenching of enzyme bound PLP ($_{325}F_{392}$) measured as described in
the methods. The enzyme concentration was (71.0µ g/ml). A, B, C and
symbols as in Fig. 3

Table 1. Coenzyme binding to the native and pyridoxyated G6PD

Ligand	Native enzyme			PLP- Modified enzyme		
	K_d (mM)	n	ΔF_{max} (%)	K_d (mM)	n	ΔF_{max} (%)
NAD$^+$	6.5	2	100	1.0	2	20.0%
NADP$^+$	0.092 0.095[a]	1.98 1.8[a]	10.0	0.301	1.82	57.0%
S-NADPH	0.122	2.1	21.0	0.151	1.84	96.0%

For NAD$^+$ and NADP$^+$ the data were taken from Figs. (3-4). For S-NADPH titration was done as described in
the methods
a) Measured by equilibrium dialysis
b) Number of moles ligand bound per mole of enzyme dimer

d) Interaction of coenzymes with G6PD using S-NADPH probe

S-NADPH was added to the enzyme dimer in such a concentration that only one subunit site was
occupied leaving the other subunit free. The fluorescence of S-NADPH enhanced about (40-50) %
when it was bound to the native enzyme. Adding NAD$^+$ at about 0.5 K_d concentration to enzyme-
S-NADPH complex quenched S-NADPH fluorescence by about 25% with a 10 nm blue shift in the
emission spectrum (Fig. 5A). NADP$^+$ binding (0.5 K_d), however, increased S-NADPH fluorescence
(17%) with no shift in the emission spectrum (Fig. 5B). Both coenzymes at a higher concentration

displaced S-NADPH as judged by the change in the fluorescence of S-NADPH or protein fluorescence simultaneously (data not shown). Glucose 6-phosphate (14mM) binding to enzyme-S-NADPH complex decreased S-NADPH fluorescence (9%) with a 5 nm blue shift in the emission spectrum (Fig. 5C). Fluorescence of S-NADPH enhanced about 18% upon binding to pyridoxylated enzyme with a 5 nm blue shift. When NAD^+ was bound to S-NADPH-pyridoxylated enzyme complex the fluorescence of S-NADPH increased (11%) with a 5 nm blue shift. $NADP^+$, however, did not show any effect (Fig. 6).

Adding of NAD^+ or $NADP^+$ to the S-NADPH-enzyme complex for native and pyridoxylated enzymes and monitoring protein fluorescence and PLP fluorescence also demonstrated distinct effects of two coenzymes   (Table 2). The addition of glucose 6-phosphate up to 30 mM to the PLP-modified enzyme did not produce any fluorescence changes.
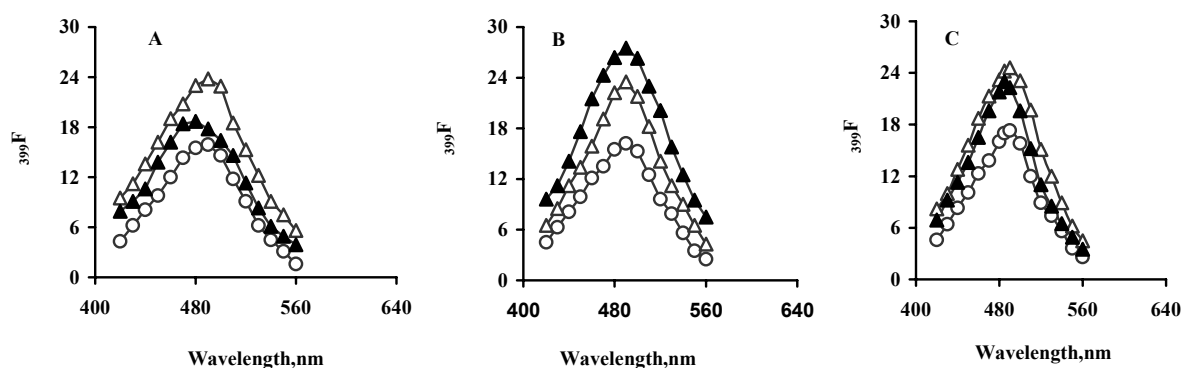

