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Inactivation of Rabbit Liver Carbonyl Reductase by Phenylglyoxal and 2,3,4-Trinitrobenzenesulfonate Sodium

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The chemical modifications of rabbit liver carbonyl reductase (RLCR) with phenylglyoxal (PGO) and 2,3,4-trinitrobenzenesulfonate sodium (TNBS), which are respective chemical modifiers of arginine and lysine residues, were examined. RLCR was rapidly inactivated by these modifiers. Kinetic data for the inactivation demonstrated that each one of arginine and lysine residues is essential for catalytic activity of the enzyme. Furthermore, based on the protective effects of $NADP^+$, NAD^+ and their constituents against the inactivation of RLCR by PGO and TNBS, we propose the possibility that the functional arginine and lysine residues are located in the coenzyme-binding domain of RLCR and interact with the 2′-phosphate group of NADPH.

Keywords: Carbonyl reductase; Chemical modification; Rabbit liver; Arginine residue; Lysine residue; Coenzyme-binding domain

INTRODUCTION

Carbonyl reductase (EC 1.1.1.184) is considered as a drug-metabolizing enzyme catalyzing the ketone-reduction of drugs such as acetohexamide, befunolol and daunorubicin.[1–3] A variety of carbonyl reductases have been purified from the liver, kidney, lung, brain and heart of mammalian species.[4–10] We have purified a carbonyl reductase from the cytosolic fraction of rabbit liver, using befunolol as a substrate,[11] since the liver is most important organ in the metabolism of drugs. As expected, the purified rabbit liver carbonyl reductase (RLCR) had the ability to effectively reduce ketone-containing drugs.[11]

It has been reported that several NADPH-dependent enzymes including carbonyl reductase have arginine and lysine residues located in their coenzyme-binding domain.[12–15] These amino acid residues have been demonstrated to serve a principal role in the binding and specificity of coenzyme. For example, NADPH-dependent aldehyde reductase purified from pig kidney has functional arginine and lysine residues in its coenzyme-binding domain.[12,16] Furthermore, our work[17] has revealed that indomethacin, a nonsteroidal anti-inflammatory drug, interacts with or near one essential arginine residue located in the coenzyme-binding domain of rabbit kidney carbonyl reductase. It is possible that nonsteroidal anti-inflammatory drugs inhibit carbonyl reductases, by interacting with an arginine or lysine residue located in the coenzyme-binding domain. The purpose of the present study is to elucidate that the functional amino acid residue is located in the coenzyme-binding domain of RLCR, as well as rabbit kidney carbonyl reductase. Consequently we attempted the inactivation of RLCR by phenylglyoxal (PGO) and 2,3,4-trinitrobenzenesulfonate sodium (TNBS), which are respective chemical modifiers of arginine and lysine residues. The protective effects of $NADP^+$, NAD^+ and their constituents against the inactivation of RLCR by PGO and TNBS were also examined.

MATERIALS AND METHODS

Materials

Carbonyl reductase was purified from the cytosolic fraction of rabbit liver as described previously.[11] Acetohexamide used as the substrate was obtained

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from Shionogi (Osaka, Japan). The purified enzyme (rabbit liver carbonyl reductase, RLCR) was a homogeneous protein on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Befunolol (Kaken Pharmaceutical, Tokyo, Japan), loxoprofen (Sankyo, Tokyo, Japan) and daunorubicin (Meiji Seika, Tokyo, Japan) were provided by the manufacturers. PGO, TNBS and 4-acetylpyridine (4-AP) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). 2′-AMP, 5′-AMP, 5′-ADP, 2′,5′-ADP, 2′-phospho-5′-ADP-ribose, nicotinamide mononucleotide (NMN) and NAD^+ were obtained from Sigma (St. Louis, MO, USA). NADPH and $NADP^+$ were products from Oriental Yeast (Tokyo, Japan). All other chemicals were of reagent grade.

