Role of Methionine-239, an Amino Acid Residue in the Mobile-Loop Region of the NADH-Binding Domain (Domain I) of Proton-Translocating Transhydrogenase

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ABSTRACT: Transhydrogenase couples the transfer of hydride equivalents between NAD(H) and NADP(H) to proton translocation across a membrane. The one-dimensional proton NMR spectrum of the recombinant NAD(H)-binding domain (domain I) of transhydrogenase from *Rhodospirillum rubrum* reveals well-defined resonances, several of which arise from a mobile loop at the protein surface. Four have been assigned to Met residues (MetA−MetD). Substitution of Met239 with either Ile (dI.M239I) or Phe (dI.M239F) resulted in loss of MetA from the NMR spectrum. Broadening and shifting of the mobile loop resonances consequent on NAD(H) binding indicate that the loop closes down on the protein surface. More NAD(H) had to be added to mutant domain I than to wild type to give comparable resonance broadening. The K_d of domain I for NADH, measured by equilibrium dialysis, was increased about threefold by the Met239 mutations. Mutant and wild-type domain I were reconstituted with domain I-depleted membranes from *R. rubrum*, and with recombinant domain III of transhydrogenase. With membranes, the K_m for acetylpyridine adenine dinucleotide during reverse transhydrogenation was 5× and >6× greater in dI.M239I and dI.M239F, respectively, than in wild-type. Cyclic transhydrogenation (in membranes and the recombinant system) was substantially more inhibited (70% in dI.M239I, and 84% in dI.M239F) than either forward or reverse transhydrogenation. The docking affinities of dI.M239I and dI.M239F to the depleted membranes were similar to those of wild-type. It is concluded that Met239 is MetA in the mobile loop of domain I, and that in proteins with amino acid substitutions at this position, the binding affinity of NAD(H) is decreased, and the hydride transfer step is inhibited.

Transhydrogenase is a membrane protein. It couples the transfer of hydride equivalents between NAD(H) and NADP(H) to proton translocation. For reviews, see (*1*−*3*).

$$\mathrm{NADH} + \mathrm{NADP^+} + \mathrm{H^+_{out}} \Leftrightarrow \mathrm{NAD^+} + \mathrm{NADPH} + \mathrm{H^+_{in}}$$

In animal mitochondria the enzyme might function in the regulation of flux through the tricarboxylic acid cycle (*4*), and in bacteria in the provision of NADPH for biosynthesis (*5*).

Transhydrogenase comprises three large domains. The NAD^+/NADH binding site is located in domain I, and the $NADP^+$/NADPH binding site in domain III. These two domains protrude from the membrane (on the cytoplasmic side in bacteria, on the matrix side in mitochondria), and domain II spans the membrane.

Recombinant domains I and III of *Rhodospirillum rubrum* transhydrogenase have been separately prepared and purified (*6*−*8*); they bind NAD^+/NADH and $NADP^+$/NADPH, respectively. Transhydrogenation activity can be reconstituted in different ways from the component domains, thus providing a useful approach for biochemical analysis of the enzyme. (a) Recombinant domain I can restore full transhydrogenation activity to everted *R. rubrum* membranes depleted of their native domain I. (b) A mixture of recombinant domains I and III forms a complex, which catalyses transhydrogenation, even in the absence of domain II. Hydride transfer and associated conformational rearrangements proceed at about the same rate in the I:III complex as in the complete enzyme, but the net rate of transhydrogenation is limited by the extremely slow rate of release of $NADP^+$ or NADPH from domain III (*7*).

Observations on the pattern of cleavage of the protein with proteases, and experiments in NMR spectroscopy, indicated that domain I of transhydrogenase has a mobile loop of perhaps 20 amino acid residues, which emanates from the surface of the protein (*7*, *9*, *10*). The loop is absent from the water-soluble alanine dehydrogenase, which is otherwise similar in sequence to domain I (*11*). The amino acid sequence in this region of *R. rubrum* domain I protein is

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211        221        231        241        251
VESLGGKFIT VDDEAMKTAE TAGGYAKEMG EEFRKKQAEA VLKELVKTDI
          ↑----------approximate----------↑
          position of the mobile loop
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Because of the high segmental mobility of the loop, its residues are detectable by ^{1}H-NMR spectroscopy of isolated recombinant domain I protein. The loop resonances broaden considerably when domain I binds NADH, indicating that the structure loses mobility, perhaps closing down on the surface of the protein. Mutations in the domain I protein at Tyr235 (dI.Y235F and dI.Y235N) led to a decrease in the NAD^+/NADH binding affinities (*9*), and to pronounced inhibition of the rate of hydride transfer by the reconstituted complete enzyme and by the I:III complex [up to 98% of k_{cat}, see (*12*)]. This suggests that Tyr235 has a role in

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catalysis by transhydrogenase. The well-defined aromatic ring resonances at 6.82 and 7.12 ppm in the spectrum of wild-type domain I protein were lost in dI.Y235F and dI.Y235N. This shows that of the four Tyr residues of domain I, only the resonances from Tyr235 contribute significantly to the ^{1}H-NMR spectrum. It was then established from two-dimensional NOESY experiments that Tyr235 is positioned within 0.5 nm of bound 5′-AMP (*10*). This is strong evidence that the mobile loop closes down on nucleotide bound in the catalytic site. It was proposed that the loop has a role in providing the appropriate environment for the nicotinamide ring of domain I-bound NADH during transfer of the hydride equivalent to domain III-bound $NADP^+$.

The objective of the experiments described in this communication was to examine the effects of mutations at Met239 on nucleotide binding, on catalysis, and on the dynamics of loop closure. Although there are fifteen Met residues in domain I, there are only four clearly defined sets of resonances that are attributable to methyl groups of methionine. They were designated MetA−MetD (*10*). MetA at 1.97 ppm is particularly interesting because it is the most sensitive to broadening, and shows a pronounced upfield change in its chemical shift, when the protein binds NAD^+ or NADH or their analogues. Its chemical shift was also affected by mutation of Tyr 235. It was tentatively suggested that the MetA resonance might derive from Met239 in the *R. rubrum* domain I sequence, rather than another candidate in the loop, Met226 (*13*). This was because there seems to be an equivalent resonance to MetA in the NMR spectrum of *Escherichia coli* domain I, and, whereas Met239 is conserved in the *E. coli* protein (as Met230), Met226 is not conserved. The question has been resolved by the mutagenesis experiments described below.

