Nucleotide binding affinities of the intact proton-translocating transhydrogenase from *Escherichia coli*

Tania Bizouarn[a], Gijs I. van Boxel[b], Tina Bhakta[b], J. Baz Jackson[b],*

[a]*Laboratoire de Chimie Physique, Bat 350, Université Paris XI-Orsay, 91405 Orsay, France*
[b]*School of Biosciences, University of Birmingham, Edgbaston Birmingham B15 2TT, UK*

Abstract

Transhydrogenase (E.C. 1.6.1.1) couples the redox reaction between NAD(H) and NADP(H) to the transport of protons across a membrane. The enzyme is composed of three components. The dI and dIII components, which house the binding site for NAD(H) and NADP(H), respectively, are peripheral to the membrane, and dII spans the membrane. We have estimated dissociation constants (K_d values) for NADPH (0.87 μM), NADP$^+$ (16 μM), NADH (50 μM), and NAD$^+$ (100–500 μM) for intact, detergent-dispersed transhydrogenase from *Escherichia coli* using micro-calorimetry. This is the first complete set of dissociation constants of the physiological nucleotides for any intact transhydrogenase. The K_d values for NAD$^+$ and NADH are similar to those previously reported with isolated dI, but the K_d values for NADP$^+$ and NADPH are much larger than those previously reported with isolated dIII. There is negative co-operativity between the binding sites of the intact, detergent-dispersed transhydrogenase when both nucleotides are reduced or both are oxidised.
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1. Introduction

The proton-translocating, nicotinamide–nucleotide transhydrogenase is located in the inner membrane of animal mitochondria and in the cytoplasmic membrane of bacteria (for review, see [1]). It couples the transfer of a hydride-ion equivalent between NAD(H) and NADP(H) to translocation of a proton across the membrane:

$$H^+_{out} + NADH + NADP^+ \leftrightarrow H^+_{in} + NAD^+ + NADPH \qquad (1)$$
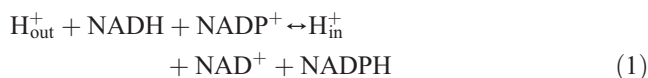
where "in" denotes the cytoplasm and "out", the periplasm, of bacteria (the mitochondrial matrix and the cytoplasm,

respectively, of mammalian cells). Transhydrogenase provides NADPH for biosynthesis and in defence against oxidative damage. In mitochondria, it probably also participates in the control of flux through the tricarboxylic acid cycle (reviewed in [2]).

Amino acid sequences indicate that the global structures of transhydrogenases from different species are very similar. The protein is composed of three components, dI, dII, and dIII (see Fig. 1). The dI component (~400 amino acid residues) binds NAD$^+$/NADH, and the dIII component (~200 residues) binds NADP$^+$/NADPH. These components are exposed to the cytosol in bacteria (the matrix in mitochondria). The transmembrane component, dII (350–400 residues), houses at least a part of the pathway for proton transport. Despite the ubiquitous tri-partite architecture of transhydrogenase, the polypeptide composition of the protein varies amongst species. That from animal mitochondria comprises only a single polypeptide and is dimeric. Bacteria can have either two polypeptides (which, in *Escherichia coli*, are called α and β, and where the
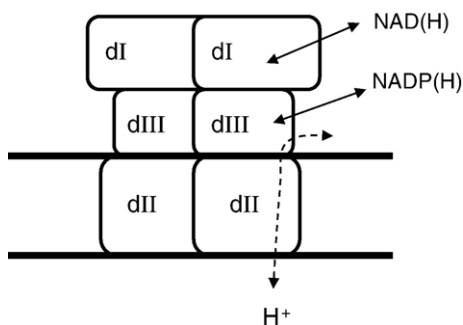
Fig. 1. Schematic representation of bacterial transhydrogenase. The organisation is based on the symmetry of the X-ray structure of dI_2dIII_1 complexes and the ready dissociation of the dI component from the dII/dIII components of the *R. rubrum* enzyme (reviewed [7]).

enzyme is an $\alpha_2\beta_2$ assembly) or three polypeptides (which, in *Rhodospirillum rubrum*, are called PntAA, PntAB, and PntB, where the enzyme is a $(PntAA)_2(PntAB)_2(PntB)_2$ assembly). The molecular features of the nucleotide binding sites were established by solving the crystal structures of isolated dI [3,4] and dIII [5,6]. These show unambiguously that there is one NAD(H)-binding site per dI and one NADP(H) site per dIII. X-ray structures of a complex of *R. rubrum* dI and dIII (the dI_2dIII_1 complex) [7,8], backed up by solution studies [9], revealed a profound asymmetry in the interaction between equivalent subunits and in the nucleotide-binding properties of the protein. There is no structure for the dII component of transhydrogenase, although predictions based on amino acid sequence and biochemical determinations suggest 12–14 transmembrane helices per monomer in different species [10,11].

