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Thermodynamic characterization of substrate and inhibitor binding to *Trypanosoma brucei* 6-phosphogluconate dehydrogenase

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6-Phosphogluconate dehydrogenase is a potential target for new drugs against African trypanosomiasis. Phosphorylated aldonic acids are strong inhibitors of 6-phosphogluconate dehydrogenase, and 4-phospho-D-erythronate (4PE) and 4-phospho-D-erythronohydroxamate are two of the strongest inhibitors of the *Trypanosoma brucei* enzyme. Binding of the substrate 6-phospho-D-gluconate (6PG), the inhibitors 5-phospho-D-ribonate (5PR) and 4PE, and the coenzymes NADP, NADPH and NADP analogue 3-amino-pyridine adenine dinucleotide phosphate to 6-phospho-D-gluconate dehydrogenase from *T. brucei* was studied using isothermal titration calorimetry. Binding of the substrate ($K_d = 5$ μM) and its analogues ($K_d =$ 1.3 μM and $K_d = 2.8$ μM for 5PR and 4PE, respectively) is entropy driven, whereas binding of the coenzymes is enthalpy driven. Oxidized coenzyme and its analogue, but not reduced coenzyme, display a half-site reactivity in the ternary complex with the substrate or inhibitors. Binding of 6PG and 5PR poorly affects the dissociation constant of the coenzymes, whereas binding of 4PE decreases the dissociation constant of the coenzymes by two orders of magnitude. In a similar manner, the K_d value of 4PE decreases by two orders of magnitude in the presence of the coenzymes. The results suggest that 5PR acts as a substrate analogue, whereas 4PE mimics the transition state of dehydrogenation. The stronger affinity of 4PE is interpreted on the basis of the mechanism of the enzyme, suggesting that the inhibitor forces the catalytic lysine 185 into the protonated state.

Drugs designed to combat African trypanosomiasis are often based on the pentose phosphate pathway enzyme, 6-phosphogluconate dehydrogenase (decarboxylating, 6PGDH, EC 1.1.1.44) [1,2]. This tropical infectious disease is caused by protozoan parasites of the *Trypanosoma brucei* species, of the order Kinetoplastida, to which *Leishmania* and the American *Trypanosoma cruzi* also belong. They are insect-transmitted pathogens affecting millions of humans and other mammals, against which few drugs exist, and those which do can lead to serious side-effects and possible resistance [2].

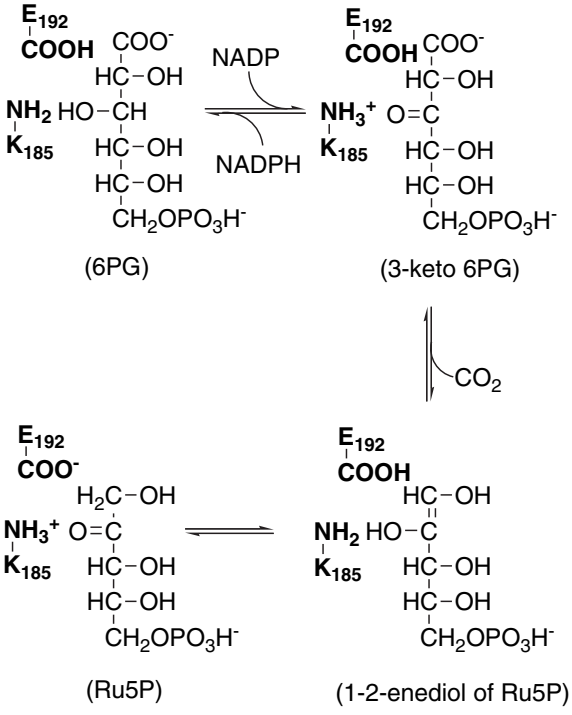
One way to approach the development of good *T. brucei* 6PGDH inhibitors has been to explore the difference in affinity for many substrate-competitive inhibitors between the parasite and the mammalian correspondent enzyme from sheep liver. Thus compounds such as 5-phospho-D-ribonate (5PR), 4-phospho-D-erythronate (4PE) and 4-phospho-D-erythronohydroxamate (4PEX) have been found to have a

Abbreviations
aPyADP, 3-amino-pyridine adenine dinucleotide phosphate; ITC, isothermal titration calorimetry; 4PE, 4-phospho-D-erythronate; 4PEA, 4-phospho-D-erythronamide; 4PEX, 4-phospho-D-erythronohydroxamate; 6PG, 6-phospho-D-gluconate; 6PGDH, 6-phosphogluconate dehydrogenase; 5PR, 5-phospho-D-ribonate; Ru5P, D-ribulose 5-phosphate.

73-, 80- and 254-fold selectivity for *T. brucei* 6PGDH, respectively [3,4]. Some recently developed 4PEX parent compounds with phosphate masking groups, which are able to deliver active compounds into parasites, show a good level of trypanotoxicity [5].

The structural comparison of sheep and *T. brucei* 6PGDHs shows some differences between the two enzymes, which can explain the different affinity in substrate analogues [6]. Furthermore, recent comparison between sheep and *Lactococcus lactis* 6PGDH–6-phospho-D-gluconate (6PG) binary complexes has revealed significant differences in the conformation of 6PG bound to the enzyme between these two moieties [7].

