Site-Directed Mutagenesis of Charged and Potentially Proton-Carrying Residues in the β Subunit of the Proton-Translocating Nicotinamide Nucleotide Transhydrogenase from *Escherichia coli*. Characterization of the βH91, βD392, and βK424 Mutants†

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ABSTRACT: Conserved and semiconserved acidic and basic residues of the β subunit of the proton-pumping nicotinamide nucleotide transhydrogenase from *Escherichia coli* potentially involved in proton pumping were investigated. Out of 16 charged residues studied, 6 have not been previously investigated. The most dramatic effects of mutation were observed with βH91, βD392, and βK424. βH91E showed a pronounced shift of the pH optimum for both reduction of thio-$NADP^+$ by NADH (forward reaction) and reduction of 3-acetylpyridine-NAD^+ by NADPH (reverse reaction) to lower pH. This mutant catalyzed a cyclic reduction of 3-acetylpyridine-NAD^+ by NADH in the presence of NADP(H) with a pH profile also shifted toward a lower pH. These results are consistent with a mechanism where the normal forward and reverse reactions are indeed limited by protonation/deprotonation of βH91. The cyclic reaction was affected by mutations of βH91, probably through conformational changes involving the active NADP(H) site. The βD392A mutant was inactive with regard to forward and reverse reactions, but showed a wild-type-like pH dependence for the partly active cyclic reaction. However, $K_{m,app}$ for NADP(H) in this reaction was elevated 50−100-fold, suggesting that βD392 is located in or near the NADP(H)-binding site. Transhydrogenases contain a conserved βK424-βR425-βS426 sequence that has been proposed to be important for NADP(H) binding. βK424R was strongly inhibited and showed an 18-fold increased $K_{m,app}$ for NADPH in the reverse reaction as compared to wild type. Consequently, this mutation affected all NADP(H)-linked activities and essentially abolished the unspecific interaction of NAD(H) with this site. The pH dependences of the forward and reverse reactions, as well as the cyclic reaction, were shifted to a lower pH as compared to the wild-type enzyme, and the salt dependence was also altered.

Proton-pumping nicotinamide nucleotide transhydrogenase (EC 1.6.1.1) from *Escherichia coli* is composed of an α subunit of 54 kDa and a β subunit of 48 kDa, and an active form of $\alpha_2\beta_2$. The enzyme catalyzes the reversible reduction of $NADP^+$ by NADH linked to the translocation of one proton (*1*, *2*) according to the reaction:

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

where "out" and "in" denote the periplasmic space and cytosol, respectively. AB-transhydrogenases are composed of three domains, i.e., the hydrophilic domain I (α1−α404) containing the NAD(H)-binding site, the hydrophobic domain II (α405−β260) containing the membrane-spanning α-helices, and the hydrophilic domain III (β261−β462) containing the NADP(H)-binding site. About 10 AB-transhydrogenase genes have been cloned and expressed to date, and they show an overall sequence identity of about 21%. The genes for the transhydrogenases from *E. coli* and *R. rubrum*[1] have been overexpressed, and the enzymes have been reconstituted in liposomes and characterized extensively regarding function but less so regarding structure (for reviews, see *3*, *4*).

Recently, the genes of the soluble AA subunit of *R. rubrum* transhydrogenase (*5*), domain I of *E. coli* (*6*, *7*), and domain III of *E. coli* (*7*), bovine (*8*, see also *9*), and *R. rubrum* (*8*, *10*) have been overexpressed and the gene products isolated and characterized. Interestingly, with the exception of the *E. coli* preparation, the combination of the expressed domains I and III, i.e., in the absence of the proton-conducting domain II, results in low forward/reverse transhydrogenase activities, but a highly active so-called cyclic reduction of $AcPyAD^+$ by NADH mediated by bound NADP(H) (*7*−*10*). In contrast to domain I and the wild-type enzyme, all domain III preparations contained the expected dinucleotide, i.e., NADP(H), suggesting a pronounced regulation of the NADP(H) site by domain II. Based on these and other results, it is now generally agreed that the proton translocation through domain II catalyzed by AB-transhydrogenases is driven by conformational changes presumably triggered by

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[1] Abbreviations: DTT, dithiothreitol; $AcPyAD^+$, 3-acetylpyridine-NAD^+; *R. rubrum*; *Rhodospirillum rubrum*.

