Volume 335, number 2, 151–154 FEBS 13311 December 1993

Autocatalytic modification of human carbonyl reductase by 2-oxocarboxylic acids

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Received 28 September 1993; revised version received 15 October 1993

Carbonyl reductase occurs in multiple molecular forms. Sequence analysis has yielded a carboxyethyllysine residue in one of the enzyme forms, suggesting that pyruvate has been incorporated in a posttranslational enzymatic reaction [Krook, M., Ghosh, D., Strömberg R., Carlquist, M. and Jörnvall, H. (1993) Proc. Natl. Acad. Sci. USA 90, 502–506]. Using highly purified carbonyl reductase from human brain we show that pyruvate and other 2-oxocarboxylic acids are bound to the enzyme in an autocatalytic reaction. The resulting enzyme forms were indistinguishable from the native enzyme forms by electrophoresis and isoelectric focusing.

Carbonyl reductase; Enzyme modification; Pyruvate; 2-Oxoglutarate; Human

1. INTRODUCTION

Carbonyl reductase (EC 1.1.1.184) is a cytosolic, monomeric oxidoreductase that catalyzes the NADPH-dependent reduction of a variety of quinones, ketones and aldehydes. It is widely distributed in human tissues, and enzymes with similar properties are present in other mammalian and nonmammalian species [1]. Characteristically, the enzyme occurs in multiple molecular forms differing in apparent molecular weight and charge but with apparently identical enzymatic properties [2–7]. Three forms, designated according to their isoelectric points as CR-8.5, CR-8 and CR-7, respectively, have been purified from human brain [2]. Sequence analysis of cDNAs isolated from human placenta, liver and MCF-7 breast cancer cell line cDNA libraries, respectively, revealed no heterogeneity of the corresponding proteins [8,9], suggesting posttranslational modification rather than genetic diversity to be the cause of the multiple forms.

Forrest et al. first reported the occurrence of a modified lysine residue at position 239 of carbonyl reductase from human placenta, although the nature of the modification was not clarified [9]. More recently, Jörnvall and coworkers identified an N^6-(1-carboxyethyl)lysine residue at the same position and suggested that this compound was formed from the lysine and pyruvate via a Schiff base and subsequent reduction, where at least the second step would require enzymatic catalysis [10]. The nature of the reductase was not invesitigated, but in view of the broad specificity of carbonyl reductase for aldehydes and ketones some mechanistic connection between enzyme actvity and enzyme modification was considered an intriguing possibility. In the present study, we used highly purified enzyme from human brain to verify this possibility and to investigate the structural basis of other molecular forms.

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2. EXPERIMENTAL

Carbonyl reductase was purified to apparent homogeneity from human brain as described previously [2]. Antibodies were raised in rabbits and further purified by ammonium sulfate precipitation and DEAE-Sephadex chromatography [11]. Their specificity has been demonstrated [11,12]. Coenzymes, 2′-AMP and carbonyl compounds were purchased from Sigma (St. Louis, MO, USA) and Fluka (Buchs, Switzerland).

Carbonyl reductase activity was determined spectrophotometrically at 340 nm in 80 mM sodium phosphate (pH 7.0), 0.05 mM NADPH and 0.25 mM menadione.

Modification of carbonyl reductase was carried out under standard conditions in 100 mM sodium phosphate (pH 7) in the presence of 50 μM NADPH and 10 mM carbonyl compound at 30°C for 20 h or at reagent concentrations and conditions as indicated in the text.

Polyacrylamide gel electrophoresis was carried out in 12% acrylamide gels in the presence of 0.1% sodium dodecylsulfate according to Laemmli [13]. Proteins were blotted on Immobilon sheets (Millipore, Milford, MA, USA) using a Bio-Rad electroblotting apparatus (Richmond, CA, USA), and the membranes were incubated with anti-carbonyl reductase antibodies in 50 mM Tris (pH 8.3) overnight. Bound antibodies were made visible using horseradish peroxidase coupled to protein A (Dr. Bommeli, Bern, Switzerland), H_2O_2 and diaminobenzidine [11].

Isoelectric focusing on thin-layer polyacrylamide gels (Pharmacia, Uppsala, Sweden) in the pH range 3.5–9 was carried out as described by the manufacturer. Carbonyl reductase was detected on the basis of enzyme activity in the presence of NADPH, menadione and nitroblue tetrazolium chloride [2].

