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Involvement of Two Basic Residues (Lys-17 and Arg-39) of Mouse Lung Carbonyl Reductase in NADP(H)-Binding and Fatty Acid Activation: Site-Directed Mutagenesis and Kinetic Analyses[1]

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Mouse lung carbonyl reductase, a member of the short-chain dehydrogenase/reductase (SDR) family, shows a strong coenzyme preference for NADP(H) over NAD(H), and is uniquely activated by fatty acids. Previous chemical modification and X-ray crystallography studies show that interactions responsible for the coenzyme specificity include salt linkages between the 2′-phosphate of NADPH and side-chains of Lys-17 and Arg-39 of the enzyme. Although Arg-39 is highly conserved in NADP(H)-dependent enzymes of the SDR family, Lys-17 is substituted with Arg in about half of the NADP(H)-dependent enzymes. The present study shows that mutations of Lys-17 to His (K17H) or Ser (K17S) and of Arg-39 to Ala (R39A) bring about decreases (from 5 to 90-fold) of the affinities for NADP(H), but minor changes in the affinity for NAD^+. The binding energy arising from the mutations on the binding of the 2′-phosphate of $NADP^+$ was decreased by 38-66% from the value of 4.8 kcal/mol calculated for the wild-type enzyme. In contrast, the mutation of Lys-17 to Arg (K17R) had little effect on the kinetic or thermodynamic properties. The activation by fatty acids was completely attenuated by the mutations of K17H and K17S, but not by K17R or R39A. These results indicate that the 2′-phosphate group of NADP(H) is recognized by both Lys-17 and Arg-39, of which Lys-17 is a component of the binding site for the activator, probably interacting with the negatively charged carboxylate group of fatty acids, and also suggest that the existence of a positively charged residue (either Lys or Arg) at position 17 is required for both NADP(H) specificity of the SDR family enzymes and fatty acid activation of the pulmonary carbonyl reductase.

Key words: fatty acid activation, carbonyl reductase, NADP(H) specificity, short-chain dehydrogenase/reductase family, site-directed mutagenesis.

Carbonyl reductase (CR) [EC 1.1.1.184] catalyzes the NADPH-dependent reduction of various carbonyl compounds to the corresponding alcohols. Among CRs purified from many mammalian tissues (*1-3*), pulmonary CR of guinea-pig (*4*), mouse (*5*), and pig (*6*) is unique with regard to intracellular localization, subunit structure and catalytic properties. It is the only tetrameric CR identified thus far that is localized mainly in mitochondria (*7, 8*) and efficiently oxidizes aliphatic and alicyclic alcohols with $NAD(P)^+$ as the coenzyme (*5, 6, 9*). Moreover, the activity of pulmonary CR is activated up to 5-fold by fatty acids, particularly *cis*-unsaturated fatty acids (*10*).

The cDNAs for pulmonary CRs of pig (*11*) and mouse (*12*) have been cloned, and the cDNA for mouse lung CR is identical with that for an mRNA which is increased in differentiation of murine adipocytes (*13*). Their deduced amino acid sequences, composed of 244 residues (85% identity between them), are structurally related to members of the short-chain dehydrogenase/reductase (SDR) family, which includes a large number of prokaryotic and eukaryotic enzymes with different specificities for coenzymes and substrates (*14*). The pulmonary CRs conserve two sequences, Tyr-X-X-X-Lys and Gly-X-X-X-Gly-X-Gly, which were demonstrated to be the active site and the coenzyme binding domain, respectively, by site-directed mutagenesis (*14* and references cited therein) and X-ray crystallographic studies (*15-17*) of several SDR family proteins.

Pulmonary CR utilizes both NADP(H) and NAD(H) as coenzymes, but K_m values for NADP(H) are much lower than those for NAD(H) (*4-6, 9*). Kinetic study has shown that pulmonary CR follows an ordered sequential mechanism with coenzyme-induced isomerization (*9*). The activation of the enzyme by fatty acids results in an increase of the K_m values for NADPH and substrate (*10*). Therefore,

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Abbreviations: CR, carbonyl reductase; SDR, short-chain dehydrogenase/reductase; K17H, Lys-17→His; K17R, Lys-17→Arg; K17S, Lys-17→Ser; R39A, Arg-39→Ala; CHX, cyclohex-2-en-1-ol; P3A, pyridine-3-aldehyde.

