Modification of an arginine residue in pig kidney general acyl-coenzyme A dehydrogenase by cyclohexane-1,2-dione

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The flavoenzyme pig kidney general acyl-CoA dehydrogenase (EC 1.3.99.3) is inactivated by cyclohexane-1,2-dione in borate buffer in a reaction that exhibits pseudo-first-order kinetics. Strong protection is afforded by the substrate octanoyl-CoA, as well as by heptadecyl-CoA, a potent competitive inhibitor of the dehydrogenase that does not reduce enzyme flavin. Enzyme exhibiting 10% residual activity in borate buffer contains about 1.3 modified arginine residues per flavin molecule. Very little reduction of the modified enzyme in borate buffer occurs at high concentrations of octanoyl-CoA, in marked contrast with the stoichiometric reduction of the native enzyme. However, in phosphate buffer alone, the modified enzyme exhibits 55% residual activity and, although binding of substrate is still seriously impaired (apparent $K_d = 14 \mu\text{M}$), excess substrate effects the formation of the characteristic reduced flavin·enoyl-CoA charge-transfer complex. These results suggest that the susceptible arginine residue, though not catalytically essential, is probably within the acyl-CoA-binding site of general acyl-CoA dehydrogenase.

Mammalian acyl-CoA dehydrogenases are soluble mitochondrial flavoproteins that catalyse the first dehydrogenation step of β -oxidation with the oxidation of acyl-CoA derivatives to the corresponding *trans*-2,3-enoyl-CoA products (Seubert & Lynen, 1953; Beinert *et al.*, 1953; Beinert, 1963). The reduced dehydrogenase is then re-oxidized by a second flavoprotein, electron-transferring flavo-protein, which mediates the transfer of these reducing equivalents to the electron-transport chain (Crane & Beinert, 1956; Ruzicka & Beinert, 1977).

Recently, protein modification techniques have been directed towards the identification of amino acid residues that are essential for catalytic activity in the general acyl-CoA dehydrogenases. For example, iodoacetate inactivates pig kidney general acyl-CoA dehydrogenase with modification of a single methionine residue (Mizzer & Thorpe, 1980). The enzyme derivative can still bind octanoyl-CoA, but the characteristic reduced flavin·enoyl-CoA complex is not observed. Pig kidney general acyl-CoA dehydrogenase does not have exposed rapidly reacting cysteine residues that are essential for

catalytic activity (Mizzer & Thorpe, 1980). The water-soluble carbodi-imide, 1-(3-dimethylamino-propyl)-3-ethylcarbodi-imide, modifies several carboxy residues in the pig liver enzyme, resulting in an impaired interaction with electron-transferring flavo-protein, but no significant effect on the reductive half-reaction with octanoyl-CoA (Frerman *et al.*, 1980a). The present paper describes the modification of an arginine residue in pig kidney general acyl-CoA dehydrogenase by reaction with cyclohexane-1,2-dione. Interestingly, this reaction yields a partially active enzyme derivative that is sensitive to further inhibition by borate ion, whereas the native protein is not inhibited by borate.

Experimental

Materials

General acyl-CoA dehydrogenase was purified from pig kidneys as described previously (Thorpe *et al.*, 1979; Thorpe, 1981). CoA, octanoyl-CoA and acetoacetyl-CoA were obtained from P-L Biochemicals. Heptadecyl-CoA, prepared as described by Ciardelli *et al.* (1981), was a gift from Dr. Theodor Wieland. Octyl-CoA was synthesized from 1-bromo-octane and CoA (Blaschkowski *et al.*,

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1979). Cyclohexane-1,2-dione and butane-2,3-dione were from Aldrich Chemical Co. Phenylglyoxal was obtained from Sigma Chemical Co. All other chemicals were of analytical grade from commercial sources.

Methods

The quoted enzyme concentrations refer to enzyme flavin determined by using the experimentally determined absorption coefficient of $15.4 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ at 446 nm (Thorpe *et al.*, 1979). U.v.- and visible-absorption spectra were recorded on a Cary 219 spectrophotometer. Unless otherwise noted, all buffers contained 0.3 mM-EDTA.

