Organization of citric acid cycle enzymes into a multienzyme cluster

Sarah J. Barnes and P.D.J. Weitzman*

Department of Biochemistry, University of Bath, Bath BA2 7AY, England

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The possibility that some of the enzymes of the citric acid cycle may be loosely associated into a multienzyme cluster has been investigated using extracts prepared by gentle disruption of cells. Gel filtration and sucrose density gradient centrifugation have shown that five sequential enzymes of the cycle specifically associate into a cluster: fumarase, malate dehydrogenase, citrate synthase, aconitase and isocitrate dehydrogenase. Ultrasonication destroys the abilities of the enzymes to associate. The cluster could catalyse the sequence of reactions leading from fumarate to oxoglutarate and has been found in extracts of several bacterial species as well as rat liver mitochondria.

Citric acid cycle *Metabolic organization* *Bacteria* *Enzyme association* *Multienzyme cluster* *Mitochondria* *Enzyme organization*

1. INTRODUCTION

The idea that the enzymes of the citric acid (Krebs) cycle may exist in the cell bound together in some form of complex has long provoked interest. Many years ago, Green et al. [1] reported such a complex to which they gave the name 'cyclophorase', but this was soon shown to be identical with the intact mitochondrion [2]. Srere [3] later proposed that the citric acid cycle enzymes are not randomly distributed in the mitochondrial matrix but are organized in assemblies, bearing a fixed relation to each other and to the inner membrane.

Several investigators have studied mixtures of certain pairs of purified citric acid cycle enzymes and observed interactions between them [4–7]. The alternative approach, i.e. to demonstrate inter-enzymic complexes in cell extracts, has not hitherto been successful. Thus, for example, Srere et al. [8] examined mitochondrial extracts by both sucrose density gradient centrifugation and gel filtration but failed to detect any high-molecular-mass enzyme complexes.

In view of the absence of organellar compartmentation of the citric acid cycle in bacteria and of our previous studies on cycle enzymes from bacteria [9] we examined extracts of gently disrupted bacteria for evidence of multienzyme association. We here report our discovery of a 'cluster' of five sequential enzymes in extracts of several bacterial species and describe some of its properties. A similar cluster may also be isolated from mitochondria.

2. EXPERIMENTAL

Bacteria were grown overnight in nutrient broth (Oxoid) with shaking at 37°C and spheroplasts were prepared and lysed as described in [11] except that the lysis buffer used was 10 mM Tris-acetate, pH 7.5, containing 20% (v/v) glycerol (TAG). After centrifugation, a sample of the supernatant was subjected to gel filtration on a column of Sepharose 4B (Pharmacia) equilibrated with TAG and elution was continued with the same buffer.

* To whom correspondence should be addressed

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Enzyme assays were carried out by standard spectrophotometric methods: aconitase (340 nm), formation of isocitrate from citrate, coupled to NADP-linked isocitrate dehydrogenase; citrate synthase (412 nm), formation of coenzyme A in the presence of 5,5′-dithiobis(2-nitrobenzoate) (DTNB); fumarase (250 nm), formation of fumarate from malate; isocitrate dehydrogenase (340 nm), oxidation of isocitrate by $NADP^+$; malate dehydrogenase (340 nm), reduction of oxaloacetate by NADH; pyruvate and oxoglutarate dehydrogenases (340 nm), oxidation of pyruvate or oxoglutarate by NAD^+ in the presence of coenzyme A; succinate dehydrogenase (600 nm), oxidation of succinate in the presence of phenazine methosulphate and dichlorophenolindophenol; succinate thiokinase (412 nm), formation of coenzyme A in the presence of DTNB.

3. RESULTS AND DISCUSSION

A multienzyme aggregate containing the enzymes of glycolysis has been isolated from *Escherichia coli* by first forming spheroplasts and then subjecting them to osmotic lysis [10,11]. Gel filtration of a lysate prepared similarly from *E. coli* gave the elution profiles shown in fig.1. Both pyruvate and oxoglutarate dehydrogenases were eluted as single peaks but the other enzymes each emerged in two clear peaks. The major portions were eluted at distinct positions corresponding to their individual molecular masses, but a small proportion was eluted earlier as a high-molecular-mass cluster along with the pyruvate and oxoglutarate dehydrogenase complexes. This result suggests that the enzymes citrate synthase, aconitase, isocitrate dehydrogenase, succinate thiokinase, fumarase and malate dehydrogenase have some tendency to aggregate together, perhaps in association with one, or both, of the enzyme complexes pyruvate and oxoglutarate dehydrogenases. No succinate dehydrogenase activity was detected in the supernatant of the spheroplast lysate; activity was measurable in the pellet fraction, consistent with the membrane location of this enzyme.