MATERIALS AND METHODS

Domain I mutants were isolated, either using the gapped duplex procedure (*9*, *14*), or using the Quickchange "kit" supplied by Stratagene (dI.M239F) , following the manufacturer's instructions. In the former instance mutagenesis was performed on a 700-base pair *Eco*RI-*Hin*dIII fragment of the domain I gene cloned into the pMa vector, as described (*9*). Following mutagenesis the 700-base pair fragment was sequenced to check for polymerase errors, using an Applied Biosystems model 373A employing *Taq* polymerase and dye terminators, according to the manufacturer's instructions. Mutagenesis with the Stratagene kit was carried out on complete preparations of pCD1 (*6*), and the whole of the domain I DNA was subsequently sequenced. Routine operations, including agarose-gel electrophoresis, preparation of plasmid DNA, growth and handling of *E. coli* strains and preparation of competent cells and transformation, were carried out as described (*15*).

Recombinant wild-type domain I and domain III proteins from *R. rubrum* transhydrogenase were expressed in *E. coli* and purified, as described (*6*, *7*). Mutant proteins were purified by the same procedures; their behavior on column chromatography was indistinguishable from that of wild-type.

[1] Nomenclature: dI.M239F and dI.M239I, recombinant forms of transhydrogenase domain I, in which Met239 has been susbstituted with Phe and with Ile, respectively.

Everted membrane vesicles (chromatophores) from phototrophically grown cells of *R. rubrum* were prepared and depleted of their native domain I protein, as described (*11*). Chromatophores of strains that over-express transhydrogenase (*16*) were depleted of domain I by washing 3 or 4 times in 2 M NaCl, 10 mM Tris-HCl, pH 8.0.

Protein was estimated by the microtannin assay (*17*). Bacteriochlorophyll was measured using the *in vivo* absorbance coefficient (*18*). SDS/PAGE was carried out as described (*19*).

Biophysical Procedures

Reverse transhydrogenation activity of reconstituted membranes was measured at 375−450 nm as reduction of $AcPdAD^+$ by NADPH. Forward transhydrogenation activity was measured at 395−450 nm as reduction of thio-$NADP^+$ by NADH by membranes irradiated with photosynthetically-active light (*20*). Absorbance coefficients are given (*21*). The reconstitution of depleted membranes and domain I protein, and the production of the domain I:III transhydrogenase complex, were carried out by simple mixing followed by a few seconds incubation at 30 °C (*7*, *11*).

Fluorescence (*6*) and NMR (*13*) spectroscopy of purified domain I protein and equilibrium dialysis (*22*) were carried out as described. Binding constants were calculated from equilibrium dialysis data using the Scatchard plot, and from NMR data by nonlinear regression analysis of the binding curve using the computer program Enzfitter (Elsevier, Cambridge, U.K.).

RESULTS

The MetA Resonance of Domain I Arises from Met239

One-dimensional ^{1}H-NMR spectra of wild-type domain I protein and the dI.M239I and dI.M239F mutants, in the absence of nucleotides, are shown in Figure 1. The experiments were performed at 37 °C, where the resonances of amino acids in the mobile loop were better defined than in earlier work at 20 °C (*9*, *10*, *13*). Chemical shifts and provisional assignments of the well-defined resonances are listed in Table 1. Note that resonances arising from the vast majority of the amino acid residues in domain I were broad and unresolved due to the long correlation time of the protein (M_r 80 000 if, as thought, it is dimeric; see ref 6).

In both mutants the MetA resonance was clearly missing, and this strongly supports the suggestion that in the wild-type it derives from Met239. In dI.M239F new resonances appeared at 7.23 and 7.09 ppm, characteristic of the ring protons of Phe. The fact that the resonances of the new Phe239 in the mutant protein were quite well defined indicates that the residue adopts the substantial segmental mobility of the loop. The 7.09 ppm component partly overlapped the resonances due to the ring 2,6 protons of Tyr235, but neither component overlapped the original Phe resonances of the wild-type protein. Interestingly, the original Phe resonances were shifted significantly upfield in dI.M239F (Table 1). Their response to the substitutions at residue 239 suggests that they may arise from Phe243 rather than, for example, Phe218, which is some distance away in the amino acid sequence. In dI.M239I, a new resonance appeared in the region of 0.8 ppm, and this might arise from the CH_3 groups of the new Ile.