TABLE I. **Comparison of sequences around the coenzyme binding fold of the SDR family proteins, grouped according to their coenzyme specificity.**[a]

Enzyme		Sequence from the numbered residue	Ref.
NADP(H)-dependent		↓ ↓	
Mouse lung CR	9	RALVT**G**AG**K**G**I**G**I**GRDTVKALHAS.GAKVVAVT**R**T	(12)
Pig lung CR	9	RALVT**G**AG**K**G**I**G**I**GRDTVKALHVS.GARVVAVT**R**T	(11)
Human CR	7	VALVT**G**GN**K**G**I**G**I**LAIVRDLCRLFSGDVVLTA**R**D	(21)
Rat CR	7	VALVT**G**AN**K**G**I**G**I**FAIVRDLCRQFAGDVVLTA**R**D	(22)
Pig 20β-HSD	7	VALVT**G**AN**K**G**I**G**I**FAIVRDLCRKFLGDVVLTA**R**D	(21)
Mouse 11β-HSD/CR	36	KVIVT**G**AS**K**G**I**G**I**REMAYHLSKM.GAHVVLTA**R**S	(23)
Datura TR-I	23	TALVT**G**GS**K**G**I**G**I**YAIVEELAGL.GARVYTCS**R**N	(24)
Datura TR-II	11	TALVT**G**GS**R**G**I**G**I**YGIVEELASL.GASVYTCS**R**N	(24)
Eubacterium 7α-HSD	7	VILVTAST**R**G**I**G**I**LAIAQACAKE.GAKVYMGA**R**N	(25)
Magnaporthe ThnR	30	VALVT**G**AG**R**G**I**G**I**REMAMELGRRGCKVIVNYANS	(26)
Arabidopsis PcR	90	NVVVT**G**ASS**G**L**G**I**G**LATAKALAETGKWNVIMAC**R**D	(21)
NAD(H)-dependent		ββββ αααααααααααα ββββ	
3α,20β-HSD	8	TVIIT**G**GA**R**G**L**G**I**AEAARQAVAA.GARVVLADVL	(27)
E. coli 7α-HSD	13	CAIIT**G**AGA**G**I**G**K**E**IAITFATA.GASVVVSDIN	(28)
Rat DhpR	9	RVLVY**G**GRGAL**G**SRCVQAFRAR.NWWVASIDVV	(29)
Human 15-HPGD	7	VALVT**G**AAQ**G**I**G**RAFAEALLLK.GAKVALVDWN	(30)
Drosophila ADH	9	VIFVA**G**.LG**G**I**G**LDTSKQLLKRDLKNLVILD**R**I	(31)

[a] The consensus glycines and conserved Lys and Arg residues in the βαβ-fold are illustrated in bold face, and Lys-17 and Arg-39 of mouse lung CR by arrows. The secondary structure of the βαβ-fold is indicated above the *Streptomyces* 3α,20β-hydroxysteroid dehydrogenase (3α,20β-HSD) sequence on the basis of its crystal structure (*16*). A gap (.) is introduced to maximize alignment. Abbreviations: HSD, hydroxysteroid dehydrogenase; TR, tropinone reductase; ThnR, tetrahydroxynaphthalene reductase; PcR, protochlorophyllide reductase; DhpR, dihydropteridine reductase; 15-HPGD, 15-hydroxyprostaglandin dehydrogenase; ADH, alcohol dehydrogenase.

