

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

B. HAGHIGHI**, A. M. AGHATABAR AND GH. SHAHSAVARI

Department of Clinical Biochemistry, Isfahan University of Medical Sciences, Isfahan, I. R. of Iran

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 obtained 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, Cu²⁺ decreased NAD⁺- linked activity (40%), but increased the NADP⁺- linked reaction (10%). Ni²⁺ did not affect NAD⁺- linked, but stimulated NADP⁺- linked activity. Other cations such as Zn²⁺ and Mn²⁺ 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.

*Received by the editor June 22, 2004 and in final revised form January 12, 2005

**Corresponding author

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 Spectrofluorometer 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.

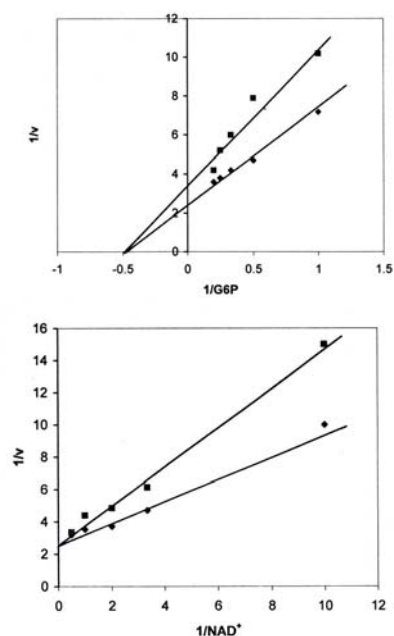


Fig.1. Inhibition of G6PD by NADH. (Up), assays were conducted using 2 mM NAD^+ , 1 to 5 mM glucose 6-phosphate and in the absence (Δ), and presence of 125 mM NADH (\blacksquare). (Bottom), assays were conducted using 5 mM glucose 6-phosphate, 0.1 to 2 mM NAD^+ and in the absence (Δ) and presence of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

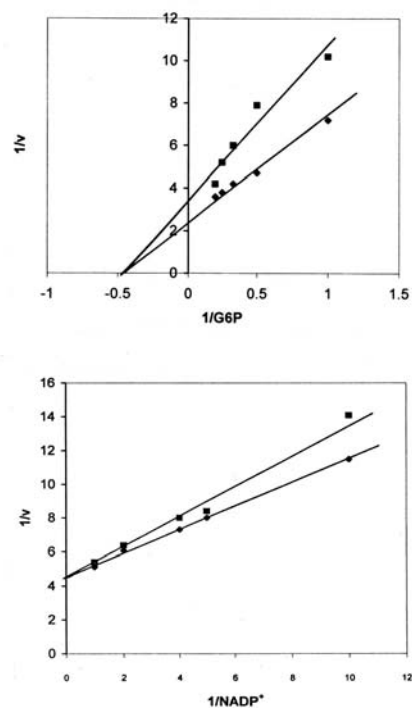


Fig.2. Inhibition of G6PD by NADPH. (Up), assays were performed using 1 mM NADP^+ , 1 to 5 mM glucose 6-phosphate and in the absence (Δ) and presence of 0.25 mM NADPH (\blacksquare). (Bottom), assays were performed using 5 mM glucose 6-phosphate, 0.1 to 1 mM NADP^+ and in the absence (Δ) and presence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

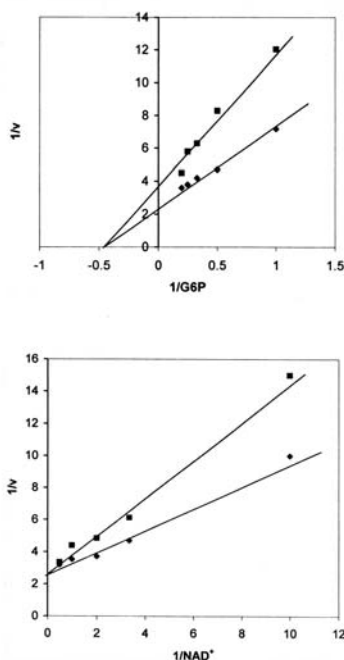


Fig. 3. Inhibition of G6PD by ATP. (Up), assays were conducted using 2 mM NAD⁺, 1 to 5 mM glucose 6-phosphate and in the absence (Δ) and presence of 5 mM ATP (\blacksquare), (Bottom), assays were conducted using 5 mM glucose 6-phosphate, 0.1 to 2 mM NAD⁺ and in the absence (Δ) and presence of 4 mM ATP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