Early experiments on transhydrogenase from various sources suggested a random-ordered binding of nucleotides and the formation of ternary complexes (e.g. $NADP^+ \cdot E \cdot NADH$, where E is transhydrogenase) [12–15]. It was later shown that the binding of nucleotides to dI was in rapid-equilibrium during turnover, whereas nucleotide dissociation from dIII was a limiting step of the reaction [16,17]. These conclusions were supported by stopped-flow experiments on the dI_2dIII_1 complex, which also showed that the transfer of hydride-ion equivalents between the two nucleotides is direct—there are no redox intermediates in the reaction [18,19]. In vivo, the proton electrochemical gradient (Δp) drives the reaction from left to right (Eq. (1)), greatly stimulating the rate and shifting the mass action ratio towards NADPH formation from unity to about 500 [20,21].

Together with a wealth of older data showing that the proteolysis of transhydrogenase and the access to chemical-modification reagents are altered by nucleotide binding (reviewed in [22]), the kinetic analyses have led to a consensus view that the coupling between proton trans-location and the redox reaction is achieved through conformational changes in the protein, an idea that originates from the early suggestions of Skulachev [23] and Rydstrom [24]. Recent discussion has focussed on the mechanism of gating of the hydride-transfer reaction and the

factors controlling the switch in proton access during translocation [1,17]. Importantly, the X-ray structures are beginning to provide indications as to the character of the coupling conformational changes, at least in the membrane-peripheral components of the enzyme [8].

Despite the detailed information that is now available on the isolated dI and dIII components of transhydrogenase, little is known about the thermodynamics and kinetics of nucleotide binding in the intact enzyme. Further work in this area is essential if we are to understand better the changes in nucleotide binding energy that are thought to occur during enzyme turnover and proton translocation. Approximate binding constants may be estimated from experiments on the protection from, or the stimulation by, nucleotides during enzyme modification, but often, this data is difficult to interpret on a quantitative basis. Hatefi and Yamaguchi used a centrifugation procedure to study the binding of NADH and NADPH to detergent-dispersed transhydrogenase from beef heart mitochondria [25]. In isolated dI and dIII, and in dI_2dIII_1 complexes, the measurement of Trp fluorescence changes [26], chemical shift changes of ^1H in NMR experiments [27,28], and equilibrium dialysis [29] have all made a useful contribution to binding studies, but, in our hands, none of these procedures have provided a satisfactory way forward with the intact bacterial enzyme. Thus, the fluorescence emission of intact transhydrogenases is complicated by multiple Trp residues and the proteins are too large for ligand-binding analysis by NMR. Satisfactory data can be obtained by equilibrium dialysis only for low K_d values and with large amounts of protein. However, we have now successfully applied the technique of micro-calorimetry to the intact *E. coli* transhydrogenase to estimate binding constants for all the physiological nucleotide substrates and products (NAD^+, NADH, $NADP^+$, and NADPH).

2. Materials and methods

2.1. Protein purification

Transhydrogenase was expressed from the plasmid pCLNH (kindly given by the J. Rydstrom group) in the *E. coli* strain JM109 and purified as described [30], but with some modifications. All steps were carried out at 4 °C. The pelleted cells from a 3 L culture were re-suspended in 130 ml 50 mM Tris–HCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.8, supplemented with 1 mg DNase, and 1 mM phenyl-methylsulfonyl fluoride, and they were disrupted by sonication. The unbroken cells were removed by centrifugation at 12,000 g for 25 min and the supernatant was then further centrifuged at 160,000 g for 1.5 h. The resulting pellet (2.5 g) was re-suspended in 10 ml 30 mM sodium phosphate, pH 7.5, and added to 10 ml of similar buffer supplemented with 2 M KCl, 60 mM cholate, and 60 mM deoxycholate. The mixture was incubated for 30 min with