Enzyme Assay

RLCR activity was assayed spectrophotometrically by monitoring NADPH oxidation at 340 nm. The reaction mixture in a total volume of 0.7 ml consisted of 0.1 M sodium potassium phosphate buffer (pH 6.5), 0.25 mM NADPH, substrate at various concentrations and the purified enzyme. The reaction was initiated by adding the enzyme sample, and the decrease in absorbance at 340 nm was monitored with a Shimadzu UV-240 spectrophotometer. One unit of enzyme activity was defined as the amount catalyzing the oxidation of 1 μmole/min of NADPH at 30°C. Protein concentration was determined by the method of Lowry *et al.*[18] using bovine serum albumin as the standard. The K_m and V_{max} values of the enzyme for ketone-containing drugs were calculated using a computer program for least-squares linear regression of double-reciprocal plots.

Chemical Modification

After pre-incubation for 3 min, the enzyme (4.2–6.6 μM) was incubated with PGO (2.5–10 mM) or TNBS (0.25–2.0 mM) in 0.1 M sodium potassium phosphate buffer (pH 7.4) at 30°C. In the case of modification with TNBS, the reaction mixture was kept in the dark. Aliquots (10–20 μl) were withdrawn at appropriate intervals and diluted, then the enzyme activities were assayed using 1.0 mM acetohexamide as the substrate. A control containing no modifier was routinely included and the residual activity (%) was calculated relative to the control.

RESULTS

Substrate Specificities of RLCR for Drugs having a Ketone Group

RLCR has been purified from cytosolic fraction of rabbit liver using befunolol as the substrate.[11] In this study, acetohexamide was used as a substrate instead of befunolol. Thus, we re-examined the substrate specificity of RLCR for drugs having a ketone group within their chemical structures. RLCR was confirmed to catalyze the ketone-reduction of various drugs such as befunolol, loxoprofen and daunorubicin including acetohexamide, and function as a drug-metabolizing enzyme (Table I).

Inactivation of RLCR by PGO

We investigated the chemical modification of RLCR with PGO, a typical chemical modifier of arginine residues. RLCR was rapidly inactivated by PGO and the inactivation reaction was time- and concentration-dependent (Figure 1A). The secondary plots of the pseudo-first-order rate constants *versus* the concentrations of PGO gave a straight line through the origin (Figure 1B), indicating that the modification is the result of a simple bimolecular reaction, in which a reversible PGO-enzyme complex is not formed before inactivation. Furthermore, a straight line with a slope of 1.1 was obtained from the double logarithmic plots according to the method of Levy *et al.*[19] (Figure 1C). This implies that 1 mol of the enzyme is inactivated by 1 mol of PGO, that is, that one arginine residue is essential for the enzyme activity.

Inactivation of RLCR by TNBS

The chemical modification of RLCR with TNBS, a typical chemical modifier of lysine residues, also caused time- and concentration-dependent loss of the enzyme activity, as shown in Figure 2A. Kinetic data for the inactivation were similar to those obtained in the inactivation of RLCR by PGO (Figures 2B and 2C), indicating that one lysine residue is essential for the enzyme activity.

Protective Effects of $NADP^+$ and 4-Acetylpyridine (4-AP) against the Inactivation of RLCR by PGO and TNBS

The protective effects of $NADP^+$ (coenzyme) and 4-AP (substrate) against the inactivation of RLCR by PGO were examined. $NADP^+$ was used as a coenzyme instead of NADPH, because PGO is

TABLE I Substrate specificity of RLCR for ketone-containing drugs

Drugs	K_m (mM)	V_{max} (units/mg)
Acetohexamide	0.92	3.39
Befunolol	1.44	4.08
Loxoprofen	0.52	1.06
Daunorubicin	3.00	41.0

The values are the means of at least two experiments.

