Interaction of Nucleotides with the NAD(H)-binding Domain of the Proton-translocating Transhydrogenase of Rhodospirillum rubrum*

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Transhydrogenase catalyzes the reduction of NADP⁺ by NADH coupled to the translocation of protons across a membrane. The polypeptide composition of the enzyme in Rhodospirillum rubrum is unique in that the NAD(H)-binding domain (called Th₁) exists as a separate polypeptide. Th₁ was expressed in Escherichia coli and purified. The binding of nucleotide substrates and analogues to Th₁ was examined by one-dimensional proton nuclear magnetic resonance (NMR) spectroscopy and by measuring the quenching of fluorescence of its lone Trp residue. NADH and reduced acetylpyridine adenine dinucleotide bound tightly to Th₁ whereas NAD⁺, oxidized acetylpyridine adenine dinucleotide, deaminonicotinamide mononucleotide, NADP⁺ and adenosine bound less tightly. Reduced nicotinamide mononucleotide, NADH and 2'-AMP bound only very weakly to Th₁. The difference in the binding affinity between NADH and NAD⁺ indicates that there may be an energy requirement for the transfer of reduced equivalents into this site in the complete enzyme under physiological conditions. Earlier results had revealed a mobile loop at the surface of Th₁ (Diggle, C., Cotton, N. P. J., Grimley, R. L., Quirk, P. G., Thomas, C. M., and Jackson, J. B. (1995) Eur. J. Biochem. 232, 315-326); the loop loses mobility when Th₁ binds nucleotide; the reaction involves two steps. This was more clearly evident, even for tight-binding nucleotides, when experiments were carried out at higher temperatures (37 °C), where the resonances of the mobile loop were substantially narrower. The binding of adenosine was sufficient to initiate loop closure; the presence of a reduced nicotinamide moiety in the dinucleotide apparently serves to tighten the binding. Two-dimensional 1H NMR spectroscopy of the Th₁-5'-AMP complex revealed nuclear Overhauser effect interactions between protons of amino acid residues in the mobile loop (including those in a Tyr residue) and the nucleotide. This suggests that, in the complex, the loop has closed down to within 0.5 nm of the nucleotide.

Recombinant Th₁ from R. rubrum was expressed at high levels in Escherichia coli (14). The purified recombinant protein completely restored transhydrogenation activity to membranes of R. rubrum that had been washed to remove native domain I polypeptide. The characteristics of NADH binding to Th₁ were investigated by monitoring fluorescence quenching of a sole Trp residue at position 72 in the recombinant protein (14). From an analysis of the one-dimensional 1H NMR spectrum of Th₁, it was proposed that a region, which straddles sites that are highly sensitive to cleavage by proteases (Lys²⁷² and Thr²²₉ and Lys³³₇-Glu³³₈), has a significantly greater segmental flexibility than the remainder of the molecule (15). This segment might be a mobile loop emanating from the surface of the protein. Some of the sharper resonances in the NMR spectrum were provisionally assigned to specific amino acids. The well defined 1H resonances attributed to amino acid residues within the mobile loop were considerably broadened and some were slightly shifted when either NAD⁺ or NADH were added to recombinant Th₁ (15). This indicates that nucleotide binding causes a loss of loop mobility, and alters the rates of chemical exchange processes between conformations as the protons sample a range of environments on the NMR time scale. Titration with NAD⁺ revealed a two-step binding reaction. At low concentrations of nucleotide predominantly one

The enzyme is located in the cytoplasmic membrane of bacteria, where it is responsible for NADPH production during biosynthesis, and in the inner mitochondrial membrane, where it may serve in regulation of the tricarboxylic acid cycle by the protonmotive force (4).