6PGDH catalyses the NADP-dependent oxidative decarboxylation of 6PG to D-ribulose 5-phosphate (Ru5P) via 3-keto 6PG and a probable 1,2-enediol as intermediates (Scheme 1) [8,9]. Two residues, one acting as an acid and the other as a base, are postulated to assist all three catalytic steps of the reaction: dehydrogenation, decarboxylation and keto–enol tautomerization. These residues, which in the *T. brucei* enzyme are Glu192 and Lys185, have been identified on the basis of crystallographic evidence and site-directed mutagenesis [10–12]. The lysine residue is thought to be protonated in the free enzyme and unprotonated in the enzyme–substrate complex, where it receives a proton from the 3-hydroxyl group of 6PG as a hydride is transferred from C3 of 6PG to NADP. The resulting 3-keto-6PG intermediate is then decarboxylated to form the enediol of 5-phospho-ribulose (Scheme 1). At this stage, an acid, which is thought to be the same Lys185, is required to donate a proton to the C3 carbonyl group of the keto-intermediate to facilitate decarboxylation. Both a base and an acid are needed in the tautomerization of the enediol intermediate to yield the ketone ribulose 5-phosphate product, with the acid (Glu192) required to donate a proton to C1 of the enediol intermediate and the base (the same Lys185) accepting a proton from its 2-hydroxyl group. At the end of the reaction, the protonation state of the two catalytic groups is the opposite to that at the beginning of the reaction; thus, an intramolecular proton transfer is required for another cycle of enzyme activity.

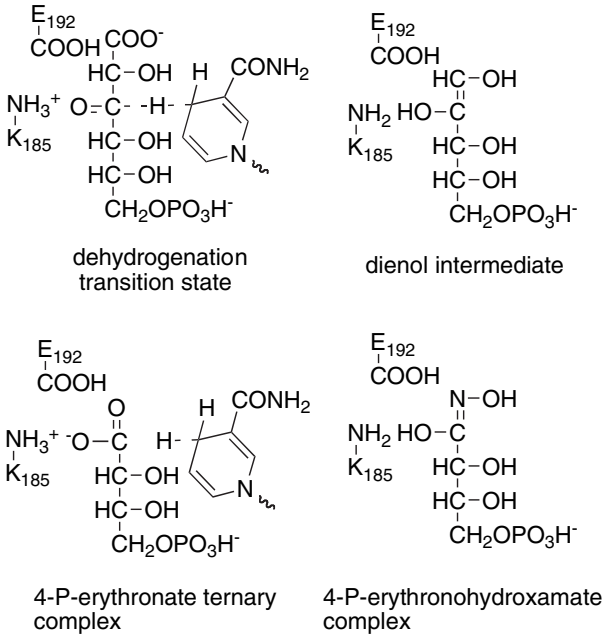


Scheme 1. 6PGDH-catalysed reaction and the two main amino acid residues involved.

6PGDH is a homodimer, but, in many species, it shows functional asymmetry [13–19]. For instance, both the yeast and sheep liver enzyme bind covalently two molecules of periodate-oxidized NADP, but, in the presence of 6PG, a half-site reactivity is acquired with only one subunit binding the NADP analogue, with the other subunit unable to bind even the adenylic moiety of the coenzyme [13,14]. Moreover, negative cooperativity for NADP has been found in human erythrocyte [16] and rat liver [17] 6PGDHs, and stopped-flow experiments have indicated in the first turnover the formation of only one NADPH molecule per enzyme dimer [15]. The substrate binding site is made up of residues from both subunits, allowing the communication between the two active sites, which has also been shown by the decarboxylation activation of 6-phospho-3-keto-2-deoxygluconate by 6PG [18], found in addition in the *T. brucei* 6PGDH [19]. Differences in the cofactor binding domains of each subunit were finally shown in the *T. brucei* and *L. lactis* 6PGDH crystal structures [6,7], the latter showing the ternary complexes enzyme–Ru5P–NADP and enzyme–4PEX–NADP only in one subunit of the three present in the asymmetric unit.

Not only 4PEX [3], but also the substrate-competitive inhibitors 4PE (Scheme 2) and 5PR [4], present K_i values for *T. brucei* 6PGDH lower than the K_m value for 6PG (Table 1), which strongly suggests that they mimic high-energy reaction intermediates rather than the substrate *per se*.

To better understand why these analogues have high affinity and to help in rational drug design, we undertook a thermodynamic characterization of substrate and analogue binding to *T. brucei* 6PGDH, in both



Scheme 2. Protonation states of the two main active site amino acid residues in different reaction steps and in the complexes with 4PE or 4PEX.

binary and ternary complexes, with NADP, the coenzyme analogue 3-amino-pyridine adenine dinucleotide phosphate (aPyADP) or NADPH. We show that the ternary complexes with the oxidized coenzyme and with aPyADP display half-site reactivity, and that 4PE, but not 5PR, is a transition state analogue.

