CHEMICAL MODIFICATION OF CITRATE SYNTHASE

Evidence against two geometrically separated catalytic sites per monomer

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1. Introduction

Citrate synthase (EC 4.1.3.7) from pig heart consists of two identical monomers each of which has been suggested to contain two different and geometrically separated active sites (lyase and hydrolase) [1,2]. Only one active site could suffice if corresponding conformational changes would occur during the catalytic cycle. Evidence in favour of such conformational changes has been presented but did not exclude the existence of 2 active sites/monomer [3]. If equipped with these sites the synthase would belong to the group of polyfunctional enzymes. One can then expect that chemical modification of the synthase would yield different degrees of inactivation of the hydrolase and lyase activities. In the laboratory of F. Lynen as an example, experiments of this kind, performed with yeast fatty acid synthetase, have shown different degrees of inactivation of the enzymes acetyl transferase, enoyl reductase and malonyl transferase [4] which are integrated into one of the 2 polyfunctional protein chains from which the synthase consists. Some chemical modifications of citrate synthase and their effect on the enzymic activity are presented here.

2. Materials and methods

Citrate synthase (pig heart) was from Boehringer Mannheim GmbH, coenzyme A (Ultra Pure) from PL Biochemicals (St Goar) and DEAE–Sepharose from Pharmacia, Freiburg. (3*S*)-Citryl-CoA and prepared enzymically [5] and 4-nitrophenyl-2-diazoacetate as in [6]. Acetonyl dethio-CoA [7] was a gift from Professors C. J. Stewart and Th. Wieland.

2.1. *Preparation of diazoacetyl thioester derivatives*

2.1.1. *N*-Acetyl-*S*-diazoacetyl cysteamine

N-Acetyl cysteamine (0.8 mmol), dissolved in 10 ml 0.1 M sodium methylate in absolute methanol, was treated for 1 h at 0°C with 1 ml 1.35 M 4-nitrophenyl diazoacetate in absolute tetrahydrofuran. The residue obtained on evaporation was suspended in 1 ml benzene and applied on a column of aluminium oxide (Woelm Alumina N, grade IV, 1 × 15 cm) which was washed with 100 ml benzene and eluted with 100 ml benzene/methylene chloride (1:1; v/v). The diazoacetyl thioester-containing fractions were combined and evaporated. Recrystallization of the residue from ethyl acetate/petroleum ether yielded 59 mg (39% yield), m.p. 57–59°C. $C_6H_9O_2N_3S$ (187.2) requires C 38.49, H 4.84, N 22.45%; found: C 38.47, H 4.84, N 22.39%. $\epsilon_{237} = 7.2 \times 10^3\ M^{-1} \cdot cm^{-1}$, $\epsilon_{281} = 16.5 \times 10^3\ M^{-1} \cdot cm^{-1}$ (methanol; thioester and diazo group, respectively). Stable in 1 M hydroxylamine (pH 7; 25°C; 60 min); slowly hydrolyzed in 50 mM NaOH ($t_{1/2} \sim 3$ h); decomposed in methanolic 5 mM HCl within 3 h to yield the absorbance ($\epsilon_{234} = 4 \times 10^3\ M^{-1} \cdot cm^{-1}$) of *N*-acetyl-*S*-glycolyl cysteamine. Photolysis at 280 nm (Aminco SPF 500 fluorimeter; slit width 40 nm) decomposed the diazo and thioester groups (100% and 90%, respectively; thioester analysis performed as in [3]) within 60 s. Thus ~90% of the photolytically generated carbene appears to undergo a Wolff-rearrangement with formation of *N*-acetyl-*S*-carboxymethyl cysteamine. This product was identified as in [3]. The results are similar as described for the photolysis of methyl diazothioacetate [8].

2.1.2. Diazoacetyl-CoA

Coenzyme A (10 mg; Li_3^+) dissolved in 0.1 ml abso-

lute methanol was mixed at 0°C with 0.2 ml 0.1 M sodium methylate in absolute methanol and treated without delay with 0.1 ml 0.2 M 4-nitrophenyl-2-diazoacetate in absolute tetrahydrofuran. The reaction was stopped 5 min later by dilution with 1.5 ml 10 mM Tris buffer (pH 8.0) followed by extraction with ethyl ether. Residual mercaptan (20% of initial) was oxidized by adding excess 5,5′-dithio-bis(2-nitrobenzoate). Diazoacetyl-CoA was purified by chromatography on a DEAE–Sepharose column as in [3] but using the LiCl gradient (0–300 mM) in 5 mM Tris buffer (pH 8.0). The fractions were analyzed from their ultraviolet absorbance and yielded 7 μmol diazoacetyl-CoA (~80% pure; spectroscopic determinations). $\epsilon_{263} = 2 \times 10^4\ M^{-1} . cm^{-1}$, ϵ_{281} (shoulder) = $1.7 \times 10^4\ M^{-1} . cm^{-1}$ (80% pure; 0.1 M Tris buffer (pH 7.0)); the shoulder disappeared in acidic solution. Photolysis at 280 nm (3 min; Aminco SPF 500 fluorimeter; slit width 40 nm) yielded the absorbance of adenosine. Diazoacetyl-CoA was no substrate for citrate synthase (80 nkat; 2 h).

