Switch of Coenzyme Specificity of Mouse Lung Carbonyl Reductase by Substitution of Threonine 38 with Aspartic Acid

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Mouse lung carbonyl reductase, a member of the short-chain dehydrogenase/reductase (SDR) family, exhibits coenzyme specificity for NADP(H) over NAD(H). Crystal structure of the enzyme-NADPH complex shows that Thr-38 interacts with the 2'-phosphate of NADPH and occupies the position spatially similar to an Asp residue of the NAD(H)-dependent SDRs that hydrogen-bonds to the hydroxyl groups of the adenine ribose of the coenzymes. Using site-directed mutagenesis, we constructed a mutant mouse lung carbonyl reductase in which Thr-38 was replaced by Asp (T38D), and we compared kinetic properties of the mutant and wild-type enzymes in both forward and reverse reactions. The mutation resulted in increases of more than 200-fold in the \( K_m \) values for NADP(H) and decreases of more than 7-fold in those for NAD(H), but few changes in the \( K_m \) values for substrates or in the \( k_{cat} \) values of the reactions. NAD(H) provided maximal protection against thermal and urea denaturation of T38D, in contrast to the effective protection by NADP(H) for the wild-type enzyme. Thus, the single mutation converted the coenzyme specificity from NADP(H) to NAD(H). Calculation of free energy changes showed that the 2'-phosphate of NADP(H) contributes to its interaction with the wild-type enzyme. Changing Thr-38 to Asp destabilized the binding energies of NADP(H) by 3.9–4.5 kcal/mol and stabilized those of NAD(H) by 1.2–1.4 kcal/mol. These results indicate a significant role of Thr-38 in NADP(H) binding for the mouse lung enzyme and provide further evidence for the key role of Asp at this position in NAD(H) specificity of the SDR family proteins.

In several animal lungs (1–5), carbonyl reductase (NADPH) (CR; EC 1.1.1.184) catalyzes the reduction of various aliphatic and aromatic carbonyl compounds and the oxidation of secondary alcohols and aliphatic aldehydes. The enzyme is highly expressed in bronchiolar and alveolar epithelial cells (3, 6) and has been thought to function in pulmonary metabolism of endogenous carbonyl compounds such as aliphatic aldehydes and ketones derived from lipid peroxidation, 3-ketosteroids, and fatty aldehydes, as well as in xenobiotic metabolism.

The cDNAs for pulmonary CRs of mouse (7) and pig (8) have been cloned, and their deduced amino acid sequences (composed of 244 residues with 85% identity between them) indicate that the enzymes belong to the short-chain dehydrogenase/reductase (SDR) family, which includes a large number of prokaryotic and eukaryotic enzymes with different specificities for coenzymes and substrates (9, 10). The pulmonary CR sequences contain two consensus sequences of Tyr-X-X-Lys and Gly-X-X-Gly-X-Gly that are demonstrated to be the active site and coenzyme binding domains, respectively, by site-directed mutagenesis (Ref. 9 and references therein) and x-ray crystallography studies (11–14) of several SDR family proteins. The region around the latter sequence of the SDRs forms a \( \beta\beta\beta \) fold that is characteristic of the coenzyme-binding fold in dehydrogenases of other families (15, 16). The NAD(H)- and NADP(H)-dependent dehydrogenases of other families have different fingerprint sequences for coenzyme binding. The NAD(H)-dependent enzymes have an invariant Gly-X-Gly-X-Gly sequence and an acidic residue (usually Asp) at the C terminus of the second \( \beta \) strand, whereas another consensus sequence has been proposed for the NADP(H)-dependent enzymes in which the third Gly of the NAD(H)-binding fingerprint is replaced by Ala, and a positively charged residue is usually included in the neighborhood of the C terminus of the \( \beta\beta\beta \) fold (15–20). For the SDR family, both NAD(H)- and NADP(H)-dependent enzymes possess the same Gly-X-X-Gly-X-Gly pattern. Although a factor determining the specificity for NAD(H) has been proposed to be Asp at the C terminus of the second \( \beta \) strand of the coenzyme-binding fold (9–14), the residue(s) responsible for NADP(H) specificity remain unknown.

We have recently solved the three-dimensional structure of mouse lung CR, which exhibits high coenzyme preference for NADP(H) over NAD(H), and have shown that Lys-17 and Arg-39, which exist before the second Gly of the Gly-rich pattern and at the C terminus of the \( \beta\beta\beta \) fold, respectively, are responsible for the coenzyme specificity (21). The roles of the two basic amino acids have been confirmed by their site-directed mutagenesis (22). In addition, comparison of crystal structures between mouse lung CR and the NAD(H)-dependent SDRs has suggested that, while the Asp residue at the coenzyme-binding fold forms a bifurcated hydrogen bond to the adenine ribose for the NAD(H)-dependent enzymes (11–14), Thr-38 of CR at a position corresponding to the Asp residue hydrogen-bonds to the 2'-phosphate of NADPH through a water molecule (21).