gentle shaking at 4 °C and centrifuged at 160,000 g for 25 min. The supernatant, containing the solubilised protein, was incubated with 15 ml of Ni-NTA resin (Qiagen) for 1.5 h. Purification was continued as in [30], but with the omission of thesit from the buffers. Purified protein was stored on ice, with little loss of activity for at least 10 days. The yield was typically 15 mg of transhydrogenase, with a purity of around 95% as judged by SDS-PAGE (Nupage 4–12%, Novex). The specific activities of the enzyme were estimated from the rate of reduction of acetylpyridine adenine dinucleotide, an NAD^+ analogue (200 μM), by NADPH (200 μM) in 10 mM Mops, 10 mM Tris, 50 mM NaCl, 0.01% Brij-35, pH 7.0, at 20 °C, by following the absorbance change at 375 nm ($\varepsilon = 6.1$ mM^{-1} cm^{-1}) at 25 °C. The specific activity was generally around 5–7 μmol min^{-1} mg^{-1}.

Protein concentrations were estimated using the bicinchoninic acid assay, with bovine serum albumin as standard [31].

2.2. Determination of K_d values by microcalorimetry

Protein was brought to a concentration of around 2.4 mg ml^{-1} (approximately 22 μM in α/β polypeptide units) by centrifugation through a filter (10 k cut-off, Vivascience) and dialysed against 30 mM sodium phosphate, 50 mM NaCl, 0.1% Brij-35, pH 7.5 (unless indicated otherwise). The same buffer was used to prepare the stock solutions of nucleotides.

Isothermal titration calorimetry experiments were performed at 15 °C using the Microcal MCS. Thus, heat changes were measured when small quantities of nucleotide were sequentially injected into the protein solution. Each incremental heat change is assumed to be proportional to the increase in the amount of bound ligand. Data were analysed using the instrument's ORIGIN software, which uses standard Marquardt algorithms and iterative procedures to derive the best-fitting K_d, binding stoichiometry, and molar heat change for ligand binding.

3. Results

3.1. The dissociation constants of intact E. coli transhydrogenase and its nucleotide substrates

Heat changes were recorded during titrations of a detergent-dispersed solution of intact *E. coli* transhydrogenase with NAD$^+$, NADH, NADP$^+$, and NADPH. Figs. 2 and 3 show representative data for NADH and NADPH. Following an appropriate correction for heats of nucleotide dilution determined in control experiments in the absence of protein (they were relatively small), analysis of the data provided reproducible K_d, ΔH, and ΔS values (Table 1). All the single-titration data were satisfactorily fitted on the assumption that the protein has just a single class of binding
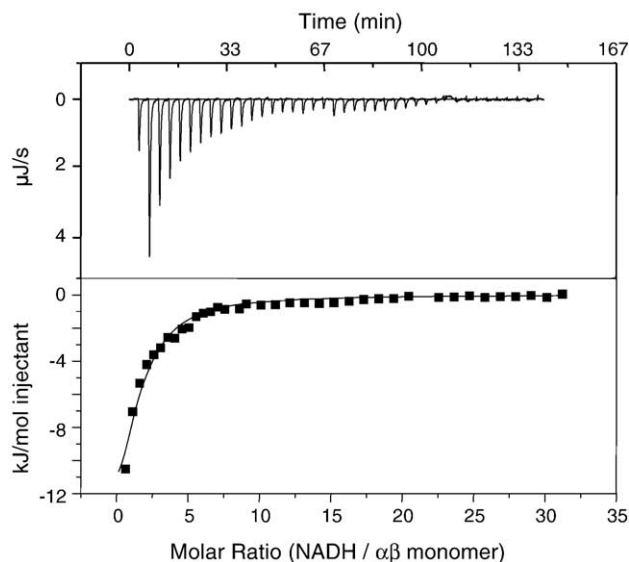

Fig. 2. Heat changes accompanying the binding of NADH to a detergent-dispersed solution of intact *E. coli* transhydrogenase. The measurements were carried out as described in Materials and methods. Titrations were performed on 2.5 ml samples containing 2.4 mg/ml of protein in 30 mM sodium phosphate, 50 mM NaCl, and 0.1% Brij-35, pH 7.5. The injection syringe contained 5.2 mM NADH in identical buffer to the protein. The upper panel shows heat changes corresponding to a single addition of 1.0 μl NADH solution, followed by 18 additions of 3.0 μl, and then by 21 additions of 6.0 μl. The lower panel shows the computer fit to the experimental data. Binding constants and enthalpy changes are given in the Table 1.