Enzyme assays. Assays were conducted at 25°C, with the use of a Beckman DB spectrophotometer, in 50 mM-potassium phosphate buffer, pH 7.6, with 30 μM -octanoyl-CoA as substrate, 1.4 mM-phenazine methosulphate as a mediator and 30 μM -2,6-dichlorophenol-indophenol as terminal acceptor (Thorpe, 1981). Alternatively, 40 mM-sodium borate buffer, pH 7.6 (Gomori, 1955), replaced the phosphate buffer. Assays were started by the addition of enzyme, unless otherwise noted.

Amino acid analysis. Samples of acyl-CoA dehydrogenase in 160 mM-borate buffer, pH 8.4, incubated in the dark with 25 mM-cyclohexanedione, were diluted at various times by the addition of 6 M-HCl to a concentration of 2 M, to halt the reaction and to prevent regeneration of free arginine (Riordan, 1973). The precipitated protein was dialysed for 18 h against 1 M-HCl at 4°C, dried and hydrolysed under vacuum in 6 M-HCl for 20 h at 110°C. Analysis of hydrolysates of native and modified enzymes were performed on a Beckman 120C amino acid analyser modified to allow either short-column separation of basic amino acids or single-column operation.

Gel filtration. Where noted, modified enzyme was desalted by using a 1 cm $\times$ 22 cm Sephadex G-25 column with either 20 mM- or 50 mM-borate or 50 mM-phosphate buffer, pH 7.6, at 4°C. Samples of native and cyclohexanedione-modified dehydrogenase and human γ -globulin were applied separately to a 1 cm $\times$ 52 cm Sephacryl S-200 (Pharmacia) column equilibrated with 100 mM-borate buffer, pH 7.6, at 20°C and eluted at 6 ml/h. The apparent molecular weight of the native enzyme has been previously estimated by using Sephacryl S-200 (Thorpe *et al.*, 1979).

Hydroxylamine treatment of native and modified enzymes. Dehydrogenase (0.5 ml, 20 μM) was inactivated to 20% residual activity in 160 mM-borate buffer, pH 8.4, containing 25 mM-cyclohexanedione. The mixture was then dialysed for 18 h against 1 litre of 100 mM-borate buffer, pH 7.6, 4°C. A separate experiment showed that enzyme activity is not significantly recovered after this dialysis step. The

modified enzyme was then treated with 0.2 M-hydroxylamine for 18 h in 50 mM-borate buffer, pH 7.3, at 4°C, and finally dialysed for 18 h against 1 litre of 100 mM-borate buffer to remove hydroxylamine. Recovery of activity is expressed versus a control of native enzyme minus cyclohexanedione, which was carried through the above steps.

Results

Fig. 1 shows the inactivation of pig kidney general acyl-CoA dehydrogenase by 25 mM-cyclohexanedione in borate buffer, pH 8.4. Under these conditions the decline in activity is first-order over 3 half-lives ($t_{1/2} = 18 \text{ min}$). Octanoyl-CoA strongly protects the dehydrogenase against this activation, but the observed rates are 20% higher than those of a corresponding control incubation with enzyme alone (Fig. 1). This slight stimulation can be duplicated in control experiments without cyclohexanedione, and is thus not due to an effect of cyclohexanedione on the octanoyl-CoA-reduced enzyme.

Several other CoA derivatives afford marked protection against inactivation of the dehydrogenase by cyclohexanedione (Table 1). Reduc-

Fig. 1. Inactivation of pig kidney general acyl-CoA dehydrogenase with cyclohexane-1,2-dione

The dehydrogenase (20 μM enzyme flavin) was incubated in the dark with 25 mM-cyclohexanedione in 1.0 ml of 160 mM-borate buffer, pH 8.4, at 25°C, alone (●), or plus 220 μM -octanoyl-CoA (▲), or in a control incubation mixture with cyclohexanedione and octanoyl-CoA omitted (■). Samples (5 μl) from these incubation mixtures were taken at the times indicated, each was diluted with 20 μl of 50 mM-borate buffer, pH 7.6, and 4 μl of the mixture was added to 0.7 ml of assay medium in borate buffer (see the Experimental section).