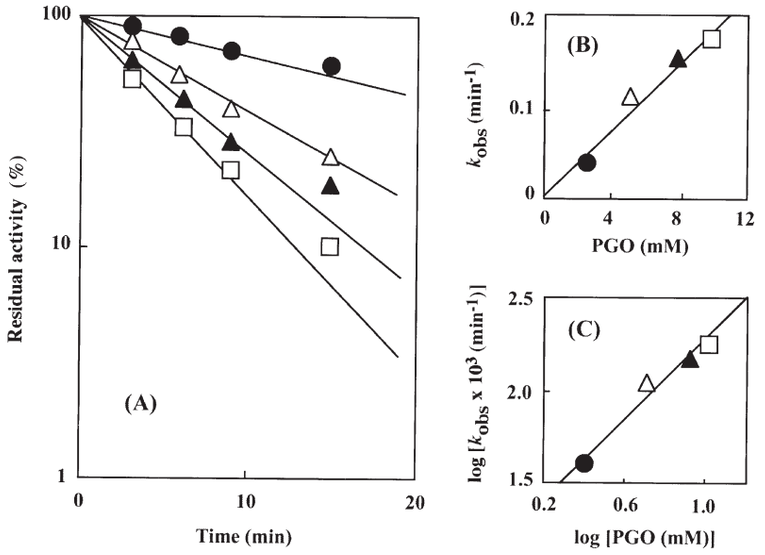

FIGURE 1 Inactivation of RLCR by PGO. (A) The enzyme was incubated with 2.5 mM (●), 5.0 mM (△), 7.5 mM(▲) and 10 mM (□) PGO. (B) Secondary plots of the pseudo-first-order constant (k_{obs}) versus the concentration of PGO. (C) Double logarithmic plots for the determination of the order of the inactivation reaction.

a substrate for the enzyme in the presence of NADPH. Our work has revealed that the enzyme reaction follows an ordered Bi–Bi mechanism, in which NADPH binds to the enzyme first and $NADP^+$ leaves last.[20] Thus, if the essential amino acid residue is located in the coenzyme-binding domain, the inactivation of RLCR by PGO will be protected in the presence of only $NADP^+$. Whereas if the essential amino acid residue is located in the substrate-binding domain, the inactivation of RLCR by PGO will be protected in the presence of both $NADP^+$ and 4-AP. As shown in Figure 3, the protective effect against the inactivation was observed in the presence of only $NADP^+$, but was unaffected by the addition of 4-AP. These results indicate that one essential arginine residue is located in the coenzyme-binding domain. Similar protective effects were found against the inactivation of the enzyme by TNBS (data not shown), indicating the presence of one essential lysine residue located in coenzyme-binding domain.

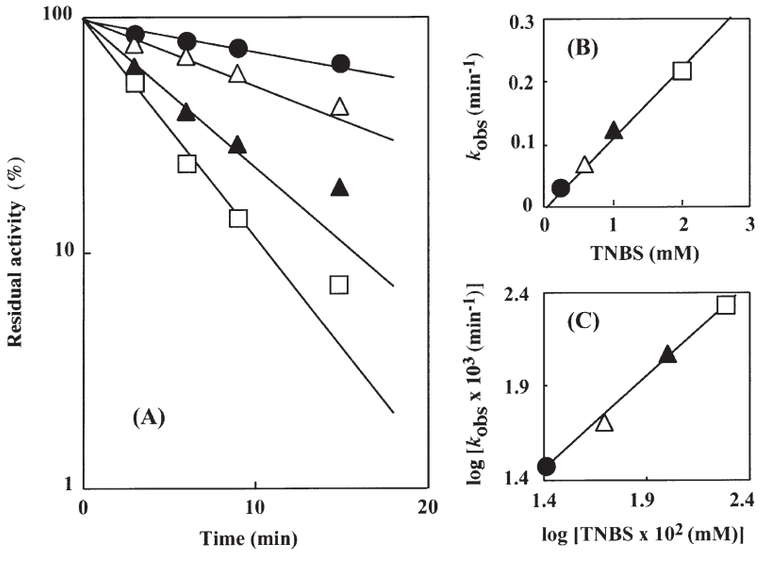

FIGURE 2 Inactivation of RLCR by TNBS. (A) The enzyme was incubated with 0.25 mM (●), 0.5 mM (△), 1.0 mM (▲) and 2.0 mM (□) TNBS. (B) Secondary plots of the pseudo-first-order constant (k_{obs}) versus the concentration of TNBS. (C) Double logarithmic plots for the determination of the order of the inactivation reaction.