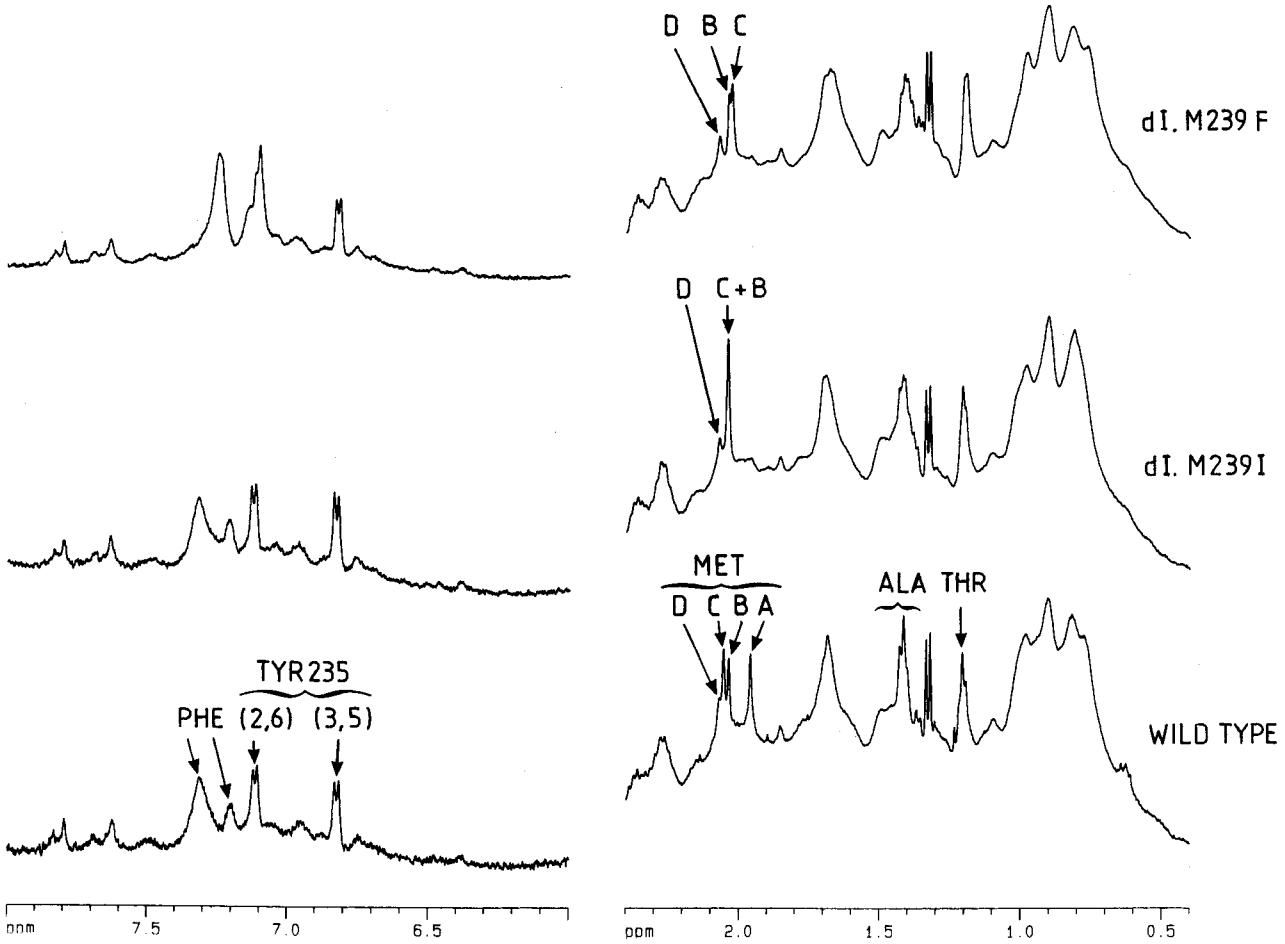

FIGURE 1: One-dimensional ^{1}H-NMR spectra of wild-type domain I of transhydrogenase from *R. rubrum* and of the mutant proteins dI.M239I and dI.M239F. Spectra (256 transients) were recorded at 37 °C in 10 mM [^{2}H]Tris-HCl, p^{2}H 7.6, 10 mM$(NH_4)_2SO_4$, 1.0 mM dithiothreitol in ^2H_2O. Protein concentrations were wild-type, 280 μM; dI.M239I, 180 μM; dI.M239F, 300 μM. The sharp doublet at 1.33 ppm is from the C-terminal Ala384 of the protein (unpublished results). The sharp signal at 0.62 ppm in the wild-type spectrum is due to added 5,5-dimethylsilapentanesulfonate; all chemical shifts are referenced to the 0.00 ppm singlet resonance of this standard.

Table 1: Chemical Shifts (ppm) of Well-Defined Resonances of Amino Acid Residues in Wild-Type Domain I, dI.M239I, and dI.M239F[a]

assignment	wild-type domain I	dI.M239I	dI.M239F
Phe243[b] ring	7.32, 7.20	7.31, 7.20	7.28, 7.14
Tyr235	7.12, 6.83	7.12, 6.83	7.11, 6.82
MetA (Met239)	1.96	nd[c]	nd
MetB	2.04	2.04	2.04
MetC	2.06	2.04	2.03
MetD	2.07	2.07	2.07

[a] Data taken from ^{1}H-NMR spectra similar to those shown in Figure 1. The amino acid residues listed in the first column refer to those identified in the wild-type domain I protein (*13*), *not* the residues introduced by the mutation (see Figure 1). [b] This tentative assignment is discussed in the text. [c] ND, not detectable.

Another feature of the spectra shown in Figure 1 is that replacement of Met239, with Ile or with Phe, resulted in an upfield shift of the MetC resonance (Table 1), but the MetB and MetD resonances were unaffected by either substitution. The outcome was that in the mutant spectra MetC moved toward MetB (the two superimpose in dI.M239I), and, whereas in the wild-type spectrum it was a shoulder, MetD was left in both mutants as a distinct peak. This interpretation of the spectra is supported by the effects of nucleotide binding on the chemical shifts of the Met resonances (see below).

Other changes in the NMR spectra of the mutants were small (Figure 1 and Table 1). These observations indicate that the overall fold of the protein is unaffected by the mutations, and that changes are localized within the loop region of domain I.

Nucleotide Binding to Domain I Proteins with Substitutions at Met239

Figure 2 shows that the addition of NADH to dI.M239I and to dI.M239F, though having little effect on resonances corresponding to the molecular core of the proteins (e.g., the upfield CH_3 protons), led to varying degrees of broadening and shifting of the resonances assigned to Thr, Ala, MetC, Glu, Tyr, and Phe (including, in dI.M239F, the new Phe), see above. Factors contributing to the resonance broadening upon NADH binding probably include (a) loss of segmental mobility of the loop and (b) alterations in chemical exchange processes as the protons sample a range of environments on the NMR time scale (*13*). Substantially higher concentrations of NADH (about 10-fold for equivalent protein concentrations) were required to produce comparable resonance broadening in the two mutants to that in wild-type domain I, consistent with an increase in the K_d values of the proteins for the nucleotide (see below). Otherwise, the effects of NADH addition on the NMR spectra were rather similar in the wild-type and mutant proteins. The MetC