Transhydrogenase has separate catalytic binding sites for NADH and for NADP⁺ (5-8). These reside in relatively hydrophilic domains of the protein that protrude from the cytoplasmic side of the membrane (in bacteria). The NADH site resides in domain I and the NADP⁺ site in domain III (9). The hydrophobic domain II spans the membrane. Several primary sequences of transhydrogenase are known. All are similar, although the polypeptide composition is variable (see Refs. 3 and 10). Uniquely, in Rhodospirillum rubrum transhydrogenase the NAD(H)-binding domain I exists as a separate polypeptide (called Th₁) (10-12). This polypeptide, in dimeric form, can be dissociated from the domain II/III components of the enzyme. Separated Th₁ and the domain II/III proteins lack transhydrogenation activity but reconstitution leads to full recovery.

1 The abbreviations used are: Th₁, the NAD(H)-binding peripheral membrane polypeptide (domain I) of transhydrogenase from R. rubrum; AcpAD⁺, acetyl pyridine adenine dinucleotide (oxidized form); deaminonicotinamide hypoxanthine dinucleotide; NOESY, nuclear Overhauser effect spectroscopy; Chex, 2-(cyclohexylamino)ethane-sulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Mops, 4-morpholinopropanesulfonic acid.

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resonance, the upfield Met at 1.97 ppm, was broadened. At higher concentrations, other mobile loop resonances also began to broaden. In titrations with NADH, relatively low nucleotide concentrations led to broadening of the mobile loop resonances. The resulting NMR spectrum was observed in the presence of higher concentrations of NADH.

In this report we describe the binding characteristics of NADH analogues to Ths with a view to establishing nucleotide specificity and defining the conformational dynamics of the mobile loop during the nucleotide-binding reaction. Two-dimensional proton NMR spectra of the Ths-5'-AMP complex reveal interactions between nucleotide and protein.

MATERIALS AND METHODS

Preparation of the strain of E. coli bearing the recombinant Ths gene from R. rubrum, growth and induction of bacteria, and purification of the protein were carried out as described (14). The procedure for preparing R. rubrum membrane vesicles depleted of native Ths was described (11).

The factor used to correct protein concentrations in colorimetric assays (14) was found to be unreliable because traces of glycerol that were present, even after dialysis, resulted in an underestimate in the amino acid analysis of samples hydrolyzed with HCl. To ensure that protein estimations were correct, Ths (more than 95% pure according to SDS-polyacrylamide gel electrophoresis; Ref. 14) was estimated using two procedures, the microtannin assay (16) and the biuret reaction (17), which rely on different reactions of the protein, using bovine serum albumin as standard. The results were similar within 2%. In turn these concentrations were similar within 25% and 5%, respectively, to concentrations of solutions of native protein, and of protein denatured in 8 M urea, calculated from the absorbance at 280 nm, using an extinction coefficient predicted from the content of aromatic amino acids in Ths. Routine measurements of the protein were subsequently estimated by the microtannin assay.

Fluorescence spectroscopy was performed with a Spex Fluoromax. Fluorescence signals were recorded at 25 °C in a stirred 1 × 1-cm cuvette using excitation and emission wavelengths of 280 nm and 310 nm, respectively. Slit widths were 1 nm. In titrations with nucleotides, inner filtering effects were compensated as described (14).

Samples of Ths for NMR spectroscopy were prepared as described (15). 1H NMR spectra were recorded on a Bruker AMX500 spectrometer. One-dimensional pulse and collect spectra were acquired using a 10-ppm sweep width and comprised 256, 512, or 1024 transients of 16,000 data points with a total acquisition time of 45 min (or less; see figure legends). The temperature was 20 °C unless otherwise indicated. Spectral data were processed, prior to Fourier transformation, by application of an exponential multiplication factor corresponding to a line broadening of 1 Hz. In two-dimensional nuclear Overhauser effect spectroscopy (NOESY) experiments, 2000 data points were collected for 224 rows, each of 128 transients. Total acquisition time was 17 h.

RESULTS

Binding of Analogue Nucleotides to Ths.—Acetylpyridine adenine dinucleotide is similar to NADH, except that it has an acetyl group at position 3 of the nicotinamide ring instead of a carboxamide (18). The oxidized form, AcPdAD⁺, is a good analogue of NAD⁺ in assays of “reverse” transhydrogenation. One-dimensional proton NMR experiments showed that binding of AcPdAD⁺ to Ths altered the conformational dynamics of the mobile loop in a similar way to physiological substrate (representative spectra are shown in Fig. 1; compare Ref. 15).