Fig.5. Emission spectra of S-NADPH in G6PD. S-NADPH complex upon binding of NAD^+ (A), $NADP^+$ (B) and G6P(C). S-NADPH (132.0μM) was added to the enzyme (78.5μg/ml) and the emission spectrum generated when excited at399nm in the presence of NAD^+ (2.5 mM), $NADP^+$ (35.6μM) and G6P (14.0mM). Each point represents the average of two independent experiments. ○, S-NADPH alone; Δ, S-NADPH. enzyme; ▲, S-NADPH.enzyme. ligand . For details see text
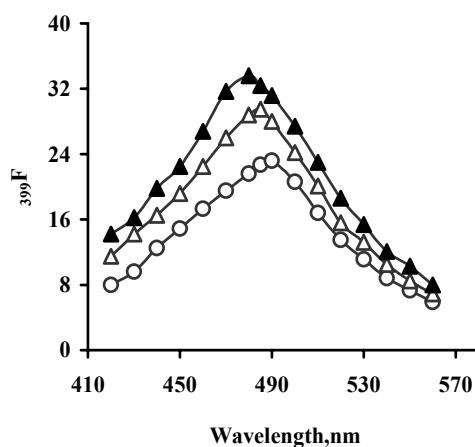

Fig.6. Emission spectra of S-NADPH in PLP–modified G6PD. S-NADPH complex upon binding of NAD^+. *S*-NADPH (185μM) was added to the modified enzyme (100μg/ml) and the emission spectrum obtained when excited at 399 nm in the presence of NAD^+ (0.36 mM). Each point shows the average of duplicate experiments. The figure legends as in Fig. 5

Table 2. Fluorescence changes induced by ligand binding to native and pyridoxylated enzyme

Ligand added	Native enzyme[a]	PLP- Modified enzyme[b]	
	Protein fluorescence quenching (%)	Protein fluorescence quenching (%)	PLP fluorescence quenching (%)
S-NADPH	13.5	34.5	54.3
NAD$^+$	34.3	6.3	3.9
S-NADPH+NAD$^+$	44.0	42.4	60.8
S-NADPH	14.5	39.4	56.7
NADP$^+$	3.7	12.7	19.4
S-NADPH+NADP$^+$	20	54.7	80.3
S-NADPH	12.5	36.7	55.1
G6P	0	0	0
S-NADPH+G6P	21	36.7	55.1

The fluorescence of protein and the PLP group was measured for native and PLP-modified enzymes as described in the methods
a) NAD$^+$, NADP$^+$, S-NADPH, G6P and enzyme concentration were 2.5 mM, 35.6μM, 132.0μM, 14.0 mM and 78.5μg/ml, respectively
b) NAD$^+$, NADP$^+$, S-NADPH and enzyme concentration were 0.36 mM, 126.1μM, 185.0 μM and 100μg/ml, respectively

e) Effect of glucose 1-phosphate on binding of NAD$^+$ and NADP$^+$

Glucose 1-phosphate inhibited the G6PD competitively with respect to glucose 6-phosphate and uncompetitively with respect to NAD$^+$. K_i values of 28 mM for glucose 6-phosphate and 52 mM for NAD$^+$ were calculated. NAD$^+$ binding to the G6PD –glucose 1-phosphate complex decreased protein fluorescence by 42%. A K_d value of 2.2 mM was calculated from these data. The fluorescence quenching by NADP$^+$ was 28% with a K_d value of 209 μM (unpublished data).

4. DISCUSSION

Comparison of absorption and emission spectra in two modified and native enzymes indicates that the conformation of pyridoxylated enzyme is different from the native form (Fig. 1). Earlier experiments have shown that incubation of *S. aureofaciens* G6PD with PLP and subsequent reduction with NaBH$_4$ causes enzyme inactivation and modification of two lysine residues per enzyme dimer that are thought to be involved in glucose 6-phosphate binding to the enzyme (20). PLP is used as a chemical modification reagent for identification of lysine residues in numerous proteins [31-35]. The appearance of new peaks in absorption and emission spectra at 325 and 392nm, respectively, corresponds to N6-phosphopyridoxyllysine and formation of reduced Schiff base [20, 24]. Similar experiments in *L.mesenteroides* G6PD indicate that the PLP-modified enzyme is structurally different from the native form [24, 36].

