CATALYTIC ACTIVITY OF CITRATE SYNTHASE FRAGMENTS

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Received 10 December 1980

1. Introduction

Citrate synthase from pig heart catalyzes the formation of citrate from acetyl-CoA and oxaloacetate [1] as well as the hydrolysis and cleavage of (3*S*)-citryl-CoA [2]. Attempts to produce functionally active domain of the enzyme by limited proteolysis remained unsuccessful [3] until the procedure in [4] was used. The synthase in the presence of palmitoyl-CoA and trypsin is primarily cleaved into two fragments of M_r ~9000 and 35 000 [4,5]. We found that these fragments form an enzymically active entity with kinetic properties that are different from those of the native synthase.

2. Materials and methods

Citrate synthase (pig heart) was from Boehringer, Mannheim; bovine trypsin (58 nkat/mg; TPCK-treated) and trypsin inhibitor (soy beans) were from Merck, Darmstadt. Sephacryl S-200 and BrCN-treated Sepharose 4B were purchased from Pharmacia, Freiburg. ATP–Sepharose was prepared as in [6]; (3*S*)-citryl-CoA was prepared enzymically [7].

2.1. *Enzyme assays*

Citrate synthase activity was determined in the presence of 0.2 mM 5,5′-dithiobis-(2-nitrobenzoate), 0.1 mM (3*S*)-citryl-CoA or 0.1 mM acetyl-CoA and 0.5 mM oxaloacetate (citryl-CoA hydrolysis or overall reaction [8]) or in the presence of 0.1 mM (3*S*)-citryl-CoA, 0.2 mM NADH and malate dehydrogenase (citryl-CoA cleavage).

2.2. *Formation and isolation of citrate synthase fragments*

Citrate synthase (1.76 mg protein; 3.18 μkat) and 0.7 mg trypsin were dissolved in 750 μl 0.1 M Tris buffer (pH 8.0). The proteolytic reaction was started by adding 250 μl 0.4 M palmitoyl-CoA and terminated 70 s later by adding 250 μl 0.1 M Tris buffer (pH 8.0) containing 3 mg trypsin inhibitor. The total synthase activity was then 118 nkat (overall reaction) and 412 nkat (citryl-CoA hydrolysis). The digest was applied on a Sephacryl S-200 column (1.5 × 60 cm) and eluted with 10 mM Tris buffer (pH 7.5); 2 ml fractions were collected (12 min/fraction). Fraction numbers 13–17 containing the activity (85 nkat; overall reaction) were combined (10 ml) and applied on ATP–Sepharose (1 × 15 cm). The column was washed with 10 mM Tris buffer (pH 7.5) for removal of trypsin and trypsin inhibitor but the effluent (30 ml) also contained minor synthase activity (15 nkat). Elution was performed with a gradient formed between 10 mM Tris buffer (pH 7.5) and the same buffer solution containing 200 μM CoA and 300 μM oxaloacetate [6], at 3 ml fractions/3 min. Activity appeared in fraction numbers 7–24. The main activity (17 nkat each) was present in the combined fraction numbers 11–14 (11.6 ml; 170 μg protein) and 15–19 (14.5 ml; 200 μg protein). The ratio of activities for overall reaction and citryl-CoA hydrolysis of these fractions was 1:3.2. Samples (3 ml each) were lyophylized prior to the determination of protein. Other samples (5 ml each) were treated likewise for the gel electrophoretic analysis; the residue was dissolved in 0.2 ml water; 50 and 100 μl were applied on the gels. All operations were checked by the gel electrophoretic procedure.

2.3. *Other methods*

Sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis was performed on a 10% gel as in [9]. Protein was determined as in [10].

Coenzyme A was found to interfere with the assay. If present in the probes the concentration of CoA was

determined spectrophotometrically and corrections were made from a calibration curve.

3. Results and discussion

3.1. *Enzymic activity correlated with limited proteolysis of citrate synthase*

The time dependence of the limited proteolysis of the synthase is shown in fig.1A. The results confirm those in [4,5] in that primarily two fragments are formed (28 s), the smaller one being preferentially degraded further on prolonged incubation. The enzymic activity for the overall reaction decreased in parallel to the progress of the limited proteolysis but that for (3*S*)-citryl-CoA hydrolysis as well as cleavage became stimulated drastically, with a maximum at ~28 s (fig.1B). At this time the native synthase was present in trace amounts only but the residual activity for the overall reaction was ~5–10% of its initial value.