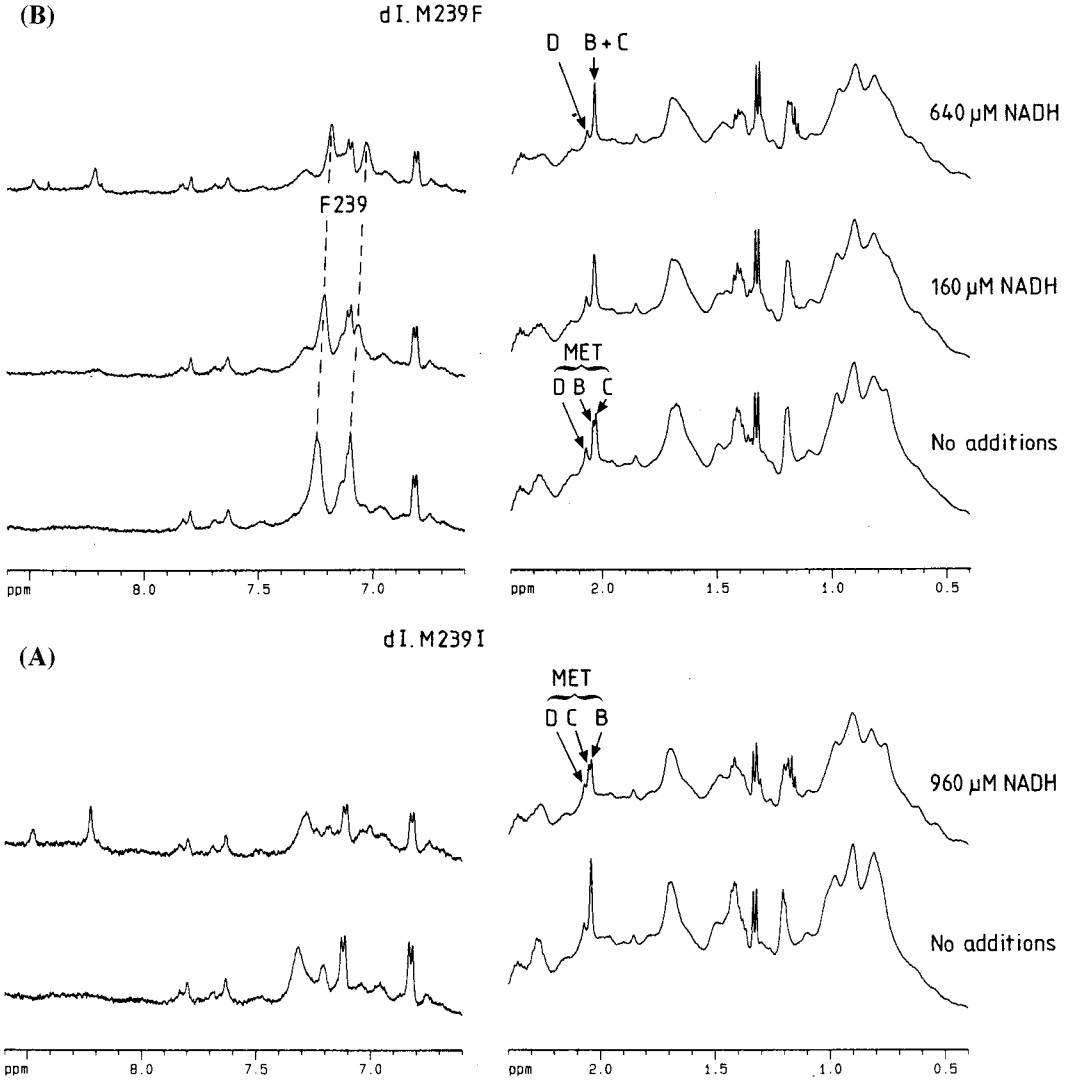

FIGURE 2: Illustrative one-dimensional ^{1}H-NMR spectra of dI.M239I and dI.M239F recorded during titrations with NADH. Conditions as in Figure 1, but with the NADH concentration shown on the right. (A) dI.M239I. (B) dI.M239F. The resonances appearing at 8.46 and 8.21 ppm are due to the A8 and A2 protons of the NADH (the N2 resonance at 6.96 ppm is barely evident at these protein and nucleotide concentrations).

resonance of both mutants was shifted downfield by NADH, but less than in the wild-type domain I.

In titrations of wild-type recombinant domain I with NADH (*10*, *13*) the nucleotide resonances, as they emerged from the base-line noise, were considerably broadened, and in some cases slightly shifted, relative to those in the absence of protein. These observations, and the behavior of the MetA resonance during the titrations, suggested that the NADH and domain I protein were in intermediate exchange. In similar titrations with dI.M239I and dI.M239F the NADH resonances began to emerge from the base-line noise at 2−3-fold lower nucleotide concentrations than with wild-type protein (for equivalent protein concentrations). This indicates that the exchange rate had increased in both mutants, probably as a result of an increase in the off rate constant for the nucleotide.

In contrast to the intermediate exchange observed for MetA in wild-type domain I, the signals attributed to Phe239 in dI.M239F did not broaden significantly during titration with NADH, suggesting the occurrence of a faster exchange process. This made it possible to plot the dependence of chemical shift upon NADH concentration for the 7.23 ppm signal (Figure 3) and to estimate K_d. A value of 70 μM was obtained, in good agreement with the results from equilibrium dialysis measurements (see below).

Addition of NAD^+ also led to broadening of the mobile-loop resonances of the wild-type protein, although higher concentrations of this nucleotide than of NADH were needed (*10*, *13*). In both the dI.M239I and dI.M239F mutants NAD^+, at concentrations up to 2 mM, caused very little resonance broadening (Figure 4 shows representative data for dI.M239F). Thus, in the mutant proteins as well as in the wild-type, the affinity for NAD^+ appears to be substantially lower than that for NADH, and the mutants might have lower affinities for NAD^+ than the wild-type.

The implication of lower nucleotide binding affinities of dI.M239I and of dI.M239F for NADH was confirmed by measurements using equilibrium dialysis. Thus, the K_d values were 85 ± 15 and 120 ± 30 μM for dI.M239I and dI.M239F, respectively (Figure 5) compared with 30 μM in wild-type (*22*). The binding capacity, approximately one NADH per monomer of the domain I protein, was unchanged by the mutations. The addition of NADH (but not NAD^+) up to 50 μM to either dI.M239I or dI.M239F resulted in a decrease in the fluorescence of Trp72 (data not shown, see

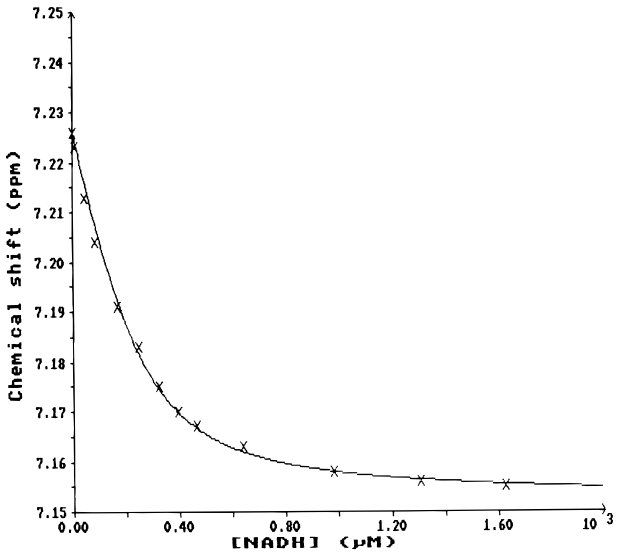

FIGURE 3: Dependence of the chemical shift of Phe 239 in dI.M239F on NADH concentration. Data were taken from the titration used for the examples of spectra shown in Figure 2. The chemical shift (δ_{obs}) was plotted against NADH concentration for the signal at 7.23 ppm, and K_d was estimated according to the equation,

$$\delta_{obs} = \delta_{free} + (\delta_{bound} - \delta_{free}) \left\{ \frac{(E_T + L_T + K_d) \pm \sqrt{(E_T + L_T + K_d)^2 - 4E_T L_T}}{2E_T} \right\}$$

where E_T and L_T are the concentrations of total enzyme and total ligand, respectively (modified from (*24*)). The Phe resonance remained resolvable from the Tyr Hδ resonance throughout the titration.

