Mutation of threonine-241 to proline eliminates autocatalytic modification of human carbonyl reductase

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Carbonyl reductase catalyses the reduction of steroids, prostaglandins and a variety of xenobiotics. An unusual property of human and rat carbonyl reductases is that they undergo modification at lysine-239 by an autocatalytic process involving 2-oxocarboxylic acids, such as pyruvate and 2-oxoglutarate. Comparison of human carbonyl reductase with the pig enzyme, which does not undergo autocatalytic modification, identified three sites, alanine-236, threonine-241 and glutamic acid-246, on human carbonyl reductase that could be important in the reaction of lysine-239 with 2-oxocarboxylic acids. Mutagenesis experiments show that replacement of threonine-241 with proline (T241P) in human carbonyl reductase eliminates the formation of carboxyethyl-lysine-239. In contrast, the T241A mutant has autocatalytic activity similar to wild-type carbonyl reductase. The T241P mutant retains catalytic activity towards menadione, although with one-fifth the catalytic efficiency of wild-type carbonyl reductase. Replacement of threonine-241 with proline is likely to disrupt the local structure near lysine-239. We propose that integrity of this local environment is essential for chemical modification of lysine-239, but not absolutely required for carbonyl reductase activity.

Key words: lysine-pyruvate adduct, Schiff base, SDR, short-chain dehydrogenase/reductase.

INTRODUCTION

Carbonyl reductase is an NADPH-dependent enzyme that catalyses the reduction of steroids, prostaglandins and a variety of xenobiotics [1–3]. Sequence analysis [4,5] reveals that carbonyl reductase belongs to the short-chain dehydrogenase/reductase (SDR) family, a very large family of enzymes that regulates the concentration of steroids, prostaglandins and retinoids in mammals, as well as catalysing the oxidation or reduction of a wide variety of other biologically important compounds [6,7].

An unusual property of human and rat carbonyl reductases is that they exist *in vivo* in three forms with different charges and slightly different molecular masses [1,8–10]. The three isoforms have about the same catalytic activity towards menadione [8,9]. These multiple isoforms of carbonyl reductase result from covalent modification of a lysine residue (lysine-239 in the human enzyme) by an autocatalytic process involving the formation of a Schiff base between the ϵ-amino group of lysine and 2-oxocarboxylic acids, such as pyruvate and 2-oxoglutarate [5,9]. Reduction of this bond yields a covalent adduct. To the best of our knowledge, this chemical modification is unique to carbonyl reductase and has not been found in any other SDR, nor in another oxidoreductase.

The mechanistic details of this reaction are unknown. In particular, is there a specific structure around human lysine-239 that is required for formation of carboxyethyl-lysine in the presence of pyruvate or 2-oxoglutarate? Or is reduction of the Schiff base at lysine-239 by carbonyl reductase independent of conformation?

Interestingly, unlike human and rat carbonyl reductases, the pig homologue is isolated from testes in an unmodified form [11]. This prompted us to compare the three sequences near lysine-239 and to construct the appropriate human carbonyl reductase mutants and examine them for modification by 2-oxocarboxylates. Here we report that replacement of threonine-241 by proline (T241P) in human carbonyl reductase eliminates the modification of lysine-239 in the presence of either pyruvate or 2-oxoglutarate. T241P retains catalytic activity for menadione. We propose that replacement of threonine-241 with proline disrupts the local structure around lysine-239 and prevents covalent modification with 2-oxocarboxylic acids, because proline lacks a side chain that can interact with nearby amino acids. However, although a specific local structure on human carbonyl reductase is required for chemical modification of lysine-239, this structure is not essential for carbonyl reductase activity.

EXPERIMENTAL

Mutagenesis of human carbonyl reductase

Human placental carbonyl reductase cDNA was cloned into a pET-11 vector [12] and used to construct mutant carbonyl reductases A236G, T241P, T241A and E246V by the method of Higuchi et al. [13] with 5′ and 3′ primers containing an *Nde*I and a *Sal*I restriction site, respectively. These mutants were subcloned into the pET21a+ expression vector for production of carbonyl reductase and its mutants in *Escherichia coli*.

Enzyme purification

E. coli cells in which wild-type carbonyl reductase or one of its mutants was expressed were collected by centrifugation in 10 mM Tris/HCl, pH 8.6/1 mM EDTA, and disrupted by freeze-thawing and sonication. Cell debris was removed by centrifugation; the supernatant was incubated with RNase and DNase, 10 μg/ml each, for 5 min on ice. Various carbonyl reductases were purified on a DEAE-cellulose column, using an NaCl gradient from 0 to 200 mM. Homogeneity of the most active fractions was visualized with SDS/PAGE using silver staining and Western-blot analysis.