3. RESULTS

3.1. *Reaction with pyruvate*

Assuming the modification of Lys-239 to be the cause of the multiple forms [9,10] the unmodified enzyme corresponds to the most basic form, CR-8.5, and the pyruvate-modified enzyme to either CR-8 or CR-7. The three forms are best distinguished on the basis of differences in apparent molecular weight by sodium dodecylsulfate-polyacrylamide gel electrophoresis or in charge by isoelectric focusing [2]. Figs. 1 and 2 show electrophoresis and isoelectric focusing gels, respectively, of carbonyl reductase after incubation with pyruvate under different conditions. In the absence of NADPH, pyruvate did not affect the mobility of CR-8.5, whereas in the presence of the coenzyme two forms, one corresponding to the native enzyme and the other exhibiting the same mobility as CR-8, were detectable. Treatment of the reaction mixture with borate before electrophoresis to dissociate Schiff bases did not alter the electrophoresis pattern. On the other hand, replacement of NADPH by $NADP^+$, 2′-AMP or NADH resulted in the absence of the second form. These results show that NADPH is essential for enzyme modification in agreement with an autocatalytic reaction. To clarify the question whether the reaction is catalyzed by the same or by other enzyme molecules, carbonyl reductase was inactivated to various degrees by 4-OH- mercuribenzoate [2] before incubation with pyruvate and NADPH. As expected for an enzyme-catalyzed reaction, total loss of activity prevented the formation of the slower migrating form. On the other hand, a larger portion of the partially inactivated enzyme than the fraction of enzymatically active molecules was converted to CR-8 (Fig. 3A), indicating that the active molecules catalyzed the modification inactivated molecules.

Opposite effects were observed when the concentration of the reaction partners, pyruvate and carbonyl reductase, was varied (cf. Fig. 3). Following the law of mass action, the proportions of the two forms changed in favor of CR-8 with increasing concentrations of pyruvate. In contrast, increasing the concentration of the enzyme decreased the portion of the modified enzyme. Addition of the nonionic detergent Triton X- 100 to the incubation mixture did not alter the proportions of the two forms but stabilized the enzyme at low concentrations. Virtually complete modification could thus be obtained at low enzyme and high pyruvate concentrations. Under no conditions, however, we observed an additional, third enzyme form. Similarly, no additional form became detectable if CR-8 from brain was incubated with pyruvate and NADPH (Fig. 1B). These findings indicate that only one lysine residue is susceptible to autocatalytic modification. Other enzyme forms probably arise from modification of the same lysine residue by other ligands. Various carbonyl compounds, in particular 2-oxocarboxylic acids, were therefore tested as possible modifying agents.

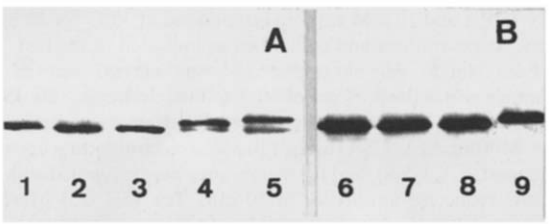

Fig. 1. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of carbonyl reductase after incubation with 2-oxocarboxylic acids. (A) CR-8.5 and (B) CR-8 were incubated under standard conditions with the following compounds: lane 1, control CR-8.5; lane 2, pyruvate without NADPH; lane 3, 2-oxoglutarate without NADPH; lanes 4,7, pyruvate plus NADPH; lanes 5, 8, 2-oxoglutarate plus NADPH; lane 6, control CR-8; lane 9, control CR-7. Enzyme forms were made visible by immuno-staining after transfer to Immobilon membranes.

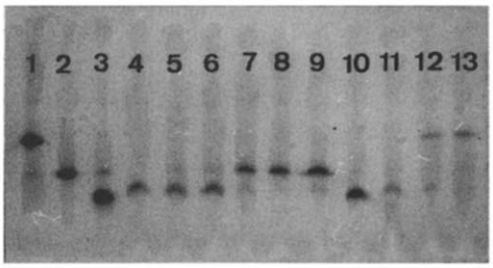

Fig. 2. Polyacrylamide gel isoelectric focusing of carbonyl reductase after incubation with 2-oxocarboxylic acids. CR-8.5 was incubated under standard conditions with NADPH and the following compounds: lanes 1–3, controls CR-7, CR-8, CR-8.5; lane 4, acetoacetate; lane 5, 2-oxo-3-methylbutyrate; lane 6, 2-oxo-3-methylvalerate; lane 7, 2-oxoisocaproate; lane 8, 2-oxobutyrate; lane 9, pyruvate; lane 10, pyruvate without NADPH; lane 11, 3-oxoglutarate; lane 12, 2-oxoglutarate; lane 13, 2-oxoadipate. The gel was stained for enzyme activity.