Equilibrium dialysis experiments (Fig. 2) showed two sites per enzyme dimer for coenzyme binding. This finding is consistent with the results obtained by fluorescence studies (Fig.3. ΠC). SDS-PAGE results revealed that the enzyme consists of two identical subunits. It is, therefore, believed that one coenzyme site is present per enzyme subunit. Our inhibition studies (unpublished data) showed identical binding sites for NAD$^+$ and NADP$^+$ on this enzyme. Similar findings have been reported for *L. mesenteroides* G6PD [11]. An equilibrium dialysis experiment for NAD$^+$ and glucose

6-phosphate was not performed due to high K_m or K_d for these ligands, which lowers the accuracy of the technique [11].

To investigate different conformational changes induced by NAD^+ or $NADP^+$ binding, S-NADPH, a coenzyme analog was used as a fluorescent probe. Reduced pyridine nucleotide coenzymes and their thioanalogs have been used extensively as fluorescent probes to monitor conformational changes in different enzymes [11, 27, 28, 30]. S-NADPH binding to *S. aureofaciens* G6PD enhanced its fluorescence (Fig.5). This fluorescence enhancement has also been reported for other dehydrogenases [27, 28].

When NAD^+ was bound to the enzyme on which only one coenzyme site was occupied by S-NADPH, the fluorescence of bound S-NADPH decreased with about a 10 nm blue shift in its emission spectrum (Fig. 5A). $NADP^+$ binding, however, enhanced S-NADPH fluorescence (Fig. 5B). Monitoring of the protein fluorescence upon NAD^+ and $NADP^+$ binding to the enzyme- S-NADPH complex also showed different fluorescence changes (Table 2). These results provide evidence for distinct conformational changes upon NAD^+ and $NADP^+$ binding. The finding that glucose 6-phosphate binding to S-NADPH-enzyme complex induced similar changes as NAD^+ binding in the S-NADPH fluorescence, suggested that glucose 6-phosphate pulls the enzyme into a conformation suitable for NAD^+ binding. Haghighi and Levy [11] have also shown similar results for *L. mesenteroides* G6PD.

Coenzyme binding was also investigated using the fluorescence of PLP as a reporter group in the pyridoxylated enzyme. NAD^+ and $NADP^+$ binding to the modified enzyme induced different conformational changes that were opposite to that of the native enzyme. Since PLP binds to the glucose 6-phosphate site, as judged by the competitive inhibition of the enzyme by PLP with respect to G6P [20], the lower K_d obtained for NAD^+ binding to the pyridoxylated enzyme, compared to that of native form (Table 2), again supports the idea that glucose 6-phosphate binding induced a conformation resembling that generated by NAD^+. This was also supported by binding the glucose 1-phosphate substrate analog to the native enzyme and monitoring the intrinsic protein fluorescence upon NAD^+ or $NADP^+$ binding to the binary complex. The lower K_d value for NAD^+ binding to enzyme-glucose 1-phosphate complex and higher K_d for binding of $NADP^+$ to both native enzyme-glucose 1-phosphate complex and pyridoxylated enzyme again illustrated that substrate binding facilitates NAD^+ binding.

The following conclusions may be drawn from our kinetic and fluorescence studies. First, binding NAD^+ and $NADP^+$ induced different conformational changes in *S. aureofaciens* G6PD; second, occupation of glucose 6-phosphate site by PLP or glucose 1-phosphate produces a similar conformation change in such a way that the NAD^+ binding is enhanced and the $NADP^+$ binding is decreased; third, since the concentration of NAD^+ inside the cells is usually lower than its K_d or K_m values [10], $NADP^+$-linked reaction is favored unless increased concentration of glucose 6-phosphate causes a conformational change in G6PD, to which NAD^+ binds with a higher affinity.

Our results also strengthen the previous reports that NAD^+ and $NADP^+$ are consumed by two different conformational isomers of the enzyme [17-20].

NOMENCLATURE

G6PD	glucose 6-phosphate dehydrogenase
PLP	pyridoxal phosphate
NAD^+	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
S-NADPH	thionicotinamideadenine dinucleotide phosphate

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