Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*

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Glucose-6-phosphate dehydrogenase (G6PD) from *Leuconostoc mesenteroides* is one of a small number of dehydrogenases that can utilize either NAD or NADP. The physiological roles of these two coenzymes are quite different and the enzymes that utilize them are generally specific for one or the other coenzyme. An essential function of G6PDs in animals, for example, is to generate NADPH for reductive biosyntheses. Although these enzymes can utilize NAD+, they do so only at non-physiological NAD+ concentrations [1, 2]. Presumably the coenzyme-binding sites of dehydrogenases have evolved so that they can distinguish between NAD and NADP. *L. mesenteroides* is a facultatively anaerobic micro-organism which metabolizes glucose to generate lactic acid, ethanol and CO₂ [3]. The NADH generated in the reaction catalysed by G6PD is utilized in the reductive steps of this heterolactic fermentation; when NADPH is produced by G6PD it is used for the biosynthesis of fatty acids [4]. Thus, G6PD plays an essential, amphiphilic role in the metabolism of *L. mesenteroides*.

Two questions that have motivated our studies of *L. mesenteroides* G6PD are: what protein structural features enable this enzyme to bind both NAD+ and NADP+ to the same site? and how are the catalytic activities with NAD+ and NADP+ regulated? Unlike glutamate dehydrogenase from animal tissues, another dehydrogenase that utilizes either NAD+ or NADP+, *L. mesenteroides* G6PD is a relatively simple protein consisting of two identical subunits of molecular mass 54 800 Da, with no propensity to form larger aggregates [5, 6]. Kinetic studies revealed that the enzyme obeys Michaelis–Menten kinetics with no evidence of cooperative kinetics.

The *Kₘ* for NAD+ is nearly 20 times greater than the *Kₘ* for NADP+, whereas *kₐₕ* for the NAD-linked reaction is nearly twice *kₐₕ* for the reaction with NADP+ [Table 1] [7, 8]. Kinetic and binding studies showed that NAD+ and NADP+ bind to the same site on the enzyme [7, 9], although it is clear that their modes of binding differ. The steady-state kinetic mechanisms of the NAD- and NADP-linked reactions differ. When NADP+ is the coenzyme, the mechanism is ordered sequential, with coenzyme binding first; the NAD-linked reaction proceeds via a random-order mechanism [10]. This difference appears to be an essential factor underlying the enzyme’s ability to regulate the utilization of NAD+ or NADP+. Our initial attempts to find metabolic intermediates or other ligands that could affect the NAD- and NADP-linked reactions differentially were unsuccessful. We therefore devised an assay method that enabled us to measure the rates of both the NAD-linked and the NADP-linked reaction simultaneously. This technique, the dual wavelength assay [8], makes use of the fact that the thionicotinamide analogues of the reduced coenzymes, S-NADH and S-NADPH, absorb maximally at 400 nm, and of the high reactivity of S-NAD* and S-NADP* with *L. mesenteroides* G6PD. The *Kₘ* values for the thionicotinamide analogues are substantially lower than, and *kₐₕ/Kₘ* values are comparable with, those for the natural coenzymes (Table 1). Using mixtures of NAD* and S-NADP*, or NADP* and S-NAD*, and measuring the simultaneous increase in absorbance at 340 nm and 400 nm, we measured both the NAD- and NADP-linked reactions simultaneously. Using this technique, we found that high concentrations of glucose 6-phosphate and high concentration ratios of NADPH to NADP* inhibit the NADP-linked, but stimulate the NAD-linked reaction [8, 11]. It must be emphasized that these differential effects on the two activities are only evident when both reactions are proceeding simultaneously, as they depend on the difference in kinetic mechanisms for the two reactions. Although we have no evidence that G6PD is regulated in this manner *in vivo*, the effects of glucose 6-phosphate and NADPH to NADP* ratio on the NAD- and NADP-linked reactions are consistent with the roles of the two reactions in the metabolism of *L. mesenteroides* [8, 10, 11].

Although the *kₐₕ/Kₘ* values for NAD* and NADP* differ only 10-fold, the *Kₐₕ* values for the two coenzymes differ approximately 1000-fold (Table 1). Dissociation constants for both coenzymes have been measured by quenching of protein fluorescence [9, 12] and from protection against thermolysin inactivation [13]; the *Kₐₕ* for NADP* was also determined by equilibrium dialysis [12]. From these *Kₐₕ* values one can calculate that the apparent binding energy of the extra phosphate group of NADP* is 4.16 kcal/mol at

**Table 1. Kinetic and binding constants for coenzymes and their thionicotinamide analogues**

Data are from Levy & Daouk [8] and Haghighi & Levy [12]; ND, Not determined.

