MODIFICATION OF PIG HEART CITRATE SYNTHASE

Anders MÅHLÉN
Department of Chemistry, National Bacteriological Laboratory, S-105 21 Stockholm, Sweden

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1. Introduction

Citrate synthase has been shown to be inhibited by photo-oxidation [1] and by acylation with acetic anhydride [2] of succinic anhydride [3], where the last reagent acts by dissociating the two peptide chains. Lysine and arginine residues would seem likely to be essential in the binding of oxalacetate and the three phosphate groups of CoA. The existence of a pyrophosphate binding site has been suggested in inhibition studies by Lee and Kosicki [4]. To gain further information about residues essential for citrate synthase activity the effects of some modifying reagents have been investigated. A specific protection by oxalacetate was found with histidine and arginine reagents.

2. Materials and methods

Pig heart citrate synthase was purchased from Boehringer, had a specific activity of 80 U/mg, and was used without further purification. Propionyl-CoA was from Sigma, and acetyl-CoA was prepared as described by Stadtman [5]. 2,3-Butanedione was redistilled and used in fresh solutions. Trimeric butanedione was prepared according to Yankeelov [6] and recrystallised to m.p. 112–116°C. Kethoxal was from Serva and nitromalondialdehyde was prepared as described by Fanta [7]. Diethylpyrocarbonate was from Carlo Erba.

Modifications were performed at 2.5 μM enzyme concentration for about 1 hr and substrates were added at 800 μM for acetyl-CoA and 1 mM for oxalacetate. Propionyl-CoA was used at 50 μM concentration. Aliquots of the mixtures were diluted with 0.1 M potassium phosphate pH 8.1 and assayed with 5.5-dithiobis (2-nitrobenzoic acid) (Nbs_2) at 22°C with 25 μM acetyl-CoA and 1 mM oxalacetate in 0.1 M phosphate buffer pH 8.1 [8].

3. Results and discussion

The stability of the enzyme during the various treatments was adequate in the absence of reagent. The substrate concentrations were selected so as to be well above K_D [9–11]. The ternary complex with propionyl-CoA is not fully nonreactive [12], however, at pH 8.1 only about 25% of the propionyl-CoA was consumed after 30 min during the conditions chosen, which justifies its use as an inhibitor. No inhibition was found with the amino group reagents KNCO at 0.2 M in 0.1 M Tris–HCl pH 7.6 [13] or *N*-acetyl homocystein thiolactone at 0.1 M in 0.1 M phosphate pH 8.1 [14]. 5 mM citraconic anhydride in 0.1 M Tris–HCl pH 8.2 gave a rapid inactivation which was independent of added ligands, and subunit dissociation as caused by succinic anhydride seems likely [3]. Diethylpyrocarbonate, which is considered to be specific for histidine [15] inactivated citrate synthase rapidly at 5 mM concentration in 0.1 M potassium phosphate pH 6.0. 100 μM oxalacetate protected the enzyme partially, and addition of propionyl-CoA gave no enhanced protection. similarly, acetyl-CoA alone did not affect the rate of inactivation significantly (fig.1). The reaction with diethylpyrocarbonate gradually caused protein denaturation. Assuming an absorptivity of 3200 at 242 nm [16] for the carbethoxyhistidine, a 50% inhibited enzyme preparation had about 3 histidines out of about 13 per subunit [17] modified.

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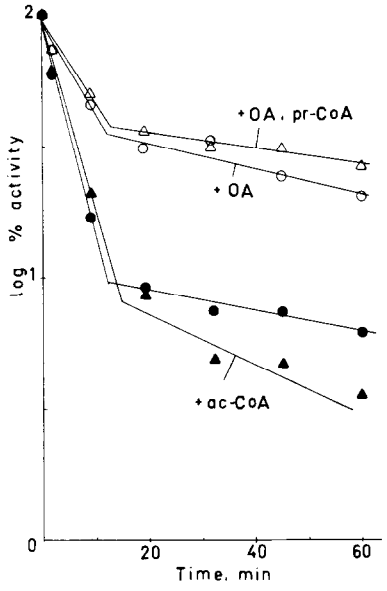

Fig.1. Effect of diethylpyrocarbonate on citrate synthase activity. 2.5 μM enzyme was reacted with 5 mM reagent at 0°C in 0.1 M potassium phosphate pH 6.0 with the following additions: (●) none, (▲) 0.8 mM acetyl-CoA, (○) 0.1 mM oxalacetate, (△) 0.1 mM oxalacetate + 50 μM propionyl-CoA. The activity is expressed as log % initial rate.

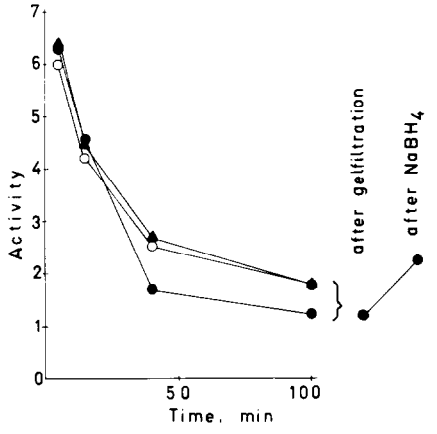

Fig.2. Effect of nitromalondialdehyde on citrate synthase activity. 2.5 μM enzyme was reacted with 15 mM reagent at 0°C in 0.1 M sodium borate pH 9.2 with the following additions (●) none, (▲) 0.8 mM acetyl-CoA,(○) 1 mM oxalacetate. After 100 min the mixtures were pooled and freed of excess reagent by Sephadex G 25 filtration. The solution was then made 1% in $NaBH_4$ and assayed after 1 hr at 0°C. The activity is given in arbitrary units.

