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Citrate synthases: Allosteric regulation and molecular size

We have previously reported on the regulatory properties of citrate synthase (EC 4.1.3.7), a key enzyme of the tricarboxylic acid cycle, from a large number of organisms[1,2]. These findings indicate a clear distinction in regulatory behaviour between, on the one hand, Gram-negative bacteria and, on the other, Gram-positive bacteria and higher, eucaryotic, organisms. Citrate synthases from the former group are all sensitive to inhibition by NADH (an "end-product" of the tricarboxylic acid cycle) while no such inhibition has been observed with the enzymes of the latter group. A further variation was found amongst the citrate synthases of Gram-negative bacteria, with some of which the NADH inhibition is specifically overcome by AMP. Significantly these variations in regulatory pattern follow established taxonomic divisions.

The allosteric nature of the action of NADH on citrate synthase was demonstrated by the criterion of selective "desensitization" of the enzyme to the regulator[3]. Moreover, the kinetics of the NADH inhibition and AMP reactivation[4,5] are in accord with the model proposed for regulatory enzymes[6]. A property of such enzymes which is fundamental to this model is their polymeric state. It is proposed that the ability to mediate allosteric interactions depends on the presence, in the enzyme molecule, of several sub-units arranged in a specific quaternary structure. Electron micrographs of citrate synthase from *Acinetobacter lwoffi* do suggest the presence of sub-units and indicate that the interactions of this enzyme with NADH and AMP lead to changes in molecular conformation[7].

In contrast, however, one might expect that those citrate synthases which are insensitive to regulation by NADH would be simpler, nonpolymeric molecules of considerably smaller size. This does appear to be the case, the molecular weight of the *A. lwoffi* enzyme being of the order of 250 000, while that of the mammalian (pig heart) enzyme has been estimated[8] to be around 80 000.

Such large differences should be demonstrable by gel filtration. It is generally accepted that gel-filtration behaviour is a good reflection of molecular weight, or more accurately, molecular size (Stokes radius)[9,10]. In the present work we have used this technique to examine a number of selected citrate synthases from various organisms in the hope of discerning a correlation between molecular size and regulatory properties.

Twelve citrate synthases of the three different regulatory types were used in this study (Table I). Of the six NADH-insensitive enzymes, three were obtained from Gram-positive bacteria and three from eucaryotic organisms. The other six enzymes were obtained from Gram-negative bacteria; three showed inhibition by NADH and three showed both NADH inhibition and AMP reactivation.

The bacterial enzymes were all partially purified in the following way. The cells were grown aerobically at 30° or 37° in nutrient broth or salts-acetate medium. Disruption was effected by ultrasonication and nucleic acid was removed with protamine sulphate. The supernatant was then fractionated with ammonium sulphate, the citrate synthase being precipitated at 50-70% saturation. This material was redissolved in buffer solution (see below) and used in the gel filtration experiments.

The enzyme from baker's yeast was obtained by suspending 20 g of yeast in 10 ml of 0.1 M sodium bicarbonate and passing the chilled suspension twice through a French press. After centrifugation the supernatant was treated with protamine sulphate and ammonium sulphate as above. Wheat germ citrate synthase was obtained by making a slurry of "Bemax" wheat germ in distilled water and allowing it to stand for 30 min before passage twice through a French press. The extract was then fractionated as for the yeast enzyme. Purified pig heart citrate synthase was obtained commercially (Boehringer).

A column of Sephadex G-200 (2.5 cm × 35 cm) was equilibrated at 4° with buffer of composition 20 mM Tris, 1 mM EDTA, 0.1 M KCl, pH 8.0. Each citrate synthase was run on the column with two marker proteins—bovine liver catalase (EC 1.11.1.6; mol. wt. approx. 250 000) and rabbit muscle lactate dehydrogenase (EC 1.1.1.27; mol. wt. approx. 140 000). 2 ml of buffer solution containing 10% sucrose, 8 mg catalase, 0.1 mg lactate dehydrogenase and an appropriate quantity of citrate synthase were applied to the column, the effluent collected in 2-ml fractions, and the fractions analysed for the three enzymes. Catalase was measured by its absorbance at 400 mμ; lactate dehydrogenase was assayed at 340 mμ by following the rate of oxidation of NADH by pyruvate, and citrate synthase was assayed spectrophotometrically[11] as previously described[4].

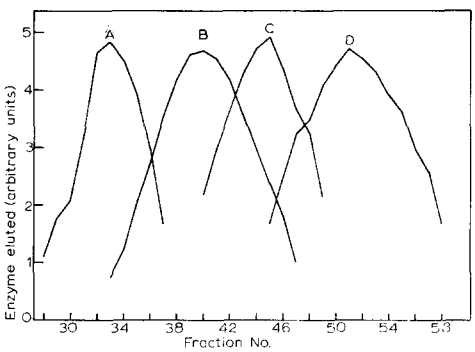

Fig. 1. Elution pattern of citrate synthases on Sephadex G-200. Experimental details are given in the text. A, "large" citrate synthase (*Pseudomonas fluorescens*); B, catalase; C, lactate dehydrogenase; D, "small" citrate synthase (pig heart).

The citrate synthases examined fall clearly into two types which we have called "large" and "small". The "large" enzymes were eluted from the column ahead of catalase, while the "small" enzymes emerged after lactate dehydrogenase. Typical elution patterns are shown in Fig. 1. The significant result is that the "large" citrate synthases are all sensitive to regulation by NADH, while the "small" ones are insensitive to NADH (Table I). There is thus a clear correlation between the molecular size of a citrate synthase and its allosteric regulatory behaviour. Although those enzymes which are NADH-sensitive but show no response to AMP are, in a sense, intermediate between those which are regulated by both NADH and AMP and those which are totally insensitive to NADH, we have not observed any corresponding intermediate

TABLE I

MOLECULAR SIZE OF CITRATE SYNTHASES

Strains of bacteria are those quoted by WEITZMAN AND JONES[2].

"Large" enzyme		*"Small" enzyme*	
Aerobacter aerogenes *Escherichia coli* *Salmonella anatum*	NADH-sensitive	*Kurthia zopfii* *Mycobacterium rhodocrous* *Streptomyces somaliensis*	NADH-insensitive
Acinetobacter lwoffi *Moraxella calcoacetica* *Pseudomonas fluorescens*	NADH/AMP-sensitive	Baker's yeast Wheat germ Pig heart	

size. Lack of AMP sensitivity may result from minor molecular differences rather than from gross differences in molecular complexity.

The existence of "small" and "large" citrate synthases is reminiscent of the pair of proteins myoglobin and haemoglobin. Both these proteins perform an oxygen-carrying function, but only the larger haemoglobin, comprising four myoglobin-like subunits, exhibits allosteric properties.

We are now investigating the sub-unit composition of the "large" citrate synthases to determine whether their regulatory properties derive from a specific association of identical or similar subunits, as in haemoglobin, or from the coexistence of distinct catalytic and regulatory subunits, as in aspartate transcarbamylase[12].

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Department of Biochemistry,
School of Biology,
University of Leicester,
Leicester (Great Britain)

P. D. J. WEITZMAN
PATRICIA DUNMORE

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