Abbreviation used: SDR, short-chain dehydrogenase/reductase.

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SDS/PAGE, Western analysis and isoelectric focusing

PAGE was carried out on 10 % acrylamide gels in the presence of 0.1 % SDS [9,11,12]. Protein bands were detected by Coomassie Brilliant Blue or silver staining. For immunodetection, the proteins were transferred to PVDF sheets by electroblotting. Membranes were incubated overnight with rabbit anti-(human carbonyl reductase) antibody in 50 mM Tris/HCl, pH 8.0/150 mM NaCl. For detection, the membrane was incubated with peroxidase-conjugated anti-rabbit IgG antibodies (Dr. Bommeli AG, Berne, Switzerland). Protein was visualized after addition of 0.04 % 3,3′-diaminobenzidine and 0.01 % H_2O_2 in Tris/HCl, pH 8.0/150 mM NaCl.

Enzymic assay and kinetic analysis

Carbonyl reductase activity was determined by measuring the oxidation of NADPH at 340 nm in the presence of 0.25 mM menadione in 100 mM phosphate buffer, pH 7.0. For kinetic characterization, the enzymes were incubated with 0.05 mM NADPH and varying concentrations of menadione. K_m and k_{cat} values were estimated by graphical extrapolation from Lineweaver–Burk plots.

Autocatalytic modification of human carbonyl reductase by 2-oxocarboxylates

Autocatalytic activity was tested by incubating human carbonyl reductase and its mutants in 100 mM phosphate buffer, pH 7.0, in the presence of 0.05 mM NADPH and 10 mM 2-oxoglutarate or pyruvate at 37 °C for 24 h. Reaction of enzyme with either 2-oxoglutarate or pyruvate was visualized on a silver-stained SDS/PAGE gel and on a Western blot.

Autocatalytic modification of pig carbonyl reductase by 2-oxocarboxylates

Purified pig carbonyl reductase was incubated with 20 mM 2-oxocarboxylic acid (pyruvate or 2-oxoglutarate) in 0.1 M potassium phosphate buffer (pH 7.0) in the presence of 100 μM NADPH at 30 °C, for 20 h [9]. Treated samples were concentrated using an Amicon concentrator and analysed with PAGE for a change in mobility due to reaction with either pyruvate or 2-oxoglutarate.

RESULTS

Pig testicular carbonyl reductase does not undergo autocatalytic modification

Although pig carbonyl reductase is found in only one form when isolated from testis [11] this could be due to the concentration *in vivo* of 2-oxocarboxylates in pig testis being too low to react with lysine-239 on pig carbonyl reductase. Thus we investigated autocatalytic modification of pig carbonyl reductase *in vitro* under conditions that result in reaction of 2-oxocarboxylates with lysine-239 on human and rat carbonyl reductases [9]. As seen in Figure 1, pig carbonyl reductase does not react with either pyruvate or 2-oxoglutaric acid under conditions in which the rat carbonyl reductase does.

Sequence analysis of mammalian carbonyl reductases

To understand the structural basis for this difference between pig, rat and human carbonyl reductases, we compared the amino acid sequences near lysine-239 in these proteins. As seen in Figure 2, compared with human and rat carbonyl reductases, pig carbonyl reductase has different amino acids at positions 236 (Gly versus Ala), 241 (Pro versus Thr) and 246 (Val versus Glu), suggesting that one or more of these amino acid replacements could account for the lack of autocatalytic activity in pig carbonyl reductase.

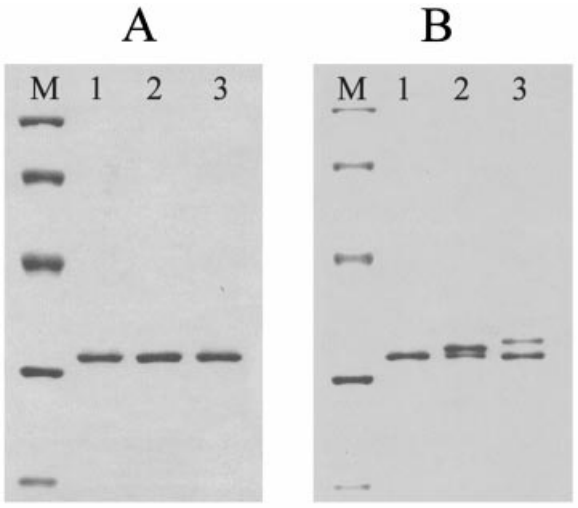

Figure 1 SDS/PAGE of pig carbonyl reductase treated with pyruvate or 2-oxoglutalate

(**A**) Pig carbonyl reductase; (**B**) rat carbonyl reductase. Pig and rat carbonyl reductases were incubated with NADPH and pyruvate (lanes 2), 2-oxoglutarate (lanes 3) or without 2-oxocarboxylic acid (lanes 1) as described in the Experimental section. Both 2-oxocarboxylates changed the mobility of rat carbonyl reductase. Under the same conditions, the pig enzyme was unmodified. M, molecular-mass markers.