our current studies were focused on the binding interactions of the 2′-phosphate of NADP(H). Chemical modification study has suggested that Lys-17, which exists before the second Gly of the coenzyme-binding sequence of mouse lung CR, interacts with the 2′-phosphate of NADPH (*18*). The crystal structure of the ternary complex of mouse lung CR (with NADPH and 2-propanol) shows that, in addition to Lys-17, the side chain of Arg-39 has an electrostatic interaction with the 2′-phosphate (*19, 20*). The latter residue is highly conserved only in the NADP(H)-dependent enzymes of the SDR family, whereas the former residue is seen in about half of the NADP(H)-dependent enzymes and is substituted with Arg in the remainder (Table I). We have investigated the contributions of Lys-17 and Arg-39 to NADP(H) binding and to fatty acid activation by means of site-directed mutagenesis and comparing the kinetic and thermodynamic properties of binding of NADPH and NAD(P)$^+$ among the wild-type CR (WT) and mutant CRs. In this paper, we show that the 2′-phosphate of NADP(H) is recognized by the two positively charged residues, of which Lys-17 is required for the activation by fatty acids.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis—Four CR mutants, Lys-17 to His (K17H), Lys-17 to Arg (K17R), Lys-17 to Ser (K17S), and Arg-39 to Ala (R39A), were generated by means of the overlap-extension technique (*32*), using *Pfu* polymerase and partially complementary primer pairs that contain the codon of an altered amino acid (Table II). The entire coding region of the CR cDNA was amplified using the flanking primers, M3 and M6, which also contain recognition sites for *Eco*RI and *Hin*dIII, respectively (*12*). The complete coding regions of the mutated cDNAs were sequenced as previously described (*11*), in order to confirm the presence of the desired mutation and to ensure that no other mutation had occurred.

TABLE II. **Oligonucleotide primers used for site-directed mutagenesis.**

Mutation	Primer	Sequence[a]
K17H	Forward	5′-GGGGCAGGG<u>CAC</u>GGGATTGGA
	Reverse	5′-TCCAATCCC<u>GTG</u>CCCTGCCCC
K17R	Forward	5′-GGGGCAGGG<u>AGA</u>GGGATTGGA
	Reverse	5′-TCCAATCCC<u>TCT</u>CCCTGCCCC
K17S	Forward	5′-GGGGCAGGG<u>TCA</u>GGGATTGGA
	Reverse	5′-TCCAATCCC<u>TGA</u>CCCTGCCCC
R39A	Forward	5′-GCGGTGACT<u>GCG</u>ACCAACTC
	Reverse	5′-GAGTTGGT<u>CGC</u>AGTCACCGC

[a] The mutated codons of the primer pairs are underlined.

Expression and Purification—The WT and mutated cDNAs were expressed in *Escherichia coli* (JM105) cells, and the recombinant enzymes were purified from the 12,000×*g* supernatants of the homogenates of the cells (each 1 liter culture) as previously described (*12*).

Characterization of Protein Samples—Protein concentration of the crude extract and enzyme preparations during the purification was determined by Bradford's method (*33*) using bovine serum albumin as the standard.

The molecular mass of the CR subunit was assessed by SDS-polyacrylamide gel electrophoresis (*34*) on 12.5% gels using molecular mass markers (Pharmacia). The pI value of the purified enzyme was determined by isoelectric focusing (*35*) on 7.5% polyacrylamide gels containing 2% Ampholyte (Pharmacia) and 8 M urea using pI markers (Oriental

Yeast). Western-blot immunoanalysis using the anti-CR serum was carried out as previously described (*12*).

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. The standard reaction mixture for the dehydrogenase activity consisted of 80 mM potassium phosphate buffer, pH 7.0, 0.5 mM $NADP^+$, 2.0 mM cyclohex-2-en-1-ol (CHX), and enzyme, in a total volume of 2.0 ml. The activity of recombinant CRs in *E. coli* extracts and during the purification was assayed with 80 mM glycine-NaOH buffer, pH 10.0, instead of the phosphate buffer in the standard reaction mixture. The reductase activity was determined with NADPH and pyridine-3-aldehyde (P3A) as the coenzyme and substrate, respectively, unless otherwise noted. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation and oxidation of 1 μmol of NAD(P)H per min at 25°C.

The kinetic constants were determined by direct fitting to the Michaelis-Menten equation. Initially, two diagnostic plots for inhibition type were made, $1/V$ *versus* inhibitor concentration and S/V *versus* inhibitor concentration, at various NADP(H) concentrations. Values of K_i were determined by assuming the appropriate kinetic model for inhibition as described (*36*). 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%.