Results and Discussion

Substrate and inhibitor binary complexes

The binding parameters for 6PG, 5PR and 4PE are reported in Table 1. The best fit of the average number of binding sites is slightly lower than two sites per dimer, reflecting the presence of some inactive enzyme. Although the observed K_d values for 6PG and 5PR are very close to their K_m and K_i values, respectively, 4PE has a higher K_d value than the K_i value measured previously [4,20].

The enthalpy change measured experimentally in titrations with 6PG arises primarily from the buffer protonation (Table 1); indeed, the release of 0.4 hydrogen ions was calculated from measurements in different buffers. The buffer-independent enthalpy change for the binding of 6PG is low and positive, 0.174 kcal·mol^{-1}, and the binding is totally entropy driven.

The buffer-independent enthalpy change for the binding of 5PR and 4PE is negative (Table 1), with the release of only a small fraction of hydrogen ions (0.029 for 4PE and 0.018 for 5PR); however, for the substrate analogues also, the main contribution to binding comes from an increase in entropy.

In all cases, the binding is entropy driven, and desolvation of the phosphorylated sugars appears to give the major contribution to the binding entropy. It has been shown that the binding of inorganic phosphate to the complex between porcine elastase and the turkey ovomucoid third domain has favourable entropy and unfavourable enthalpy as a result of the release of strongly immobilized water molecules [21]. Phosphorylated sugars should show a similar behaviour, and the major part of the entropy gain observed could arise from the phosphate group. Furthermore, we observed that $T\Delta S$ decreases by about 500–700 cal by shortening the carbohydrate chain for each carbon atom, probably reflecting the water molecules immobilized by hydrogen bonds with the sugar hydroxyl. Thus, the high entropy gain obtained by the desolvation of the ligands can overcome the entropy loss caused by the immobilization of the carbohydrate chain.

The enthalpy changes should also be discussed. The binding enthalpy for the inorganic phosphate to the elastase–ovomucoid third domain complex is about + 3 kcal·mol^{-1} [21]. In this complex, there is only one ionic bond, whereas, in 6PGDH, the phosphate group of 6PG forms two ionic bonds with R289 (R287 in the sheep liver sequence) and R453 (R446 in the sheep liver sequence). It has been shown by site-directed mutagenesis [22] of sheep liver 6PGDH that these two arginine residues can contribute to the binding free energy by −4.0 and −2.8 kcal·mol^{-1}, respectively. Thus, the additional enthalpy gain generated by a second ionic bond could overcome the positive enthalpy change generated by desolvation of the phosphate group.

Nevertheless, although the binding enthalpy of the inhibitors is negative, the binding enthalpy of 6PG is

Table 1. Binding parameters of substrate and substrate analogues to 6PGDH from *Trypanosoma brucei*. K_mK_i values taken from [4,20].

Ligand	K_d (μM)	K_mK_i (μM)	ΔH_0 (cal·mol^{-1})	$T\Delta S_0$ (cal·mol^{-1})	nH$^+$	Sites/dimer
6PG	4.96 ± 0.69	3.5	173.8	7398	− 0.46	1.47 ± 0.06
5PR	1.35 ± 0.19	0.95	− 1330	6626	− 0.018	1.33 ± 0.1
4PE	2.86 ± 0.79	0.13	− 2381	5111	− 0.029	1.83 ± 0.3

small and positive. This correlates with the proton release during binding. 6PG releases about 0.4 H^+, and this can account for up to 2–3 kcal·mol^{-1} if the hydrogen ion is removed from a nitrogen acid. Both 5PR and 4PE release a very small amount of H^+, and so the measured binding enthalpy is not shielded by the cost of proton release. The H^+ release is observed only in the enzyme–6PG complex, indicating that some rearrangement of the enzyme occurs when the substrate binds, whereas inhibitors are not able to induce the same changes. The selective action of the substrate could be correlated with the change in the protonation state of Lys185, the residue involved in the catalytic activity, that is supposed to release H^+ on binding of the substrate [10,11,20] (Scheme 1). The hydroxyl group at C2 of 5PR and the carboxylate group of 4PE (Scheme 2) correspond to the hydroxyl group at C3 of 6PG, which faces the amino group of catalytic K185 [6,7,10]. 5PR does not release H^+, probably because it does not fit the active site in the same conformation of 6PG; indeed, it has an inverted configuration at C2, so that the hydroxyl group could be misaligned to K185. 4PE does not release H^+ either, probably because the negatively charged carboxylate group facing K185 requires a positively charged group. 4PE (and its derivative 4PEX) is a very powerful inhibitor of 6PGDH, and it has been suggested that it might resemble the dienol intermediate [4]. If 4PE binds to protonated K185, the inhibitor resembles more closely the 3-keto intermediate, which has been suggested to be next to K185 in the protonated state (Scheme 2). As discussed below, 4PE strongly affects the binding of both NADP and NADPH, again suggesting that this inhibitor can mimic some features of the 3-keto intermediate.