In this study, we used site-directed mutagenesis to replace Thr-38 with Asp (T38D) and compared the kinetic and thermo-
dynamic properties of coenzyme binding to the wild-type CR and T38D. The results show that the single mutation converts the coenzyme specificity of mouse lung CR from NAD(P)H to NAD(H).

EXPERIMENTAL PROCEDURES

Materials—Pyridine nucleotide coenzymes and PI markers were obtained from Oriental Yeast (Tokyo, Japan); restriction and DNA-modifying enzymes were from Nippon Gene (Tokyo, Japan) and Takara Shuzou (Osaka, Japan); Escherichia coli cells and plasmids were from Stratagene. The cDNA for mouse lung CR and the antibody against the enzyme previously prepared (7) were used. The oligonucleotide primers for the site-directed mutagenesis were synthesized as described (7, 22). All other chemicals were of the highest grade that could be obtained commercially.

Site-directed Mutagenesis, Expression, and Purification—The cDNA for T38D was generated using a modified overlap-extension technique (25) as described previously (22), using the partially complementary primer pairs (forward 5'-GGCGGGTGGATCTGGAGCAA-3' and reverse 5'-TGTGCCGCCACCACCGCAC-3') that contained a codon of an altered amino acid (underlined). The complete coding region of the mutated cDNA was sequenced as described previously (8) to confirm the presence of the desired mutation and to ensure that no other mutation had occurred.

The wild-type and mutated cDNAs were expressed in E. coli (JM105) cells, and the recombinant enzymes were purified from the 12,000 g supernatants of the homogenates of the cells (each a 1-liter culture) as described previously (7).

Characterization of Protein Samples—Protein concentration of the crude extract and enzyme preparations during the purification was determined by the Bradford method (24) using bovine serum albumin as the standard. The molecular mass of the CR subunit was determined by SDS-polyacrylamide gel electrophoresis (25) on 12.5% gels. The PI value of the purified enzyme was assessed by isoelectric focusing (26) on 7.5% polyacrylamide gels containing 2% Ampholite (Pharmacia Biotech Inc.) and 8 μl urea using the pl markers. Western blot immunanalysis using the antibody against CR was carried out as described previously (7).

Enzyme Assay and Kinetic Analysis—Reductase and dehydrogenase activities of CR were assayed by recording the rate of change in NAD(P)H absorbance at 340 nm, except that an absorbance at 366 nm was monitored in the assay with high concentrations of NAD(P)H. The reductase activity was determined with NAD(P)H and pyridine-3-aldehyde (P3A) as the coenzyme and substrate, respectively. The kinetic effect of the mutation was assessed by comparing the kinetic parameters in the forward and reverse (CHX as the substrate) directions between WT and T38D (Table I). In the forward reaction, the most striking alteration by the mutation was the increased oxidation with a fixed saturated concentration of substrate or coenzyme (the kinetic effect of the mutation was assessed by comparing the kinetic parameters in the forward and reverse (CHX as the substrate) directions between WT and T38D (Table I). In the forward reaction, the most striking alteration by the mutation was the increase in the dissociation constant of substrate A.

RESULTS AND DISCUSSION

Expression and Purification of Wild-type and Mutant CRs—When the CHX dehydrogenase activity in the lysate of the E. coli cells was assayed with NADP+ and NAD+ as the coenzymes, the mutant enzyme T38D showed pronounced preference for NAD+. The ratio of the NAD+-linked to the NADP+-linked activities was 18, which was much higher than the value of 0.1 for WT. The mutant enzyme was purified to apparent homogeneity on SDS-polyacrylamide gel electrophoresis (Fig. 1A) and isoelectric focusing (Fig. 1B). The NADP+ - and NAD+-linked activities of the purified T38D were 0.39 unit/mg and 6.6 units/mg, respectively, whereas the respective values of the purified WT were 4.5 units/mg and 0.70 unit/mg. Although the T38D and WT showed the identical subunit molecular mass and reactivity with the CR antibody on Western blot immunanalysis (data not shown), the PI value of 8.8 for T38D was lower than the PI value of 9.3 for WT due to the introduction of an acidic charge of Asp.