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binding/release of NADP(H) from domain III. In the *E. coli* enzyme, it was suggested that metal ions and other salts inhibit the cyclic reaction and stimulate the reverse reaction by increasing the release of NADP(H) (*11*, *12*).

In the *E. coli* enzyme, βH91, located in the predicted transmembrane helix 3 of the β subunit part of domain II (*13*, *14*) and βD392, in domain III (*15*), have been demonstrated to be important. Together, these residues were proposed to form a proton wire (*15*). Some mutants of βH91 show limited activities with regard to proton pumping and the forward and reverse reactions (*13*, *14*). Mutants of βD392 are essentially inactive with regard to the forward/reverse reactions although some catalyze a slow cyclic reaction (*15*). βH91 is the only conserved charged residue and potential proton-carrier in the predicted transmembranous domain II and is as such comparable to the single Asp61 of the c-subunit of, e.g., *E. coli* F_1F_o-ATPase (*16*). Recently, conserved histidines and arginines in the proposed NADP(H)-binding region, e.g., βH345 and βR350, were studied, of which the latter was suggested to be important for catalysis but not for binding (*17*, *18*).

The present investigation concerns the potential role of charged residues in transhydrogenase-catalyzed reactions, e.g., proton translocation. In addition to βH91 and βD392, conserved and semiconserved acidic residues as well as previously uncharacterized arginine and lysine residues in domain III were mutated and characterized. The results are consistent with the assumption that protonation/deprotonation of βH91 indeed is limiting to various degrees in all reactions, that βH91 constitutes part of a proton wire, and that βD392 is important for binding of NADP(H). Mutation of βK424 strongly affected the affinity of transhydrogenase for NADP(H) as well as the salt and pH dependence, suggesting that this residue is located in the NADP(H) site and possibly involved in the effects of salt on the enzyme.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The *pnt* gene was introduced into the constructs pDC21 or pSA2, which were used to transform the *E. coli* K12 strain TG1 or JM109, respectively. pDC21 and pSA2 were used throughout this study as a source of wild-type and mutant transhydrogenases (*1*, *13*, *19*). The His-tagged βH91E mutant was produced by replacement of the *Sph*I and *Bss*HII cleaved gene fragment in pNHis (*7*) construct with the one containing the βH91E mutant from pDC21.

Mutagenesis. Transformation of *E. coli* K12 strain TG1 or JM109 carrying wild-type *pnt* genes, or genes mutated by PCR, was carried out as described (*3*, *13*, *20*, *21*). Mutants were routinely established by complete sequencing.

Preparation of Cytoplasmic Membrane Vesicles. Inside-out cytoplasmic vesicles were prepared as described previously (*3*).

Purification of Wild-Type and Mutant E. coli Transhydrogenases. Wild-type and mutant transhydrogenases were purified as described (*1*). Preparations were routinely at least 90% pure as judged by SDS−PAGE in the presence of urea, and stored at −20 °C.

The His-tagged βH91E mutant transhydrogenase was purified as follows. About 8−10 g of *E. coli* cells were disrupted by passage through an X-press (AB Biox, Göteborg, Sweden) after equilibration at −25 to −30 °C for at least 30 min. The pellet was resuspended in P-buffer (30 mM sodium phosphate, pH 7.5), 5 mL per gram wet cells, to which 2 mM Pefabloc protease inhibitor (Boehringer) had been added from a 100 mM stock solution in 30 mM sodium phosphate buffer (pH 7.5). The suspension was homogenized and sonicated 3 × 1 min at a power setting of 60 W. Unbroken cells were removed by low-speed centrifugation (20 min at 10 000 rpm in a JA20-rotor), and the membrane vesicles were pelleted by centrifuging the supernatant for 1 h at 45 000 rpm (250000*g*) in a Beckman 70i rotor. Membranes were resuspended in 2.5 mL/g of P-buffer to which 2 mM Pefabloc protease inhibitor had been added. Another 2.5 mL/g of P-buffer containing 2 M KCl, 60 mM sodium cholate, and 60 mM sodium deoxycholate was added. The mixture was gently stirred at 4 °C for 25 min in an ultracentrifuge tube. The tube was filled to 18 mL with the same buffer as in the solubilization mixture and centrifuged at 45 000 rpm in a Beckman 70i rotor for 1 h.