Low concentrations of nucleotide (e.g., 50–100 μM for 200 μM protein; Fig. 1B) led to an almost specific broadening of the upfield Met resonance at 1.97 ppm (denoted MetA; Ref. 22), whereas somewhat higher concentrations (100–1000 μM; Fig. 1C) were required to broaden other resonances assigned to the mobile loop, namely those at 1.22 ppm (CH₃ of Thr), 1.43 ppm (CH₃ of Ala), 2.06 ppm (CH₃ of MetC; see Ref. 22), 2.28 ppm (γCH₂ of Glu), 6.82 and 7.12 ppm (3.5 and 2,6 ring protons, respectively, of Tyr), and 7.33 ppm (ring protons of Phe). In this higher concentration range, there were also small changes in the chemical shift of resonances, especially of aromatic protons, similar to those observed in the NAD⁺ titration. It was evident that AcPdAD⁺, like NAD⁺, binds to Ths in a two-step reaction. The first step is characterized by broadening of the upfield Met resonance, and the second by broadening of other mobile loop resonances. AcPdAD⁺ resonses became detectable even at low concentrations (200 μM, Fig. 1), as described for NAD⁺ (15). Thus, the sharp resonance at 2.22 ppm of CH₃ protons of the acetyl group of AcPdAD⁺, in particular, but also resonances at 9.41, 9.14, 8.86, 8.45, 8.18, and 8.14 ppm (corresponding to the N-2, N-6, N-4, A-8, N-5, and A-2 protons) became clearly resolved at 200 μM nucleotide. Emerging nucleotide resonances were only slightly shifted and, with the interesting exception of A8 proton, not apparently broadened relative to those of nucleotide in buffer.

The reduced nucleotide, AcPdADH, binds to Ths and produces conformational changes in the mobile loop similar to those observed during NADH binding. 1) Addition of AcPdADH to a solution of Ths led to quenching of Trp fluorescence (Fig. 2). For similar concentrations of nucleotide, quenching was less extensive with AcPdADH than with NADH. Because of limitations imposed by inner-filtering effects of the nucleotides (14), the Kᵣ of the analogue was difficult to determine with precision, but assuming the extent of fluorescence quenching at saturating AcPdADH was similar to that with NADH, the Kᵣ was approximately 40 μM (cf. approximately 20 μM for NADH; Ref. 14). 2) Titration of Ths with AcPdADH led to changes in the NMR spectrum similar to those observed in NADH titrations (Fig. 3; compare Ref. 15). Thus, low concentrations of nucleotide (30–100 μM for 122 μM protein) led to extensive broadening of resonances assigned to the mobile loop, i.e. those at 1.22, 1.43, 1.97, 2.06, 2.28, 6.82, 7.12, and 7.33 ppm (as-signed as above). Also similar to NADH titration spectra, resonances of nucleotide protons were undetectable (due to line broadening) until high concentrations (>400 μM for 122 μM protein) of AcPdADH were added.

We speculated (15), although it was not clearly evident from NMR spectra, that NADH as well as NAD⁺ might bind to Ths in a two-step process. Detection of the intermediate state might be difficult at 20 °C because of differences in rates of chemical exchange between reaction intermediates that involve reduced nucleotide. Experiments at 37 °C indicate that for both NADH and AcPdADH a two-step reaction can indeed be resolved. Reconstitution experiments with depleted membrane vesicles showed that Ths retains activity when incubated at 37 °C for >24 h. The NMR spectrum of Ths at 37 °C in the absence of
nucleotides is shown in Fig. 4A. The resonances were much sharper than those at 20 °C (compare Figs. 1 and 3), indicating that at higher temperatures the loop takes on even greater segmental mobility. Titration of Ths, at 37 °C with either NADH (representative data in Fig. 4, B and C) or AcPdADH (data not shown) revealed the two-step binding reaction. At low concentrations of reduced nucleotide (20 μM; Fig. 4B), the MetA resonance at 1.97 ppm was shifted (0.02 ppm) upfield and was broadened more extensively than those attributable to Thr, Ala, Tyr, etc. in the mobile loop. At slightly higher concentrations of NADH (100 μM; Fig. 4C) or AcPdADH (data not shown), other loop resonances did become broadened.