Table 1. Effect of CoA derivatives on inactivation of general acyl-CoA dehydrogenase by cyclohexane-1,2-dione

The compounds were added in the concentrations shown to 4 μ M general acyl-CoA dehydrogenase in 0.7 ml of 160 mM-borate buffer, pH 8.4, at 25°C in the presence or in the absence of 25 mM-cyclohexanedione. After 30 min the activities were measured and are expressed as percentages of corresponding values obtained without cyclohexanedione but with protecting agent present (see the text).

CoA derivative added	Residual activity (%)
None	28
Octanoyl-CoA (200 μ M)	116
Heptadecyl-CoA (50 μ M)	111
Octyl-CoA (160 μ M)	64
Acetoacetyl-CoA (160 μ M)	60

tion of the flavin is therefore not a requirement for protection, since the thioether analogues octyl-CoA (Frerman *et al.*, 1980b) and heptadecyl-CoA (Thorpe *et al.*, 1981) are not substrates but are competitive inhibitors of the dehydrogenase. Heptadecyl-CoA is a particularly potent inhibitor ($K_i < 40$ nM; Thorpe *et al.*, 1981), and the amounts of this thioether carried over from incubation to assay are appreciably inhibitory. The values in Table 1 are thus expressed as percentages of a corresponding control containing the CoA derivative, but without cyclohexanedione.

As is observed with several other enzymes, the inactivation of acyl-CoA dehydrogenase by cyclohexanedione does not require the presence of borate buffer. Inactivation with 25 mM-cyclohexanedione in 50 mM-pyrophosphate buffer, pH 8.4, at 25°C is equally rapid as that in borate buffer of the same pH (see Fig. 1). In both cases, assays were performed in borate buffer (see the Experimental section). Enzyme activity is not significantly regained on gel filtration of a sample inactivated to 10% residual activity on a column equilibrated with 20 mM-borate buffer, pH 7.6, or on prolonged dialysis against 50 mM-borate buffer, pH 7.6. A small recovery of activity (about 10%) is observed on gel filtration in 50 mM-phosphate buffer, pH 7.6 (see below). Neutral hydroxylamine treatment has been reported to re-activate several enzymes inactivated by cyclohexanedione (cf., e.g., Patthy & Smith, 1975a,b; Blumenthal & Smith, 1975). Enzyme inactivated by cyclohexanedione treatment to 20% residual activity was treated with 0.2 M-hydroxylamine for 18 h at 4°C, with a recovery of activity to 49% of a corresponding control (see the Experimental section).

A comparison of the amino acid analysis of enzyme treated to 10% residual activity with the composition of the native enzyme showed that only

the arginine content was decreased: by 1.3 ± 0.4 residues (mean $\pm$ S.D. for 11 determinations) per flavin molecule. The native pig kidney enzyme contains 23 arginine residues per flavin molecule (Thorpe *et al.*, 1979).

Fig. 2 shows that the only notable difference between the visible-absorption spectra of native and cyclohexanedione-modified forms in borate buffer, pH 7.6, is a significantly increased absorbance in the near-u.v. region. However, octanoyl-CoA titrations reveal striking differences in behaviour between the two enzymes (Fig. 2 inset). The native dehydrogenase is almost fully reduced by 1 equiv. of octanoyl-CoA in borate buffer (Fig. 2), as was previously observed in phosphate buffer (Thorpe *et al.*, 1979). The long-wavelength band, $\lambda_{\max} = 570$ nm, has been ascribed to a charge-transfer complex between reduced flavin as the donor and octenoyl-CoA as acceptor (Engel & Massey, 1971; Massey & Ghisla, 1974; Thorpe & Massey, 1982). In contrast, 1 equiv. of substrate effects very little bleaching of the modified enzyme, although a 20-fold excess (180 μ M) does produce the partially reduced spectrum shown in Fig. 2 with the appearance of a corresponding increase at long wavelengths. The modified enzyme is readily reduced by the addition of a small amount of solid $\text{Na}_2\text{S}_2\text{O}_4$ to the cuvette to give a spectrum very similar to that of dithionite-reduced native enzyme (Thorpe *et al.*, 1979).