2.2. *Enzyme assays*

Citrate synthase activities (assays 1–3) were determined as in [3,9].

3. Results

3.1. *Assays*

Three assays were performed in parallel throughout this work for the detection of modification-dependent changes of the synthase activities.

(1) Overall reaction

Acetyl-CoA + oxaloacetate + $H_2O \rightleftharpoons$ citrate + CoASH

(2) Citryl-CoA hydrolysis

(3*S*)-Citryl-CoA + $H_2O \rightleftharpoons$ citrate + CoASH

(3) Citryl-CoA cleavage

(3*S*)-Citryl-CoA $\rightleftharpoons$ acetyl-CoA + oxaloacetate

3.2. *Modifications*

3.2.1. Diazoacetyl-CoA

The efficiency of diazoacetyl-CoA to produce enzyme inactivation on photolysis was low. In a series of reactions performed under several different conditions in the absence of presence of oxaloacetate the quantitative photolytic reaction of diazoacetyl-CoA at 280 nm (1–2 min) or 365 nm (60 min) never yielded an inactivation of the synthase ≳25%. A typical result is shown in fig.1. The 3 assays yielded identical results.

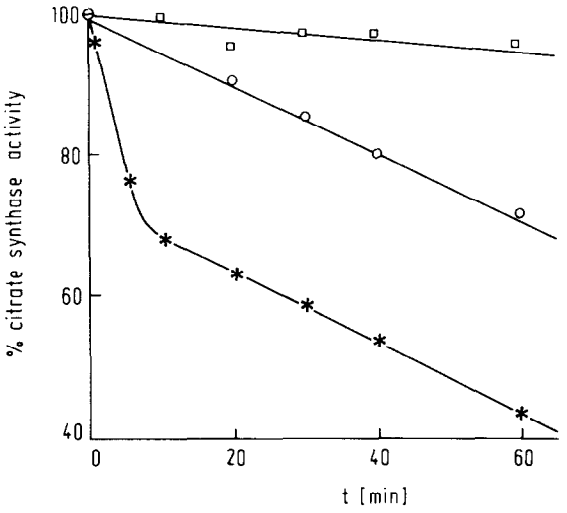

Fig.1. Inactivation of citrate synthase by photolysis of diazoacetyl-CoA. The reaction mixture in 2 ml total vol. Tris buffer (pH 8.0) contained 0.05 mM oxaloacetate, 0.1 μmol diazoacetyl-CoA and 0.17 μkat citrate synthase. Irradiation (365 nm) was performed at 10°C in an Aminco SPF 500 fluorimeter (slit width 40 nm) in the presence (*) and absence (○) of diazoacetyl-CoA; control without irradiation (□).

3.2.2. Diethylpyrocarbonate

Ethoxyformylation of the synthase with diethyl pyrocarbonate had been shown [10] to produce 3 ethoxyformylated histidine residues/monomer, concomitant with 50% loss of enzymic activity. Under our conditions (0.3 μkat citrate synthase, 5 μmol diethyl pyrocarbonate in 1.0 ml 50 mM phosphate buffer (pH 6.0); 45 min; 25°C) the reaction yielded ~2.2 ethoxyformylated histidine residues/monomer (calculated from the difference spectra using $\epsilon_{242} = 3.2 \times 10^3\ M^{-1} . cm^{-1}$ for 1-*N*(ethoxyformyl)histidine [11]), concomitant with 80% loss of enzymic activity. The 3 assays yielded identical results.

3.2.3. Acetylation

Treatment at pH 5 of the synthase with acetic anhydride results in complete loss of enzymic activity which can be restored with hydroxylamine [12]. The

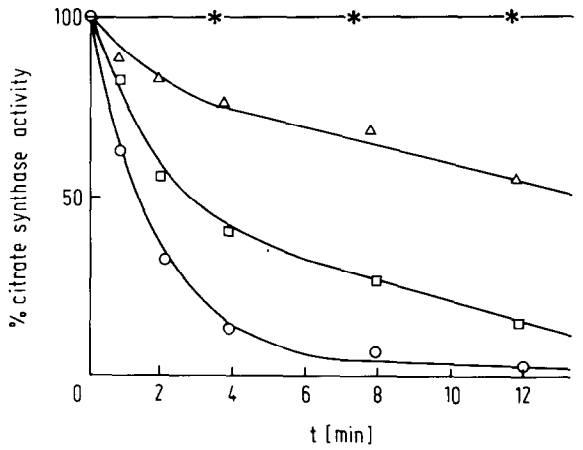

Fig.2. Inactivation of citrate synthase by acetylation. The reaction mixture in 0.4 ml total vol. contained 0.2 M acetate buffer (pH 5.1); 1 mg serum albumine; 18 nkat citrate synthase and no (*) or 5 μmol acetic anhydride (○); additionally 0.7 μmol oxaloacetate (□) or 0.7 μmol oxaloacetate and 0.05 μmol acetonyl dethio-CoA (△).

kinetics of inactivation by acetic anhydride at pH 5 is shown in fig.2. Oxaloacetate provided a shielding effect that was increased in the additional presence of the acetyl-CoA analogue acetonyl dethio-CoA. About 60% of initial activity was recoverable with hydroxylamine (75 mM). All activities of the synthase were likewise affected on inactivation and reactivation.