The stability of the high-molecular-mass aggregate was tested by a second gel filtration of the appropriate fractions; the aggregate was again observed, though considerable dissociation to the 'free' enzymes took place.

When the low-molecular-mass enzyme fractions (30–55 of fig.1) were pooled, concentrated by ultrafiltration (Amicon cell) and re-run on gel filtration, a high-molecular-mass cluster was again observed, containing the activities of fumarase, malate dehydrogenase, citrate synthase, aconitase and isocitrate dehydrogenase. This result clearly indicates the ability of the individual enzymes to re-associate (at least partially). Succinate thiokinase, although present in the pooled fractions, did not emerge with the other enzymes in the high-molecular-mass peak; this may be due to the absence of oxoglutarate dehydrogenase in the pooled fractions, especially in the light of the reported interaction of succinate thiokinase and oxoglutarate dehydrogenase from animal sources [7]. In addition, the production of the high-molecular-mass cluster in the absence of the pyruvate or oxoglutarate dehydrogenases indicates that these two complexes are not necessary for its formation.

That the formation of cluster is not an entirely non-specific association was shown by adding lactate dehydrogenase (rabbit muscle; Boehringer) to the preparation applied to the gel filtration column; no lactate dehydrogenase was detected at the position of elution of the high-molecular-mass cluster.

Some effects of changing the solution environment on the extent of enzyme association to the cluster were examined. In all experiments it was found necessary to include 20% (v/v) glycerol in the buffers for spheroplast lysis and gel filtration as very little cluster material was observed without it. Simulation of the intracellular milieu by the additional presence of polyethylene glycol (Sigma, approx. M_r 8000; 14% w/v) or bovine serum albumin (10 mg/ml) produced a 2–3-fold increase in the yield of cluster from gel filtration. Conversely, high salt (1 M KCl) almost eliminated the cluster and shifts of pH away from the standard pH 7.5, to 6.5 or 8.9, decreased the yield by around 50%.

When the *E. coli* cells were disrupted by ultrasonication or with the French press, no multienzyme cluster was found on gel filtration. Similarly, if the isolated cluster was subjected to ultrasonication, or if the pooled low-molecular-