RESULTS AND DISCUSSION

Expression and Purification of CR Mutants—To elucidate the roles of Lys-17 and Arg-39 in NADP(H) binding, Arg and Ser were first chosen to replace Lys-17 because Arg or Ser is the residue at position 17 of mouse lung CR in some NADP(H)-dependent enzymes of the SDR family (Table I). Since, of the two mutant enzymes, K17R showed no significant change in the kinetic parameters (described below), we further replaced Lys-17 with His, which is a basic amino acid, but is not positively charged under the assay conditions of pH 7.0. The Ala mutant was chosen to examine the effect of removing the charge at position 39.

The five forms of mouse lung CR were expressed in *E. coli*: K17H, K17R, K17S, R39A, and WT. Although the activity of native CR and WT has been determined with 4-nitroacetophenone as the substrate, the enzymes efficiently catalyze the $NADP^+$-linked oxidation of alicyclic alcohols such as CHX (*5, 12*). The CHX dehydrogenase activity was assayed to detect the expression of CR in the 12,000 × *g* supernatants of the cell homogenates, since no endogenous CHX dehydrogenase activity could be detected in *E. coli* cells that exhibit low 4-nitroacetophenone reductase activity (*12*). The specific activities of the recombinant enzymes and their percentages in the total soluble proteins in the *E. coli* extracts were different depending on the mutations, and the specific activity of the purified enzymes ranged from 2.5 to 7.1 units/mg (Table III). No apparent effects of the mutations were observed on the purification of the recombinant CRs: The five enzymes showed almost the same elution patterns from the three column chromatographies on Blue-Sepharose, Sephadex G-100 and hydroxylapatite as those of WT, which is known to be tetrameric (*12*). The purified enzymes showed single bands with the same molecular mass of 29 kDa on SDS-polyacrylamide gel electrophoresis (Fig. 1A), and reacted similarly with the CR antibody on Western blot immunoanalysis (Fig. 1B). The purified enzymes also showed single bands on isoelectric focusing (Fig. 1C). The pI value of K17R was similar to that of WT, whereas the values of K17H, K17S, and R39A were lower than that of WT due to the loss of the positive charge of Lys-17 or Arg-39.

Kinetic Characterization—The kinetic effects of the mutations were assessed by comparing their kinetic parameters in the forward (with P3A as the substrate) and reverse (with CHX as the substrate) directions with those of WT (Table IV). In the forward reaction, the mutagenesis of Lys-17 and Arg-39 to uncharged residues (His, Ser, or Ala) produced 3- to 7-fold increases in the K_m for NADPH, and the K_m values for P3A increased more than 10-fold for K17H and K17S. The effect of the mutation on the k_{cat} values was relatively small for R39A, but was greater for

TABLE III. **Specific activities of WT and mutant enzymes in the extract of *E. coli* cells, and summary of their purification.**

	WT	K17H	K17R	K17S	R39A
In the extract					
Specific activity[a]	0.2	0.03	0.2	0.1	0.03
% of soluble protein	3.3	1.1	3.5	0.8	0.9
Purified enzyme					
Recovery (%)	23	8	10	14	20
Total yield (mg)	4.1	0.9	2.5	1.2	1.5
Specific activity[a]	4.6	2.5	4.5	7.1	3.8

[a] The activity (units/mg) was assayed with CHX as the substrate at pH 10.

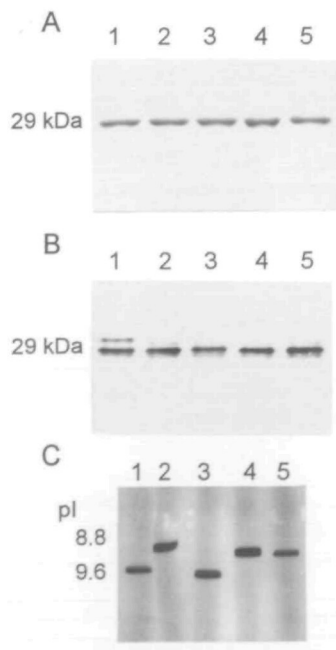

Fig. 1. **SDS-polyacrylamide gel electrophoresis (A), Western blot immunoanalysis (B), and isoelectric focusing (C) of purified WT and mutant enzymes.** Lanes: 1, WT; 2, K17H; 3, K17R; 4, K17S; 5, R39A. Lanes contain 1 μg (A), 0.1 μg (B), or 5 μg (C) of enzyme. Enzymes in gels were stained with Coomassie Brilliant Blue (A and C), or with the anti-CR serum as the first antibody (B). The molecular mass or pI values of the enzymes are indicated at the left of the respective panels.