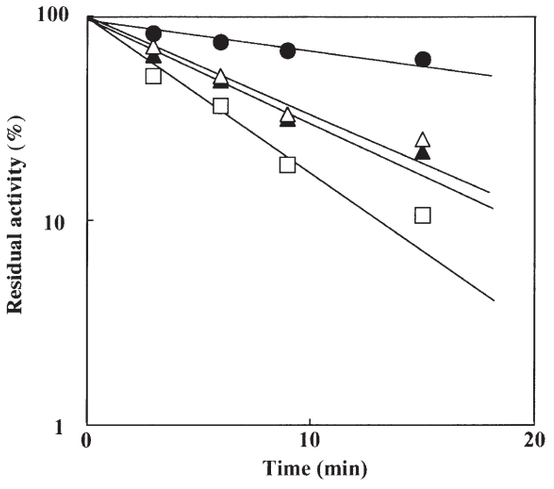

FIGURE 3 Protective effects of $NADP^+$ and substrate 4-AP against the inactivation of RLCR by PGO. The enzyme was incubated with 10 mM PGO (□), 10 mM PGO + 1 μM $NADP^+$ (▲), 10 mM PGO + 1 μM $NADP^+$ + 1.0 mM 4-AP (△) and 10 mM PGO + 5 μM $NADP^+$ (●).

Protective Effects of $NADP^+$, NAD^+ and their Constituents against the Inactivation of RLCR by PGO and TNBS

Table II summarizes the protective effects of $NADP^+$, NAD^+ and their constituents against the inactivation of RLCR by PGO and TNBS. The half-life ($t_{1/2}$) was estimated from the apparent pseudo-first-order rate constant. As described above, $NADP^+$ effectively protected the inactivation of the enzyme by PGO and TNBS, but NAD^+ did not. 2′-AMP, 2′,5′-ADP and 2′-phospho-5′-ADP-ribose afforded protective effects against the inactivation of the enzyme by PGO and TNBS, whereas 5′-AMP, 5′-ADP or NMN, which lack a 2′-phosphate group, were without effect. The results suggest that the functional arginine and lysine residues located in the coenzyme-binding domain of RLCR interact with the 2′-phosphate group of NADPH.

DISCUSSION

Kinetic data for the inactivation of RLCR by PGO and TNBS provide evidence that each one of the arginine and lysine residues is essential for the enzyme activity. Furthermore, this study demonstrates that the functional arginine and lysine residues are located in the coenzyme-binding domain of RLCR, based on the protective effect of $NADP^+$ against the inactivation of RLCR by PGO and TNBS.

Sciotti and Wermuth[15] have shown the importance of arginine and lysine residues at positions of 38 and 15, respectively, for NADPH binding in human carbonyl reductase. The mutant enzyme R38Q (substitution of Gln for Arg-38 of human carbonyl reductase) exhibits a significant enzyme activity. Interestingly, R38Q is inactivated by PGO to the same extent as the native enzyme, suggesting that, in addition to Arg-38, at least one other arginine residue contributes to the binding of NADPH. As is evident from Figure 4, the amino acid sequence deduced from the RLCR gene (*RCBR5*) has a high identity with that of human carbonyl reductase.[21,22] However, in the amino acid sequence of RLCR, position 38, unlike that of human carbonyl reductase, is Gln. One essential arginine residue of RLCR probably corresponds to an arginine residue other than position 38 responsible for NADPH binding in human carbonyl reductase, although the position number in the amino acid sequence remains to be determined.

