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# GLUCOSE 6-PHOSPHATE DEHYDROGENASE FROM *STREPTOMYCES AUREOFACIENS:* LIGAND-INDUCED CONFORMATIONAL CHANG*

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**Abstract –** Some kinetic properties of $NAD^+$- and $NADP^+$- dependent glucose 6-phosphate dehydrogenase (G6PD) purified from streptomyces aureofaciens were studied. Both NADH and NADPH inhibited the enzyme competitively and noncompetitively, with respect to the corresponding coenzymes and glucose 6-phosphate, respectively. ATP inhibited the $NAD^+$ - linked reaction but not that of the $NADP^+$- linked activity. The inhibition was competitive with respect to $NAD^+$ and noncompetitive with respect to glucose 6-phosphate. $K_m$ values were 0.14 mM for $NAD^+$ and 0.075 mM for $NADP^+$. Similar $K_m$ values (0.75-0.79 mM) were obtaind for glucose 6-phosphate using either $NAD^+$ or $NADP^+$ as a coenzyme. The optimum pH was 6.6 for $NAD^+$- and 7.4 for $NADP^+$- dependent activity. Maximum protein fluorescence was increased by $NAD^+$ (49%) and $NADP^+$ (8%). Among bivalent cations studied, $Cu^{2+}$ decreased $NAD^+$- linked activity (40%), but increased the $NADP^+$- linked reaction (10%). $Ni^{2+}$ did not affect $NAD^+$- linked, but stimulated $NADP^+$- linked activity. Other cations such as $Zn^{2+}$ and $Mn^{2+}$ also differently affected the two reactions. The data suggested that binding of $NAD^+$ and $NADP^+$ produces a different conformational change in S. aureofaciens G6PD or an isomerisation process regulates coenzyme utilization.

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

## 1. INTRODUCTION

Glucose 6-phosphate dehydrogenase (G6PD, EC.1.1.1.49) in eukaryotes and many prokaryotes are either $NAD^+$- or $NADP^+$- dependent, generating reduced NADH or NADPH, respectively [1]. In cells NADH provides reducing power for catabolism, while NADPH is utilized in biosynthetic pathways. In some organisms, however, a single dual nucleotide specific enzyme can catalyze both $NAD^+$- and $NADP^+$- linked reactions [1]. Among these, the best studied example is G6PD from *leuconostoc mesenteroides* [2-5]. Other dual nucleotide specific enzymes such as mitochondrial glutamate dehydrogenase [6] and plant homoserine dehydrogenase [7] have also been reported. Another G6PD that utilizes both $NAD^+$and $NADP^+$ is that from *S. aureofaciens* [8], primary kinetic and inhibition studies have shown two different forms of *S. aureofaciens* G6PD for binding to $NAD^+$ and $NADP^+$ [8-9]. We have recently found, by denaturation and renaturation [10] and by fluorescence [11] studies, that $NAD^+$ and $NADP^+$ binding to this enzyme produces different conformational structures. However more information is needed to elucidate the mechanism which controls the utilization of $NAD^+$ and $NADP^+$ in *S. aureofaciens* G6PD. In the present work kinetic parameters, cation effects, enzyme inhibition and other enzymological characteristics of this enzyme for both $NAD^+$- and $NADP^+$-linked activities were compared.

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## 2. MATERIALS AND METHODS

*Materials:* G6PD was isolated from *streptomyces aureofaciens* (#1119, Iranian Scientific Research Organization), Glucose 6-phosphate, $NAD^+$, $NADP^+$, NADH, NADPH, ATP, DEAE-Cellulose, Sephadex G-100 and Tris purchased from Sigma Co. (U. S. A). All other chemicals were of the highest grade commercially available.

*Enzyme purification: Streptomyces aureofaciens* was grown and purified from the cells as described before [10]. The purification procedure included ammonium sulfate fractionation, DEAE-Cellulose chromatography and gel filtration on the Sephadex G-100 column. Specific activity of the purified enzyme was 2.1U/mg protein.