When enzyme inactivated in borate buffer, and showing about 10% residual activity in 40 mM-borate buffer, pH 7.6 (see the Experimental section), is assayed in 50 mM-phosphate buffer, pH 7.6, apparently 55% residual activity remains. Unlike the assays in borate buffer, assays performed in phosphate buffer show a pronounced lag phase: about 40 s is required before the attainment of the maximal rate. This lag is abolished if the enzyme is incubated with the assay mixture minus octanoyl-CoA for 1 min before the addition of substrate. Two simple explanations could account for the increased activity observed on assay in phosphate buffer: a comparatively rapidly, but partial, dissociation of cyclohexanedione to regenerate native enzyme, or dissociation of borate yielding a partially active enzyme-cyclohexanedione adduct. The points described below favour the latter explanation. (1) The cyclohexanedione-modified enzyme, after gel filtration in 50 mM-phosphate buffer, pH 7.6, retains sensitivity to inhibition by borate, exhibiting 63% activity in phosphate buffer, but only 23% in 40 mM-borate buffer. In contrast, the native enzyme is insensitive to borate under these conditions. A rapid dissociation of the dione is also inconsistent with the only slight recovery of activity (from 55% to 63%) observed on gel filtration in phosphate buffer at pH 7.6. (2) If dissociation of cyclohexane-

Fig. 2. Octanoyl-CoA titration of native and cyclohexane-1,2-dione-modified general acyl-CoA dehydrogenase. The dehydrogenase was inactivated to 10% residual activity as indicated in Fig. 1 and gel-filtered on Sephadex G-25 equilibrated with 20 mM-borate buffer, pH 7.6. The modified enzyme (—; $9\mu\text{M}$ enzyme FAD in 0.7 ml of 20 mM-borate buffer, pH 7.6, at 25°C) was titrated with octanoyl-CoA to a final concentration of $180\mu\text{M}$ (· · · · ·). Intermediate A_{446} values are shown in the inset (●). The results of a corresponding titration of the native enzyme in borate buffer (· · · · ·) to $26\mu\text{M}$ -octanoyl-CoA (— · — · —) are also shown in the inset (○). A sample of modified enzyme was also gel-filtered and titrated with octanoyl-CoA in 50 mM-phosphate buffer (■, see the text).

dione occurred during the lag phase of the assay (see above), the subsequent addition of 20 mM-borate buffer should not influence the rate significantly, since the native enzyme is insensitive to borate under these conditions. However, the enzyme actually remains equally sensitive to borate inhibition, whether borate buffer is added before the enzyme, or after the maximal rate has been attained. (3) Further evidence supporting the interaction of borate with the modified enzyme is presented in the inset to Fig. 2. As described above, reduction of the cyclohexanedione-modified enzyme in borate buffer by octanoyl-CoA appears to be thermodynamically unfavourable, whereas reduction of the native enzyme in borate buffer (Fig. 2) or in phosphate buffer (Thorpe *et al.*, 1979) is virtually stoichiometric. Freshly prepared modified enzyme was therefore gel-filtered in 50 mM-phosphate buffer to remove borate and titrated with octanoyl-CoA as before. The inset to Fig. 2 shows that the behaviour of the modified enzyme in phosphate buffer is intermediate between those of the native and modified enzymes when titrated in borate buffer. This plot does not show the pronounced biphasicity that would be expected for the stoichiometric reduction of a sizeable proportion of native enzyme molecules. A double-reciprocal plot of the octanoyl-CoA titration of the modified enzyme in phosphate buffer (A_{446} versus substrate concentration) is linear,

Table 2. Activity of general acyl-CoA dehydrogenase treated with dicarbonyl reagents

General acyl-CoA dehydrogenase ($4\mu\text{M}$) was treated with 25 mM reagent in 160 mM-borate buffer, pH 8.4, at 25°C in the dark. Samples ($4\mu\text{l}$) were withdrawn at the times shown and diluted into the appropriate assay medium (see the Experimental section).