The limited data for the protective effects of $NADP^+$, NAD^+ and their constituents against the inactivation of RLCR by PGO and TNBS lead us to suggest that arginine and lysine residues serve as positively charged sites and interact with the 2′-phosphate group of NADPH. Similar results are observed for arginine and lysine residues in the coenzyme-domain of enzymes belonging to the families of aldo–keto reductase and short-chain dehydrogenase/reductase (SDR): aldose and aldehyde reductases, and carbonyl reductase are members of aldo-keto reductases and SDRs, respectively.[23,24] For example, crystallographic analysis of recombinant human placenta aldose reductase complexed with NADPH reveals that Arg-268 and Lys-262 residues are involved in the interaction with the 2′-phosphate group of NADPH.[25] Two basic residues, Arg-39 and Lys-17, in mouse tetrameric carbonyl reductase also promote NADPH binding by interacting with the 2′-phosphate group.[14,26] It is reasonable to assume that arginine and lysine residues located in the coenzyme-binding domain of aldo-keto reductases and SDRs play a role in the binding and specificity of coenzyme.

TABLE II Protective effects of $NADP^+$, NAD^+ and their constituents against the inactivation of RLCR by PGO and TNBS

	$t_{1/2}$ (min)	
Coenzyme (concentration)	PGO	TNBS
Control	4	3
$NADP^+$ (5 μM)	48	39
NAD^+ (1 mM)	4	4
2′-AMP (1 mM)	12	10
5′-AMP (1 mM)	4	3
5′-ADP (1 mM)	4	4
2′,5′-ADP (1 mM)	12	31
2′-phospho-5′-ADP-ribose (1 mM)	21	48
NMN (1 mM)	5	3

The enzyme was incubated with 10 mM PGO or 2.0 mM TNBS. The values are the means of at least two experiments.

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                                                *
HCR     MSSGIHVALVTGGNKGIGLAIVRDLCRLFSGDVVLTARDVTRGQAAVQQL   50
RLCR    MPSDRRVALVTGANKGVGFAITRALCRLFSGDVLLTAQDEAQGQAAVQQL   50

HCR     QAEGLSPRFHQLDIDDLQSIRALRDFLRKEYGGLDVLVNNAGIAFKVADP  100
RLCR    QAEGLSPRFHQLDITDLQSIRALRDFLRRAYGGLNVLVNNAVIAFKMEDT  100

HCR     TPFHIQAEVTMKTNFFGTRDVCTELLPLIKPQGRVVNVSSIMSVRALKSC  150
RLCR    TPFHIQAEVTMKTNFDGTRDVCTELLPLMRPGGRVVNVSSMTCLRALKSC  150

HCR     SPELQQKFRSETITEEELVGLMNKFVEDTKKGVHQKEGWPSSAYGVTKIG  200
RLCR    SPELQQKFRSETITEEELVGLMKKFVEDTKKGVHQTEGWPDTAYGVTKMG  200

HCR     VTVLSRIHARKLSEQRKGDKILLNACCPGWVRTDMAGPKATKSPEEGAET  250
RLCR    VTVLSRIQARHLSEHRGGDKILVNACCPGWVRTDMGGPNATKSPEEGAET  250

HCR     PVYLALLPPDAEGPHGQFVSEKRVEQW                          277
RLCR    PVYLALLPPDAEGPHGQFVMDKKVEQW                          277
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FIGURE 4 Amino acid sequences of RLCR and human carbonyl reductase (HCR). The sequence data were cited from references 21 and 22. Asterisk shows Arg-38 of HCR.

It has been reported that nonsteroidal anti-inflammatory drugs including indomethacin are potent inhibitors of carbonyl reductases.[27,28] RLCR is also known to be inhibited by a variety of nonsteroidal anti-inflammatory drugs.[29] Further studies are in progress to elucidate whether or not nonsteroidal anti-inflammatory drugs interact with the functional arginine and lysine residues located in the coenzyme-binding domain of RLCR.

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