The Ni−NTA resin (3 mL of resin/g of membrane) was packed in a column with an inner diameter of 2.6 cm and washed with 10 volumes of double-distilled H_2O and equilibrated with buffer [60% PNI-buffer (30 mM sodium phosphate, 0.8 M NaCl, 12 mM imidazole, pH 7.5); 40% P-buffer (1 mg/mL Brij 35, 1 mg/mL Thesit)]. The supernatant, containing solubilized enzymes, was diluted with 1.5 volumes of PNI-buffer, sterile-filtered, and applied to the equilibrated column. The column was first washed with M1-buffer (30 mM sodium phosphate, 0.7 M NaCl, 35 mM imidazole, 0.1% Brij 35, 0.1% Thesit, pH 7.5) until the base line was stable, and thereafter with 3−4 column volumes of M2-buffer (30 mM sodium phosphate, 0.1 M NaCl, 10 mM imidazole, 0.1% Brij 35, 0.1% Thesit, pH 7.5). M3-buffer (30 mM sodium phosphate, 0.1 M NaCl, 150 mM imidazole, 0.1% Brij 35, 0.1% Thesit, pH 7.5) was then used for elution. Flow rates were approximately 1 mL/min. Active fractions were pooled and further purified with a FPLC Resource Q column as described previously (*1*). All steps were performed at 4 °C.

Activity Assays. The catalytic activities of the wild-type and mutant transhydrogenases, assayed at room temperature either with cytoplasmic vesicles or with the purified enzyme, were determined spectrophotometrically at 375 nm as reduction of 3-acetyl-NAD^+ ($AcPyAD^+$) by NADPH using a molar absorption coefficient of 5100 $M^{-1}\cdot cm^{-1}$; the forward reaction was assayed at 400−460 nm as reduction of thio-$NADP^+$ by NADH using a molar absorption coefficient of 11 200 $M^{-1}\cdot cm^{-1}$ (*1*, *22*). The cyclic reduction of $AcPyAD^+$ by NADH in the absence and presence of NADP(H) was assayed as described (*1*). Unless indicated otherwise, the assay medium for the forward and reverse reactions contained 17 mM Mes, 17 mM Hepes, 17 mM Tris, 0.01% Brij35, 50 mM NaCl, and 5 mM $MgCl_2$, with a pH of 6.0. pH dependences were investigated using this medium adjusted to pH 5.5−9.0. In the assay of the cyclic reaction, NaCl and $MgCl_2$ were omitted. The concentrations of $AcPyAD^+$, NADH, NADPH, thio-$NADP^+$, and $NADP^+$ were normally 200 μM.

Reconstitution and the assay for proton pumping were carried out essentially as described (*21*), using the Mes−Hepes−Tris buffer (pH 6.0) described above.

Table 1: Properties of Mutant *E. coli* Transhydrogenases Containing Modified Conserved and Semiconserved Basic and Acidic Residues in the β Subunit[a]

residue	mutant	activity (%)			reference
		reverse	cyclic	H^+-pumping	
βH91	S, T, C, K, N, D	4−80	30−120	7−20	(*13*) (*14*) (*24*)
βH91	K, E	4	3−20	ND	this study
βK145	T	29	ND[b]	71	P. D. Bragg, unpublished
βH161	S, T, C	59−90	ND	94−108	(*13*)
βD213	N, H	82−92	ND	34−44	(*13*)
	I, N	10−30	75	10−30	(*9*)
βH345	N, Q	7−17	ND	ND	(*18*)
βR350	G, S, C	3−4	19−38	ND	(*18*)
βE361	Q	20	113	40	(*15*)
βE374	L	63	33	108	(*15*)
βD383	L, R	49−92	ND	79−123	(*15*)
βD392	A, K, N, Q, T	1−3	2−44	0	(*15*)
βD401	G, E, V	0−102	3−76	0−88	this study
βE413	G, D, V	70−106	ND	89−99	this study
βK416	G	33	27	82	this study
βK424	G, R	1−5	7−36	0	this study
βR425	G, E, K	0−13	1−38	0−98	this study
βK452	D, G	4−44	8−39	0−99	this study

[a] Mutants were prepared and their properties determined as described under Materials and Methods. Percent activities relate to those of wild type. [b] Not determined

Protein Determination. Protein concentration was determined by the method of Peterson (*23*), using BSA as standard.

Chemicals. Oligonucleotides were from MedProbe (Oslo, Norway). Enzymes were purchased from Life Technologies or Boehringer Mannheim. Other biochemicals were obtained from Sigma or Boehringer Mannheim.