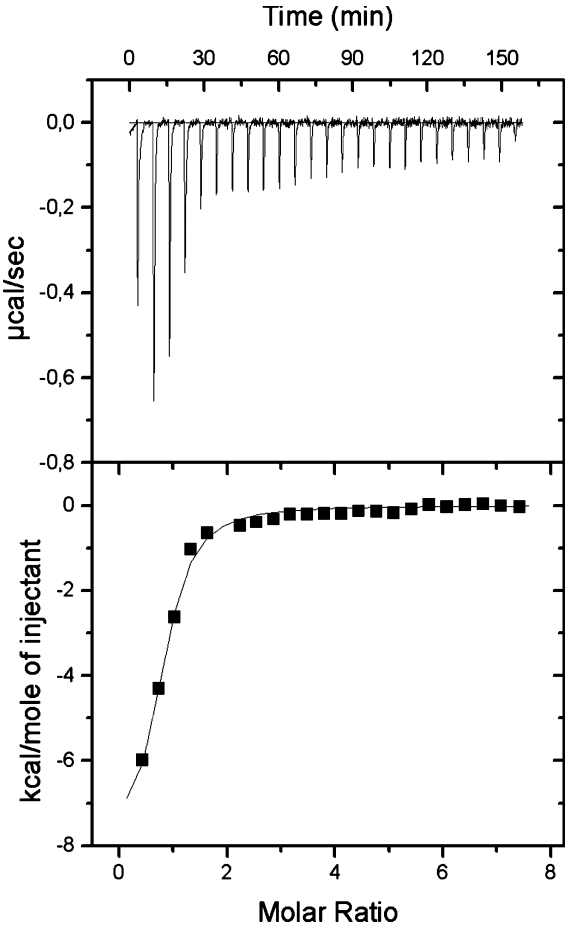


Fig. 1. Titration of *Trypanosoma brucei* 6PGDH with aPyADP. The cell contained 5.2 µM dimer concentration in 50 mM Hepes buffer at pH 7.5, 0.1 mM EDTA and 1 mM 2-mercaptoethanol. The syringe contained 0.43 mM aPyADP in the same buffer. A total of 25 injections was made at 380 s intervals. Top panel: raw ITC data. Bottom panel: data after the subtraction of the control titration and peak integration. The full line is the fit to a single-site model.

Enzyme–coenzyme complexes

The binding parameters for NADP, NADPH and aPyADP (a nonoxidizing analogue of NADP) are reported in Table 2. A binding isotherm and the fitted data for the binding of aPyADP to the enzyme are shown in Fig. 1. The binding stoichiometry was close to two sites per dimer for all the coenzymes tested. A quite surprising result is the relatively high value of K_d for NADP, around an order of magnitude higher than the K_m value of the coenzyme [20]. The enthalpy change for NADP binding is relatively low, and a positive entropy change contributes to binding. For NADPH and aPyADP, the binding appears to be totally enthalpic, and a negative entropy change is associated with complex formation. It is known that NADP and NADPH bind in a different way to sheep liver 6PGDH [10]. The differences in the thermodynamic parameters between oxidized and reduced coenzyme suggest that, also in the *T. brucei* enzyme, coenzyme binding involves different interactions with the protein.

Table 2. Binding parameters of coenzymes to 6PGDH from *Trypanosoma brucei*.

Ligand	K_d (µM)	ΔH_0 (cal·mol^{-1})	$T\Delta S_0$ (cal·mol^{-1})	nH$^+$	Sites/dimer
NADP	7.54 ± 0.19	− 5382	1486	− 0.18	1.86 ± 0.13
NADPH	1.05 ± 0.05	− 11819	− 3093	0.08	2.07 ± 0.05
aPyADP	1.56 ± 0.1	− 10581	− 2838	0.45	1.65 ± 0.012

With regard to the thermodynamic parameters, aPyADP resembles more closely NADPH, even though

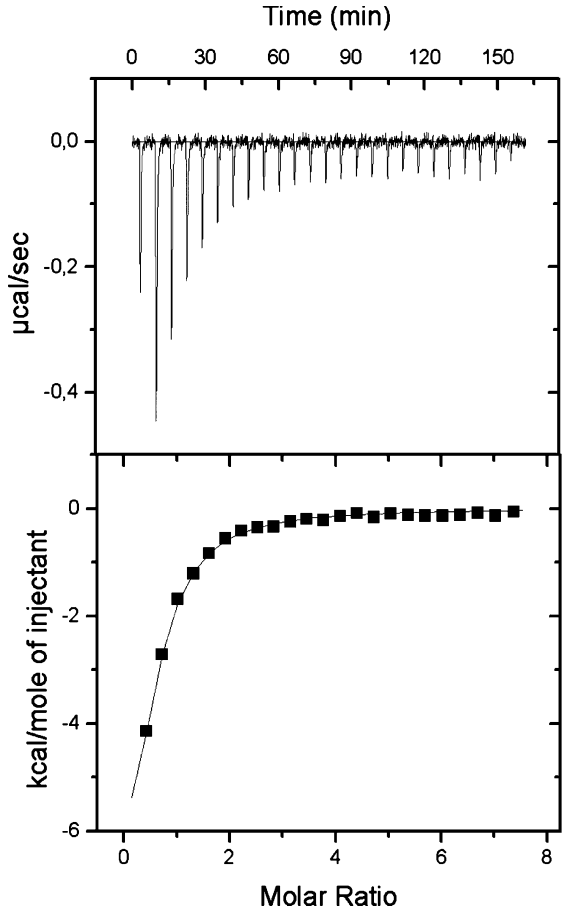


Fig. 2. Titration of the *Trypanosoma brucei* 6PGDH–6PG complex with aPyADP. The cell contained 5.2 µM dimer concentration and 1.2 mM 6PG in 50 mM Hepes buffer at pH 7.5, 0.1 mM EDTA and 1 mM 2-mercaptoethanol. The syringe contained 0.43 mM aPyADP and 1.2 mM 6PG in the same buffer. A total of 25 injections was made at 380 s intervals. Top panel: raw ITC data. Bottom panel: data after subtraction of the control titration and peak integration. The full line is the fit to a single-site model.