site for each nucleotide. For the weak-binding NAD$^+$, we assumed 1.0 nucleotide binding site per α/β polypeptide unit, although the estimated K_d was not greatly affected by small changes in the selected binding stoichiometry (e.g., down to 0.5 binding sites per α/β unit). The fit in NAD$^+$ titrations was rather poor, and we give a range of K_d values that would be consistent with the data. The reduced nucleotides (NADH and NADPH) bound more tightly than did the respective oxidised nucleotides (NAD$^+$ and NADP$^+$). The triphosphonucleotides (NADP$^+$ and NADPH) bound more tightly than did the respective diphosphonucleotides (NAD$^+$ and NADH).

In general, in ligand-binding experiments, stoichiometries are only reliably determined when the working protein concentration needed to observe a full titration is substantially greater than the K_d (i.e., the experiment is performed in the "tight-binding region"). Thus, in our experiments, only the titration with NADPH was found to give a reproducible and unambiguous nucleotide-binding stoichiometry (0.84 ± 0.01 per α/β polypeptide unit). Even here, there may be uncertainties over the estimated value arising from difficulties in the accurate determination of membrane-protein concentration [29], but we suggest that the result indicates that each single α/β polypeptide unit binds a single nucleotide.

The binding affinity of NADPH at pH 6.5 was barely different from that at pH 7.5 (Table 1). In view of the fact
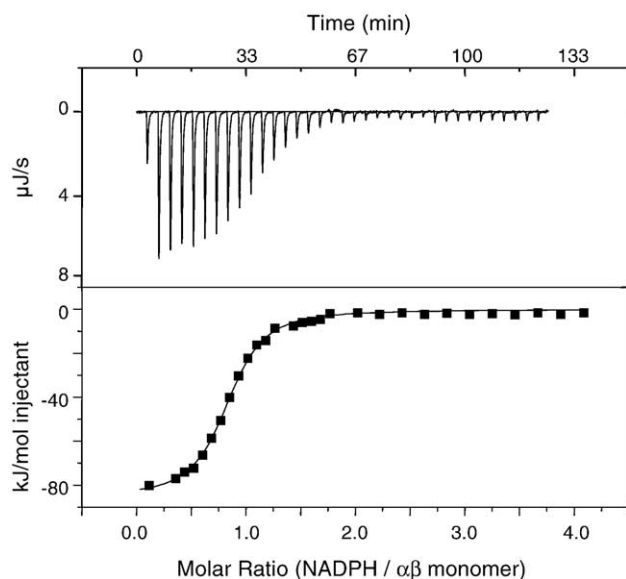
Fig. 3. Heat changes accompanying the binding of NADPH to a detergent-dispersed solution of intact *E. coli* transhydrogenase. See Fig. 2. The injection syringe contained 1.04 mM NADPH. The upper panel shows heat changes corresponding to a single addition of 1.0 μl NADPH solution, followed by 24 additions of 2.5 μl, and then by 10 additions of 6.0 μl. The lower panel shows the computer fit to the experimental data. The binding constants and enthalpy changes are given in the Table 1.

that the binding affinity of NADH to isolated dI is pH independent in the range 6.0 to 9.0 [32], and that the NAD^+- and NADH-binding affinities of isolated dI at pH 7.5 [9,25,26,29] closely resemble those of the intact enzyme (see below), we have not carried out further experiments at other pH values with these nucleotides.

A set of experiments was carried out to investigate nucleotide binding during the formation of "dead-end" ternary complexes (NADH·E·NADPH and NAD^+·E·$NADP^+$) of the intact transhydrogenase. Catalysis is not possible, of course, within these complexes because the nucleotides are either both oxidised or both reduced. The simple finding was that titration with NADPH in the presence of pre-bound NADH, or with NADH in the presence of pre-bound NADPH, or with $NADP^+$ in the presence of pre-bound NAD^+, all led to a decrease in affinity relative to that found in the equivalent titrations in the absence of the pre-bound nucleotides. That is, for pairs of reduced nucleotides (NADH and NADPH) and for pairs of oxidised nucleotides (NAD^+ and $NADP^+$), there is negative co-operativity between the binding sites. Note that titration with NAD^+ in the presence of $NADP^+$ was not carried out since the affinity of the former nucleotide, even on its own, is already very low and difficult to analyse (see above). Because of the interaction between the NAD(H) and NADP(H) sites, analysis of the single data sets for the dead-end complexes leads only to apparent dissociation constants (Table 1). True K_d values (i.e., at saturating concentrations of the pre-bound nucleotide) will be higher. The titration with NADPH in the presence of pre-bound NADH was distorted to the extent

that the data could not be satisfactorily fitted with a single class of NADPH-binding sites.