RESULTS

As shown in Table 1, the conserved and semiconserved basic or acidic residues investigated in the β subunit of *E. coli* transhydrogenase are not essential in the sense that mutagenesis of the residue results in a total abolishment of all transhydrogenase-catalyzed activities. These activities are the reduction of thio-$NADP^+$ (an analogue of $NADP^+$) by NADH (forward reaction), reduction of $AcPyAD^+$ (an analogue of NAD^+) by NADPH (reverse reaction), the so-called cyclic reaction, i.e., reduction of $AcPyAD^+$ by NADH in the absence or presence of NADP(H), and proton-pumping driven by the reduction of $AcPyAD^+$ by NADPH. However, five residues, βH91, βR350, βD392, βK424, and βR425, emerge as important since mutagenesis of these strongly affected all activities and particularly the reverse reaction which normally is related to the activity for proton translocation. However, in our hands βR350 mutants showed ambiguous properties when isolated, and they are therefore still being characterized (O. Fjellström, V. Rotter, and J. Rydström, unpublished). Both βH91 (*13*, *14*, *24*) and βD392 (*15*) have been characterized partially previously. βK424 and βR425 have been proposed to be parts of an essential KRS sequence in or close to the NADP(H) site (*7*). Mutants of the remaining residues, βK145, βH161, βD213, βH345, βE361, βE374, βD383, βD401, βE413, βK416, and βK452, all catalyze the reverse and/or cyclic reactions as well as proton-pumping to various degrees.

Two mutants of βH91, i.e., βH91K and βH91E, were selected for further characterization because their protonation properties and/or charge differ as compared to the native histidine. The pH dependences of the forward and reverse reactions catalyzed by wild-type transhydrogenase are shown in Figure 1. In agreement with previous results, these reactions had their pH optima around pH 6.5−7.0 (*12*, *21*). However, the pH optima of the purified βH91E mutant were below 5.5 for the forward reaction and about 6.0 for the reverse reaction (Figure 2). The corresponding activities of βH91E at pH 6.0 were approximately 7% and 20%, respectively, of those of the wild-type enzyme, with the reverse reaction being about 10−15-fold faster than the forward reaction, which is typical for all transhydrogenases (cf. *3*). However, at pH 5.5, these values were shifted to 17% and 50%, respectively. Proton pumping by the βH91E mutant proved difficult to estimate because of too low activity when reconstituted in liposomes, and the βH91K mutant was essentially inactive in the reverse reaction regardless of pH (not shown). Thus, these results are consistent with the possibility that protonation/deprotonation of βH91 as the major proton wire component in domain II

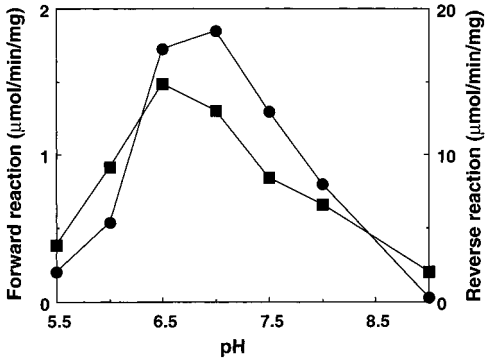

FIGURE 1: pH dependences of the forward and reverse transhydrogenase reactions, catalyzed by wild-type transhydrogenase. Assays were carried out as described under Materials and Methods using a medium containing Mes−Hepes−Tris buffer (pH 5.5−9.0) and purified transhydrogenase. Symbols denote: (■) forward reaction; (●) reverse reaction.

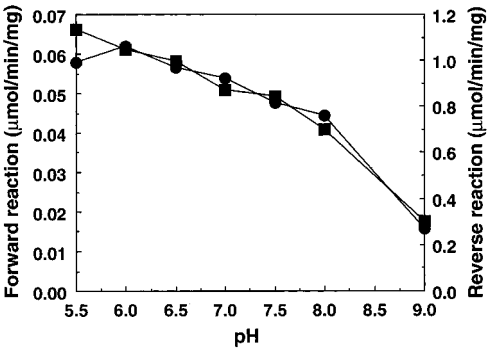

FIGURE 2: pH dependences of the forward and reverse reactions catalyzed by the βH91E mutant transhydrogenase. Conditions were as in the legend of Figure 1. Symbols denote: (■) forward reaction; (●) reverse reaction.

is limiting and depends largely on the pK_a of the residue in this position in both the forward and reverse transhydrogenase reactions.