TABLE IV. **Kinetic parameters for P3A reduction and CHX oxidation by WT and mutant enzymes.**

Parameter	WT	K17H	K17H/WT	K17R	K17R/WT	K17S	K17S/WT	R39A	R39A/WT
(Reduction)[a]									
K_m for NADPH (μM)	1.0	4.5	4.5	1.2	1.2	7.2	7.2	3.5	3.5
K_m for P3A (μM)	16	160	10	33	2.1	230	14	24	1.5
k_{cat} NADPH[b] (s^{-1})	1.2	3.9	3.3	1.7	1.4	5.9	4.9	1.4	1.2
k_{cat}/K_m for NADPH ($s^{-1}\cdot mM^{-1}$)	1,200	870	0.7	1,400	1.2	820	0.7	400	0.3
k_{cat}/K_m for P3A ($s^{-1}\cdot mM^{-1}$)	75	24	0.3	52	0.7	26	0.3	58	0.8
(Oxidation)[a]									
K_m for $NADP^+$ (μM)	3.0	34	11	8.2	2.7	25	8.3	23	7.7
K_m for CHX (μM)	280	400	1.4	330	1.2	560	2.0	300	1.1
k_{cat} $NADP^+$[b] (s^{-1})	2.4	5.1	2.1	4.1	1.7	4.9	2.0	3.6	1.5
k_{cat}/K_m for $NADP^+$ ($s^{-1}\cdot mM^{-1}$)	800	150	0.2	500	0.6	200	0.2	160	0.2
k_{cat}/K_m for CHX ($s^{-1}\cdot mM^{-1}$)	8.6	13	1.5	12	1.4	8.8	1.0	12	1.4
K_m for NAD^+ (mM)	1.8	3.9	2.2	1.1	0.6	1.0	0.6	3.6	2.0
K_m for CHX (μM)	700	880	1.3	580	0.8	740	1.1	660	0.9
k_{cat} NAD^+[b] (s^{-1})	1.4	1.6	1.1	2.1	1.5	1.8	1.3	2.1	1.5
k_{cat}/K_m for NAD^+ ($s^{-1}\cdot mM^{-1}$)	0.8	0.4	0.5	1.9	2.5	1.8	2.3	0.6	0.8
k_{cat}/K_m for CHX ($s^{-1}\cdot mM^{-1}$)	2.0	1.8	0.9	3.6	1.8	2.4	1.2	3.2	1.6

[a]The fixed substrate and coenzyme were 5 mM P3A and 0.1 mM NADPH (in the reduction reaction), and 5 mM CHX and 0.5 mM $NADP^+$ or 10 mM NAD^+ (in the oxidation reaction), respectively. [b]The values were calculated from the V_{max} values obtained with the coenzymes as the variable coenzyme substrates and on the basis of the CR subunit molecular weight of 26 kDa (*12*).

TABLE V. **K_i values for coenzymes.**

Inhibitor	WT	K17H	K17H/WT	K17R	K17R/WT	K17S	K17S/WT	R39A	R39A/WT
$NADP^+$[a] (μM)	1.0	30	30	6.8	6.8	92	92	18	18
NAD^+[a] (mM)	3.5	4.2	1.2	5.6	1.6	2.0	0.6	4.0	1.1
NADPH[b] (μM)	1.2	6.1	5.1	1.5	1.3	8.8	7.4	5.4	4.5

[a]The values for $NAD(P)^+$ were determined in the reduction reaction with NADPH (varied coenzyme) and 5 mM P3A (fixed substrate). [b]The values for NADPH were obtained in the dehydrogenase reaction with $NADP^+$ (varied coenzyme) and 5 mM CHX (fixed substrate), except that 10 mM CHX was used for determination of the values of K17S and R39A enzymes.