(*6*)), but the parameter is not sensitive enough to determine K_d values in the required range (*9*).

Behavior of the MetA (Met239) Resonance of Wild-Type Domain I During Nucleotide Titrations

The MetA resonance is particularly sensitive to broadening, and to changes in its chemical shift, during titrations of wild-type domain I with nucleotides (*9*, *10*, *13*). We have now carried out experiments with NAD^+ at the higher temperature of 37 °C. At this temperature the loop resonances are narrower than at 20 °C, probably because of their greater mobility, and because NAD^+ is in faster exchange than NADH with the protein, the loop resonances are also better-defined at higher concentrations of nucleotide. These two factors enabled us to measure the dependence of the chemical shift of the MetA resonance on the NAD^+ concentration with greater accuracy than before (Figure 6). Note (panel A), that the upfield shift is very extensive during the nucleotide titration; the resonance is located at 1.97 ppm in the absence of nucleotide and reaches 1.83 ppm before becoming hidden among other resonances at above 720 μM NAD^+. Unfortunately, it was still not possible to resolve the final position of the resonance at saturating NAD^+, making it difficult to obtain an accurate estimate of K_d. However, the data could be fitted quite well to a simple binding curve with a single K_d component of approximately 500 μM (panel B), somewhat higher than the value determined by equilibrium dialysis ($K_d \approx 300$ μM (*22*)).

Catalytic Properties of dI.M239I and dI.M239F

dI.M239I, dI.M239F, and wild-type protein were reconstituted with domain I-depleted chromatophore membranes to give the complete transhydrogenase, and Michaelis constants were then determined from the dependence of steady-state rates of reaction on nucleotide concentrations (Table 2). For "forward" transhydrogenation, measured as the reduction of thio-$NADP^+$ by NADH in illuminated membranes, the V_{max} values were decreased by 34% and 36%, respectively, for dI.M239I and dI.M239F relative to wild-type. The K_m values for thio-$NADP^+$ were not significantly altered, but those for NADH were slightly increased by the mutation. For "reverse" transhydrogenation (the reduction of $AcPdAD^+$ by NADPH), the V_{max} values were decreased by 20% and 42%, respectively, for dI.M239I and dI.M239F. The K_m values for NADPH were slightly

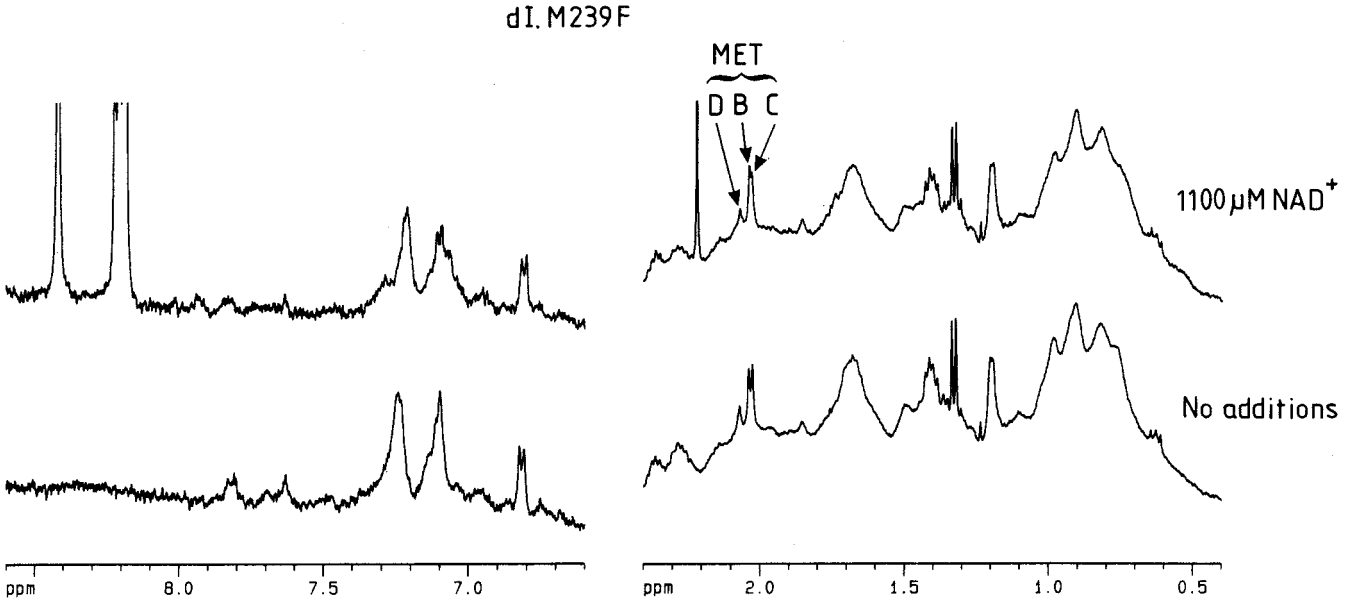

FIGURE 4: Illustrative one-dimensional ^{1}H-NMR spectra of dI.M239F recorded during titration with NAD^+. Conditions as Figure 1, except that the protein concentration was 128 μM, and the NAD^+ concentration is shown on the right. The resonances appearing at 8.42, 8.21, and 8.19 ppm are due to the A8, N5, and A2 protons, respectively, of the NAD^+. The sharp singlet at 2.2 ppm is from acetone present in the NAD^+.