Cyclic activities of wild-type, βH91K, and βH91E transhydrogenases varied as a function of pH approximately in the same manner, i.e., increased with decreasing pH, except that wild type and especially βH91E, in contrast to βH91K, showed a significantly lower activity with $NADP^+$ than NADPH (Figure 3). Overall rates for the cyclic reaction were about 6-fold lower with βH91K (Figure 3B) and more than 10-fold lower with βH91E (Figure 3C) as compared to wild-type activities (Figure 3A). Apparently, the pH dependence of βH91E suggests a change in the apparent pK_a to a lower value.

In contrast to the βH91E mutant, the pH profile of the cyclic reaction assayed for the βD392A mutant was similar to that of wild type but with a 65-fold increased $K_{m,app}$ for NADP(H); no forward or reverse reaction was catalyzed by the βD392A mutant (not shown).

Like the βH91E mutant, the pH optima of the forward and reverse reactions for the βK424R mutant were significantly shifted toward a lower pH as compared to wild type (Figure 4). As shown in Figure 5, the cyclic reaction showed a more pronounced pH dependence below pH 6.5 than wild type (cf. Figure 3A); cyclic activities of this mutant were approximately 50% of those of the wild type. The difference in activities of the βK424R mutant with $NADP^+$ and NADPH was partly due to the presence of nonsaturating concentrations of $NADP^+$ (see below)

The cyclic reaction catalyzed by wild-type and βK424R transhydrogenases was measured as a function of the concentration of $NADP^+$ and NADPH, respectively (not shown). The $K_{m,app}$ values for NADPH and $NADP^+$ were quite different for the two enzymes. With the wild-type enzyme, the $K_{m,app}$ values for NADPH and $NADP^+$ were very low, approximately 30 and 250 nM, respectively, whereas the corresponding values for the βK424R mutant were dramatically increased some 1500-fold to about 50 and 400 μM, respectively. Remarkably, the rate of reduction of $AcPyAD^+$ by NADH in the *absence* of NADP(H) was about 40−50% of the maximal activity in the presence of saturating NADP(H) for the wild-type transhydrogenase, but virtually nil for the βK424R mutant enzyme. This was interpreted to indicate that mutation of βK424 strongly decreased the affinity for NADP(H) and that the unspecific binding of NADH to the NADP(H) site was essentially eliminated in

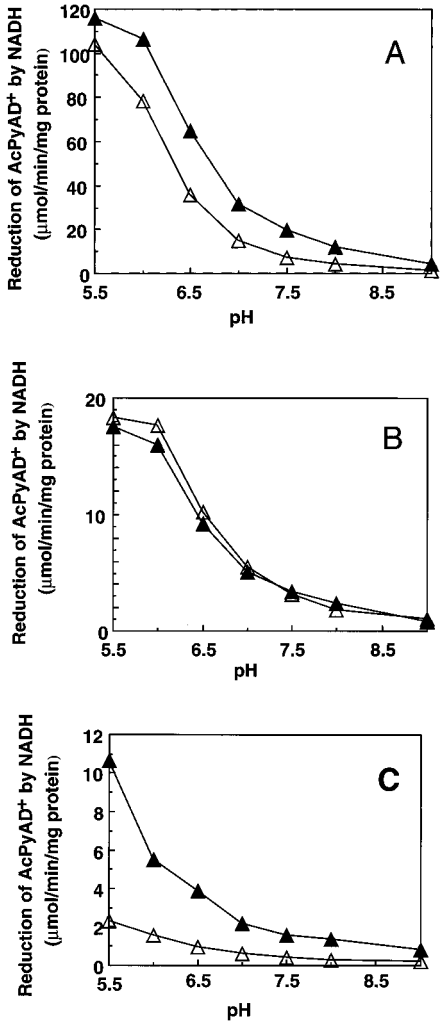

FIGURE 3: Determination of the activity of the cyclic reaction catalyzed by the wild-type (A), βH91K (B), and βH91E (C) transhydrogenases. Conditions were as described under Materials and Methods. Closed symbols represent the reaction carried out in the presence of 200 μM NADPH, and open symbols the reaction carried out in the presence of 200 μM $NADP^+$. The concentrations of NADP(H) were saturating.