Deamino-NADH is modified in the adenine part and not in the nicotinamide part with respect to Ths. It had a small quenching effect on fluorescence from Trp72 of Ths (Fig. 2), but the Kd was too large to measure. The weak binding of this analogue to Ths was also reflected by NMR data. Thus, features observed in NADH titrations were also seen with deamino-NADH, but at higher concentrations (about 2-fold for equivalent protein concentrations, not shown). The two-step reaction was evident, the MetA resonance broadening at lower nucleotide concentrations than other mobile loop resonances.

5'-AMP and adenosine are weak inhibitors of transhydrogenase (19). Inhibition with 5'-AMP is competitive with respect to AcPdADH+ and mixed with respect to NADPH (5, 6, 8). Remarkably, the effects of 5'-AMP and adenosine on the NMR spectrum of Ths were qualitatively very similar to those of NAD+ and AcPdADH+: low concentrations specifically broadened and shifted slightly upfield the MetA resonance, and higher concentrations led to broadening of the other mobile loop resonances (data not shown). The nucleotide resonances became evident as narrow bands in the spectrum even at quite low concentrations of 5'-AMP and adenosine (e.g. 150 μM 5'-AMP for 200 μM protein).

Reduced nicotinamide mononucleotide (NMNH) corresponds to the complementary half of NADH to 5'-AMP. It did not quench fluorescence of Trp72, and, up to 1 mM, either on its own or in combination with 400 μM 5'-AMP, NMNH had no effect on the NMR spectrum of Ths (data not shown).

In contrast to NADH, NADPH up to about 50 μM did not quench the fluorescence from Trp72 of Ths and addition of NADPH up to 200 μM had little effect on the NMR spectrum (data not shown). High concentrations of NADPH (around 1 mM) led to slight broadening. Again in contrast to NADH, the nucleotide resonances became evident as sharp bands, even at low concentrations of NADPH (50 μM), during the titration with Ths (208 μM). Together, these data indicate that NADPH can bind into the NADH site, at best with only very low affinity, and the possibility is not ruled out that other contaminating nucleotide(s) in the NADPH solution cause the protein resonance broadening.

2'-AMP is an inhibitor of transhydrogenase (8, 5, 6, 19), competitive with respect to NADPH and mixed with respect to AcPdADH+. 2'-AMP had no effect on the NMR spectrum of Ths, and nucleotide resonances were detectable at very low nucleotide concentrations (data not shown).

Effect of pH on NADH Binding to Ths—It has been speculated that coupling of transhydrogenation to the protonmotive force might be mediated through changes in the affinity of the enzyme for NAD(H) (20, 21). If NAD(H) binding were coupled to proton translocation, then the binding affinity would have a strong pH dependence. However, Fig. 5 shows that the Kd for NADH as determined from the quenching of fluorescence of Trp72, was independent of pH between 5.5 and 9.0. Because of inner filtering by NADH, reliable data can be collected only for the early part of the binding curve. This has the effect that the larger the value of Kd, the more it is subject to error, and the result shown in Fig. 5 therefore reflects more reliably the lack of an increase in binding affinity with changing pH than the lack of a decrease in affinity.

The one-dimensional proton NMR spectrum of Ths at pH 6.3 in cacodylate buffer (data not shown) was similar to that recorded in Tris-Cl buffer at pH 7.6. The unassigned resonances between 7.6 and 7.9 ppm were slightly broader at the lower pH, which might indicate that they arise from His residues, but
Fig. 5. The pH dependence of NADH binding to Ths. Experiments were performed as in Fig. 2, except that instead of Tris-HCl, the medium contained 20 mM Ches, 20 mM Tricine, 20 mM Mops with the pH adjusted to that shown in the figure with NaOH. The concentration of Ths was 1.0 μM. Kd values were calculated from double-reciprocal plots of the corrected data.