the amino-pyridine ring should be more similar in geometry and charge to that of NADP. Indeed, aPyADP has been used as an analogue of the oxidized coenzyme in 6PGDH from *Candida utilis* [23]. The anomalous behaviour of aPyADP could result from the lack of the carboxamide group, allowing a conformation of the binary complex closer to that of the reduced coenzyme. The different conformation, and the lower steric hindrance, could slightly perturb the pK value of the ionizable groups surrounding the amino-pyridine moiety, resulting in the uptake of 0.45 H^+.

Half-site reactivity of ternary complexes

Titration of the enzyme–6PG complex with aPyADP (Fig. 2) shows a small increase in the dissociation constant of the coenzyme analogue, a more negative binding enthalpy and, more interestingly, a decrease in the binding stoichiometry of the coenzyme (Table 3). Indeed, only one coenzyme molecule per enzyme dimer is bound. Titration of the same enzyme–6PG complex with NADPH gives a stoichiometry of 1.55 coenzyme molecules per dimer, a value similar to that observed in binary complexes, which could be accounted for by the partially inactivated enzyme. Thus, the differences between aPyADP and NADPH binding reflect a real change in the stoichiometry.

Titration with NADP of the binary complexes of the enzyme with the inhibitors 5PR or 4PE again shows a binding stoichiometry of about one coenzyme molecule per dimer, confirming the presence of half-site reactivity. Likewise, for 4PE, the binding stoichiometry of NADPH is 1.58 coenzyme molecules per dimer, indicating that the half-site reactivity is strictly limited to the oxidized coenzyme.

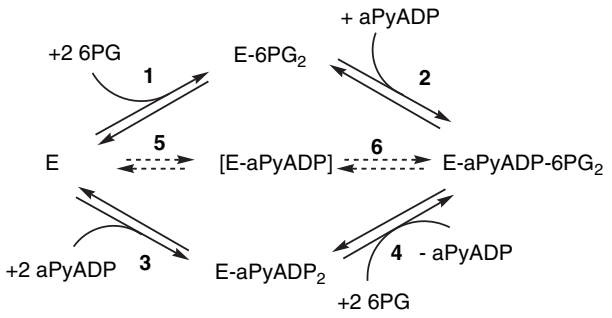
To test whether the half-site reactivity involves only the coenzyme, or both NADP and substrate, 6PGDH was titrated with 6PG and 4PE in the presence of saturating concentrations of aPyADP and NADP, respectively.

The titration of the enzyme–aPyADP complex with 6PG gives small signals, whose values are so close to

Table 3. Ternary complexes of 6PGDH from *Trypanosoma brucei*. ND, not determined.

Titrant	Binary complex	K_d (µM)	ΔH_0 (cal·mol^{-1})	$T\Delta S_0$ (cal·mol^{-1})	Sites/dimer
aPyADP	6PGDH–6PG	3.62 ± 0.27	– 12645	– 5467	1.07 ± 0.08
NADPH	6PGDH–6PG	2.04 ± 0.58	– 15670	– 8000	1.55 ± 0.064
NADP	6PGDH–5PR	12.9 ± 3.38	– 18599	– 12273	0.879 ± 0.013
NADP	6PGDH–4PE	0.043 ± 0.04	– 15742	– 6010	1.0 ± 0.003
NADPH	6PGDH–4PE	0.0203 ± 0.0103	– 22299	– 12089	1.58 ± 0.09
6PG	6PGDH–aPyADP	10.2 ± 0.7[a]	ND	ND	ND
4PE	6PGDH–NADP	0.177 ± 0.015	– 8741	285.6	1.0 ± 0.1
		1.62 ± 0.062	– 2232	5601	1.0 ± 0.1

[a] From the fluorescence measurements.



Scheme 3. Kinetic mechanism of the binding to 6PGDH of the substrate 6PG and the NADP analogue aPyADP. The enzyme is a homodimer with a NADP half-site reactivity in the presence of 6PG.

blank titrations that it is impossible to handle the experimental data. As binding stoichiometry suggests that only one coenzyme molecule per dimer is bound in the ternary complex, titration of the enzyme–$(aPyADP)_2$ complex with 6PG should cause the release of a coenzyme molecule from the dimer (Scheme 3, step 4). aPyADP release has a large positive ΔH value and is accompanied by H^+ release (Table 2). 6PG binding has a small positive ΔH value and is accompanied by H^+ release (Table 1). Thus, during the formation of the enzyme–6PG–aPyADP ternary complex from the enzyme–$(aPyADP)_2$ complex, there are two opposite effects: a positive ΔH value for aPyADP release and 6PG binding, and a negative ΔH value for buffer protonation. These opposite effects can result in an experimental value close to blank data.

