Carbonyl Reductase Purified from Rabbit Liver Is Not the Product of a Carbonyl Reductase Gene (*RCBR5* or *RCBR6*) Cloned from the Rabbit Liver cDNA Library

Yorishige IMAMURA,^{*,a} Toshihisa KOGA,^a Masaki OTAGIRI,^a Kumiko SATOH,^b and Akira HARA^b

Faculty of Pharmaceutical Sciences, Kumamoto University,^a 5-1, Oe-honmachi, Kumamoto 862, Japan and Laboratory of Biochemistry, Gifu Pharmaceutical University,^b 5-6-1, Mitahora-higashi, Gifu 502, Japan.

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Six peptides were obtained by the digestion of carbonyl reductase purified from rabbit liver. The amino acid sequences of the six peptides were virtually identical to the corresponding regions in amino acid sequences deduced from two cloned carbonyl reductase genes (*RCBR5* and *RCBR6*). However, there was a difference of one amino acid residue between the sequences of peptides from the purified enzyme and the corresponding region in the amino acid sequences deduced from the two cDNAs. The purified carbonyl reductase was confirmed to exhibit no reactivity towards menadione, even though the transient expression of the two cDNA for rabbit liver carbonyl reductase has been reported to cause a marked increase of menadione reductase activity in COS7 cells. The enzyme purified from rabbit liver was inactivated by thiol-specific reagents, 5,5'-dithiobis(2-nitrobenzoic acid) and sodium tetrathionate, suggesting that menadione probably interacts with the functional cysteine residue(s), and cannot serve as a substrate of the purified enzyme. Based on these results, it is concluded that the carbonyl reductase purified from rabbit liver is not the product of cloned carbonyl reductase gene (*RCBR5* or *RCBR6*).

Key words carbonyl reductase; peptide sequence; functional cysteine residue; menadione; rabbit liver

Carbonyl reductase [EC 1.1.1.184] is an enzyme responsible for the reduction of drugs which have a ketone group within their chemical structures.^{1,2)} We have recently purified a carbonyl reductase from the cytosolic fraction of rabbit liver by using acetohexamide, an oral antidiabetic drug, as a substrate.^{3,4)} The carbonyl reductase purified from rabbit liver was found to reduce many drugs such as befunolol and daunorubicin,³⁾ indicating that it functions as a drug-metabolizing enzyme. In general, carbonyl reductase can catalyze not only the reduction of ketones and aldehydes, but also the reduction of quinones. More recently, two cDNAs (*RCBR5* and *RCBR6*)⁵⁾ for rabbit liver carbonyl reductase were cloned and found to code for proteins with menadione (2-methyl-1,4-naphthoquinone, vitamin K₃) reductase activity, based on the expression of the two cDNAs in COS7 cells.⁶⁾ However, the carbonyl reductase purified from rabbit liver by us had no ability to reduce menadione,^{3,4)} which is an effective substrate of carbonyl reductase.^{7–9)} Thus, the enzyme purified from rabbit liver is likely to be a specific form of carbonyl reductase present in rabbit liver.

The present study was undertaken to compare peptide sequences of the carbonyl reductase purified from rabbit liver with amino acid sequences deduced from two cDNAs (*RCBR5* and *RCBR6*) for rabbit liver carbonyl reductase. We further examined whether the carbonyl reductase purified from rabbit liver has functional cysteine residue(s), because menadione is known to interact with cysteine residue(s) of various proteins and enzymes.^{10–12)}

MATERIALS AND METHODS

Materials Lysyl endopeptidase was purchased from Wako Pure Chemicals (Osaka, Japan). Acetohexamide was supplied by Shionogi Co. (Osaka, Japan). NADPH was obtained from Oriental Yeast Co. (Tokyo, Japan). 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) and dithiothreitol (DTT)

were products of Nacalai Tesque (Kyoto, Japan). Sodium tetrathionate dehydrate (STT) was obtained from Wako Pure Chemicals (Osaka, Japan). All other chemicals were of reagent grade.

Purification of Carbonyl Reductases Two carbonyl reductases were purified to homogeneity from the cytosolic fractions of rabbit liver and kidney according to our previous procedures.^{3,4,9)}

Analysis of Peptide Sequences The analysis of peptide sequences, including the reductive pyridylethylation of the enzyme (0.2 mg), its digestion with lysyl endopeptidase, isolation of the peptides by reversed-phase high performance liquid chromatography (HPLC), and sequencing by automated Edman degradation was carried out as described previously.¹³⁾

Enzyme Assay The enzyme activity was assayed spectrophotometrically as described previously.⁹⁾ Acetohexamide was used as a substrate. The reaction mixture in a total volume of 0.7 ml consisted of 0.1 M sodium-potassium phosphate buffer (pH 6.5), 1.0 mM acetohexamide, 0.25 mM NADPH and the enzyme sample. The reaction was initiated by adding the enzyme, 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 oxidation of 1 μ mol of NADPH/min at 30 °C. Protein concentration was determined by the method of Lowry *et al.*¹⁴⁾ with bovine serum albumin as a standard.