### *a) Enzyme assay and kinetic studies*

Standard assays of G6PD for $NAD^+$- and $NADP^+$- linked reactions were performed according to Neuzil et al. [8] in a Perkin-Elmer Spectrophotometer model 551S at 25°C. Kinetic constants and the type of enzyme inhibition were determined in a 40mM Tris-HCl buffer pH7.4 for $NADP^+$- linked and in a 50mM Imidazole-HCl buffer pH 6.6 for $NAD^+$- linked reactions using double reciprocal plots and the corresponding replots. Initial velocities were measured at four concentrations each of coenzyme and substrate.

The concentration ranges used for the variable substrates were 0.5-10 mM, 0.2-2 mM and 0.02-0.5 mM for glucose 6-phosphate, $NAD^+$ and $NADP^+$, respectively. Changing fixed substrate concentrations were 0.5-5 mM for glucose 6-phosphate and 0.05-1 mM for either $NAD^+$ or $NADP^+$. Inhibitor studies were NADH (0.75 mM), NADPH (0.6 mM) and ATP (4 mM). The effects of bivalent cations such as $Cu^{2+}$, $Mn^{2+}$, $Zn^{2+}$ and $Ni^{2+}$ each at 5 μM concentration were determined on both $NAD^+$- and $NADP^+$- linked reactions. Double-reciprocal and the corresponding replots were drawn using linear regression analysis.

### *b) Other methods*

The pH profiles for $NAD^+$- and $NADP^+$- linked reactions were determined at a pH range of 5.5 to 8.8 using 0.1 M Imidazole and Tris-HCl buffers. Fluorescence studies were done in 50 mM Tris-HCl buffer pH 7.4 using a Perkin-Elmer Spectrophotofluorometer model LS-3B at 25°C. Emission spectra of intrinsic protein (0.5 mg/ml) fluorescence were determined in the absence and presence of $NAD^+$ (2 mM) or $NADP^+$ (1 mM) at a 280 nm excitation wavelength. Protein concentration was measured by the Lowry et al. method [12]. Polyacrylamide gel electrophoresis for confirmation of the enzyme purity was performed according to the standard procedure [13].

## 3. RESULTS

*S. aureofaciens* G6PD was inhibited by NADH, NADPH and ATP. Both NADH (Fig. 1) and NADPH (Fig. 2) inhibited the enzyme competitively and noncompetitively with respect to the corresponding oxidized coenzymes and glucose 6-phosphate, respectively. ATP inhibited the $NAD^+$- dependent reaction (Fig. 3), but not that of the $NADP^+$- dependent activity. The inhibition was competitive with respect to $NAD^+$ and noncompetitive with respect to glucose 6-phosphate.

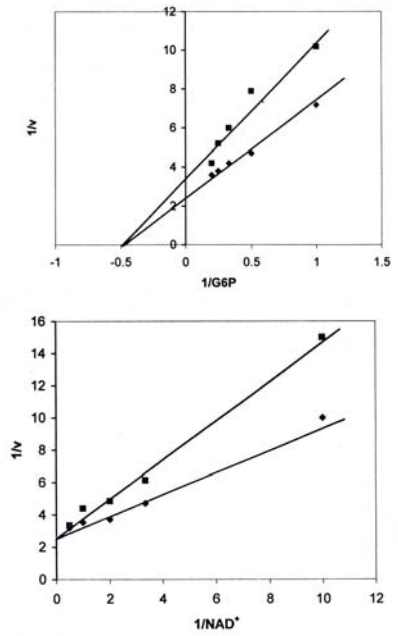


Fig.1. Inhibition of G6PD by NADH. (Up), assays were conducted using 2 mM $NAD^+$ , 1 to 5 mM glucose 6-phosphate and in the absence (Δ),and presence of 125 mM NADH (■). (Bottom), assays were conducted using 5 mM glucose 6-phosphate, 0.1 to 2 mM $NAD^+$ and in the absence (Δ) and presence of 0.75 mM NADH (■)