Computer Modeling—The subunit 1 (protein and NADP(H)) in the crystal structure of WT (21) was chosen to build the mutated model structure T38D. The Thr-38 of the subunit was replaced with an Asp (AGT to GAT) as described previously (22). The single-subunit model with the coenzyme NADPH was refined through the energy minimization routine incorporated in the program X-PLOR (29).

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Kinetic Alteration by Mutagenesis—The kinetic effect of the mutation was assessed by comparing the kinetic parameters in both forward (with P3A as the substrate) and reverse (with CHX as the substrate) directions between WT and T38D (Table I). In the forward reaction, the most striking alteration by the mutation was the increase in the dissociation constant of substrate A. The kinetic constants in the CHX dehydrogenase activity were derived from saturation curves of P3A and CHX (27), and enzyme in a total volume of 2.0 ml. The reductase activity was determined with NAD(P)H and pyridine-3-aldehyde (P3A) as the coenzyme and substrate, respectively. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation and oxidation of 1 μmol of NAD(P)H/min at 25 °C.

The kinetic mechanism and constants of the PAS reduction were analyzed according to the method of Cleland (27). The initial velocities were fitted to the equation

\[
u = V_{\text{max}}[\text{A}] + K_{\text{B}}[\text{A}] + K_{\text{D}}[\text{S}] + K_{\text{D}}[\text{S}]
\]

(Eq. 1)

where \(v\) is the initial velocity, \(V\) is the maximum velocity at saturating substrate concentrations, A and B are the two substrate concentrations, \(K_{\text{B}}\) and \(K_{\text{D}}\) are their corresponding Michaelis constants, and \(K_{\text{D}}\) is the dissociation constant of substrate A. The kinetic constants in the CHX oxidation with a fixed saturated concentration of substrate or coenzyme were directly determined by fit to the Michaelis-Menten equation. The kinetic studies in the presence of inhibitors were carried out in a similar manner, and the inhibition constants, \(K_{\text{B}}\) (slope effect) and \(K_{\text{D}}\) (intercept effect), were determined as described (28). All kinetic measurements were performed at least three times, and mean values were used for subsequent calculation. All standard errors of fits were less than 15%.

Thermal and Urea Stability Study—For thermal inactivation, the enzymes (0.1 mg/ml) were incubated at 34 °C in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.1% of Triton X-100 and 0.01% bovine serum albumin in the presence or absence of NAD(P)H or substrate. At different times, aliquots of 50 μl from each sample were taken and assayed for the dehydrogenase activity. For the denaturation by urea, the enzyme (30 μg/ml) was incubated at 25 °C for 3 h in 0.1 M Tris-HCl buffer, pH 8.0, containing 0–5 M urea in the presence or absence of NAD(P)+ or substrate. The dehydrogenase activity was expressed as a percentage of that in the absence of urea. This assay was unaffected by the presence of up to 0.1 M urea.

FIG. 1. SDS-polyacrylamide gel electrophoresis (A) and isoelectric focusing (B) of purified WT and T38D. The enzymes (2 μg) were run and stained with Coomassie Brilliant Blue.
analysis of product inhibition at the saturated concentration of the fixed substrate, noncompetitive inhibition patterns were obtained with NAD$^+$ ($K_\text{m} = 0.83 \text{ mM}$; $K_\text{m} = 1.5 \text{ mM}$) and pyridine-3-methanol ($K_\text{m} = 31 \text{ mM}$; $K_\text{m} = 90 \text{ mM}$) when P3A was the variable substrate. The patterns found with NAD$^+$ and pyridine-3-methanol were competitive ($K_\text{m} = 0.19 \text{ mM}$) and uncompetitive ($K_\text{m} = 75 \text{ mM}$), respectively, with respect to NADH. In addition to NAD$^+$, its analogs NADP$^+$, 2'-AMP, and 5'-AMP inhibited both WT and T38D competitively with respect to NADP$^+$ (Table II). From these data, the kinetic mechanism inhibited both WT and T38D competitively with respect to variable substrate. The patterns found with NAD$^+$ for NAD(H) in the column of T38D/WT. The product inhibition patterns for T38D appears most likely to be an Ordered Bi Bi mechanism. The product inhibition patterns for T38D were competitive ($k_{\text{cat}}/K_\text{m} = 1.1 \text{ mM}$) and uncompetitive ($k_{\text{cat}}/K_\text{m} = 190 \text{ mM}$) with respect to the coenzyme, implying their dissociation constants in this kinetic mechanism. The small changes in $K_\text{m}$ values for the competitive inhibitors with respect to the coenzyme imply their dissociation constants in this kinetic mechanism, and thus the effects of the mutation on the affinities for the oxidized coenzymes and their analogs can be assessed by comparing their $K_\text{m}$ values between WT and T38D. The mutation decreased the affinities for NAD$^+$ and 2'-AMP and increased the affinities for NAD$^+$ and 5'-AMP, although the changes in the affinities for the AMPs were low because of the absence of other parts of NAD(P)$^+$ molecules that interacted with several residues of the enzyme (21). The results indicate that the adenine ribose moiety of the coenzymes interacts with Thr-38 and the replaced Asp.