K17H and K17S. The combined effects of these changes were to decrease the k_{cat}/K_m values for the coenzyme and substrate. The mutation of Lys-17 to Arg produced only minor changes in the kinetic parameters. In the $NADP^+$-linked oxidation of CHX, the mutations, except the replacement of Lys-17 to Arg, resulted specifically in more than 7-fold increases in the K_m for $NADP^+$ and about 5-fold decreases in the k_{cat}/K_m for $NADP^+$. In contrast, such great effects of the mutations were not observed in the kinetic parameters of the NAD^+-linked oxidation of CHX.

As the reaction catalyzed by guinea-pig lung CR follows a di-iso ordered Bi Bi mechanism (*9*), double reciprocal plots of initial velocity *versus* NADPH concentration at four fixed levels of P3A yielded a series of intersecting lines for WT, K17H, and R39A (data not shown). The pattern is consistent with a sequential mechanism for the mouse lung CRs. Such experiments were not carried out for the other mutant enzymes, but product inhibition studies confirmed that all the recombinant mouse lung CRs followed a sequential mechanism. $NADP^+$ gave a linear competitive inhibition pattern with respect to NADPH, and, in the reverse reaction, NADPH was a competitive inhibitor with respect to $NADP^+$. In addition, NAD^+ was also a competitive inhibitor with respect to NADPH. The K_i values for the coenzymes are summarized in Table V. Since the K_i value implies the dissociation constant for the competitive inhibitor in this kinetic mechanism, the effects of the mutations on the affinities for the coenzymes can be assessed by comparing the K_i values among the recombinant CRs. There were more than 18-fold increases in the K_i for $NADP^+$ for K17H, K17S, and R39A, but K17R showed a relatively small increase of 6-fold. Similarly, the K_i for NADPH increased more than 4-fold for K17H, K17S, and R39A, and little effect was seen for K17R. If Lys-17 and Arg-39 interact with the 2′-phosphate of $NADP^+$, the mutations would be expected to have no effect on the K_i for NAD^+, which lacks the 2′-phosphate group. As the mutations had little effect on the kinetic parameters of the NAD^+-linked dehydrogenase activity (Table IV), the K_i values for NAD^+ were similar among WT and the mutant enzymes. These results indicate that both Lys-17 and Arg-39 interact with the 2′-phosphate of NADP(H). Of the mutant enzymes, K17H and K17S also increased the K_m values for P3A in the forward reaction, but not those for CHX in the reverse reaction. This may be caused by the use of substrates with different structures in the two reactions. Alternatively, the two mutations might affect the catalytic action of the enzyme by the loss of potential conformational changes in the enzyme normally induced by NADPH binding in the forward reaction (*9*).

The present results have confirmed the X-ray crystallography finding that both Lys-17 and Arg-39 have electrostatic interactions with the 2′-phosphate of NADPH (*20*). In addition, the relatively small effect of the mutation of Lys-17 to Arg on the K_m and K_i values for NADP(H) suggests that a positively charged group of either Lys or Arg at this position interacts with the 2′-phosphate of NADP(H). In addition to Arg-39, either Lys or Arg at position 17 of mouse lung CR sequence is highly conserved in many NADP(H)-dependent enzymes of the SDR family (Table I), so the positively charged residue may play a role in the specificity of the SDR family enzymes for NADP(H) over NAD(H), like Lys-17 of mouse lung CR. Of the NADP(H)-dependent SDR family proteins, some enzymes

TABLE VI. **Thermodynamic effects of removing K-17 or R-39 of MLCR.**

Energy*	Modification	Coenzyme	WT	K17H	K17R	K17S	R39A
$\Delta\Delta G_{(-2P)}$	$NADP^+ \rightarrow NAD^+$	—	4.8	2.9	4.0	1.8	3.2
$\Delta\Delta G_{(-K \text{ or } R)}$	Removing K17 or R39	$NADP^+$	—	2.0	1.2	2.7	1.7
$\Delta\Delta G_{(-K \text{ or } R)}$	Removing K17 or R39	NAD^+	—	0.11	0.28	−0.33	0.08