Fig. 6. Two-dimensional ¹H NMR of Ths. Experiments were performed as described in Fig. 1 and under “Materials and Methods.” A, 200 μM Ths alone; B, plus 600 μM 5'-AMP. Controls with 600 μM 5'-AMP alone are not shown.

other resonances displayed a similar chemical shift and line-width. Consistent with the lack of effect of pH on Kd, NMR spectra recorded during an NADH titration of Ths at pH 6.3 (data not shown) were similar to those at pH 7.6 (15).

Two-dimensional Proton NMR of the Ths-AMP Complex—The two-dimensional NOESY experiment detects interactions between protons close to one another in space (<0.5 nm), and can be used to build up a picture of the molecular structure in solution. The diagonal of the two-dimensional spectrum represents a contour plot of the one-dimensional spectrum, and NOE interactions appear as cross peaks located away from the diagonal. The interactions in our system may be either intramolecular (Ths-Ths or nucleotide-nucleotide) or intermolecular (Ths-nucleotide) “transferred” NOEs.

Ths bound to NADH was not suitable for NOESY experiments because of the extreme broadening of the resonances from both molecules at the high concentrations of NADH required (15). The analogue 5'-AMP, with a lower binding affinity, was preferred. Experiments were performed on a mixture of Ths and 5'-AMP, control experiments on Ths and 5'-AMP alone. Very few NOE interactions were detected in Ths alone; interesting exceptions were from Tyr235 ring protons (see Ref. 22 for confirmation of this assignment) to a resonance at 4.52 ppm, likely to be the Tyr235 CαH (Fig. 6A). Additional cross-peaks were detected in the Ths/5'-AMP mixture, with the H8, H2, H1', H4', H5', and H5' protons of 5'-AMP all showing interactions with protons of Ths (Fig. 6). Although absolute assignments are not possible, the H8 proton in particular appeared to interact with CαH of Tyr235, a further CαH (possibly Lys or Ala), and side chain methylene and methyl groups. The Tyr239 ring proton-CαH interaction at 4.52 ppm in the absence of nucleotide (see above) was shifted to 4.58 ppm, and decreased in intensity, in the presence of 5'-AMP.

DISCUSSION

The reduced nucleotides, NADH and AcPdADH, bind more tightly to Ths than do the oxidized nucleotides, NAD+ and AcPdAD+. (a) Low concentrations of NADH and AcPdADH quenched the fluorescence of Trp72 of the protein, whereas NAD+ and AcPdAD+ up to 50 μM did not; (b) equilibrium dialysis measurements give Kd values for NADH consistent with those measured from Trp72 fluorescence quenching, and indicate higher values for NAD+; (c) in NMR spectra recorded during titrations, the resonances of NADH and AcPdADH were broad until quite high concentrations were added to Ths, but those of NAD+ and AcPdAD+ appeared as sharp bands even at quite low concentrations; and (d) resonances attributable to the mobile loop of Ths were broadened at relatively low concentrations of NADH and AcPdADH, but only at high concentrations of NAD+ and AcPdAD+. In principle, NMR spectroscopy can be used to measure binding constants, but the mobile loop resonances of Ths are insufficiently resolved to allow the detailed analysis of linewidth and lineshape as a function of nucleotide concentration required for determination of the type of exchange and therefore accurate values of Kd. Nevertheless, the dependence of the amplitude of the reasonably resolved resonances (e.g., Thr, Ala, and Tyr) on nucleotide concentration should give a comparative indication of binding affinities: there could be a 5-10-fold difference in the Kd values (Kd of NADH and Kd of AcPdADH are higher than Kd of deamino-NADH). Supporting the conclusions from kinetic analyses (4–8), the experiments with analogues indicate: (a) the adenosine moiety is crucial in the binding reaction and (b) the reduced nicotinamide moiety increases the binding affinity when it is part of the NADH molecule, but that, as a free entity, NMMNH cannot occupy the binding site. The oxidized nicotinamide moiety seems barely to contribute to the affinity since, on the basis of the NMR spectra, NAD+ appeared to bind only a little more tightly than 5'-AMP and adenosine. The importance of the adenosine part is also highlighted by the observation that deamino-NADH bound more weakly than NADH. Different patterns of inhibition of the transhydrogenation reaction by 5'-AMP (an NAD(H) analogue) and 2'-AMP (an NADP(H) analogue) were instrumental in the development of the now established concept that the complete enzyme has separate sites for NAD(H) and NADP(H) (4–8, 19). The observation from NMR spectra that, in contrast to 5'-AMP, 2'-AMP fails to bind to Ths is a complementary view of those inhibition patterns. That adenosine binds to Ths with the same order of affinity as 5'-AMP indicates that lack of the 5'-phosphate is not critical for binding, but comparison with the 2'-AMP results shows that presence of the 2'-phosphate blocks nucleotide binding at this site.