The presence of bivalent cations such as Cu²⁺, Mn²⁺, Zn²⁺ or Ni²⁺ in the assay mixture at a low concentration (5 μ M) affected NAD⁺- and NADP⁺- linked reactions differently (Table 1).

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

Cation	Activity, %	
	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 (5 μ M) 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 Mg²⁺ 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 Cu²⁺ decreased NAD⁺- linked activity (40%), but increased the NADP⁺- linked reaction (10%). Nickel did not affect the NAD⁺- linked reaction, but significantly stimulated that of the NADP⁺-linked (50%). The NAD⁺- dependent reaction was stimulated (10%) by Zn²⁺, while the NADP⁺-dependent one was inhibited (30%) by this cation. Both NAD⁺- and NADP⁺- linked activities were stimulated in the presence of Mn²⁺ 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 , mM	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).

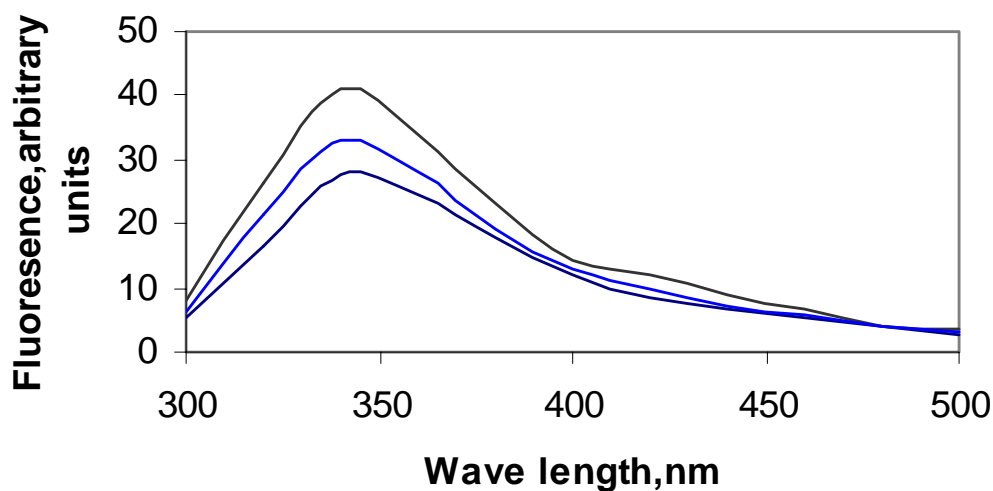


Fig.4. Intrinsic protein fluorescence of G6PD, excitation wave length was 280 nm. Emission spectra of the enzyme (0.5 mg/ml) were produced in 50 mM Tris-HCl buffer pH 7.4 in the absence (Bottom) and presence of 1 mM NADP⁺ (Middle) or 2 mM NAD⁺ (Up)

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 consistent with that reported by other workers using partially purified enzymes [8]. For other dual nucleotide specific G6PDs such as *L. mesenteroides* [15] and *Pseudomonas* [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, Cu²⁺ 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 conformational changes in *streptomyces aureofaciens* 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 amino acids involved in the catalytic functions of *Leuconostoc mesenteroides* glucose 6-phosphate dehydrogenase. *Biochemistry*, *49*, 15012-15021.
4. Levy, H. R. (1989). Glucose 6-phosphate dehydrogenase from *Leuconostoc 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 mechanism for oxidoreductase. *Nat. Struct. 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. Haghghi, 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. Haghghi, B. & Akmal, 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 *Leuconostoc 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. Haghghi, B. & Levy, H. R. (1982). Glucose 6-phosphate dehydrogenase from *Leuconostoc 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.