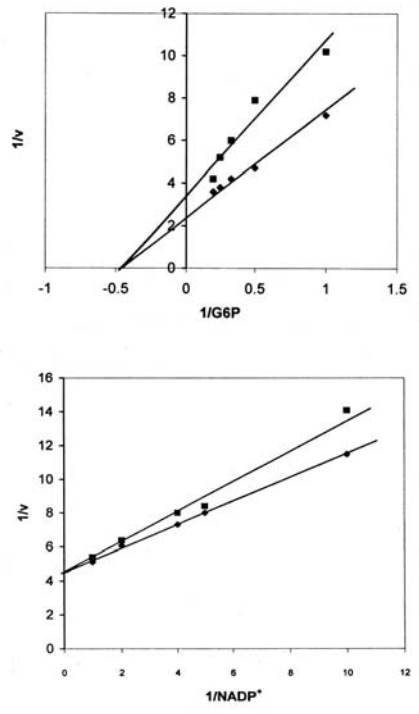


Fig.2. Inhibition of G6PD by NADPH. (Up), assays were performed using 1 mM $NADP^+$, 1 to 5 mM glucose 6-phosphate and in the absence (Δ) and presence of 0.25 mM NADPH (■). (Bottom), assays were performed using 5 mM glucose 6-phosphate, 0.1 to 1 mM $NADP^+$ and in the absence (Δ) and presence of 0.6 mM NADPH (■)

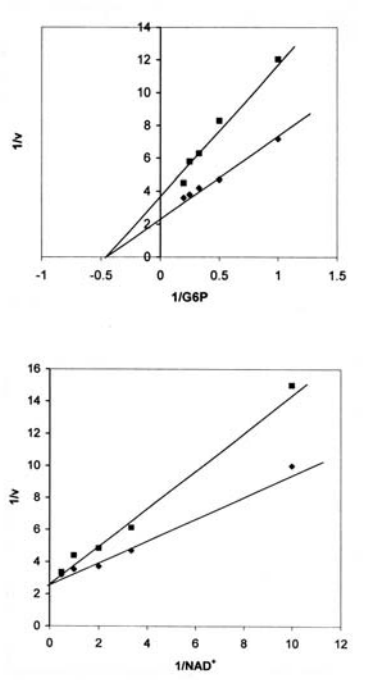


Fig. 3. Inhibition of G6PD by ATP. (Up), assays were conducted using 2 mM $NAD^+$, 1 to 5 mM glucose 6-phosphate and in the absence (Δ) and presence of 5 mM ATP (■), (Bottom), assays were conducted using 5 mM glucose 6-phosphate, 0.1 to 2 mM $NAD^+$ and in the absence (Δ)and presence of 4 mM ATP (■)

The presence of bivalent cations such as $Cu^{2+}$, $Mn^{2+}$, $Zn^{2+}$ or $Ni^{2+}$ in the assay mixture at a low concentration (5 μM) affected $NAD^+$- and $NADP^+$- linked reactions differently (Table 1).

Table 1. The Effects of bivalent cations on streptomyces aureofaciens G6PD

| | **Activity, %** | |
|---|---|---|
| **Cation** | **$NAD^+$- linked** | **$NADP^+$- linked** |
| None | 100 | 100 |
| $Cu^{2+}$ | 60 | 110 |
| $Mn^{2+}$ | 120 | 140 |
| $Zn^{2+}$ | 110 | 70 |
| $Ni^{2+}$ | 100 | 150 |

Each cation (5μM) was added to the assay mixture and the enzyme activity measured as described in the methods. The enzyme activity in the absence of the cations (except $Mg^{2+}$ present in the assay mixture) was taken as 100. Each value represents the average of two independent experiments.

The activity in the absence of the above cations was taken as 100. The results showed that $Cu^{2+}$ decreased $NAD^+$ - linked activity (40%), but increased the $NADP^+$ - linked reaction (10%). Nickle did not affect the $NAD^+$ - linked reaction, but significantly stimulated that of the $NADP^+$ -linked (50%). The $NAD^+$ - dependent reaction was stimulated (10%) by $Zn^{2+}$, while the $NADP^+$ -dependent one was inhibited (30%) by this cation. Both $NAD^+$ - and $NADP^+$ - linked activities were stimulated in the presence of $Mn^{2+}$ by 20% and 40%, respectively.

Other differences in kinetic characteristics between $NAD^+$- and $NADP^+$ - linked reactions are summarized in Table 2.