*Free energy changes (kcal/mol) for removing the 2′-phosphate of NADPH ($\Delta\Delta G_{(-2P)}$) and for removing K-17 or R-39 ($\Delta\Delta G_{(-K \text{ or } R)}$) were calculated using the following equations: $\Delta\Delta G_{(-2P)} = -RT \ln(K_{NADP^+}/K_{NAD^+})$ or $\Delta\Delta G_{(-K \text{ or } R)} = -RT \ln(K_{WT}/K_{mutant})$, where K is the dissociation constant of the coenzyme binding to either WT or one of the mutant enzymes, R is the gas constant, and T is the temperature in kelvins (*38*).

such as protochlorophyllide reductase and tetrahydroxynaphthalene reductase lack one of the two basic residues at positions 17 and 39, but they conserve another basic residue (Table I). Since K17S and R39A of mouse lung CR still showed preference for NADP(H) over NAD(H), the remaining one positively charged residue may contribute to the coenzyme specificity of the two SDR family enzymes, although other residues of the enzymes would also interact with the 2′-phosphate group. In conformity with this view, the two basic residues are not conserved in many NAD(H)-dependent enzymes of this family. Although some NAD(H)-dependent enzymes such as 3α,20β-hydroxysteroid dehydrogenase and *Drosophila* alcohol dehydrogenase include an Arg residue, at positions corresponding to Lys-17 and Arg-39, respectively, of mouse lung CR sequence, site-directed mutagenesis and crystallographic studies have shown that Asp (corresponding to the residue at position 37 of mouse lung sequence) confers the coenzyme specificity upon the two NAD(H)-dependent enzymes by forming hydrogen bonds to the hydroxyl groups of the adenine ribose as well as by introducing electrostatic repulsion between the negative charges of its carboxylate group and the 2′-phosphate of NADP(H) (*16, 37*).

Thermodynamic Contribution of Lys-17 and Arg-39—We also analyzed the thermodynamic effects of removing the 2′-phosphate of $NADP^+$ or removing Lys-17 and Arg-39 on the coenzyme binding energy, in order to gain insight into the strength and specificity of the coenzyme binding to mouse lung CR (Table VI). The change in the binding energy for 2′-phosphate removal in the presence of both Lys-17 and Arg-39 (*i.e.*, WT) was 4.8 kcal/mol, whereas the values were lowered by 1.8-3.2 kcal/mol when one of the two basic residues was mutated to the uncharged residues (*i.e.*, K17H, K17S, and R39A). In addition, the change in the binding energy toward $NADP^+$ on replacing Lys-17 or Arg-39 with the uncharged residues was much larger than that toward NAD^+. This indicates that both Lys-17 and Arg-39 contribute significantly to the binding energy of $NADP^+$ through the electrostatic forces between their positively charged groups and the negatively charged phosphate group of the coenzyme.

The change in the binding energy for 2′-phosphate removal with K17R was larger than the values obtained with the other mutants, and the change in the binding energy toward $NADP^+$ on this mutation was smallest, which also supports the importance of the presence of a positively charged group of either Lys or Arg at position 17 for the coenzyme binding. When the values calculated for the other mutants were compared, the change in the binding energy for 2′-phosphate removal in the absence of the positively charged residue at position 17 (*i.e.*, K17H and K17S) was smaller than that in the absence of Arg-39 (*i.e.*, R39A). Moreover, the change in the binding energy

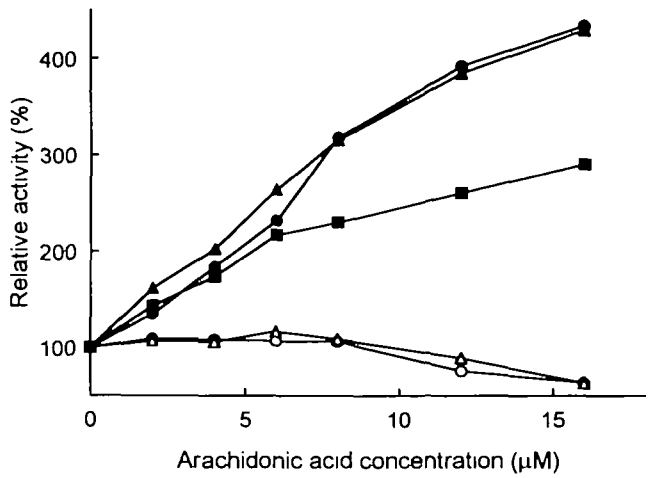

Fig. 2. **Effects of arachidonic acid on reductase activities of WT and mutant enzymes.** Enzymes: WT (●), K17H (○), K17R (▲), K17S (△), and R39A (■). The activity was assayed with 0.1 mM NADPH and 1 mM 4-nitroacetophenone as the coenzyme and substrate, respectively, at pH 7.0, as described (*12*).