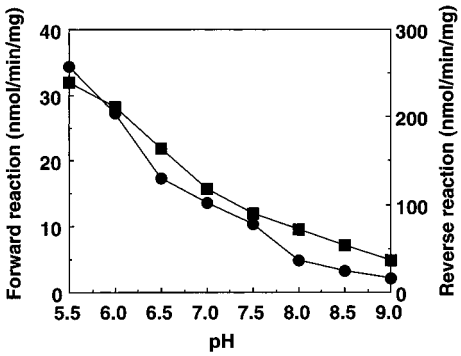

FIGURE 4: pH dependences of the forward and reverse reactions catalyzed by the βK424R mutant transhydrogenase. Conditions were as in the legend of Figure 1. Symbols denote: (■) forward reaction; (●) reverse reaction.

the βK424R mutant (cf. *11*). Indeed, the $K_{m,app}$ value of βK424R for NADPH in the reverse reaction was dramatically elevated, i.e., 180 μM as compared to about 10 μM for detergent-dispersed wild-type transhydrogenase (11); salt, e.g., $MgCl_2$, had no effect on the $K_{m,app}$ for NADPH in the reverse reaction as concluded previously for the wild-type

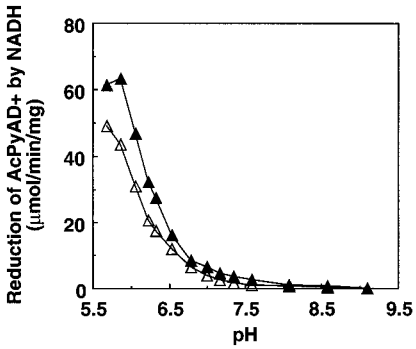

FIGURE 5: pH dependence of the cyclic reaction catalyzed by the βK424R mutant. Conditions were as in Figure 1. The cyclic reaction was supported by $NADP^+$ (△) or NADPH (▲). The concentrations of $NADP^+$ and NADPH were 600 and 200 μM, respectively.

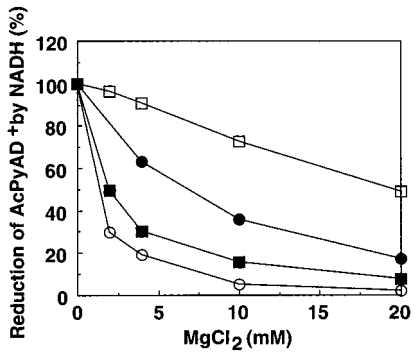

FIGURE 6: Effect of $MgCl_2$ on the cyclic reaction catalyzed by wild-type and βK424R mutant transhydrogenases. Conditions were as described under Materials and Methods. Symbols denote: (○) wild-type enzyme without added NADPH; (■) βK424R with 400 μM added NADPH; (●) wild-type with 0.2 μM added NADPH; (□) wild-type with 2 μM added NADPH.

enzyme (11). The $K_{m,app}$ for $AcPyAD^+$ was unchanged (not shown).

The cyclic activity in the absence of NADP(H) catalyzed by purified wild-type transhydrogenase was shown to be strongly inhibited by salt (*11*, *12*). In the present study this effect was found to be counteracted by increased concentrations of NADPH (Figure 6). Indeed, the cyclic reaction supported by the unspecific interaction of NADH with the NADP(H) site showed a similar salt dependence as that supported by $NADP^+$ (cf. *11*). Likewise, the βK424R mutant also showed a sensitivity to salt even in the presence of 400 μM NADPH, reflecting the low affinity of this enzyme for NADPH. This experiment did not show the slight stimulation of the cyclic reaction by low concentrations of salt (*11*). Higher concentrations of salt inhibited the cyclic reaction conceivably by increasing the dissociation of NADP(H) from the binding site, and therefore have a stimulating effect on the reverse reaction catalyzed by the wild-type enzyme (Figure 7). However, the βK424R enzyme behaved opposite to the wild-type enzyme with respect to the reverse reaction, and was actually inhibited by a range of concentrations of salt, although low concentrations of salt were slightly stimulatory (Figure 7). This inhibition was not related to the change in $K_{m,app}$ for NADPH. In the absence of salt, the activities of the reverse reaction catalyzed by the wild-type and βK424R enzymes were of the same order of magnitude.