To further study the binding of 6PG to the enzyme–aPyADP complex, we measured the changes in the fluorescence of the bound coenzyme on addition of 6PG (Fig. 3). The fluorescence changes cannot be fitted with a simple binding isotherm; however, the data are consistent with the mechanism depicted in Scheme 3, where the substrate does not bind to the enzyme–$(aPyADP)_2$ complex. The resulting K_d value, 10.2 ± 0.7 μM (Table 3), is close to 7.81 μM, the value calculated on the basis of multiple equilibrium constraints:

$$K_{6PG4} = K_{6PG1}K_{aPyADP2}/K_{aPyADP3} \quad (1)$$

where the numbers in the subscripts refer to the steps in Scheme 3.

Further support for the half-site model for *T. brucei* 6PGDH comes from enzyme kinetics. Indeed, although at high 6PG concentrations the enzyme displays the usual Michaelis–Menten kinetics towards NADP, at low 6PG concentrations the enzyme shows a marked inhibition by NADP (Fig. 4). This substrate-dependent inhibition by the coenzyme has been observed previously for the enzyme from *C. utilis*, and has been correlated with the presence of half-site reactivity. At low substrate concentrations, the coenzyme inhibits the enzyme by shifting the equilibrium towards the

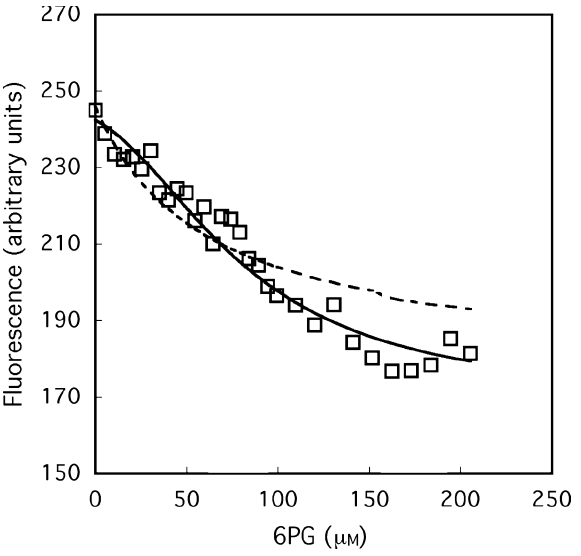


Fig. 3. Fluorescence titration of the 6PGDH–aPyADP complex with 6PG. Changes in the fluorescence of the bound coenzyme on addition of 6PG are shown. Lines were obtained by nonlinear least-squares fitting to a full-site model (broken line) or a half-site model (full line).

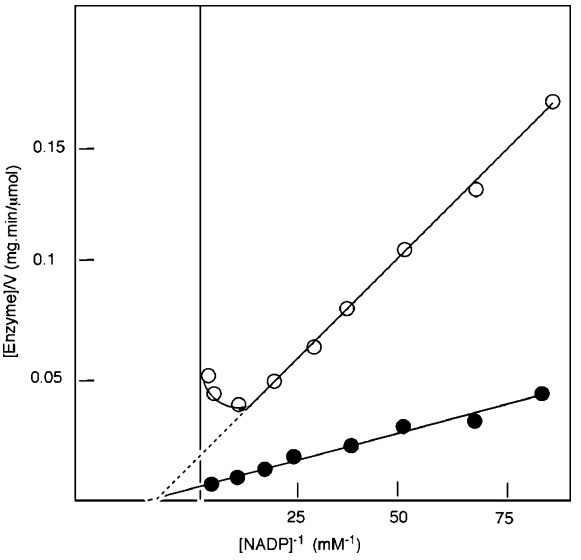


Fig. 4. Inhibition of *Trypanosoma brucei* 6PGDH by NADP. The assay mixture contained 1 mL of 50 mM triethanolamine buffer, pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, NADP at the concentration indicated on the abscissa and either 20 μM 6PG (open circles) or 2.2 mM 6PG (filled circles).

nonproductive enzyme–$(NADP)_2$ complex that cannot bind the substrate. At high substrate concentrations, the equilibrium is shifted towards the enzyme–substrate complex, preventing the binding of the second coenzyme molecule, and the inhibition is cancelled [23].

In conclusion, titration of the enzyme–aPyADP complex with 6PG [by both isothermal titration calorimetry (ITC) and fluorescence measurements], titration of the enzyme–6PG complex with aPyADP and kinetic data all support the half-site reactivity of *T. brucei* 6PGDH, where only one ternary complex can be formed on the enzyme dimer.