Inactivation by DTNB or STT The inactivation experiments were carried out according to standard procedures. Prior to the treatment with DTNB or STT, the enzyme was gel-filtered for the removal of 2-mercaptoethanol. The enzyme (3 μ M) was incubated with 10 μ M DTNB or 0.5 mM STT in 0.1 M sodium-potassium phosphate buffer (pH 8.0) at 30 °C. DTT at a final concentration of 1.0 mM was added to the sample of the inactivated enzyme. The aliquots were withdrawn at appropriate intervals, then the enzyme activities

* To whom correspondence should be addressed.

were measured as described above.

RESULTS

Amino Acid Sequence At first, the carbonyl reductase purified from rabbit liver was directly sequenced. Edman degradation of the intact enzyme failed, suggesting that the N-terminus of the enzyme is blocked. Thus, we sequenced six peptides (LK-1, 2, 3, 4, 5 and 6) obtained by digestion of the enzyme with lysyl endopeptidase. Figure 1 shows a comparison of the amino acid sequences of the six peptides with the amino acid sequences deduced from *RCBR5* and *RCBR6*. The amino acid sequences of the six peptides were virtually identical to the corresponding region in terms of amino acid sequences deduced from two cDNAs (*RCBR5* and *RCBR6*) for rabbit liver carbonyl reductase.⁶⁾ However, there was a difference of one amino acid residue between the sequence of peptides LK-3 from the purified enzyme and the corresponding region in the amino acid sequence deduced from the two cDNAs; Ile-199 of peptide LK-3 corresponds to Met-199 of the deduced sequences.

Inactivation by Thiol-Specific Reagents The inactivation of carbonyl reductase purified from rabbit liver by DTNB, a thiol-specific reagent, was examined. As shown in Fig. 2, DTNB inactivated the enzyme purified from rabbit liver, and the inactivated enzyme was completely restored by adding DTT. A similar inactivation was observed for the treatment with STT, accompanying the restoration of the inactivated enzyme by DTT. Furthermore, the inactivation by

<i>RCBR5</i>	MPSDRRVALVTGANKGVGFAITRALCRLFSGDVLTTAQDEAQQGAQVQQL	50
<i>RCBR6</i>	MPSDRRVALVTGANKGVGFAITRNLCRLFSGDVLTTAQDEAQQGAQVQQL	
<i>RCBR5</i>	QAEGLSPRFHQLDITDLQSIRALRDLRRAYGGLNVLVNNNAVIAFKMEDT	100
<i>RCBR6</i>	QAEGLSPRFHQLDITDLQSIRALRDLRRAYGGLNVLVNNNAVIAFKMEDT	
<i>RCBR5</i>	TPFHIQAEVTMKTNFDGTRDVCTELLPLMRPGGRVVNVSSMTCLRALKSC	150
<i>RCBR6</i>	TPFHIQAEVTMKTNFDGTRDVCTELLPLMRPGGRVVNVSSMTCLRALKSC	
	<div> <div>TNFDGXXD</div> <div>LK-1</div> </div>	
<i>RCBR5</i>	SPELQKKFRSETITEELVGLMKKFVEDTKKGVHQTEGWPDYAYGVTKMG	200
<i>RCBR6</i>	SPELQKKFRSETITEELVGLMKKFVEDTKKGVHQTEGWPDYAYGVTKMG	
	<div> <div>GVHQTEGXPDXYG</div> <div>LK-2</div> <div>IG</div> </div>	
<i>RCBR5</i>	VTVLSRIQARHLSEHRGGDKILVNACCPGWVRTDMGGPNATKSPEEGAET	250
<i>RCBR6</i>	VTVLSRIQARHLSEHRGGDKILVNACCPGWVRTDMGGPNATKSPEEGAET	
	<div> <div>VTVLSRIQARXL</div> <div>LK-3</div> <div>ILVNACXPGXVRTXM</div> <div>LK-4</div> <div>SPEEGAEX</div> <div>LK-5</div> </div>	
<i>RCBR5</i>	PVYLALLPPDAEGPHGQFVMDKKVEQW	277
<i>RCBR6</i>	PVYLALLPPDAEGPHGQFVMDKKVEQW	
	<div> <div>PVYLA</div> <div>LK-6</div> <div>KVEQ</div> </div>	

Fig. 1. Comparison of Amino Acid Sequences of Six Peptides Obtained by the Digestion of Carbonyl Reductase Purified from Rabbit Liver with Amino Acid Sequences Deduced from Rabbit Liver Carbonyl Reductase Genes (*RCBR5* and *RCBR6*)

Sequences of six peptides were determined with average repetitive yields of 77–98% by Edman degradation. Amino acids of peptides matching for sequences deduced from *RCBR5* and *RCBR6* are denoted by vertical lines. X is the unidentifiable amino acid. There was a difference of only three residues between the amino acid sequences deduced from *RCBR5* and *RCBR6*. An asterisk shows these three amino acid residues.