Table 2. Enzymological characteristics of $NAD^+$- and $NADP^+$- linked G6PD purified from streptomyces aureofaciens

| $K_m$, m M | $NAD^+$ - linked | $NADP^+$- linked |
|---|---|---|
| $NAD^+$ | 0.14 | - |
| $NADP^+$ | - | 0.075 |
| G6P | 0.79 | 0.75 |
| pH Optimum | 6.6 | 7.4 |
| Specificactivity,U/mg | 6.7 | 5.4 |

$K_m$ values were obtained from the replots of the double reciprocal plots. A pH range of 5.5-8.8 were used for pH profiles. For details see text. Each value represents the average of at least two independent experiments

$K_m$ values were 0.14 mM for $NAD^+$ and 0.075 mM for $NADP^+$, while that of glucose 6-phosphate was about the same for both reactions (0.75-0.79 mM). The pH optima for $NAD^+$ - and $NADP^+$ - dependent activities were 6.6 and 7.4, respectively. The difference in the specific activities of both reactions was also significant. Emission maxima of protein fluorescence were increased by 49% and 18% in the presence of $NAD^+$ and $NADP^+$, respectively (Fig. 4).

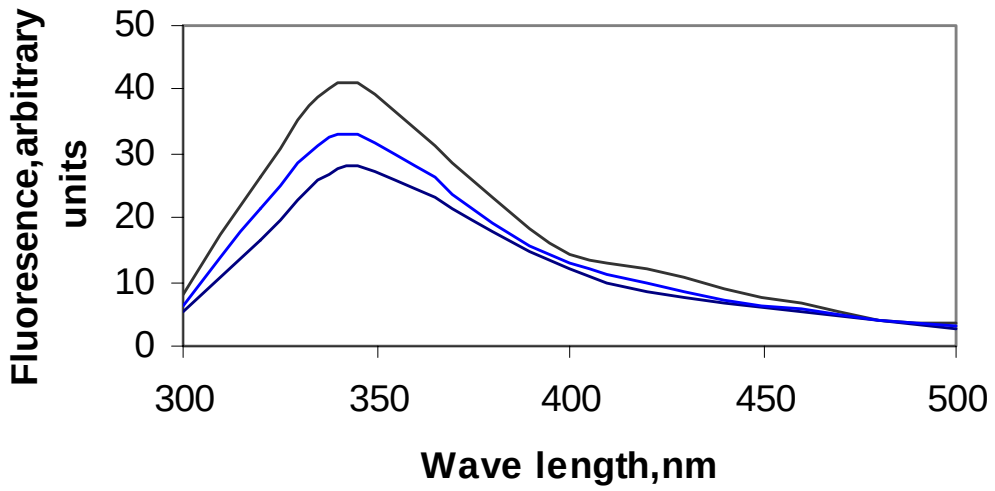


Fig.4. Intrinsic protein fluorescence of G6PD, excitation wave length was 280 nm. Emission spectra of the enzyme (0.5 mg/ml) were produced in 50 mM Tris-HCl buffer pH 7.4 in the absence (Bottom) and presence of 1 mM $NADP^+$ (Middle) or 2 mM $NAD^+$ (Up)

## 4. DISCUSSION

Among dual nucleotide specific G6PDs, the *streptomyces aureofaciens* enzyme has been the least studied. Our recent deraturation and renaturation studies suggested that this enzyme undergoes different conformational transitions upon coenzyme binding [10]. The present data also provided evidence for such conformational changes induced by $NAD^+$ and $NADP^+$. Competitive inhibition of the enzyme by NADH or NADPH versus $NAD^+$ or $NADP^+$ (Figs. 1 and 2) indicates that both coenzymes probably bind to the same site. Inhibition of a $NAD^+$-dependent reaction by ATP, but not

that of $NADP^+$- dependent, suggests two different conformations for $NAD^+$-G6PD and $NADP^+$-G6PD complexes, and that ATP as a high energy compound stops $NAD^+$ utilization and consequently catabolic pathway. Naylor et al. [14] determined crystallized structures of $NADP^+$- and $NAD^+$- G6PD complexes of *L. mesenteroides* G6PD and reported different interdomain hinge angles in these complexes. It was previously shown that for *L. mesenteroides*, G6PD glucose 6-phosphate plays a regulatory role for coenzyme utilization and pulls the enzyme toward the conformation suitable for $NAD^+$ binding [14]. The regulatory role of glucose 6-phosphate in coenzyme utilization was also shown for *Streptomyces aureofaciens* G6PD using fluorescence studies [11].