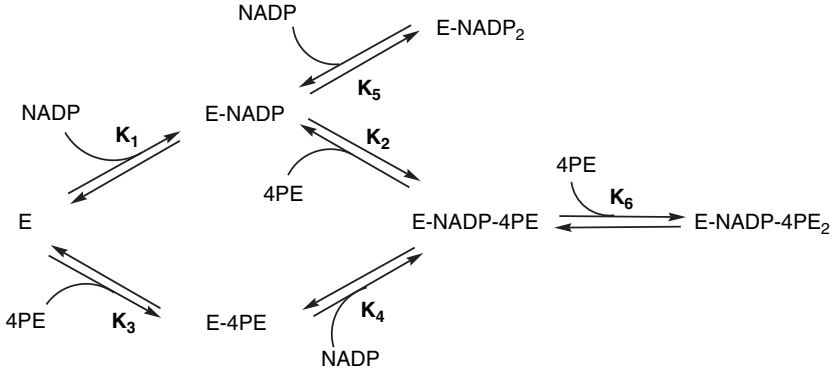
The binding of 4PE to the enzyme–$(NADP)_2$ complex gives a large measurable enthalpy change, and the best fit is obtained by assuming two sequential binding sites. The first site shows an apparent K_d value of 0.177 μM, very close to the K_i value of the inhibitor determined kinetically (0.18 μM) [4]. However, K_2 in Scheme 4 must be given by the product K_3K_4/K_1, which is 0.015 μM, much lower than the measured K_d value. To explain this discrepancy, it should be considered that only one NADP molecule can be present in the ternary complex (Table 3), so that, in the formation of the ternary complex, the excess of NADP could act as a competitive inhibitor of 4PE (Scheme 4, step 5). In other words, NADP could act as an inhibitor for 4PE binding in the same way as NADP inhibits enzymatic activity. If this holds true, the K_d value measured experimentally is an apparent dissociation constant, and the true value should be obtained by correcting the experimental value by the usual term $K_{app} = K_d(1 + [I]/K_i)$, where I is NADP and K_i is the dissociation constant of NADP for the free enzyme. In our experimental conditions, the calculated true K_d value is 17.7 nM, in good agreement with the value imposed by multiple equilibrium constraints.

The second site shows K_d and ΔH values close to those of the binary complex, suggesting that the asymmetric form of the enzyme causes only moderate effects on the substrate binding site of the subunit devoid of the coenzyme. Thus, the asymmetric ternary complex binds only one NADP molecule, but still binds two substrate molecules.

The half-site reactivity of 6PGDH has been observed previously in the enzyme from *C. utilis* and from sheep liver. In both cases, the evidence was obtained using an inhibitor derived from NADP, periodate-oxidized NADP [13,14]. Further support for an asymmetric functional enzyme has been obtained by studying the binding of aPyADP in the presence of 6PG [23], and by observing that 6PG enhances the decarboxylation of 3-keto-2-deoxy 6PG, an analogue of the putative intermediate 3-keto 6PG [8,18,19]. Recently, the crystallographic structure of the ternary complex of *L. lactis* 6PGDH with NADP and 4PEX/4PEA has been published, showing only one subunit filled by both coenzyme and inhibitor [7]. The superimposition of the subunit bearing NADP and the inhibitor on the other subunit shows a movement of the cofactor binding domain, resulting in a 5° rotation and a 0.7 Å translation, indicating a structural change on one subunit when the other is filled by the ternary complex [7]. Here, we have shown by direct binding experiments that 6PGDH from *T. brucei* also makes only one ternary complex per dimer. In conclusion, the half-site reactivity appears to be common behaviour for 6PGDH.

Substrate analogues and transition state analogues

The ternary complexes formed by aPyADP and 6PG or NADP and 5PR are very similar. Indeed, although the binding enthalpy of the coenzymes is higher in ternary complexes than in binary complexes, the enthalpic gain is compensated by a large entropy loss, and K_d changes slightly (Tables 2 and 3). The large entropy decrease could be a consequence of the tighter binding



Scheme 4. Competition between 4PE and NADP for the binding to the enzyme with one NADP bound.

that reduces the conformational freedom of the residues interacting with the ligands [24].

The fact that the K_d value of 5PR is close to the inhibition constant (Table 1) suggests that 5PR is simply a substrate-competitive inhibitor.

Quite different behaviour is observed for the ternary complexes with 4PE, where the K_d values of NADP and NADPH show a dramatic decrease. In these complexes, the enthalpy gain overcomes the entropy loss as a result of the tighter binding.

In *L. lactis* 6PGDH, an overlay of 4PEX with 6PG and Ru5P indicates that the inhibitors adopt similar conformation in the active site [7]. However, 4PEX lacks the three hydrogen bonds formed by the carboxylate group of 6PG; nevertheless, the K_i value is far below the K_d value of the substrate. The very tight binding of 4PEX can be explained by suggesting that the planar nature of the hydroxamate group should mimic the planar structure of the dienol intermediate (Scheme 2). It is reasonable that 4PE adopts a conformation similar to that of 4PEA, with a water molecule bridging the carboxylate O1 to the catalytic E192 [7]. The observation that 4PE strongly affects both NADP and NADPH binding suggests that the inhibitor should mimic an intermediate in the dehydrogenation step, where the coenzyme structure changes from the oxidized to the reduced form (Scheme 2). Deuterium kinetic isotope effects indicate a nonsymmetric transition state for the dehydrogenation reaction, suggesting a 'late' transition state [25]. Within this hypothesis, K185 goes from the nonprotonated form in the reagents to the protonated form in the transition state, together with C3 becoming planar. The negative charge of the carboxylate group of 4PE could force K185 into the protonated form, thus supporting the conformational/charge changes that strengthen the binding of the transition state.