DTNB of carbonyl reductase purified from rabbit liver was compared with that of carbonyl reductase purified from rabbit kidney (Fig. 3). DTNB was found to much more strongly inactivate carbonyl reductase purified from rabbit liver than that from rabbit kidney.

DISCUSSION

Several multiple forms of carbonyl reductase with broad

Fig. 2. Inactivation of Carbonyl Reductase Purified from Rabbit Liver by DTNB and STT

DTT was added to the sample of the inactivated enzyme at the time indicated by the arrow.

Fig. 3. Inactivation of Carbonyl Reductases Purified from Rabbit Liver and Kidney by DTNB

Open and solid circles represent rabbit liver and kidney enzymes, respectively.

substrate specificity for aldehydes and ketones have been purified from rabbit liver.¹⁵⁾ We also purified a carbonyl reductase from rabbit liver by using acetohexamide as a substrate.⁴⁾ Recently, two carbonyl reductase genes (*RCBR5* and *RCBR6*) were cloned from the rabbit liver cDNA library.^{6,16)} The amino acid sequences of the six peptides obtained from digestion of the carbonyl reductase purified from rabbit liver were virtually identical to the corresponding regions in amino acid sequences deduced from the two cDNAs. However, there was a difference of one amino acid residue between the sequence of the peptide from the purified enzyme and the corresponding region in the amino acid sequences deduced from the two cDNAs. Furthermore, the purified carbonyl reductase was confirmed to exhibit no reactivity towards menadione, even though the transient expression of *RCBR5* and *RCBR6* has been reported to cause a marked increase of menadione reductase activity in COS7 cells.⁶⁾ Judging from these results, it is reasonable to assume that the enzyme purified from rabbit liver is not the product derived from *RCBR5* or *RCBR6*, but is one of the multiple forms of rabbit liver carbonyl reductase.

Carbonyl reductase purified from rabbit liver was inactivated by DTNB and the inactivated enzyme was completely restored by adding DTT. STT, which is a thiol-specific reagent without a bulky group,¹⁷⁾ also inactivated the enzyme purified from rabbit liver. These findings clearly indicate that cysteine residue(s) is essential for the catalytic activity of carbonyl reductase purified from rabbit liver. Menadione is known to interact with cysteine residue(s) of various proteins and enzymes.^{10–12)} We propose that menadione interacts with the functional cysteine residue(s) located in the active site of carbonyl reductase purified from rabbit liver and, as a result of the interaction, cannot serve as a substrate of the purified enzyme.

We have recently purified a carbonyl reductase from the cytosolic fraction of rabbit kidney.⁹⁾ The enzyme purified from rabbit kidney, unlike the enzyme purified from rabbit liver,^{3,4)} effectively reduced menadione.⁹⁾ Interestingly, our previous papers^{3,4,9)} have revealed that *p*-chloromercuribenzoate (*p*-CMB), a typical thiol-specific reagent, much more strongly inhibits carbonyl reductase purified from rabbit liver than that from rabbit kidney. Furthermore, the present study demonstrates that DTNB much more strongly inactivates carbonyl reductase purified from rabbit liver than that from rabbit kidney. These findings also support the idea that cysteine residue(s) is involved in the catalytic activity of carbonyl reductase purified from rabbit liver. Further studies are in progress to provide direct evidence for the interaction of menadione with the functional cysteine residue(s) of carbonyl reductase purified from rabbit liver.

It has been accepted that carbonyl reductase is a member of the aldo-keto reductase superfamily, similar to aldehyde

reductase [EC 1.1.1.2] and aldose reductase [EC 1.1.1.21].¹⁸⁾ However, recent works have demonstrated that carbonyl reductase belongs to the short-chain alcohol dehydrogenase/reductase (SDR) superfamily, based on its primary structure.^{6,16,19)} The rabbit cDNAs (*RCBR5* and *RCBR6*) are known to code for proteins with 84% identity to human carbonyl reductase, which is a member of the SDR superfamily.⁶⁾ Therefore, carbonyl reductase purified from rabbit liver, as well as some carbonyl reductases,^{6,16,19)} appear to be classified as members of the SDR superfamily,²⁰⁾ although it has no ability to reduce menadione.

In conclusion, the present study provides evidence that carbonyl reductase purified from rabbit liver is not the product of a carbonyl reductase gene (*RCBR5* or *RCBR6*) cloned from the rabbit liver cDNA library.

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