Reagent	Incubation time (min)	Activity (%)	
		Borate buffer	Phosphate buffer
Cyclohexane-1,2-dione	60	10	55
Butane-2,3-dione	7	10	47
Phenylglyoxal	100	19	48

yielding an apparent K_d of about $14\mu\text{M}$; the corresponding value in the native enzyme is in the 20 nM region (Thorpe *et al.*, 1981). If the extent of bleaching with $30\mu\text{M}$ -octanoyl-CoA (the substrate concentration used for the assays) is expressed as a percentage of the corresponding bleaching with the native enzyme, the modified enzyme shows 14% of the maximal bleaching in borate buffer and 62% in phosphate buffer. These values correlate quite well with the enzymic activity determined in the appropriate assay system after gel filtration.

It was decided to determine whether the activity of enzyme treated with butane-2,3-dione and phenylglyoxal was also dependent on the presence of borate in the assay medium. Table 2 shows that in all three cases enzyme exhibiting 10–20% residual activity in borate buffer shows about 50% activity in phosphate buffer. In addition, butane-2,3-dione inactivates general acyl-CoA dehydrogenase much more rapidly than does either cyclohexane-1,2-dione or phenylglyoxal.

Discussion

The reaction of cyclohexanedione with pig kidney general acyl-CoA dehydrogenase is highly selective, with the modification of only about 1 arginine out of a total of 23 residues per flavin molecule (Thorpe *et al.*, 1979). It appears probable that the susceptible arginine residue is located within the substrate-binding site of general acyl-CoA dehydrogenase, since acyl-CoA derivatives and analogues afford marked protection against inactivation by cyclohexanedione and because binding of octanoyl-CoA to the modified enzyme is seriously impaired (Fig. 2). A body of evidence suggests that arginine residues may participate in the binding of anionic substrates and coenzymes in a variety of different enzymes (e.g. see Riordan *et al.*, 1977; Riordan, 1979). Thus the guanidinium moiety of the susceptible arginine residue in general acyl-CoA dehydrogenase may play a similar role in CoA binding, interacting with the pyrophosphate or the 3'-phosphate groups of the coenzyme. Although the protection studies suggest that the arginine residue is within the active centre, the possibility that a ligand-induced conformational change decreases the reactivity of a distant arginine residue, whose modification nevertheless impairs substrate binding, cannot be rigorously excluded. The modified enzyme, however, shows only minor differences from the native enzyme in the flavin spectrum above 400 nm and no significant difference in apparent molecular weight (see the Experimental section).

The results of the present study suggest that the cyclohexanedione-treated enzyme is itself partially active in phosphate buffer, and that activity is further suppressed by reversible complexing of the modified enzyme with borate. This borate effect might represent reversible binding to the *cis*-diol cyclohexanedione-arginine adduct (Riordan, 1973, 1979; Patthy & Smith, 1975a). The introduction of a negative charge and further steric bulk on complex-formation would be expected to render octanoyl-CoA binding much less favourable. An alternative explanation is that modification leads to a conformational change with the acquisition of a borate-

binding site not directly involving the susceptible arginine residue (e.g. with binding to the ribityl or ribose hydroxy groups of FAD). Although these possibilities cannot be distinguished at present, this work does indicate that the susceptible arginine residue, though not catalytically essential, is important in the binding of octanoyl-CoA to pig kidney general acyl-CoA dehydrogenase.

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