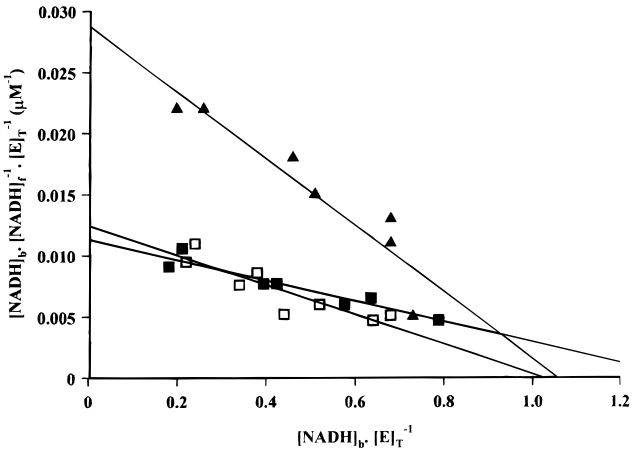

FIGURE 5: Binding of NADH to dI.M239I, dI.M239F, and wild-type domain I of transhydrogenase from *R. rubrum*. The data were obtained by equilibrium dialysis. Experiments were performed in 50 mM Tris-HCl, pH 8.0, 10 mM $(NH_4)_2SO_4$ at a protein concentration of 25 μM (wild-type, ▲), 33 μM (dI.M239I, ■), and 50 μM (dI.M239F, □). The lines were drawn by linear regression assuming equal weighting for all data points.

decreased, but there was a large increase in the K_m for AcPdAD$^+$ in both mutants.

Chromatophores catalyze a cyclic transhydrogenation reaction in which NADP$^+$, permanently bound to the protein, is alternately reduced by NADH and oxidized by AcPdAD$^+$. The reaction is inhibited by removal of the native domain I protein, and is fully recovered upon reconstitution with recombinant domain I (*23*). Table 2 shows that in the dI.M239I- and dI.M239F-reconstituted systems, the apparent V_{max} for cyclic transhydrogenation is significantly more inhibited relative to wild-type (70% and 84%, respectively), than are the forward and reverse reactions (see above). Note that, because in cyclic transhydrogenation, AcPdAD$^+$ and NADH compete for the same site on the enzyme, the cyclic reaction cannot be carried out at saturating concentrations of both nucleotide substrates (*12*, *23*). Thus, the experiments summarized in Table 2 were performed at nucleotide concentrations that were optimized to give the maximal rate of reaction; the optimal concentrations were similar for wild-type and for both mutant domain I proteins (not shown).

The above experiments on reconstituted membranes were carried out with saturating levels of the relevant domain I protein. In titrations it was established that the concentrations of the recombinant domain I proteins required to give half-maximal rates of reverse transhydrogenation with domain I-depleted membranes were similar for wild-type, dI.M239I, and dI.M239F (data not shown, but compare data with wild-type, dI.Y235N, and dI.Y235F (*9*)). This indicates that the docking affinity between domain I and domain I-depleted membranes was unaffected by the Met239 mutations.

Because the release of NADP$^+$ and of NADPH are extremely slow in the absence of domain II, a mixture of recombinant domain I and domain III proteins catalyzes only very low rates of forward and reverse transhydrogenation but high rates of the cyclic reaction (*7*). Figure 7 shows the dependence on NADH and AcPdAD$^+$ concentrations of rates of the cyclic reaction catalyzed by mixtures of dI.M239I, dI.M239F or wild-type domain I, plus wild-type domain III. As found in the reconstituted membrane system, the maximum rates of the cyclic reaction were strongly inhibited in those I:III mixtures containing the mutant proteins, about 85% relative to wild type for dI.M239I, and about 88% for dI.M239F. Substrate inhibition by high concentrations of NADH was evident in complexes with both wild-type (see (*12*)) and mutant domain I proteins, and thus the determination of K_m values for NADH was difficult. Substrate inhibition was not evident with AcPdAD$^+$ as substrate (presumably because of the weaker binding of this nucleotide (*12*)).

The experiments with mixtures of recombinant domains I and III (Figure 7 and Table 2) were carried out under conditions in which domain III was saturated with domain I. Titration profiles of the dependence of the rate of cyclic transhydrogenation on the concentration of wild-type domain I, dI.M239I, and dI.M239F at a fixed domain III concentration were all very similar (not shown); half-saturation in each case was achieved at a ratio of about 30 nM domain I to 40 nM domain III (see (*7*)). An equivalent result was reported for Tyr235 mutants (*12*).

DISCUSSION

Effect of Substitution of Met239 on the ^{1}H-NMR Spectrum of Domain I in the Absence of Added Nucleotides

The results show that the MetA resonance in the NMR spectrum of domain I of *R. rubrum* transhydrogenase arises from the methyl protons of Met239 (Figure 1). This observation supports our conclusion that this region of the protein is part of a polypeptide loop with a high segmental mobility (*9*, *10*, *13*).

Substitution of Met239 with Ile and with Phe also led to secondary changes in the chemical shift of MetC (Figure 1 and Table 1). Similarly, substitutions at Tyr235, as well as producing direct effects in the Tyr ring region of the spectrum, gave rise to shifts of the MetA and MetC resonances (*9*). These results suggest spatial proximity between amino acid residues at positions 235 and 239, and the residue giving rise to MetC. This might be Met226, the only other Met residue in the mobile-loop region. Note that the chemical shifts of MetB and MetD are unaffected by those amino acid substitutions in the loop which have been made to date, and, in contrast to MetA and MetC, are not broadened or shifted upon nucleotide binding (see Results and below). Thus, the MetB and MetD resonances probably do not arise from residues in the mobile loop, but might originate from some other mobile region, for example at the N-terminus or C-terminus of the protein.

Responsiveness of the Met239 Resonances to Nucleotide Binding

The chemical shift and linewidth of the MetA resonance are more responsive to NAD(H) binding than other resonances from the mobile loop. Evidently, the the CH_3 protons of Met239 are located in an environment which is particularly sensitive to conformational events accompanying the nucleotide binding reaction. The reason for this is not clear, but it is tempting to propose that that they are ring-shifted through proximity to the adenine.

The pronounced sensitivity of the MetA resonance during titrations of domain I with NAD(H) previously led us to suggest that the binding reaction occurred in two steps (*9*, *10*, *13*); the effects of NAD(H) on MetA appeared to be substantially complete well before those on other loop