Despite the close position of βR425 to βK424, the properties of mutants of the former residue were different

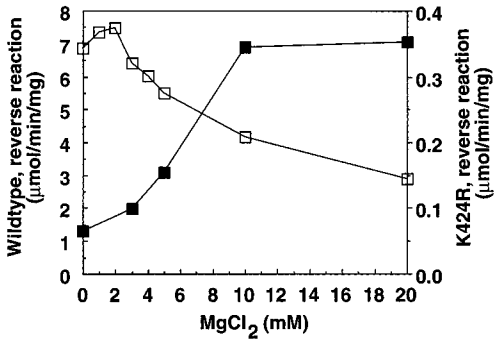

FIGURE 7: Effect of $MgCl_2$ on the reverse reaction catalyzed by wild-type and βK424R mutant transhydrogenases. Conditions were as described under Materials and Methods. Symbols denote: (■) wild-type enzyme; (□) βK424R mutant enzyme.

from those of the βK424 mutants. The pH dependences of all reactions catalyzed by βR425K were similar to those of the wild-type enzyme (not shown). However, $K_{m,app}$ values for the reverse and forward reactions were increased to 100 and 170 μM for NADPH and thio-$NADP^+$, respectively; the βR425E mutant was essentially inactive (not shown).

DISCUSSION

The present results show that among the conserved and semiconserved basic and acidic residues investigated in the β subunit of the *E. coli* transhydrogenase, none is essential. However, the mutants of the previously identified βH91 and βD392, and βK424 found in this study, show profoundly decreased activities. βR350 is probably also important, but this residue needs to be characterized further. βH91, βD392, and βK424 were therefore characterized in more detail as possible components of a transhydrogenase proton wire, especially with regard to the pH dependences of the mutants. The βK424R and βR425K mutants were of interest because of their potential roles in both proton transport and NADP(H) binding. In this context, it should be pointed out that the state of protonation of the substrates may be important for the pH dependences. However, since various mutants and isolated domains of transhydrogenases often show different pH dependences, the substrates cannot be the only contributing factors.

Hydride ion transfer is direct (*25*), and the forward and reverse reactions at neutral pH are believed to be limited by the release of NADP(H), which in turn is associated with proton translocation (*3*, *12*, *21*). A direct link has previously been established between the nature of the βH91 residue and the affinity of the NADP(H) site for NADP(H); e.g., the βH91K mutation leads to the abolishment of the forward and reverse reactions, a high cyclic activity, and an increased trypsin sensitivity, in both cases in the absence of added NADP(H) and due to bound NADP(H) (*14*, *17*, *24*). Since βH91 may be assumed to be located in the predicted helix 3 of the β subunit, i.e., presumably far from the NADP(H)-binding site in domain III, there is little doubt that βH91 regulates the properties of the NADP(H) site through long-range conformational changes and *vice versa* (*3*). It is conceivable that the pH optimum of 6.5−7.0 for both the forward and reverse reactions, catalyzed by *E. coli* transhydrogenase, reflects the limiting protonation/deprotonation of βH91. In agreement with this assumption, the pH dependences of both the forward and reverse reactions

catalyzed by the βH91E mutant were altered and the pH optima shifted downward at least 1 pH unit as compared to wild type, accompanied by a reduction in maximal activities. However, the altered pH dependence of the βH91E enzyme and the changes introduced as a consequence of this mutation suggest the involvement of several protonatable residues.

There has been a discussion (cf. *3*, *21*, *26*) regarding the possible alternating movement/exposure of the potential proton-carrying residue βH91 (or βD392 or other proton-carrying groups) to the periplasmic and cytosolic side of the enzyme during the cyclic reaction. In a thorough investigation of the influence of Δ*p* on the NADPH-supported cyclic reactions catalyzed by the *R. rubrum* and *E. coli* transhydrogenases, no evidence was found for any effect of Δ*p* on these reactions (*21*, *26*), and it was concluded that hydride transfer is separate from proton-pumping. However, internal proton movements may still occur (*21*). In contrast to the net forward and reverse reactions, the cyclic reaction was found to be limited by hydride transfer rather than, e.g., release of NADP(H) (*12*), and is therefore at least 5−10 times faster at low pH (*11*, *12*). In the present investigation, both the βH91K and βH91E mutations led to a pronounced decrease in cyclic activity, but with a similar pH profile; i.e., the activity increased with decreasing pH (however, a possible pK_a shift in these mutants for the cyclic reaction cannot be excluded). This suggests the existence of one or more protonatable groups that limit hydride transfer, i.e., the cyclic reaction, and which are separate from that/those (e.g., βH91) limiting the reactions that are dependent on binding/release of NADP(H). Since NADP(H) was saturating in the cyclic reaction, the decreased maximal rates of the βH91 mutants are assumed to reflect a constraint in the conformational interactions between βH91 and the NADP(H)-binding site. It is interesting to note that the βH91E mutant showed a considerably lower cyclic activity with $NADP^+$ than with NADPH. Wild type showed a similar but less extensive difference especially at low pH. Assuming that βH91 forms the periplasmic end of the proton channel, this difference may be interpreted to indicate a discrepancy in proton accessibility to the two ends of the proton channel.