toward $NADP^+$ on replacing Lys-17 with His or Ser was larger than that on replacing Arg-39 with Ala. Although we can not rule out the possibility that the mutation of one of the basic residues disrupts the interaction of the other residue with the 2′-phosphate of NADP(H), Lys-17 may contribute more significantly to the binding energy of $NADP^+$ than does Arg-39. This is also compatible with X-ray crystallography data of mouse lung CR, showing that the side-chain of Lys-17 is in closer contact with the oxygen atoms of the 2′-phosphate of NADPH than is the side-chain of Arg-39 (*20*).

The values of 1.7-2.7 kcal/mol for the salt bridge made by Lys-17 or Arg-39 of mouse lung CR are slightly smaller than the values reported for Lys-2′-phosphate interaction in $NADP^+$-dihydrofolate reductase (2.8 kcal/mol, *39*) and for Arg-2′-phosphate interaction in $NADP^+$-aldose reductase (4.3 kcal/mol, *40*) and NADPH-cytochrome P-450 reductase (3.0 kcal/mol, *38*). However, the sum of the values for the salt bridges made by the two basic residues of mouse lung CR is comparable to or greater than the values reported for the above enzymes. No other residue that interacts with the 2′-phosphate of NADPH has been found in the high-resolution three-dimensional structure of the enzyme (*20*). Thus, the involvement of both Lys-17 and Arg-39 in recognizing the 2′-phosphate of NADP(H) leads to the strong coenzyme specificity of the enzyme for NADP(H) over NAD(H).

Effects on Activation by Arachidonic Acid—Under conditions where WT is activated up to 4-fold (*12*), arachidonic

acid stimulated the reductase activities of K17R and R39A, although the stimulation of R39A was slightly less than those of WT and K17R at high concentrations of the activator (Fig. 2). In contrast, K17H and K17S were not activated at all. A previous study on the fatty acid activation of pig lung CR indicated that the common structural requirement for all the activator species is a negatively charged carboxylate group in addition to the hydrophobic alkyl chains, and it was proposed that a nonessential activator mechanism operates, in which fatty acid binds both the enzyme and the enzyme-coenzyme complex (*10*). The abolition of the activation by the mutations of Lys-17 to uncharged residues and the retention of this property in the presence of Arg at this position suggest that the negatively charged group of the activator electrostatically binds to a positively charged side-chain of Lys or Arg at position 17, and that the activator molecule binds within the coenzyme-binding site of CR. Since Arg-39, which is expected to be proximal to Lys-17 (*20*), was not required for the activation, other hydrophobic amino acid residues around the coenzyme-binding site of CR may also play significant roles in the binding of the activator molecule.

The activation by arachidonic acid resulted in about 8-fold increase of the K_m value of WT for NADPH (8.5 μM in the presence of 10 μM arachidonic acid), similarly to the case of pig lung CR (*10*). This is similar to the increase in the K_m for NADPH, as well as in the k_{cat} value, upon replacing Lys-17 with His or Ser. Although conformational change of the pig lung enzyme by activator binding has been suggested (*10*), we propose an additional activation mechanism, *i.e.*, that the binding of the activator to Lys-17 causes perturbation of the interaction between the 2′-phosphate of NADPH and Lys-17, which leads to rapid release of the products. Fatty acid activation has been reported for pulmonary CRs of pig (*10*) and mouse (*12*), but not for other NADP(H)-dependent enzymes of the SDR family, most of which conserve the positively charged residue at position 17 (Table I). The pulmonary CR may differ from the other NADP(H)-dependent enzymes in its hydrophobic site required for proper orientation of the activator molecule to interact with Lys-17.

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