Analysis of reaction of carbonyl reductase mutants with 2-oxocarboxylates

To test this hypothesis, we constructed three mutants of human carbonyl reductase: A236G, T241P and E246V. Each mutant was examined for autocatalytic modification by 2-oxoglutarate and pyruvate. Figure 3 shows the Western-blot analysis with 2-oxoglutarate. As seen in Figure 3, only the T241P mutant was not modified. A similar pattern was found when wild-type carbonyl reductase and each mutant were incubated with pyruvate (results not shown).

To investigate whether the loss of autocatalytic activity in T241P was due to a specific property of proline, we constructed a T241A mutant and examined it for autocatalytic activity. As shown in Figure 3, the human carbonyl reductase T241A mutant was modified by 2-oxoglutarate in the same way as wild-type carbonyl reductase. Thus some special property of proline-241 prevents chemical modification of lysine-239 with 2-oxocarboxylates.

Catalytic activity of carbonyl reductase mutants

We also studied the reduction of menadione by human carbonyl reductase mutants A236G, E246V, T241P and T241A, and present the K_m and V_{max} values for this reaction in Table 1. Compared with wild-type carbonyl reductase, there was an increase in K_m for E246V, a decrease in V_{max} for T241A, and a decrease in both K_m and V_{max} for A236G. T241P had about a 10-fold higher K_m and a 2-fold higher V_{max} for menadione or, in other words, a catalytic efficiency that was one-fifth that of wild-type carbonyl reductase.

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Pig CR              235 Met-Gly-Gly-Pro-Lys-Ala-Pro-Lys-Ser-Pro-Glu-Val-Gly
Rat and Mouse CR    235 Met-Ala-Gly-Pro-Lys-Ala-Thr-Lys-Ser-Pro-Glu-Glu-Gly
Human CR            235 Met-Ala-Gly-Pro-Lys-Ala-Thr-Lys-Ser-Pro-Glu-Glu-Gly
Rabbit CR           235 Met-Ala-Gly-Pro-Asn-Ala-Thr-Lys-Ser-Pro-Glu-Glu-Gly
Trout CR            234 Met-Ala-Gly-Ser-Lys-Ala-Pro-Lys-Ser-Pro-Glu-Glu-Gly
S. mansoni CR       231 Met-Ser-Ser-His Lys-Gly-Thr-Lys-Thr-Ala-Asp-Glu-Gly
B. floridae CR      231 Met-Gly-Gly-Pro-Asn-Ala-Gly-Arg-Ser-Val-Asp-Lys-Gly
Arabidopsis CR      255 Met-Asn-Phe-Lys-Thr-Gly-Ile-Leu-Ser-Val-Glu-Glu-Gly
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Figure 2 Alignment of mammalian, fish, invertebrate and plant carbonyl reductases

The region in human and rat carbonyl reductases (CRs) containing lysine-239, which is covalently modified by 2-oxocarboxylic acids in an autocatalytic reaction, is shown. Comparison with pig carbonyl reductase, which does not undergo autocatalytic modification, indicates that Ala-236, Thr-241 and Glu-246 could be important in this reaction. Rabbit carbonyl reductase has asparagine-239 instead of lysine-239. The rest of this region in rabbit carbonyl reductase is identical with that of its human, rat and mouse homologues. Trout carbonyl reductase has a proline at the position corresponding to threonine-241 in human carbonyl reductase. The invertebrate *Schistosoma mansoni* contains both lysine-235 and threonine-237. Putative carbonyl reductases in the invertebrate *Branchiostoma floridae* and in the plant *Arabidopsis* have diverged substantially from the other carbonyl reductases in this region.