Mutation of βD392 to βD392A and βD392N are the only amino acid replacements of this residue so far tested that give a partially active enzyme with regard to the cyclic activity as determined with cytosolic membranes (*15*). It is noteworthy that the $K_{m,app}$ values for NADP(H) in this reaction were increased substantially, possibly because binding/release of NADPH was affected.

Unexpectedly, in contrast to the βR425K mutant, the βK424R mutant showed a strong shift of the pH optima of the forward and reverse reactions toward a lower pH. The cyclic reaction rate displayed a different pH dependence below pH 6.5. $K_{m,app}$ for NADPH in the reverse reaction was increased some 18-fold, whereas that for $AcPyAD^+$ was unchanged. Likewise, the apparent $K_{m,app}$ values for $NADP^+$ and NADPH in the cyclic reaction were increased dramatically, again with a pronounced difference between $NADP^+$ and NADPH as in the case of the wild-type enzyme. These results are interpreted to indicate that there is only one NADP(H) site, and that the βK424R mutation selectively and severely affects steps associated with binding and release of NADP(H), leading to an inhibition of both the forward and reverse reactions. Despite the high $K_{m,app}$ for NADP(H), the cyclic reaction retained approximately 50% of the wild-type activity in the presence of very high and saturating NADP(H). The βK424 residue, as part of the KRS sequence, was proposed to be located close to or in the vicinity of the NADP(H) site, although its detailed position and function in the site have not yet been established (*7*). However, in view of the changes in the pH dependences of the reactions catalyzed by the βK424R mutant, it is conceivable that these changes are indirect and mediated by an unknown group.

A very interesting property of the βK424R mutant is that it no longer catalyzes a cyclic reduction of $AcPyAD^+$ by NADH in the absence of NADP(H). Based on, e.g., steady-state kinetics and the use of site-specific inhibitors, this NADP(H)-independent reaction was earlier proposed to involve an unspecific interaction of $AcPyAD^+$ or NADH with the NADP(H) site (*11*, *27*, *28*). Alternative explanations were later provided for this observation (*27*, *28*). The finding that the NADP(H) independent cyclic reaction is essentially lost in the βK424R mutant strongly supports the assumption that the affinity of the NADP(H)-site for substrates in this mutant has decreased to an extent that, under the conditions used, NADH does not bind significantly to this site. Indeed, the fact that the apparent $K_{m,app}$ value of the βK424R mutant for NADP(H) was more than 2 orders of magnitude higher than that for wild-type suggests that an increased binding of NADP(H) facilitates the cyclic reaction but is *not* a prerequisite for this reaction. Indeed, it is sufficient that the NADP(H) stays bound during the hydride transfer cycle.

A second unexpected observation with the βK424R mutant was that, when catalyzing the reverse reaction, it was inhibited by salt, whereas the wild-type enzyme is stimulated under the same conditions. Most transhydrogenases show a stimulation of the forward and reverse activities by salt followed by an inhibition at very high salt (*3*, *4*, *11*, *12*, *29*). As originally suggested by Fisher and co-workers (*30*), the salt effect may reflect interactions with amine residues. Therefore, it is possible that βK424 binds salt in the wild-type enzyme, leading to a stimulation of the forward and reverse reactions through an increased release of NADP(H), and that other residues are involved in the inhibitory effect. Metal ions may also be involved in the interactions between subunits (*31*).

In conclusion, of the conserved and semiconserved charged residues investigated in the β subunit of *E. coli* transhydrogenase, five residues emerge as important, namely, βH91, βD392, βK424, βR350, and βR425. Proton-pumping involves βH91 whereas NADP(H)-binding involves βH91, βD392, and βK424. Residues βR350 and βR425 are proposed to be close to the NADP(H)-binding site, but their roles remain to establish.

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