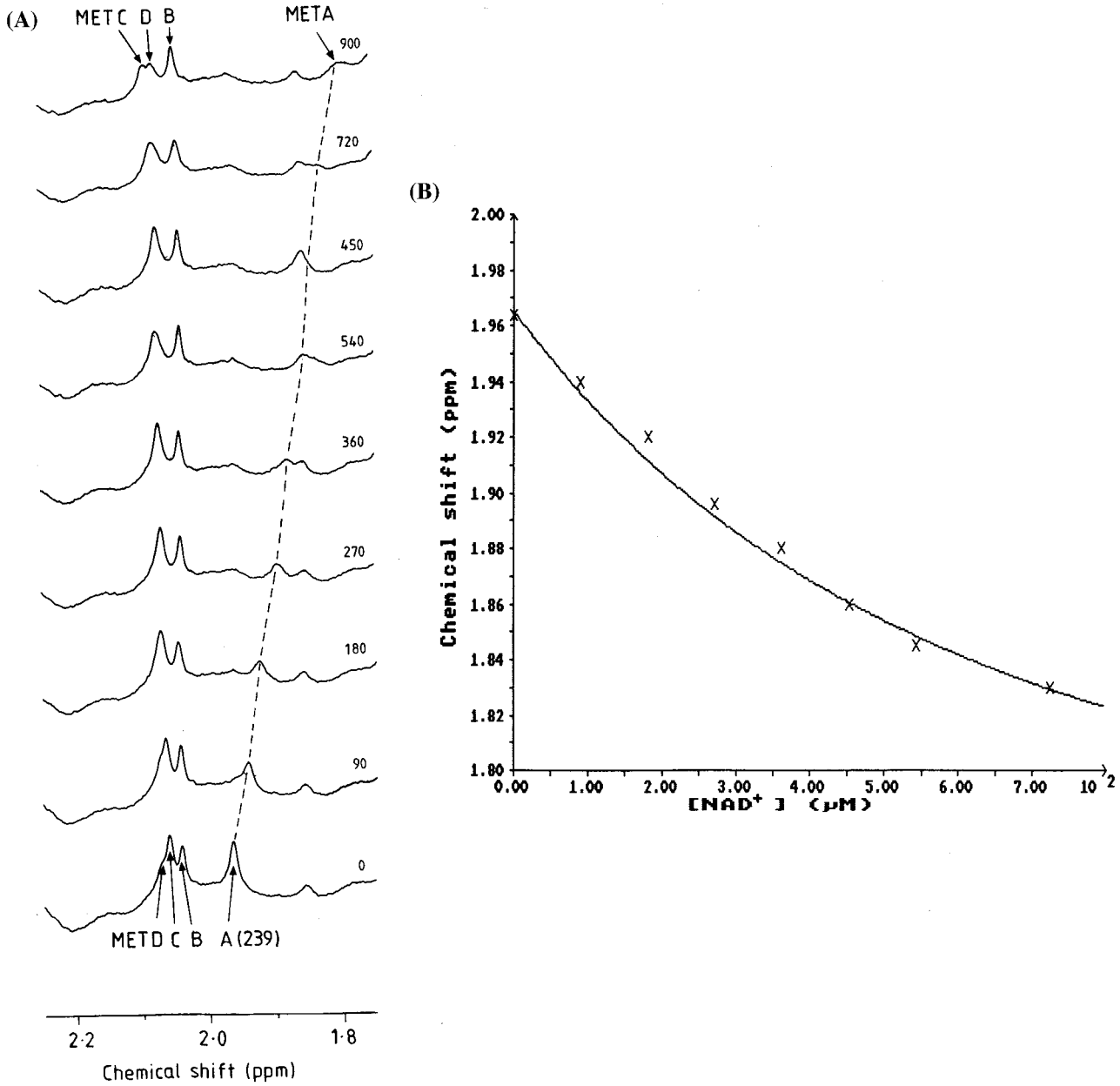

FIGURE 6: Dependence of the chemical shift of the MetA resonance of wild-type domain I on the concentration of NAD^+. (A) Experiments performed as in Figure 1 with wild-type protein at 190 μM. NAD^+ concentrations (μM) are shown on the left. (B) Replotted data from A. The curve was calculated for $\delta_{bound} = 1.72$ ppm and $K_d = 500$ μM (see text).

resonances (e.g., those due to Tyr235, Phe, MetC, Ala, and Thr). In the present work, by performing titrations of domain I protein with NAD^+ at 37 °C, we are able to clarify the description. The critical observation (Figure 6) was that the change in the chemical shift of MetA was very extensive, from 1.97 ppm in the absence of nucleotide to about 1.83 ppm in the presence of 720 μM NAD^+. This is a far greater change in position than that of any other observed resonance, and under conditions of intermediate or moderately fast exchange, would give rise to considerably more exchange broadening of this signal than of any other signal in the spectrum. When NAD^+ titrations were performed at the lower temperature of 20 °C, this extra broadening caused the MetA resonance to disappear rather early in the titration, before the other loop resonances were seen to be affected. During titrations with NADH the MetA resonances broadened considerably at both the low (*13*) and the high (*10*) temperatures, consistent with the view that the exchange rate is slower for the reduced nucleotide, and probably lies in the intermediate range on the NMR time scale. It is reasonable to conclude that the large upfield shifts of MetA that were observed in the high temperature titrations with NAD^+ (see above, Figure 6) also take place at lower temperatures with NAD^+, and in NADH titrations, but that they are obscured by the resonance broadening. The combination of extensive shifting *and* broadening of the MetA resonance in those titrations results in an identifiable response only at very low nucleotide concentrations. In contrast to the earlier description, we feel that the experimental evidence is adequately explained by a simple one-step binding reaction.

Effects of Substitutions of Met239 on Nucleotide Binding and on Catalysis

Mutation of Met239 of domain I of *R. rubrum* transhydrogenase to either Ile or Phe resulted in a lowered binding

Table 2: Catalytic Activities of Mutant Domain I Proteins after Reconstitution, either with Domain I-Depleted Membranes or with Purified Domain III[a]

transhydrogenation	wild-type	dI.M239I	dI.M239F
reverse (membranes)			
K_m^{app}(AcPdAD$^+$) (μM)	115	590	>700
K_m^{app}(NADPH) (μM)	26	20	11
V_{max}	7.6	6.1	4.4
forward (membranes)			
K_m^{app}(thio-NADP$^+$) (μM)	5	4	4
K_m^{app}(NADH) (μM)	5	11	15
V_{max}	0.30	0.20	0.19
cyclic (membranes)			
V_{max}^{app}	20.2	6.1	3.2
cyclic (I:III complex)			
V_{max}^{app}	18200	3400	2300