The tryptophane reagent dimethyl-(2-hydroxy-5-nitro-benzyl) sulfonium chloride [18] had no effect on the enzyme activity at 250 μM concentration in 0.1 M phosphate pH 6.8 or 8.1.

Arginine reagents such as kethoxal [19], nitromalondialdehyde [20], butanedione and trimeric butanedione [6] were tested with citrate synthase.

Kethoxal as a 0.5% solution in 0.1 M potassium phosphate pH 8.1 had little or no effect on the activity. Nitromalondialdehyde did not inactivate the enzyme at 15 mM concentration in 0.1 M borate pH 7.5, but at pH 9.2 a rapid inhibition occurred (fig.2). No significant protection was afforded by the substrates. Spectrophotometric investigation after gelfiltration of the 76% inhibited enzyme revealed about 6 nitropyrimidines out of 16 arginines per subunit [17], assuming an absorptivity of 16 000 at 331 nm [20]. Treatment with 1% $NaBH_4$ at 0°C for 1 hr caused the disappearence of the 331 nm peak and partially reactivated the enzyme. 60 mM Butanedione in 0.125 M borate pH 7.5 inactivated citrate synthase (fig.3), and again oxalacetate partially protected the enzyme. The inactivation was slowly reversed during

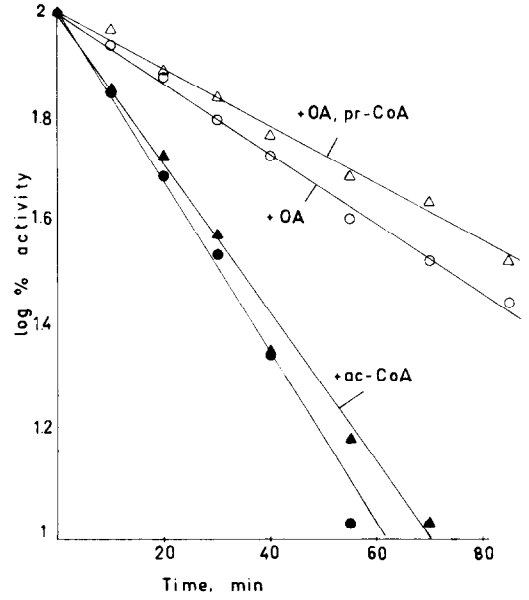

Fig 3. Effect of butanedione on citrate synthase activity. 2.5 μM enzyme was reacted with 60 mM butanedione at 22°C in 0.125 M sodium borate pH 7.5 with the following additions: (●) none, (▲) 0.8 mM acetyl-CoA, (○) 1 mM oxalacetate.

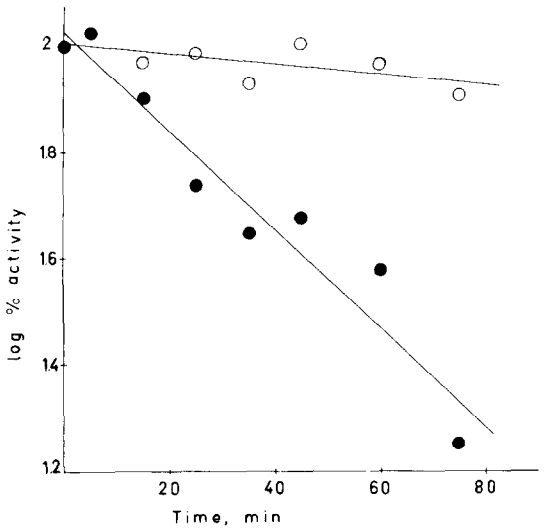

Fig.4. Effect of trimeric butanedione on citrate synthase activity. 2.5 μM enzyme was reacted with 55 mM reagent at 22°C in 0.1 potassium phosphate pH 6.0 with (○) and without (●) 1 mM oxalacetate.

the assay, hence initial rates are given in the figure. Since butanedione may also react with lysine, trimeric butanedione was also used to further assess the involvement of arginine. Thus, at 55 mM trimeric butanedione in 0.1 M potassium phosphate pH 6.0 an improved protection by oxalacetate was achieved (fig.4). No reactivation during the assay occurred with this reagent. Unfortunately the arginine reagents used form derivatives which are poorly stable during acid hydrolysis, therefore the extent of modification is difficult to determine. In conclusion arginine and histidine residues may be involved in the oxalacetate binding site of citrate synthase. On the other hand the oxalacetate binary complex has altered conformation as reflected in UV difference spectrum [21], and stability towards urea denaturation [10,22], which may involve burial of reactive groups distant from the binding site. The lack of protection by oxalacetate in the nitromalondialdehyde reaction could be explained if this reagent was a powerful competitive inhibitor. Nitromalondialdehyde is negatively charged as opposed to butanedione and might be more strongly bound. At pH 8.1, however, only weak inhibition was found kinetically, and neither K_D of oxalacetate (C.-J. Johansson, pers. commun.) nor the charge of nitromalondialdehyde [20] are drastically changed at pH 9.2. On the other hand, the reactivation after reduction is indicative of the essentiality of a positive charge. Arginine has been implicated as a carboxyl binding residue in lactate dehydrogenase [23]. Residues of the acetyl-CoA binding site were obviously inaccessible to the reagents used (cf. a recent article on alcoholdehydrogenase [24]).

Although the commercial preparation used here is not pure [17] the quantitative estimates of the modification indicate that several residues are reacted, and no major differences in reactivity between different residues is apparent from the inhibition curves.

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