The kinetic results were also supported by differences in protection against the thermal and urea denaturation by coenzymes between T38D and WT. The thermal inactivation of WT was protected completely by low concentrations of NADP(H) and moderately by high concentrations of NAD(H), whereas NAD(H) provided more efficient protection against the thermal inactivation of T38D than did NADP(H) (Fig. 2). Similar protective effects of the coenzymes appeared on the denaturation by urea, in which T38D was slightly unstable compared with WT (Fig. 3). NADP$^+$ showed greater protection against the urea denaturation of WT than NAD$^+$, whereas obvious protective effects of the coenzymes appeared on the denaturation of T38D from the denaturation was observed only by the addition of NAD$^+$. It should be noted that the substrate (2 mM P3A or CHX) did not show significant protection against the thermal and urea denaturation of the two enzymes, which supports the kinetic order addition of the coenzymes to the free enzymes followed by the substrates.

In addition to the loss of the hydrogen bridge between Thr-38 and the 2'-phosphate of NADP(H) by the mutation, other structural factors must be considered to explain the large alteration in the kinetic constants for the coenzymes. In the crystal structure of the mouse lung CR-NAD$^+$ complex (21), the 2'-phosphate of NADPH has been shown to interact with the side chains of Lys-17, Thr-38, and Arg-39, of which Thr-38 occupies the position spatially similar to the Asp of the NAD(H)-dependent SDRs that interacts with the adenine ribose of NAD(H) (11–14). In the mutated model structure (T38D), the distances between each oxygen atom of Asp-38 carboxylate and the near-

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est oxygen atom of the NAD(P)H 2’-phosphate were 2.8 Å and 3.8 Å. The calculated electrostatic energy between the Asp-38 and the 2’-phosphate moiety (T38D) was 22 kcal/mol higher than that between the Thr-38 and the 2’-phosphate moiety (WT). Thus, one of the possible structural factors for the switch of the coenzyme specificity by the mutation is an electrostatic repulsion between the negatively charged 2’-phosphate of NADPH and the carboxylic group of the replaced Asp. Another structural factor may be steric hindrance of the side chain of the replaced Asp residue against the NADP(H) binding. The presence of the Asp side chain that is larger than that of Thr-38 may interfere with the proximity of the side chains of Lys-17 and Arg-39 to 2’-phosphate of NADP(H) as suggested (21). Furthermore, the increase in the affinity for NADH by the mutation suggests that the side chain of the replaced Asp makes a hydrogen-bonded interaction with the hydroxyl groups of adenine ribose of the coenzymes as shown in the crystal structures of the NAD(H)-dependent SDRs (11–14).

**Thermodynamic Effect of Mutagenesis on Coenzyme Binding Energy and Transition State Stabilization**—Thermodynamic effects of removing the 2’-phosphate of NADP(H) or replacing Thr-38 with Asp on the coenzyme binding energy were calculated according to the relationships described by Sem and Kasper (30) (Table III). The change in the binding energy for 2’-phosphate removal of NADP(H) in the presence of Thr-38 (i.e. WT) indicates that the 2’-phosphate groups of NADPH and NADP+ contribute 3.2 and 4.5 kcal/mol, respectively, of binding energy in their interactions with the enzyme. The values for 2’-phosphate removal of NADPH and NADP+ for the mutant T38D were −1.9 and −1.3 kcal/mol, respectively. The negative values imply that the mutation not only destabilizes the NADP(H) binding but also stabilizes the NADH binding. When Thr-38 was replaced with Asp, the binding energies of NADPH and NADP+ were destabilized by 3.9 and 4.5 kcal/mol, respectively, but those of NADH and NADP− were conversely stabilized by 1.2 and 1.4 kcal/mol, respectively. Since the values of 3.9 and 4.5 kcal/mol for the hydrogen bridge made by Thr-38 of mouse lung CR are comparable to the values for the Ser-2’-phosphate interaction in cinnamyl-alcohol dehydrogenase (31) and for other salt bridges between NADP(H) and Lys or Arg reported for mouse lung CR (22) and other enzymes (30–33), Thr-38 may contribute significantly to the binding energy of NADPH by making a hydrogen bridge to the 2’-phosphate of NADPH. In addition, the stabilization of the NAD(H) binding energies by the mutation supports the hydrogen-bonded interaction of the side chain of the replaced Asp with the hydroxyl groups of adenine ribose of the coenzymes.