<table>
<thead>
<tr>
<th></th>
<th>NAD*</th>
<th>NADP*</th>
<th>NAD* / NADP*</th>
<th>S-NAD*</th>
<th>S-NADP*</th>
<th>S-NAD* / S-NADP*</th>
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</thead>
<tbody>
<tr>
<td><em>kₐₕ</em> (s⁻¹)</td>
<td>518</td>
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<td><em>Kₐₕ</em> (mM)</td>
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<td>1100</td>
<td>ND</td>
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<td>ND</td>
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<tr>
<td><em>Kₘ</em> (mM)</td>
<td>0.106</td>
<td>0.0057</td>
<td>18.6</td>
<td>0.011</td>
<td>0.0012</td>
<td>8.94</td>
</tr>
<tr>
<td><em>kₐₕ/Kₘ</em> (s⁻¹ mM⁻¹)</td>
<td>4890</td>
<td>50 500</td>
<td>0.097</td>
<td>9820</td>
<td>48 600</td>
<td>0.202</td>
</tr>
</tbody>
</table>

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25°C. One can also calculate the contribution made by the extra phosphate group of NADP⁺ in the transition state. Assuming that the chemical activation energy for the bond making and breaking steps are identical for the NAD- and NADP-linked reactions, then it can be shown [14, 15] that:

\[
\Delta G^o = RT \ln \left[ \frac{[NADP^+]}{[NAD]} \right] \left[ \frac{[NAD]}{[ADP]} \right]
\]

where 1 and 2 refer to the substrate lacking and containing the group Y, respectively, i.e. NAD⁺ and NADP⁺. From this equation one can calculate that in the transition state, the apparent binding energy of the extra phosphate group of NADP⁺ is only 1.38 kcal/mol at 25°C. This difference in the contribution of the extra phosphate group of NADP⁺ to coenzyme binding in the ground state and the transition state can be explained by assuming that the apparently weak binding of NAD⁺ to the enzyme reflects the utilization of part of the binding energy for enhanced catalysis (kcat/Km) is greater for NAD⁺ than for NADP⁺, Table 1) and for altering the energetics effectively to accommodate NAD⁺ at an NADP⁺ binding site. Thus, the binding energy calculated from the Ks for NAD⁺ would be an underestimate of its true binding energy [16].

That this explanation may be correct is suggested by two lines of evidence. First, the Ks and Kd for NADP⁺ are, within experimental error, identical (Table 1). Double-reciprocal plots in the steady-state kinetic studies showed that, for the NADP-linked reaction, the lines intersect on the abscissa, whereas for the NAD-linked reaction, the lines intersect above the abscissa [7]. Thus, NAD⁺ and glucose 6-phosphate do not influence each other’s binding during catalysis, but NAD⁺ and glucose 6-phosphate promote each other’s binding. Second, G6PD from the lactating rat mammary gland can utilize NAD⁺, but only at high, non-physiological concentrations; kcat/Km for NADP⁺ is over 20000 times that for NAD⁺ [17]. Using the same calculations as for the L. mesenteroides G6PD, and kinetically determined Ks values for Kd, it can be calculated that the extra phosphate group appears to contribute 4.74 kcal/mol towards binding NADP⁺ to the enzyme, and 5.91 kcal/mol in the transition state, both at 25°C. The mammary G6PD discriminates effectively against NAD⁺ binding and against NAD⁺ during catalysis. The difference between the bacterial and mammary G6PDs resides in the fact that only the former can utilize NAD⁺ under physiological conditions.

Thirdly, there is experimental evidence that NAD⁺ binding to L. mesenteroides G6PD engenders a different and larger conformational change in the protein than NADP⁺ binding. The intrinsic fluorescence of L. mesenteroides G6PD is quenched when either NAD⁺ or NADP⁺ binds. Titration with the coenzymes shows that the maximum fluorescence quenching is at least 10 times greater for NAD⁺ than for NADP⁺ [9, 12]. The enzyme can be covalently modified with pyridoxal 5'-phosphate, followed by sodium borohydride reduction. This leads to inactivation, accompanied by the modification of one of the 37 lysine residues per subunit, and this lysine appears to be involved in glucose 6-phosphate binding [18, 19]. The fluorescence of the covalently bound pyridoxyl group is quenched by the addition of either NAD⁺ or NADP⁺; NADP⁺ produces greater maximum quenching than NAD⁺ [12].

The fluorescence of S-NADPH is enhanced when it binds to L. mesenteroides G6PD. When NAD⁺ was added to a solution of the enzyme containing bound S-NADPH, the fluorescence of S-NADPH was quenched and blue-shifted; addition of NADP⁺ to the enzyme S-NADPH complex enhanced S-NADPH fluorescence without any shift in the maximum fluorescence emission wavelength. These experiments were conducted under conditions where none of the bound S-NADPH was displaced [12].