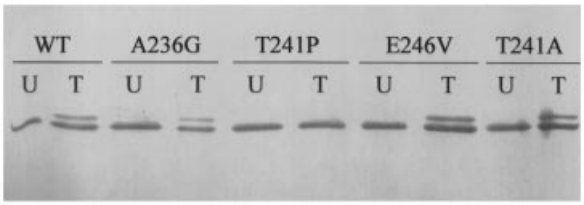

Figure 3 Western-blot analysis of native human carbonyl reductase and its mutants before and after incubation with 2-oxoglutalate

Native human carbonyl reductase (WT) and mutants A236G, T241P, E246V and T241A were incubated with NADPH and 2-oxoglutarate and analysed on PAGE as described in the Experimental section. The first lane in each pair is untreated enzyme (U). The second lane is treated enzyme (T), which was incubated with NADPH and 2-oxoglutarate. Only T241P does not have a doublet, showing reaction of lysine-239 with 2-oxoglutarate. Separate experiments showed that boiled carbonyl reductase does not react with 2-oxoglutarate in the presence of NADPH (results not shown). Also, wild-type carbonyl reductase does not react with 2-oxoglutarate in the absence of NADPH [9].

Table 1 Kinetic constants for human carbonyl reductase and its mutants

Carbonyl reductase	Substrate	K_m (μM)	V_{max} (units/mg)
Wild-type	Menadione	30	17
T241A	Menadione	25	7
T241P	Menadione	220	38
A236G	Menadione	3.5	0.6
E246V	Menadione	80	16

DISCUSSION

The original isolation of carbonyl reductase from human brain revealed the presence of three isoforms with similar catalytic activity [1]. A clue to the biochemistry of this phenomenon was discovered by Krook et al. [5], who showed that carbonyl reductase undergoes chemical modification of lysine-239 by 2-oxocarboxylates, such as pyruvate. Similar behaviour is found in rat carbonyl reductase [9], but not in pig or rabbit carbonyl reductases. Nor has this chemical modification been reported in other SDRs or other oxidoreductases, suggesting that a specific structure is required for reaction of carbonyl reductase with 2-oxocarboxylates.

The region near lysine-239 in human, rat and mouse carbonyl reductases is absolutely conserved (Figure 2), but differs from the pig enzyme at positions 236, 241 and 246. Rabbit carbonyl reductase has an asparagine instead of a lysine. Thus, rabbit carbonyl reductase cannot react with 2-oxocarboxylates.

We constructed human carbonyl reductases that were mutated to the pig sequence at positions 236, 241 or 246. Mutation of either alanine-236 or glutamic acid-246 to glycine and valine, respectively, resulted in a human carbonyl reductase that reacted at lysine-239 with 2-oxoglutarate and pyruvate. Only the T241P mutant did not react with 2-oxocarboxylates at lysine-239. The reaction of the T241A mutant with 2-oxocarboxylates indicates that one or more properties of proline cause the lack of autocatalytic activity in T241P. The most likely explanation is that the absence of a side chain on proline disrupts the local structure, interfering with either the formation of a Schiff base at lysine-239 with 2-oxoglutarate and pyruvate or its subsequent reduction.

We also investigated the catalytic activity of each mutant. Compared with wild-type carbonyl reductase, A236G had the greatest change in kinetic constants. The other mutants, E246V and T241A, had similar catalytic properties towards menadione, as did wild-type carbonyl reductase. T241P has about 5-fold lower catalytic efficiency than wild-type human carbonyl reductase. This indicates that this region in carbonyl reductase is important in catalysis.

The reaction of lysine-239 on carbonyl reductase with 2-oxocarboxylates is physiological, because the three forms of carbonyl reductase are found in untreated brain cytosol [10]. Moreover, when *E. coli* cells that are transformed with human carbonyl reductase are incubated in media with glucose the yield of pyruvate-modified carbonyl reductase increases [12]. This suggests that the degree of modification of lysine-239 in carbonyl reductase depends on the metabolic state of the cells. The physiological role of this chemical reaction is not understood at this time.

Five mammalian carbonyl reductases have been sequenced and found to be 80 % or more identical with each other over their entire lengths. In the region near lysine-239, human, rat and mouse enzymes are identical (Figure 2). Mouse carbonyl reductase has not been examined for autocatalytic modification by 2-oxocarboxylates. Absolute conservation of residues in the region near lysine-239 indicates that the mouse enzyme could

undergo autocatalytic modification. However, although the entire sequence of mouse carbonyl reductase is 89 % identical with rat carbonyl reductase, it may be that one or more of these differences perturb the positions of NADPH and 2-oxocarboxylate in the region of lysine-239 sufficiently enough to prevent reaction with 2-oxocarboxylates. If mouse carbonyl reductase reacts with 2-oxocarboxylates at lysine-239, then gene deletions and substitutions in transgenic mice could help to elucidate the biological function of this activity.

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