4. Discussion

The K_d for the binding of NADH to intact *E. coli* transhydrogenase (50 μM, Table 1) determined in microcalorimetry experiments is comparable with, though somewhat higher, that for NADH binding to the intact mitochondrial enzyme, as measured by a centrifugation procedure (9.5 μM; [25]). The K_d for NADPH binding to intact *E. coli* transhydrogenase from the current microcalorimetry studies (0.87 μM, Table 1) was similar to that determined by equilibrium dialysis (1.2 μM; [33]), and both of these values are somewhat lower than that estimated for the same enzyme (in different detergent) by the centrifugation procedure (19.7 μM; [39]). The centrifugation procedure gave a K_d for NADPH binding to intact mitochondrial enzyme of 5.7 μM [25]. There are no published K_d values for the binding of $NADP^+$ and NAD^+ to intact transhydrogenase from any species to compare with those determined in the present work (16.1 μM and 100–500 μM, respectively).

When both nucleotides are in the same redox state, there is negative co-operativity between the NAD(H)- and NADP(H)-binding sites of intact *E. coli* transhydrogenase (Table 1). For example, the binding of NADPH lowers the affinity for NADH, and the binding of $NADP^+$ lowers the affinity for NAD^+. This will have the effect of discouraging the formation of catalytically "dead-end" complexes, which, under physiological conditions, would otherwise tie up the enzyme in unproductive intermediates. The result was not anticipated from steady-state kinetics analysis of intact transhydrogenase [12–15].

Table 1
Nucleotide binding to detergent dispersions of *E. coli* transhydrogenase

Nucleotide used in the titration (pre-bound nucleotide)	K_d (μM)	ΔH (kJ mol^{-1} enzyme)	ΔS (kJ mol^{-1} K^{-1})
NADPH (none)	0.87	−84	−0.18
NADPH (NADH)	1.6	−99	−0.23
$NADP^+$ (none)	16.1	−108	−0.28
$NADP^+$ (NAD^+)	214	−88	−0.24
NADH (none)	50	−60	−0.13
NADH (NADPH)	>500	Not determined	
NAD^+ (none)	100 to 500	−4 to −10	
NADPH at pH 6.5 (none)	0.68	−81	−0.16

Conditions as described in Materials and methods at pH 7.5, unless otherwise stated. Experiments were carried out as shown in Figs. 2 and 3. The nucleotides shown in parentheses in the first column were added to the protein solution (360 μM for NADH, 590 μM for NAD^+, and 48 μM for NADPH) before the titration with the given nucleotide was initiated. For these titrations, we show only apparent K_d values (see text). ΔH and ΔS values are given for the association of the protein and nucleotide.

The K_d values for the binding of NADH to intact transhydrogenase are in the same order as those found (using several procedures) for the binding of this nucleotide to the isolated dI components of enzymes from *E. coli* (45 μM; [29]), *R. rubrum* (20 μM; [9,26,29]), and beef-heart mitochondria (9.7 μM; [25]). The implication is that the removal of dI from the intact enzyme has only a small effect on its NADH-binding affinity. It is likely that the same conclusion applies to the affinity of dI for NAD^+, although there are fewer data, and, because K_d values are high, they are less reliable. Thus, the K_d range of 100–500 μM for the binding of NAD^+ to intact *E. coli* transhydrogenase (Table 1) is comparable with values (300 μM by equilibrium dialysis [29] and 500 μM by NMR [28]) that have been determined for isolated dI from the *R. rubrum* enzyme (the only form of the protein for which data is currently available).