3.2. *Reaction with other carbonyl compounds*

The homologues of pyruvate, 2-oxobutyrate and 2-oxocaproate as well as 2-oxoisocaproate yielded an enzyme form which, similar to the reaction product with pyruvate, was indistinguishable from CR-8 by sodium dodecylsulfate-polyacrylamide gel electrophoresis and isoelectric focusing. 2-Oxo-3-methylbutyrate and 2-oxo-3-methylvalerate in which the carbon chain branches proximate to the oxo group gave only very faint bands at the position of CR-8, and acetoacetate in which the oxo group is in the β-position produced no slower migrating enzyme form (Fig. 2)

Electrophoresis patterns different from those observed with pyruvate and the other 2-oxocarboxylic compounds were obtained with the dicarboxylic acids 2-oxoglutarate and 2-oxoadipate. Both compounds yielded an enzyme form that on both electrophoresis (Fig. 1) and isoelectric focusing gels (Fig. 2) migrated at the same position as CR-7. Similarly to the results obtained with pyruvate, the degree of modification by 2-oxoglutarate was inversely related to the enzyme concentration (Fig. 3B) and proportional to the concentra-

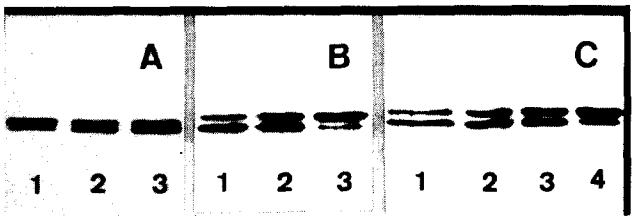

Fig. 3. Dependence of carbonyl reductase modification on (A) enzyme inactivation, (B) enzyme concentration and (C) ligand concentration, detected by sodium dodecyl-sulfate polyacrylamide gel electrophoresis. (A) CR-8.5 was incubated with 2 and 5 μM 4-OH-mercuribenzoate (pMB), respectively, dialyzed against reaction buffer and incubated with NADPH and pyruvate under standard conditions. Lane 1, control (100% activity); lane 2, enzyme incubated with 2 μM pMB (29% activity); lane 3, enzyme incubated with 5μM pMB (5% activity). (B) CR-8.5 (120 μg/ml) was diluted 4- and 10-fold, respectively, before incubation with NADPH and 2-oxoglutarate under standard conditions. Equal amounts of protein were applied to the gel. Lane 1, undiluted enzyme; lane 2, 4-fold diluted enzyme; lane 3, 10-fold diluted enzyme. (C) CR-8.5 (corresponding to the 4-fold diluted enzyme in panel (B) was incubated under standard conditions with NADPH and the following concentrations of 2-oxoglutarate: lane 1, 1 mM; lane 2, 5 mM; lane 3, 10 mM; lane 4, 20 mM. Bands on all gels were made visible by immunostaining.

tion of the ligand (Fig 3C). 3-Oxoglutarate, in agreement with the result obtained with acetoacetate, did not affect the mobility of the enzyme. Similarly, replacing CR-8.5 by CR-8 prevented the formation of CR-7 by 2-oxoglutarate (Fig. 1B), in keeping with the hypothesis that both CR-8 and CR-7 arise from modification of the same lysine residue by different ligands.

Multiple bands were also observed after incubation of the enzyme with dicarbonyl compounds, e.g. methylglyoxal, which bind to arginine residues [14]. However, the reaction also occurred in the absence of NADPH as well as in the presence of $NADP^+$. Moreover, all dicarbonyls completely abolished the enzyme activity, in contrast to the oxocarboxylic compounds which did not inactivate the enzyme. Sugar aldehydes and ketones, including glucose galactose and fructose, which are known to bind to proteins nonenzymatically had no effect on the electrophoretic mobility of the various carbonyl reductase forms. Moreover, carbonyl reductase which had been incubated with fructose or glucose was not retained on a phenylboronate column, indicating that no fructosamine had been formed.

4. DISCUSSION

The present results demonstrate the ability of carbonyl reductase to catalyze its own modification by pyruvate and other 2-oxocarboxylic compounds. The reaction occurs between two molecules, one functioning as catalyst, the other as substrate, rather than within the same molecule, in agreement with structural data from computer modeling, which located the modified lysine residue outside of the active site cleft [10]. The pyruvate-modified enzyme exhibits the same electrophoretic properties as CR-8 from human brain and most probably corresponds to the carboxyethylated enzyme form identified by Jörnvall and coworkers [10]. In addition to pyruvate, several mono-carboxylic homologues yielded an enzyme form with apparently identical electrophoretic mobility, whereas the dicarboxylic compounds, 2-oxoglutarate and 2-oxoadipate, in line with their additional negative charge, produced a more acidic enzyme form with the same electrophoretic properties as CR-7 from brain. The difference in apparent molecular weight between the native and modified enzyme forms, about 400 and 900, respectively [2], is significantly larger than, and apparently independent of, the molecular weight of the ligands. Uncomplete unfolding of the modified proteins in the presence of sodium dodecylsulfate and/or altered binding of the detergent may be responsible for the observed differences.