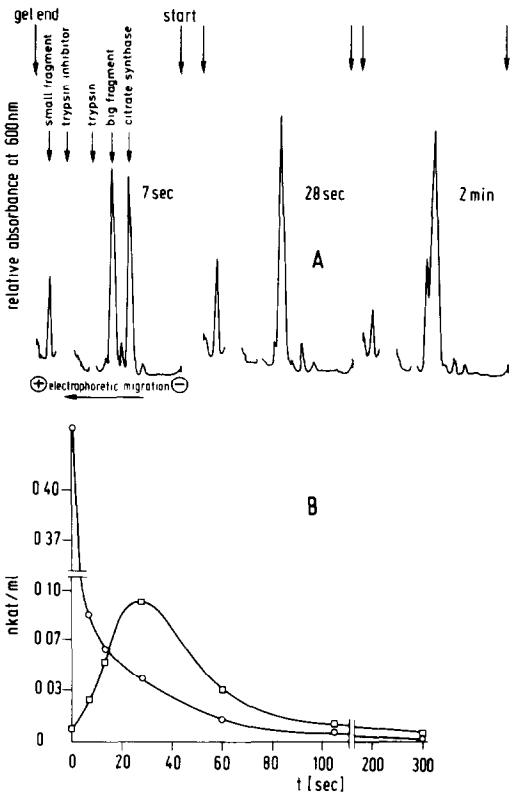

Fig.1. Correlation of limited proteolysis of citrate synthase with enzymic activity. The incubation mixture which was kept at 25°C, in 100 μl total vol. 10 mM Tris buffer (pH 8.0) contained 170 μg (0.42 μkat) citrate synthase, 20 μg trypsin and 10 nmol palmitoyl-CoA. Samples (10 μl each) were stopped at the times indicated by adding 50 μl solution of trypsin inhibitor (20 μg). (A) Electrophoresis was performed with 30 μl each of the stopped reaction mixtures (the absorbancies of trypsin and trypsin inhibitor are not shown). (B) Enzymic activity of the stopped solutions (10 μl each) was determined after appropriate dilutions: overall reaction (○); citryl-CoA hydrolysis (□); the cleavage of citryl-CoA (not shown) was stimulated in parallel to its hydrolysis ($v_{hydrolysis} : v_{cleavage} = 1:3$).

3.2. *Isolation of citrate synthase fragments*

In order to ensure complete fragmentation of the native synthase the limited proteolysis, performed as in section 2, was prolonged up to 70 s. The generated fragments were purified by chromatography on Sephacryl S-200 followed by affinity chromatography using ATP–Sepharose [6]. Citrate synthase activity-containing fractions were isolated. As judged from SDS–polyacrylamide gel electrophoresis (fig.2) these fractions contained the two fragments in a molar ratio of 1:1 and were devoid of native citrate synthase. The results also demonstrate that the two fragments were not separable by the chromatographic procedures used and hence that they form an entity with a conformation that is probably similar to that of the native enzyme [4]. This assumption is consistent with the site of proteolytic attack of the synthase [4,5] and with results obtained on prolonged proteolysis where a preferential degradation of the smaller fragment results in complete loss of enzymic activity (cf. fig.1).

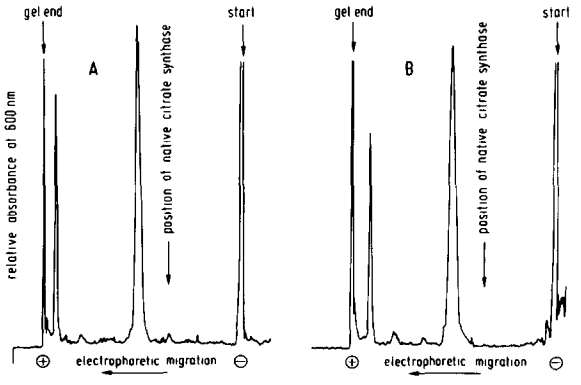

Fig.2. SDS–polyacrylamide gel electrophoresis of purified citrate synthase fragments (eluates from ATP–Sepharose). (A) Fraction numbers 11–14; 36 μg protein was applied (200 V; 60 min). (B) Fraction numbers 15–19; 34 μg protein was applied (200 V; 60 min). The stained gels were recorded at 600 nm with a Gilford spectrophotometer (model 250) equipped with a gel scanner (model 2520).

The synthase activity of the purified fragments cannot be derived from the presence of trace amounts of native enzyme. If it is assumed that the small protein peak (~0.8% of total protein) visible in fig.2A, but absent in fig.2B, represents proteolytically unattacked enzyme, its concentration could account for at most 15% of the activity of this sample found under saturating conditions.

3.3. *Catalytic properties of isolated citrate synthase fragments*

The affinity of oxaloacetate towards the fragmented enzyme was unchanged (K_m = 5 μM) but that for acetyl-CoA was increased to K_m = 60 ± 20 μM (6 μM for the native enzyme).

The fragmented enzyme catalyzed the hydrolysis and cleavage reactions of citryl-CoA ten times faster than the native enzyme (cf. fig.1). They catalyzed the hydrolysis of citryl-CoA about twice as fast and the cleavage of this substrate nearly as fast as the overall reaction. These results are entirely consistent with citryl-CoA being an intermediate and must be compared with those obtained with the native enzyme which catalyzes these reactions at rates of 2% and 0.7%, respectively, of that of the overall reaction.

4. Conclusion

The kinetic properties of the fragmented enzyme are those to be expected for a simplified citrate synthase working without cooperativity during the catalytic cycle. This conclusion is strengthened by other studies which demonstrate that the native enzyme operates indeed in a much more organized manner than was so far anticipated (in preparation).

Acknowledgement

Support of this work by the Fonds der Chemischen Industrie is gratefully acknowledged.

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