A quite different conclusion is reached for the binding of $NADP^+$ and NADPH. With one reported exception (which we believe should be provisionally discounted, see below), these nucleotides bind much more tightly to isolated dIII than they do to the intact enzyme. Thus, the K_d values for $NADP^+$ and NADPH binding to intact transhydrogenases are in the order 10^{-6}–10^{-5} M (depending on the species and experimental method, see above), but for the isolated dIII of the *R. rubrum*, *E. coli*, and human mitochondrial enzymes the K_d values are $<10^{-9}$ M [34–36]. Because they are so low, accurate K_d values for isolated dIII are unavailable but first-order rate constants for nucleotide release have been determined and are indeed extremely small. In contrast with these findings, it has been reported that an isolated dIII preparation from beef-heart mitochondria bound NADPH with a K_d unchanged from that found for the intact enzyme [25]. Note, however, that these experiments were carried out with a protease-derived dIII (as distinct from the recombinant proteins used in the other work) and there are reasons to be cautious about using the centrifugation technique with low molecular weight proteins [29].

Based on both structure and kinetics data, we have developed the view during the last decade that energy transduction between the proton-translocation pathway in dII and the hydride-transfer site at the dI/dIII interface proceeds through conformational changes in dIII [1]. A crucial step is where dIII is driven (by events associated with proton translocation) from an "open" state, in which bound NADP(H) can rapidly exchange with nucleotides in the solvent but hydride transfer is blocked, into an "occluded" state, in which NADP(H) exchange is prevented but hydride transfer is permitted. Good evidence for this step was provided by the finding that mutations in dII affect the apparent NADP(H)-binding affinity [30,37–43]. The differences in the NADP(H)-binding affinity of intact transhydrogenase and isolated dIII, described above, are also readily explained by the model. Thus, in the intact enzyme, the K_d values for NADP(H) in the region of 10^{-6}–

10^{-5} M would represent binding to the open state in equilibrium with the occluded state. The equilibrium is expected to be pH dependent, though, evidently, the pK_a value for NADPH binding is not close to 7 (Table 1). However, isolated dIII and the dI_2dIII_1 complex are locked in the occluded state (or equilibrium greatly favours the occluded state), and their very high affinities for NADP(H) are reflected in the slow dissociation of the tightly bound nucleotide. Crystal structures of isolated dIII and the dI_2dIII_1 complex reveal why NADP(H) is so tightly bound in these forms—the extended loop E folds like a lid over the bound nucleotide. It is envisaged that the retraction of loop E is required during progress to the open state of dIII.

NAD(H) in the dI site has an essentially "passive" role as a hydride donor/acceptor [16,17,44]: The nucleotide does not undergo large affinity changes during turnover and correspondingly the K_d values for both the oxidised and reduced forms are similar in the intact enzyme, isolated dI and the dI_2dIII_1 complex. There are important changes in NAD(H) conformation in dI (probably driven by events in dIII)–to gate the hydride transfer reaction [8]–but these are probably not associated with large changes in the intrinsic binding energy. The dI component has an extended loop (designated the "mobile loop"), which is somewhat analogous to loop E of dIII, but its closure over NAD(H) is dependent upon the process of nucleotide binding [45] and does not appear to be coupled to proton translocation.

The negative co-operativity between the two binding sites of the intact enzyme, when the nucleotides are either both oxidised or both reduced, is relevant to earlier discussions on the hydride transfer mechanism of transhydrogenase. It was found that two NADH molecules bind to the isolated dI dimer with identical affinities (K_d=20 μM, see above) but to the dI_2dIII_1 complex with different affinities (K_d^1=20 μM; $K_d^2 \approx 300$ μM) [9]. We can now see that this probably results from the fact that the single dIII polypeptide in the complex is loaded with NADPH (experimentally to block hydride transfer), and therefore, the affinity of the adjacent dI polypeptide for NADH is decreased by the negative co-operativity between the sites. In turn, this shows that the elevated NADH concentration needed to reach maximum rates of hydride transfer in the dI_2dIII_1 complex (much greater than the K_d value of 20 μM [46]) results not from rapid equilibrium binding at a low affinity site but from a slow step that follows nucleotide binding and precedes hydride transfer (see also [8]).

It is evident from the forgoing discussion and from earlier work that nucleotide binding to transhydrogenase is complex and may include large changes in conformation of both the protein and the ligands. The binding constants determined by micro-calorimetry are a composite of these processes and can be used only as an indication of events. Nevertheless, the data reported here represent the first "complete" set of binding constants for an intact transhydrogenase and provide a starting point for understanding how changes in nucleotide-binding energy might be

instrumental in linking the redox reaction to proton translocation.

Acknowledgements

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