The difference found in $K_m$ values of $NAD^+$ and $NADP^+$ (Table. 2) is consistant with that reported by other workers using partially purified enzymes [8]. For other dual nucleotide specific G6PDs such as *L. mesenteroides* [15]and *Psedonomas* [16] enzymes, the $K_m$ $NAD^+$/ $K_m$ $NADP^+$ ratios of 18 and 13 have been reported, respectively. If $NAD^+$ and $NADP^+$ binds to the same site, the difference observed in the $K_m$ values also demonstrated that coenzyme binding induces different conformational isomers for the enzyme. The weaker binding of $NAD^+$ to the free enzyme may result from the fact that a substantial proportion of the binding energy is used to cause a large conformational change in the protein. Such a large conformational change upon $NAD^+$ binding to the enzyme was also observed by the substantial increase in maximum emission spectrum (Fig. 6). Deweck et al. [17] attributed the different affinities of $NAD^+$ and NADH to alcohol, and lactate dehydrogenase to differences in the conformational work performed by these coenzymes on the enzyme. Inactivation of *L. mesenteroides* G6PD by several proteases, 4M urea and heating at 49°C have been protected, to varying degrees, by $NAD^+$, $NADP^+$ and glucose 6-phosphate [18]. These authors concluded that $NAD^+$ binding to the enzyme resulted in a larger global conformational change than did $NADP^+$.

The differential effects of bivalent cations on $NAD^+$- and $NADP^+$- linked activities (Table. 1) indicated that cation binding may pull the enzyme toward a conformation suitable for binding of a specific coenzyme. Such a conformational change in this enzyme was also proved by extensive fluorescence studies using different fluorescence probes [11]. These studies demonstrated that S. aureofaciens G6PD undergoes different conformational changes upon $NAD^+$, and $NADP^+$ binding and modification of glucose 6-phosphate by pyridoxal 5-phosphate induces a conformation that favours $NAD^+$ binding**.** For instance, $Cu^{2+}$ promotes utilization of $NADP^+$, but not that of $NAD^+$. Site directed mutagenesis have shown that in *L. mesenteroides* G6PD, Arg-46 is important for $NADP^+$ binding but not for $NAD^+$ binding [3], and that His-240 and Asp-177 are involved in the enzyme catalysis [19]. It is, therefore, conceivable that either the environments of amino acids involved in the enzyme catalysis are changed upon cation binding or the conformational change brought about by $NAD^+$ or $NADP^+$ binding alters cation binding.

The principle conclusion that has emerged from this work and our previous studies [10] is that binding of $NAD^+$ and $NADP^+$ probably produces different conformal changes in *streptomyces aureofaiens* G6PD. More studies are needed to clarify the mechanism by which coenzyme utilization is regulated in this dual nucleotide specific enzyme.