3.2.4. Irradiation at 280 nm

Irradiation of the synthase with ultraviolet light (280 nm) produces a tryptophan residue-specific fluorescence at 333 nm [13]. The fluorescence disappeared on prolonged irradiation thus indicating that all tryptophan residues became destroyed during this treatment. A decrease of enzymic activity (assay 1) occurred in parallel to the loss of fluorescence. Both partial reactions however were stimulated ~1.5-fold initially. The results are shown in fig.3.

3.2.5. Methyl mercuric chloride

The synthase activity (assay 1) became somewhat decreased in the presence of methyl mercuric chloride (0.1–1.0 mM) but the inhibition was not proportional to poison concentration, a 10-fold increase causing only a weak additional inhibition. Both partial reactions became stimulated. Prolonged incubations of enzyme and mercurial were without additional effect. The results summarized in table 1 point to mercurial-dependent conformational changes of the synthase.

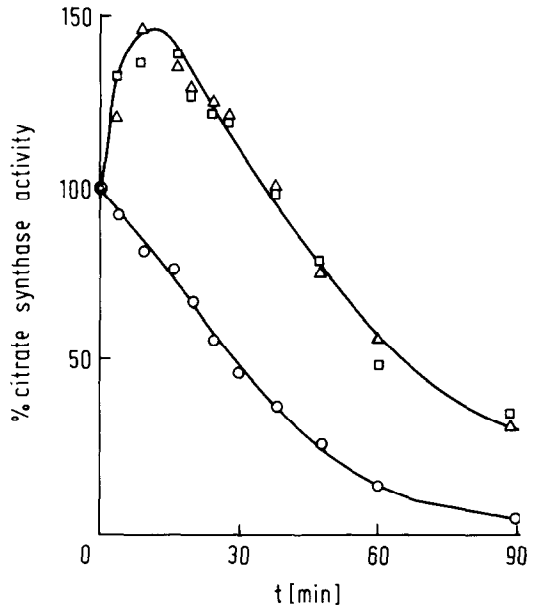

Fig.3. Inactivation of citrate synthase by irradiation at 280 nm. The reaction mixture in 1.5 ml total vol. 0.1 M Tris buffer (pH 8.0) contained 80 nkat citrate synthase. Irradiation was performed at 280 nm in an Aminco SPF 500 fluorimeter (10°C; slit width 10 nm). Samples were withdrawn at the times indicated in the figure and the synthase activities were determined: assay 1 (○); assay 2 (△); assay 3 (□).

4. Discussion

Two effects were observed upon chemical modification of the synthase. The catalytic activity determined by assays 1–3 either decreased in parallel or that for the overall reaction decreased but that for both partial reactions became stimulated. In no case could a separation of the hydrolase and lyase functions be achieved. Earlier attempts (H. E., U. Remberger, unpublished) are in accord with these results: cell-free extracts of citrate-auxotroph *E. coli* mutants, kindly

Table 1
Influence of methyl mercuric chloride on citrate synthase activities

CH_3HgCl (mM)	% Activity		
	Assay 1	Assay 2	Assay 3
0	100	100	100
0.1	75	275	135
1.0	70	180	120

provided by Professor B. D. Davies, contained no partial activity of the synthase. The kinetics of heat inactivation of the enzyme (60°C) on determination with the 3 assays was identical. The results are consistent with the presence of only one catalytic site existing in 2 different conformations [3].

The weak inhibition of the synthase by methyl mercuric chloride is of particular interest. The mercurial has been shown by X-ray crystallographic analysis to bind to the enzyme at a buried site that is accessible through a narrow 1.5 nm long channel [1]. The same site also binds, tentatively, a second citrate, the first one being bound at the mouth of the channel [1]. These results are the only available experimental evidence for the existence of 2 distinct catalytic sites/monomer. Under conditions as used for the X-ray crystallographic studies ($[CH_3HgCl]$ = 0.1 and 1.0 mM) the synthase became only weakly inhibited (assay 1) but both partial reactions became stimulated. We conclude from these results that the mercurial binding site represents none of the 2 anticipated catalytic sites. The second citrate binding site has been excluded by more recent experiments (R. Huber, personal communication). In agreement with our results the X-ray crystallographic analysis therefore indicates the existence of only 1 active site/subunit.

The stimulation of the partial reactions and the simultaneously occurring inhibition of the overall reaction found on irradiation of the enzyme as well as in the presence of methyl mercuric chloride are similar to the catalytic properties of citrate synthase fragments [9]. They are understood as a derepression of the partial reactions through a destruction of cooperativity that occurs on the native enzyme during substrate flow (in preparation).

Acknowledgements

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