Imino compounds, with the exception of dichlorophenol-indophenol, have not previously been reported as substrates of carbonyl reductase. In contrast to the carbonyl substrates which comprise a broad range of structurally different compounds, the specificity for imino compounds was rather restricted. A carboxyl group adjacent to the imino group was essential for enzyme activity, and a side chain at the β-position almost completely prevented the modification of the enzyme. Since the reduction of low molecular weight carbonyl compounds does not show comparable restrictions, interference with amino acid residues in the vicinity of Lys-239 may be responsible for the restricted specificity. Similarly, the inverse relationship between the enzyme concentration and the extent of modification suggests that protein–protein interactions either prevent the formation of the Schiff base or, more likely, impede the formation of the enzyme substrate complex between two carbonyl reductase molecules.

To our knowledge, the reductive carboxyalkylation of lysine residues by 2-oxocarboxylic acids has not previously been detected in other proteins. Under physiological conditions, the respective cytosolic concentrations of pyruvate and 2-oxoglutarate probably determine the proportions of CR-8 and CR-7. However, in some metabolic diseases, e.g. maple syrup urine disease and 2-oxoadipic aciduria, other 2-oxocarboxylic acids may also bind significantly to the enzyme. The physiological or pathophysiological significance of the reaction, however, remains unknown. With the exception of small differences in the Km values for NADPH and the specific activities, the native and the modified enzyme forms exhibit the same substrate specificities and sensitivities to inhibitors [15]. However, essentially all comparative studies have been carried out with small substrates which probably do not interact with Lys-239 [10]. In contrast, larger substrates, e.g. prostaglandins or steroids, might interact with the lysine residue and exhibit different affinities for the three enzyme forms. Alternatively, the introduction of negative charges

might affect the interaction with other protein molecules and modulate the activity and/or stability of the enzyme. More information may become available with the detection of similar modifications in other proteins.

Acknowledgements: This work was supported by a grant from the Swiss National Science Foundation to B.W.

REFERENCES

[1] Wermuth, B. (1985) in: Enzymology of Carbonyl Metabolism 2 (Flynn, T.G. and Weiner, H. eds.) pp. 209–230, Alan R. Liss, New York.
[2] Wermuth, B. (1981) J. Biol. Chem. 256, 1206–1213.
[3] Felsted, R.L. and Bachur, N.R. (1982) Prog. Clin. Biol. Res. 114, 291–305.
[4] Jarabak, J., Luncsford, A. and Berkowitz, D. (1983) Prostaglandins 26, 849–868.
[5] Nakayama, T., Hara, A., Yashiro, K. and Sawada, H. (1985) Biochem. Pharmacol. 34, 107–117.
[6] Inazu, N., Ruepp, B., Wirth, H. and Wermuth, B. (1992) Biochim. Biophys. Acta 1116, 50–56.
[7] Schieber, A., Frank, R.W. and Ghisla, S. (1992) Eur. J. Biochem. 206, 491–502.
[8] Wermuth, B., Bohren, K.M., Heinemann, G., von Wartburg, J.P. and Gabbay, K.H. (1988) J. Biol. Chem. 263, 16185–16188.
[9] Forrest, G.L., Akman, S., Krutzik, S., Paxton, R.J., Sparkes, R.S., Doroshow, J., Felsted, R.L., Glover, C.J., Mohandas, T. and Bachur, N.R. (1990) Biochim. Biophys. Acta 1048, 149–155.
[10] Krook, M., Ghosh, D., Strömberg, R., Carlquist, M. and Jörnvall, H. (1993) Proc. Natl. Acad. Sci. USA 90, 502–506.
[11] Wirth, H. and Wermuth, B. (1985) FEBS Lett. 187, 280–282.
[12] Wirth, H. and Wermuth, B. (1992) J. Histochem. Immunochem. 40, 1857–1863.
[13] Laemmli, U.K (1970) Nature 227, 680–685.
[14] Bohren, K.M., von Wartburg, J.-P. and Wermuth B. (1987) Biochim. Biophys. Acta 916, 185–192.
[15] Bohren, K.M., von Wartburg, J.-P. and Wermuth, B. (1987) Biochem. J. 244, 165–171.