We suggest that 4PE and 4PEX represent the transition state analogues of two different steps: 4PE, dehydrogenation; 4PEX, decarboxylation (Scheme 2).

Conclusions

The results presented here show some important features fruitful for the design of inhibitors specific for *T. brucei* 6PGDH.

The first observation focuses attention on the role of entropy and the phosphate group. The major contribution to the binding energy of 6PG and its analogues comes from entropy and, in particular, from the entropy gain resulting from the desolvation of the phosphate. The bonds formed by the ligand with the enzyme can only counterbalance the positive desolvation enthalpy of the phosphate. Therefore, the design of new inhibitors should firstly preserve the entropy gain.

The second observation is on the proton linkage, an aspect that can escape the analysis of crystallographic structures. Both the proton release and internal rearrangement of the ionic charges can affect negatively the binding enthalpy. By comparing the binding enthalpy of 6PG and 4PE, it appears that, despite 4PE forming a smaller number of hydrogen bonds than 6PG, the binding enthalpy is greater. This can be related to the absence of H^+ loss on binding of the inhibitor. The presence of the charged carboxylate anion of 4PE near K185 strongly suggests that this residue must be charged, whereas the catalytic mechanism requires an uncharged lysine in the complex with 6PG. The transfer of a hydrogen ion from K185 to the medium or to another functional group of the protein could have a high energy cost, which is absent in the binding of the inhibitor. This appears to be the most rational explanation of the high affinity of 4PE. Therefore, a better understanding of the catalytic mechanism is a prerequisite for the correct design of new inhibitors.

Last, but not least, the transition state involves not only the substrate analogue, but also the coenzyme. In other words, the inhibitor must be more efficient when the coenzyme is present. In the case of 4PE, the K_d value of the inhibitor is close to the K_d value of the substrate, but it decreases by two orders of magnitude in the presence of both oxidized and reduced coenzyme. This means that a powerful inhibition occurs under both normal cellular conditions, when the NADPH/NADP ratio is high, and stress conditions, when NADPH decreases and NADP increases.

Experimental procedures

Recombinant *T. brucei* 6PGDH was prepared and assayed as described previously [19]. aPyADP, NADP, NADPH, ribose-5-phosphate, erythrose-4-phosphate and 6PG were purchased from Sigma (St Louis, MO, USA). 5PR and 4PE were prepared by bromine oxidation [26] of ribose-5-phosphate and erythrose-4-phosphate, respectively. The concentrations of 5PR and 4PE were determined by measuring the concentration of organic phosphate [27], 6PG and NADP were determined enzymatically, and the concentrations of aPyADP and NADPH were determined spectrophotometrically using $\varepsilon = 3.09$ mM (at 331 nm) [28] and $\varepsilon = 6.22$ mM (at 340 nm), respectively.

ITC measurements

Before each experiment, the enzyme was dialysed exhaustively and the titrant was diluted in dialysis buffer. All

solutions were properly degassed before the titration experiments. The enzyme (4–6 μM dimer) was placed in the stirred cell and titrated with a total of 23 injections of 10 μL of ligand, at 380 s intervals. An initial preinjection of 5 μL volume was made, and the result from this injection was not used for data analysis. Heats of dilution and mixing, obtained by blank titrations, without the enzyme, were subtracted from the heats obtained with enzyme titrations. For ternary complex studies, the first ligand was added at the same concentration in both enzyme and titrant to keep the concentration constant during the experiment.

The enzymatic activity was measured before and after each experiment to verify whether enzyme inactivation occurred during titration.

All experiments were performed in 50 mM buffer, pH 7.5, with 0.1 mM EDTA and 1 mM 2-mercaptoethanol. Three buffers were used: Hepes ($\Delta H_{ion} = 5.03$ kcal·mol^{-1}), triethanolamine ($\Delta H_{ion} = 7.932$ kcal·mol^{-1}) and Tris ($\Delta H_{ion} = 11.3$ kcal·mol^{-1}). The buffer-independent binding enthalpy ΔH_0 and the number of hydrogen ions exchanged were calculated by the least-squares fitting of the experimental enthalpy in different buffers:

$$\Delta H_{exp} = \Delta H_0 + n\mathrm{H}^{+}\Delta H_{ion}$$

Measurements were performed at 20 °C in a VP-ITC microcalorimeter (Microcal, Northampton, MA, USA), and the data were fitted by nonlinear least-squares fitting using Origin™ software provided by the instrument manufacturer.

Fluorescence measurements

All experiments were performed with a Perkin-Elmer (Waltham, MA, USA) LS55 spectrofluorimeter at 20 °C, with $\lambda_{exc} = 330$ nm and $\lambda_{emi} = 410$ nm; 1 mL of solution containing 19 μM enzyme and 350 μM aPyADP was titrated with additions (1–2 μL each) of 5.54 mM 6PG.

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