## REFERENCES


1. Cosgrove, M. S., Naylor, C., Paludan, S., Adams, M. J. & Levy, H. R. (1998). On the mechanism of the reaction catalyzed by glucose 6-phosphate dehydrogenase. *Biochemistry*, *37*, 2759-2767.
2. Cosgrove, M. S., Gover, S., Naylor, C. E., Vandeputte-Rutten, L., Adams, M. L. & Levy, H. R. (2000). An examination of the role of Asp-177 in His-Asp catalytic dyad of L. mesenteroides glucose 6-phosphate dehydrogenase: X-ray structure and pH dependence of kinetic parameters of the D177 mutant enzyme. *Biochemistry*, *39*, 15002-15011.
3. Vought, Ciccone, T., Davino, M. H., Fairbarin, L., Lin, V., Cosgrove, M. S., Adams, M. J. & Levy, H. R. (2000). Delineation of the roles of mino acids involved in the catalytic functions of Leuconostos mesenteroides glucose 6-phosphate dehydrogenase. *Biochemistry, 49*, 15012-15021.
4. Levy, H. R. (1989). Glucose 6-phosphate dehydrogenase from Leuconstoc mesenteroides. *Biochem, Soc. Trans*, *17*, 313-315.
5. Ragunathan, S. & Levy, H. R. (1994). Purification and characterization of the $NAD^+$ preferring glucose 6-phosphate dehydrogenase from Acetobacter hansenii. *Arch. Biochem. Biophys*., *310*, 360-366.
6. Aubert, S., Bligny, R., Douce, R., Gout, E., Ratcliffe, R. G. & Roberts, J. K. (2001). Contribution of glutamate dehydrogenase to mitochondrial glutamate metabolism studied by 13C and 31P nuclear magnetic resonance. *J. Exp. Bot*., *52*, 37-55.
7. DeLaBarre, B., Tampson, P. R., Wright, G. D. & Berghuis, A. M. (2000). Crystal structures of homoserine dehydrogenase suggest a novel catalytic mechnism for oxidoreductase. *Nat. Sruct. Biol*., *7*, 238-244.
8. Neuzil, J., Navotna, J., Erban, V., Behal, V. & Hostalek, Z. (1988). Glucose 6-phosphate dehydrogenase from a tetracycline-producing strain of S. aureofaciens. *Biotech. Internat*., *17*, 187-196.
9. Neuzil, J., Novotna, J., Behal, V. & Hostalek, Z. (1986). Inhibition studies of glucose 6-phosphate dehydrogenase from tetracycline-producing streptomyces aureofaciens. *Biotech. Appl. Biochem*., *8*, 375-378.
10. Haghighi, B. & Atabi, F. (2003). Reassociation and reactivation of glucose 6-phosphate dehydrogenase from streptomyces aureofaciens after denaturation by 6M urea. *J. Sci. (IRI)*, *14*, 103-111.
11. Haghighi, B. & Akmaly, M. (2004). Interaction of $NAD^+$ and $NADP^+$ with native and pyridoxal phosphate-modified glucose 6-phosphate dehydrogenase from streptomyces aureofaciens. *Iranian J. Sci. Tech.*, *28*, 149-160.
12. Lowry, O. H., Rosenbrough, N. J., Fair, A. L. & Randall, R. J. (1957). Protein measurement with folin-phenol reagent. *J. Biol. Chem*., *193*, 265-273.
13. Harris, E. L. V. & Dunn, M. J. (1989*). Protein purification methods*. *A practical approach*. Oxford University Press.
14. Naylor, C. E., Grover, S., Basak, A. K., Cosgrove, M. S., Levy, H. R. & Adams, M. J. (2001). $NADP^+$ and $NAD^+$ binding to dual coenzyme specific enzyme Leucosnostoc mesenteroides glucose 6-phosphate dehydrogenase: different interdomain hinge angles are seen in different binary and ternary complexes. *Acta Crystallogr. D. Biol. Crystallogr*., *57*, 635-648.
15. Haghighi, B. & Levy, H. R. (1982). Glucose 6-phosphate dehydrogenase from Leconostoc mesenteroides. Kinetics of reassociation and reactivation from inactive subunits. *Biochemistry*, *21*, 6429-6434.
16. Bassat, A. B. & Goldberg, I. (1980). Purification and properties of glucose 6-phosphate dehydrogenase ($NADP^+/NAD^+$) from methanol grown pseudomonas C. *Biochem. Biophys. Acta*, *611*, 1-10.

17. DeWeck, Z., Pande, J. & Kagi, J. H. R. (1978). Interdependence of coenzyme-induced conformational work and binding potential in yeast alcohol and porcine heart Lactate dehydrogenase. A hydrogen-deuterium exchange study. *Biochemistry*, *26*, 4769-4776.
18. Kurlansky, S. B., Hilburger, A. C. & Levy, H. R. (1988). glucose 6-phosphate dehydrogenase from leuconostoc mesenteroides. Ligand-induced conformational change. *Arch. Biochem. Biophys*., *264*, 93-102.
19. Cosgrove, M. S., Naylor, C., Paludan, S., Adams, M. J. & Levy, H. R. (1998). On the mechanism of the reaction catalyzed by glucose 6-phosphate dehydrogenase. *Biochemistry*, *37*, 2759-2767.