These experiments indicate that NAD⁺ and NADP⁺ produce different conformational changes when they bind to L. mesenteroides G6PD. The conformations of the bound coenzymes are also different, as indicated by differences in transferred nuclear Overhauser effects [20]. Recently, we have shown that there is a striking similarity in the degree of protection afforded by NAD⁺ against a variety of enzyme inactivation procedures; the protection by NADP⁺ is much less and, again, remarkably similar for all the procedures tested. In these experiments [13], L. mesenteroides G6PD was incubated with trypsin, chymotrypsin, thermolysin, pronase or 4 M urea, or heated to 49°C. In each case, inactivation followed first-order kinetics. When 10Kp NAD⁺ was included in the incubation mixture, protection was 17 ± 9% (range: 0-35%); the addition of 10Kp NAD⁺ led to 57 ± 13% protection (range: 40-77%). A detailed discussion of these experiments will be presented elsewhere [21].

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be the result of a convergent evolution (Williams, 1988).

The genealogy of thioredoxin reductase is not clear; the protective bacterial enzyme is NADPH-dependent disulphide reductases (Fahey, 1977; Perham, 1987; Schirmer, 1987). When O2 became necessary. Key roles of these functions are played by early cells made extensive use of thiols as functional groups such as disulphides and sulphate were the chemically favoured forms of sulphur, and free thiols were threatened by such as disulphides and sulphate were the chemically favoured forms of sulphur, and free thiols were threatened by extinction. To produce and protect intracellular thiols in this increasinglly hostile environment, adaptive mechanisms became necessary. Key roles of these functions are played by NADPH-dependent flavoenzymes such as thioredoxin reductase, pantethinediphosphate reductase, glutathione reductase and trypanothione reductase. In some habitats, intracellular thiols must also be protected from Hg2+ ions (Walsh et al., 1988). The protective bacterial enzyme is NADPH-dependent mercuric ion reductase which catalyses the reaction NADPH + Hg2+ + H+ → NADP+ + Hg2+. (Fox & Walsh, 1982). The catalytic cycle of thioredoxin reductase is not clear; the mechanistic similarity (Fig. 1) between this enzyme and the family represented by lipoamide dehydrogenase might well be the result of a convergent evolution (Williams, 1988). As the work on NAD(P)H: disulphide oxidoreductases has been extensively reviewed (Williams, 1976; Holmgren, 1980; Perham, 1987; Schirmer & Schulz, 1987; Williams, 1989), the present report focuses on recent developments.

Trypanothione reductase, a recently discovered disulphide reductase

Trypanosomatid parasites are known to cause a variety of serious diseases in man and domestic animals. These include African sleeping sickness, Chagas' disease, oriental sore and kala azar in humans and nagana in cattle. The thiol–disulphide metabolism of these protozoal parasites is not based on glutathione and glutathione reductase as in most other eukaryotic cells, but on glutathionylspermidines and trypanothione reductase (Fairlamb et al., 1985; Henderson & Fairlamb, 1987). Trypanothione reductase (Fig. 2) has been isolated in crystalline form (Krauth-Siegel et al., 1987) from Trypanosoma cruzi, the causative agent of Chagas' disease in South America (Charles Darwin's mysterious disease was probably Chagas' disease (Adler, 1959; Medawar, 1972). This enzyme and glutathione reductase (GSSG + NADPH + H+ → 2GSH + NADP+), the corresponding host enzyme, share many physical and chemical properties. Both proteins are homodimers of approx. 100 kDa, contain FAD as prosthetic group and a redox active dihol at the catalytic site, and both prefer NADPH as reductant (Fig. 1). Recently the complete primary structure of trypanothione reductase from the cattle pathogen T. evansi was determined (Krauth-Siegel et al., 1987). When O2 became necessary. Key roles of these functions are played by early cells made extensive use of thiols as functional groups such as disulphides and sulphate were the chemically favoured forms of sulphur, and free thiols were threatened by extinction. To produce and protect intracellular thiols in this increasinglly hostile environment, adaptive mechanisms became necessary. Key roles of these functions are played by NADPH-dependent flavoenzymes such as thioredoxin reductase, pantethinediphosphate reductase, glutathione reductase and trypanothione reductase. In some habitats, intracellular thiols must also be protected from Hg2+ ions (Walsh et al., 1988). The protective bacterial enzyme is NADPH-dependent mercuric ion reductase which catalyses the reaction NADPH + Hg2+ + H+ → NADP+ + Hg2+. (Fox & Walsh, 1982). The catalytic cycle of thioredoxin reductase is not clear; the mechanistic similarity (Fig. 1) between this enzyme and the family represented by lipoamide dehydrogenase might well be the result of a convergent evolution (Williams, 1988). As the work on NAD(P)H: disulphide oxidoreductases has been extensively reviewed (Williams, 1976; Holmgren, 1980; Perham, 1987; Schirmer & Schulz, 1987; Williams, 1989), the present report focuses on recent developments.

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