The finding that the binding affinity of Ths for NADH is

2 T. Bizouarn, unpublished results.
that primary energy coupling in transhydrogenase is centered under physiological conditions is positive. There is evidence that binding of NADH is not coupled to proton binding and release components of the proton translocation reaction are coupled directly to NADP⁺ binding and NADPH release, respectively (23–25). Thus energy required to drive the unfavorable hydride transfer reaction (E-NADH-NADP⁺ → E-NAD⁺-NADPH) must be derived from those primary events. It is proposed that conformational changes driven by the protolytic reactions that accompany NADP(H) binding and release promote the hydride transfer from NADH to NAD⁺, either by making the NADH more reducing or by making the NADP⁺ more oxidizing (compare (26)). In structural terms, interactions between protein side chains and nucleotide in the binding site in domain I must be relatively favorable for NADH and unfavorable for NAD⁺. Δp must drive hydride transfer against these unfavorable structural changes before the protein can release NAD⁺. The ΔG required to maintain the NAD⁺/NADH ratio on the enzyme against a 10-fold increase in Kᵦ (KᵦNADH = 20 µM; KᵦNAD⁺ ~ 200 µM; see above) is approximately 5.7 kJ⋅mol⁻¹. The energy available from a Δp of 200 mV is 19.3 kJ⋅mol⁻¹ (assuming one proton translocated per H⁻ transferred between nucleotides; Ref. 27). The balance of 13.6 kJ⋅mol⁻¹ available at the primary energy-coupling reaction to generate the change in binding constants for NADP(H) that are associated with protonation/deprotonation; this ΔG is sufficient to increase KᵦNADP⁺/KᵦNADH by 230-fold.

Kᵦ values of transhydrogenase for NADH and NAD⁺ are affected by membrane energization, and this was interpreted as evidence for a different model to the one above; it was thought that changes in Kᵦ might reflect changes in affinity for NADH and NAD⁺ during energization (20, 21). However, caution is necessary when interpreting changes in Kᵦ for complex reactions whose mechanism is not understood. Thus, even within the framework of our model, in which there are no Δp-dependent changes in the affinity for NAD⁺ or NADH, changes in the Kᵦ for these nucleotides are still expected (24). Evidence that binding of NADH is not coupled to proton binding or release in the domain I protein of transhydrogenase is given under “Results”; the Kᵦ of Thᵳ for NADH (measured by quenching of Trp72 fluorescence) was independent of pH from 6.0 to 9.0 (Fig. 5), and there were no differences in NMR spectra recorded during NADH titrations at pH 6.3 and 7.6. Energy-linked changes in the binding affinity of either NAD⁺ or NADH during turnover cannot be ruled out, but (a) it is unnecessary to invoke them to describe known properties of the enzyme, and (b) Thᵳ is a very stable, water-soluble protein, which readily restores transhydrogenation activity to depleted membranes, and thus it is reasonable to assume that its properties reliably reflect those of domain I in the complete enzyme.