[a] The first three sets of experiments were performed with domain I-depleted chromatophore membranes. The reverse reaction was measured in a medium containing 50 mM KCl, 50 mM Mops, pH 7.2. For determination of the K_m^{app}(AcPdAD$^+$) the NADPH concentration was fixed at 200 μM, and for the K_m^{app}(NADPH), the AcPdAD$^+$ concentration was fixed at 960 μM. The domain I-depleted membranes (6.5 μM bacteriochlorophyll) were reconstituted with 0.15 μM domain I (wild-type or mutant). The forward reaction was measured in a medium containing 50 mM KCl, 2 mM $MgSO_4$, 1 mM Na^+-succinate, 50 mM Tris, pH 8.0. For determination of the K_m^{app}(thio-NADP$^+$) the NADH concentration was fixed at 120 μM, and for the K_m^{app}(NADH), the thio-NADP$^+$ concentration was fixed at 120 μM. The concentrations of domain I-depleted membranes and recombinant domain I (wild-type or mutant) were the same as for reverse transhydrogenation. The cyclic reaction was measured under similar conditions to those employed for the reverse reaction, except that the medium contained NADP$^+$ (20 μM). For determination of the K_m^{app}(AcPdAD$^+$) the NADH concentration was fixed at 20 μM, and for the K_m^{app}(NADH), the AcPdAD$^+$ concentration was fixed at 600 μM. For all experiments with membranes V_{max} is given in units of μmol of AcPdAD$^+$ reduced (μmol of bacteriochlorophyll)$^{-1}$ min^{-1}. The last set of data on the I:III complex is taken from Figure 7. The V_{max} is given in units of μmol of AcPdAD$^+$ reduced (nmol of domain III)$^{-1}$ min^{-1}. For forward and reverse transhydrogenation (membranes and I:III complex) K_m^{app} values were calculated from *s*/*v versus s* plots of the data. All experiments were performed in duplicate. Experiments on mutant proteins were always carried out in parallel with experiments on wild-type. The determinations on dI.M239F were performed on a single preparation of protein., those on dI.M239I on two preparations with agreement within 10%. Values of K_m^{app} and V_{max} were calculated with Enzfitter; standard deviations were always <10−15%.

affinity for both NADH and NAD$^+$. A decreased affinity of dI.M239I and dI.M239F for the reduced nucleotide was shown directly by equilibrium dialysis (Figure 5), and is consistent with the finding that much higher concentrations of NADH were required to produce comparable loop resonance broadening in the mutants than in the wild-type protein (Figure 2). Because the K_d of domain I for the oxidized nucleotide is much higher (*22*), equilibrium dialysis is not accurate in detecting changes in binding affinity. However, the finding that substantially higher concentrations of NAD$^+$ were needed to produce resonance broadening in the mutants (Figure 4), and the much larger K_m for AcPdAD$^+$ in reverse transhydrogenation by reconstituted protein (Table 2), indicate that oxidized nucleotide also binds rather more weakly to these proteins than to wild type.

Following reconstitution of purified domain I proteins, either with domain I-depleted membranes, or with recombinant domain III, the V_{max} values for transhydrogenation were decreased in the two Met239 mutants relative to wild type (Table 2). The rates of forward and reverse transhydrogenation are limited by release of NADPH and NADP$^+$, respectively, (*12*, *23*). The cyclic reaction, which is considerably faster than forward and reverse transhydrogenation, is limited by hydride transfer between bound nucleotides and associated conformational rearrangements. Because forward and reverse transhydrogenation were only slightly inhibited in the Met239 mutants, but the cyclic reaction was strongly inhibited (~80%), this is a clear indication that Met239 is involved in the hydride transfer reaction. The mutations at Met 239 had no significant effect on either (a) the docking affinity of the domain I protein with domain I-depleted membranes or (b) the K_m values for NADP(H) in either forward or reverse transhydrogenation (Table 2 and Results). It appears that changes that result from the mutation are confined to events taking place at the NAD(H) binding site of the protein.

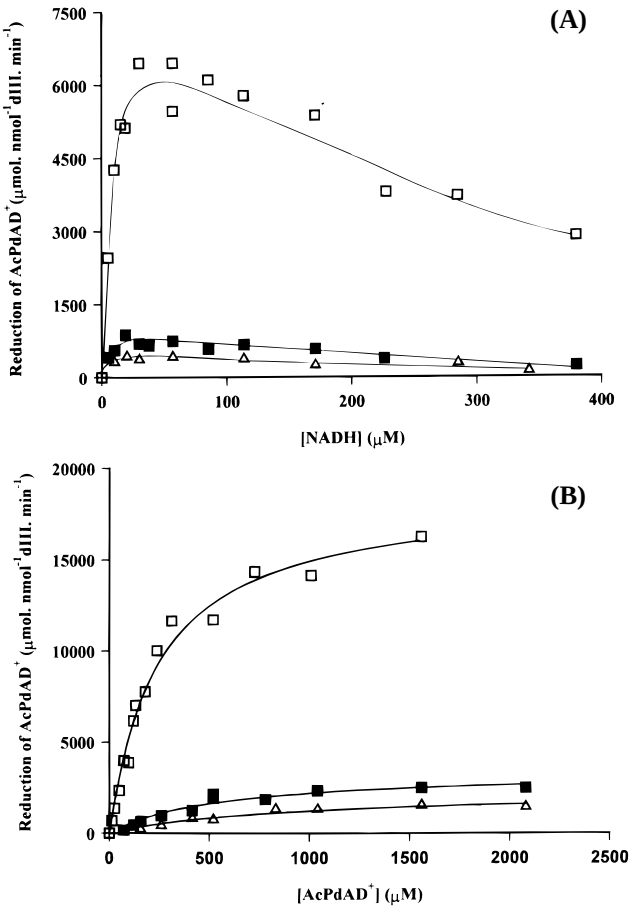

FIGURE 7: Dependence on nucleotide concentration of the rate of the cyclic reaction catalyzed by domain I:III complexes. Experiments were performed in 50 mM NaCl, 50 mM Mes, pH 6.0 and at 30 °C. The domain I protein was 37 μM and the domain III, 8.5 μM. (A) Performed at a fixed concentration of AcPdAD$^+$ (209 μM). (B) Performed at a fixed concentration of NADH (25 μM). In both sets of experiments the NADP$^+$ concentration was 25 μM: (□) wild-type domain I; (■) dI.M239I; (△) dI.M239F.

The results of a two-dimensional NMR experiment had previously shown that Tyr235 and possibly other amino acid residues in the mobile loop of domain I protein interact with adenosine of bound 5′-AMP (*10*). This gave rise to the conclusion that the mobile loop closes down on, and makes contact with, the nucleotide as it binds to the protein. Thus, there is good reason to suppose that Met239 might have direct contacts with NAD(H), that these contacts contribute to the binding energy of the ligand−protein complex, and

that they are kinetically important in the hydride transfer reaction. If hydride transfer between the NAD(H) and NADP(H) is direct (see (*1*)), then the positioning/orientation of the two nicotinamide rings, and their relative redox potentials, will be critical for rapid rates of reaction. We propose that interactions between the loop amino acid residues and the NAD(H) can modulate one or both of these parameters.

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