Thr-38 is conserved only in mouse and pig lung CRs (7, 8) of the NADPH(H)-dependent SDR enzymes, several of which have Ser or Cys (34–36) and most of which have Ala at this position (9, 21). Although the amino acids with a hydroxyl or sulfhydryl group can be anticipated to participate in NADP(H) binding in several SDRs, the role of Ala remains unknown. However, the presence of an Ala residue at this position in the other SDRs has suggested to be important for making the coenzyme-binding cleft to avoid the steric hindrance against the interactions between the 2’-phosphate of NADP(H) and the side chains of the basic residues corresponding to Lys-17 and Arg-39 of mouse lung CR (21). That is, since the interactions of the two basic residues with the 2’-phosphate of the coenzymes in the SDRs containing Ala at this position may be stronger than those in mouse lung CR, the additional interaction by Thr would no longer be required for the coenzyme specificity.

**Comparison with Coenzyme Switching Studies for Oxidoreductases of the SDR and Other Families**—Such a drastic change in coenzyme specificity by single mutation observed in the present study has not been reported yet for dehydrogenases of the SDR (37–39) and other families (17, 19, 40, 41) in which systematic replacement of a set of amino acids in their βββ motif is necessary to switch their coenzyme specificity. Although there has been no report on the mutation of the residue corresponding to Thr-38 of mouse lung CR in NADP(H)-dependent enzymes of the SDR family, the reverse-sense mutation, i.e. the replacement of the Asp with other residues, has been described for two NAD(H)-dependent enzymes of the SDR family, the reverse-sense mutation, i.e. the replacement of the Asp with other residues, has been described for two NAD(H)-dependent enzymes of this family. The mutations of Asp-37 to Ile for rat dihydropyridine reductase (37) and of Asp-39 to Asn for Drosophila alcohol dehydrogenase (38) increase the affinities for NADP(H) but do not have a significant effect on those for NAD(H); therefore, the mutant enzymes still show coenzyme preference for NAD(1H) over NADP(1).

The present study reveals not only the important role of Thr-38 in the NADP(H) binding to mouse lung CR, but also the key role of Asp at the C terminus of the second β strand of the βββ fold in the NAD(H) specificity of the SDR family proteins. Since (unlike dehydrogenases of other families (15–20)) the SDR family proteins have the same Gly-X-X-Gly-X-Gly sequence in their βββ folds (9–14), the most critical determinant for coenzyme specificity is the presence of Asp at this position. As T38D, which has Lys-17 and Arg-39 replaced for the NADP(H) binding (21, 22), showed the coenzyme preference for NADH over NADP(H), some SDRs with both the Asp and one of the basic residues corresponding to Lys-17 and/or Arg-39 of mouse lung CR are NAD(H)-specific (42–44). Conversely, the structural determinant for the NADP(H) specificity in the SDR family proteins is the replacement of the Asp with neutral amino acids with shorter side chains in addition to the presence of the two basic residue(s), as suggested by comparison of

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**TABLE III**

| Modification | Enzyme or coenzyme | ΔΔG<sub>2PH</sub> | ΔΔG<sub>TMD</sub> |
|--------------|---------------------|----------------|----------------|
| NADPH → NADH | WT                  | 3.2            |               |
| NADP<sup>+</sup> → NADP<sup>+</sup> | WT                  | 1.9            |               |
| NADP<sup>+</sup> → NADP<sup>−</sup> | T38D                | -1.3           |               |
| Thr-38 → Asp | NADPH               | -1.4           |               |
|             | NADH                | -1.2           |               |
|             | NADP<sup>+</sup>     | -4.5           |               |
|             | NAD<sup>−</sup>      | -1.3           |               |

**FIG. 3. Effects of coenzymes on urea denaturation of WT (A) or T38D (B) at pH 8.0.** The enzyme was incubated at 25°C in the absence (●) or presence of the following coenzymes: 2 mM NAD<sup>+</sup> (▲), 4 mM NAD<sup>−</sup> (△), 0.2 mM NADP<sup>+</sup> (■), and 4 mM NADP<sup>−</sup> (□).
crystal structures of mouse lung CR and several NAD(H)-
dependent SDRs (21).

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