The question arises as to the role of the mobile loop during catalytic turnover. An important observation (Fig. 6) of NOE interactions between a Tyr residue in Thᵳ and 5'-AMP bound within the NAD(H) site indicates that the nucleotide and the amino acid residue are in close proximity. It is shown by mutagenesis that this is Tyr²₃⁵ in Thᵳ (22). Thus, the earlier conclusion, that the loop loses segmental mobility when Thᵳ binds NAD(H) (15), can now be refined: upon binding nucleotide, the loop closes down on the surface of the protein such that some loop residues are very close (≤0.5 nm) to the nucleotide.

The one-dimensional NMR data show a similar pattern of behavior for different nucleotides that bind to the NAD(H) site of Thᵳ. Evidently, this behavior is not triggered by the reduced nicotinamide moiety, which in this context only increases the binding affinity of the nucleotide, but by the adenosine group. In all cases the furthest upfield of the proton resonances associated with Met residues (Metₐ) is especially sensitive to nucleotide binding; the resonance is broadened at lower concentrations of nucleotide than are required to broaden other loop resonances. The identity of this residue is not known, but Met²₃⁹ is a good candidate (15). It is proposed that, following binding of nucleotide, the protein adopts a conformation in which the chemical environment of protons in Metₐ has changed; for example the residue might lose mobility, resulting in a broader NMR signal. It is clear (e.g., Fig. 4), particularly for NADH and AcPdADH, at 37 °C, that broadening of the Metₐ resonance occurs at nucleotide concentrations considerably lower than the protein concentration. Therefore, the exchange rate between the two conformational states is probably intermediate/fast for NADH on the NMR timescale and fast for NAD⁺. In titrations with NADPH and 2'-AMP, the NMR spectrum of this conformation is evidently more related to its solution concentration, and hence exchange with the intermediate state is relatively slow. The binding process can be summarized (15) by the following reaction.

\[ \text{Thᵳ} + \text{NAD(H)} \leftrightarrow \text{Thᵳ-NAD(H)} \leftrightarrow \text{Thᵳ*-NAD(H)} \]

Reaction 2

This is consistent with the inverse relationship between the appearance of nucleotide resonances during titration of Thᵳ with different nucleotides, and the broadening of loop resonances. For those that bind tightly (NADH and AcPdADH), nucleotide resonances are not resolved from baseline noise (i.e., they remain extensively broadened) until they are present at concentrations somewhat in excess of the protein, whereas loop resonances are broadened at very low concentrations of the nucleotide. For those that bind weakly (NAD⁺ and 5'-AMP) nucleotide resonances emerge from baseline noise as sharp bands even at quite low nucleotide concentrations; higher concentrations are needed to cause broadening of loop resonances. In titrations with NADPH and 2'-AMP (which do not bind significantly), their resonances were evident as narrow bands even at very low nucleotide concentrations. The broadening of nucleotide resonances probably results from decreased mobility upon binding to the protein and, in principle, may occur in both Thᵳ-NAD(H) and Thᵳ*-NAD(H). The less extensive broadening of the more weakly-bound NAD⁺ and AcPdAD⁺ arises as a result of faster exchange, and/or because the solution concentration of the unbound nucleotide, for a given total concentration, is higher.

Fjellström et al. (28) developed a model of part of domain I of

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transhydrogenase, based on sequence similarities with known three-dimensional structures of NAD(H)-binding domains of dehydrogenases. They describe their model with a bound nucleotide, and thus it should be compared with the situation (see above) in which the NMR-visible mobile loop has closed down on the NAD(H) binding site. Unfortunately, no such feature is evident in the predicted structure of Fjellstrom et al. The segment of polypeptide chain that we define as the “mobile loop” is shown as a short loop at the C terminus of a predicted β-strand (βC) together with the N terminus of a predicted α-helix (αD) (28). This feature is envisaged as pointing away from the bound nucleotide. In contradiction with NMR data, the E. coli equivalent of Tyr 235 in the model is situated 0.5 nm from the bound nucleotide. The model of Fjellstrom et al. retains attractive features. Perhaps the choice of dihydropteridine reductase as template for αD, based only on its length and some homologies in the subsequent β-strand (28) was inappropriate. It is conceivable that this segment is unique to transhydrogenase, which unlike the templates used by Fjellstrom et al. is a transmembrane ion pump.

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