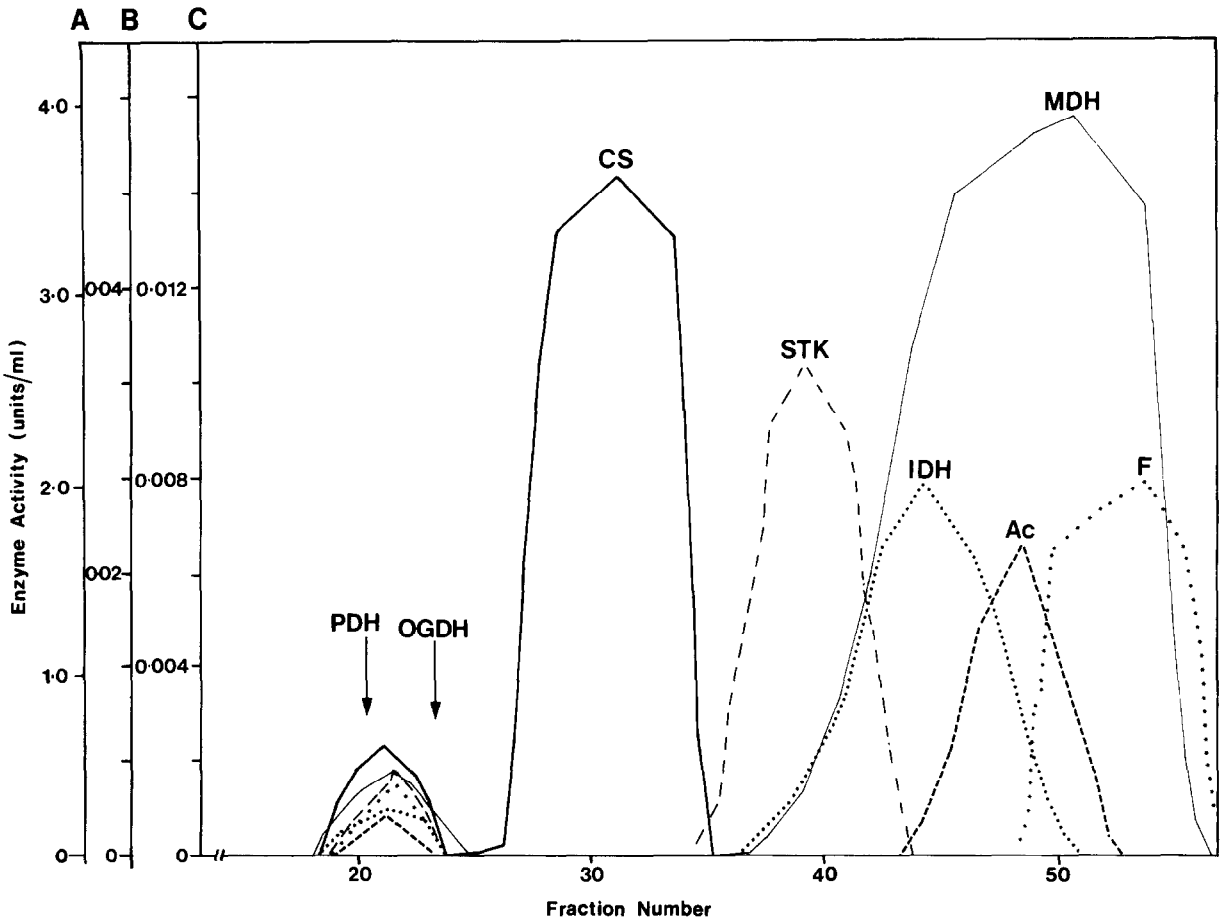

Fig.1. Enzyme elution profiles from gel filtration of *E. coli* lysate. 1 ml of lysate was applied to a column of Sepharose 4B (1.5 × 30 cm) at 4°C and 1-ml fractions were collected. Other experimental conditions are described in the text. PDH, pyruvate dehydrogenase; OGDH, oxoglutarate dehydrogenase; CS, citrate synthase; STK, succinate thiokinase; IDH, isocitrate dehydrogenase; Ac, aconitase; MDH, malate dehydrogenase; F, fumarase. The two vertical arrows indicate the elution peak positions of PDH and OGDH. Ordinate scales: A, CS and MDH; B, IDH and F; C, Ac and STK. Enzyme units are μmol/min.

mass enzyme fractions obtained by gel filtration of lysed spheroplast preparation were sonicated and then concentrated, no evidence of re-association to an aggregate was found by gel filtration. Thus the effect of sonication may be to damage either structural elements in the enzymes themselves or some other as yet unidentified component(s) required for association. The latter might, for example, be a low-molecular-mass membrane fragment, though gel filtration in the presence of 0.1% Triton X-100 still showed elution of the cluster.

The cluster might suggest that the enzymes were contained within a vesicle. This is unlikely to be the case as demonstrated by the re-association of the free enzymes into a cluster and also by the inactivation of citrate synthase within the cluster by rabbit antibody prepared to *E. coli* citrate synthase. The antibody protein would not be expected to traverse a vesicle membrane.

Further evidence for the existence of the multienzyme cluster was obtained by sucrose density gradient centrifugation. When a sample of the high-molecular-mass material from gel filtration was centrifuged through a sucrose gradient (10–50% w/v), the five enzymes of the cluster co-sedimented as a single band. By contrast, when a

mixture of the low-molecular-mass enzyme fractions was similarly examined, the enzymes were found to sediment as individual bands.

The experiments reported above were done with *E. coli*, but a similar five-enzyme cluster was isolated from lysed spheroplasts of other bacteria, both Gram-negative (*Acinetobacter calcoaceticus, Pseudomonas aeruginosa*) and Gram-positive (*Bacillus subtilis*). Preliminary experiments with lysates of mitochondria from rat liver also indicated the five-enzyme cluster. These results complement the recent report [12] that gently disrupted rat liver mitochondria produce a sedimentable membrane-bound complex of citric acid cycle enzymes. That study and the present communication are probably both concerned with slightly different aspects of the same multienzyme aggregate which may constitute a citric acid cycle 'metabolon' [13].

Our results indicate that the multienzyme cluster occurs in the citric acid cycle of a range of organisms and may well be universal throughout Nature. The cluster would be capable of catalysing the sequence of reactions:

fumarate ⟶ malate ⟶ oxaloacetate ⟶
citrate ⟶ isocitrate ⟶ oxoglutarate,

and work is in progress to investigate whether such a cluster achieves any catalytic enhancement and/or channelling of intermediates compared with a mixture of the non-associated enzymes. The dissociation–association of the cluster reported here may be of physiological significance (resembling the phenomenon of 'ambiquitous' enzymes [14]) and may add a new facet to the regulation of the citric acid cycle. The advantages which a multienzyme complex may confer have been identified by a number of authors, e.g. [15–17]. Furthermore, it is increasingly being recognised that conditions within the living cell are 'crowded', requiring cell components to be closely located and thus favouring specific inter-enzymic associations, e.g. [18–22]. The study of such associations is likely to add to our knowledge and understanding of metabolism as an integrated cellular activity.

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