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A CRYSTALLOGRAPHIC INVESTIGATION OF CITRATE SYNTHASE FROM PIG AND CHICKEN HEART MUSCLE

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SUMMARY

Citrate synthase (EC 4. 1. 3. 7.) from pig heart and chicken heart muscle can be crystallized from 10 mM phosphate buffer (pH 7. 0-7. 5) and 10-15% PEG4000 solution. The space group is $P4_12_12$ with one subunit/asymmetric unit for the pig heart enzyme. The chicken heart citrate synthase purified from Blue-dextran as well as ATP-Sepharose affinity chromatography both crystallize in space group $P2_12_12$ with one molecule/asymmetric unit, but they have different unit cell dimensions. The a-axis differs by about 17 Å between these two crystal forms, while b- and c-axis dimensions are virtually identical.

Citrate synthase (EC 4. 1. 3. 7.), also known as "citrate condensing enzyme", catalyzes the formation of citrate from oxalacetate and acetyl-CoA. The enzyme acts in the first step of the tricarboxylic acid cycle. Many metabolites have been reported to regulate its activity; its inhibition by ATP and NAD have been well-characterized (1). Citrate synthase functions physiologically in the mitochondrial matrix of eucaryotic cells. The enzyme from most animal cells shows similar physical and kinetic properties; it has a molecular weight of 100,000 and is composed of two identical subunits (2, 3)

The enzymes from pig (4) and rat (5) heart muscle have been purified by "ATP"-Sepharose affinity chromatography. Here we report the purification of citrate synthase from pig and chicken heart muscle by "ATP"-Sepharose

Abbreviations: PEG, polyethylene glycol; CoA, coenzyme A; ATP, adenosine 5'-triphosphate; NAD, nicotinamide-adenine dinucleotide; SDS, sodium dodecyl sulfate.

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as well as "Blue-dextran" Sepharose affinity chromatography (6). A total of three different crystal forms of citrate synthase have been prepared, one of which was described earlier (7). Preliminary X-ray diffraction studies indicate that these crystals are suitable for three dimensional structure analysis

METHODS

(a) Preparation of affinity chromatography gels

Both the 8-(6-aminohexyl)-amino-5'-ATP-Sepharose gel and the Blue-dextran-Sepharose gel were prepared using the procedure described by Marsh, et al. (8). Sepharose 4B and Blue dextran were obtained from Pharmacia Fine Chemicals. The 8-(6-aminohexyl)-amino-5'-ATP ligand was a gift from Dr. Chi-Yu Lee. Coupling of both ligands was done at 4°C for 24 hours.

(b) Purification

Citrate synthase was purified from pig and chicken heart muscle. Initial extraction and ammonium sulfate fractionation of the enzyme was carried out as described by Srere and Kosicki (9). The crude protein (50 to 70% ammonium sulfate precipitate) was dissolved in 10 mM phosphate buffer (pH 7.4) and dialyzed against the same.

(i) Purification of the crude pig heart citrate synthase was carried out by affinity chromatography using the ATP-Sepharose gel as described by Johansson and Lee (4). The dialysate was loaded onto the column and after thorough washing with buffer, the enzyme was specifically eluted with 0.5 mM CoA. A single peak was obtained as monitored by the absorbance at 280 nm. It corresponded to the sole peak of citrate synthase activity.

(ii) Crude citrate synthase from chicken heart muscle was purified using both the ATP-Sepharose and Blue-dextran-Sepharose columns. Specific elution of the enzyme in this case was effected with a solution of 0.1 mM oxalacetate and 0.1 mM CoA (5). Again, a single protein peak was obtained.

In both cases of (i) and (ii), the fractions containing the most active protein were pooled, dialyzed against buffer to remove oxalacetate and CoA. The enzyme solution is then concentrated by vacuum dialysis. Activity of the enzyme was assayed by the DTNB (5,5'-dithiobis (2-nitrobenzoate)) method as described by Srere, Brazil, and Gonen (10). Protein was determined using the method of Lowry, et al. (11) or by fluorescamine (12). Samples from each column were run electrophoretically on SDS-polyacrylamide gels and the major citrate synthase band comprised at least 90% of the total protein.

(c) Crystallization

Twenty μl aliquots of citrate synthase solution were placed into the depressions of glass spot plates and placed on half a disposable petri dish

in plastic sandwich boxes. To the bottom of the box was added 25 ml of a polyethylene glycol (PEG) solution of various molecular weights (13). The top of the box was sealed with stopcock grease, and the sample and reservoir allowed to equilibrate via vapor phase diffusion at 4°C.

(i) Chicken heart citrate synthase purified from the Blue-dextran-Sepharose column was crystallized from a variety of conditions. The samples producing crystals ranged from 5-10 mg protein/ml and 3-5% PEG4000, while the reservoir contained 8-16% PEG4000. A fine precipitant was evident after 24 hours from which clusters of long orthorhombic crystals formed in 1-2 weeks. The largest crystals were obtained from 7.5 mg protein/ml and 4% PEG in the sample, and with 10% PEG in the reservoir. The maximum dimensions were 2.0 mm X 0.3 mm X 0.1 mm.

(ii) In crystallization experiments with the enzyme purified from the ATP-Sepharose column, a precipitate was observed after 24 hours which did not convert to crystals even after 3 months. Later, we discovered that if microcrystals from the "Blue dextran" crystals were used to seed the samples, the precipitate would disappear and crystallization began within a few days. Growth was complete after 2 weeks. These crystals (Fig. 1a) could be obtained in abundance from all conditions. They were rectangular plates with maximum dimensions of 1.0 mm X 0.7 mm X 0.2 mm.

(iii) Nicely formed tetragonal bipyramid crystals (Fig. 1b) were obtained from samples of pig heart enzyme containing 15 mg protein/ml, 10 mM phosphate buffer (pH 7.4), and 2% PEG4000. The crystals appeared between 4 and 6 weeks with the length of the largest crystals being 1 mm. Lower quality crystals were obtained from PEG1000, 6000, and 20,000. These crystals looked similar to those made by dialysis against a high ionic strength buffer as reported by Wiegand (7).

(d) X-ray examination

Single crystals were mounted in glass capillaries with a droplet of mother liquor and the capillaries were sealed. X-ray diffraction photographs were taken on a Buerger precession camera with a crystal to film distance of 10 cm. Nickel filtered CuKα radiation (λ = 1.5418 Å) was produced by an Elliot rotating anode generator. All procedures were carried out at 6°C.

RESULTS AND DISCUSSION

The orthorhombic crystals of chicken heart muscle citrate synthase purified by Blue-dextran-Sepharose column produced diffraction spots that extended to a resolution of at least 3 Å. Based on the systematic absences ($h \neq 2n + 1$ for $h00$, $k \neq 2n + 1$ for $0k0$), the space group was established as $P2_12_12$. The dimensions of the unit cell and volume are listed in Table I.

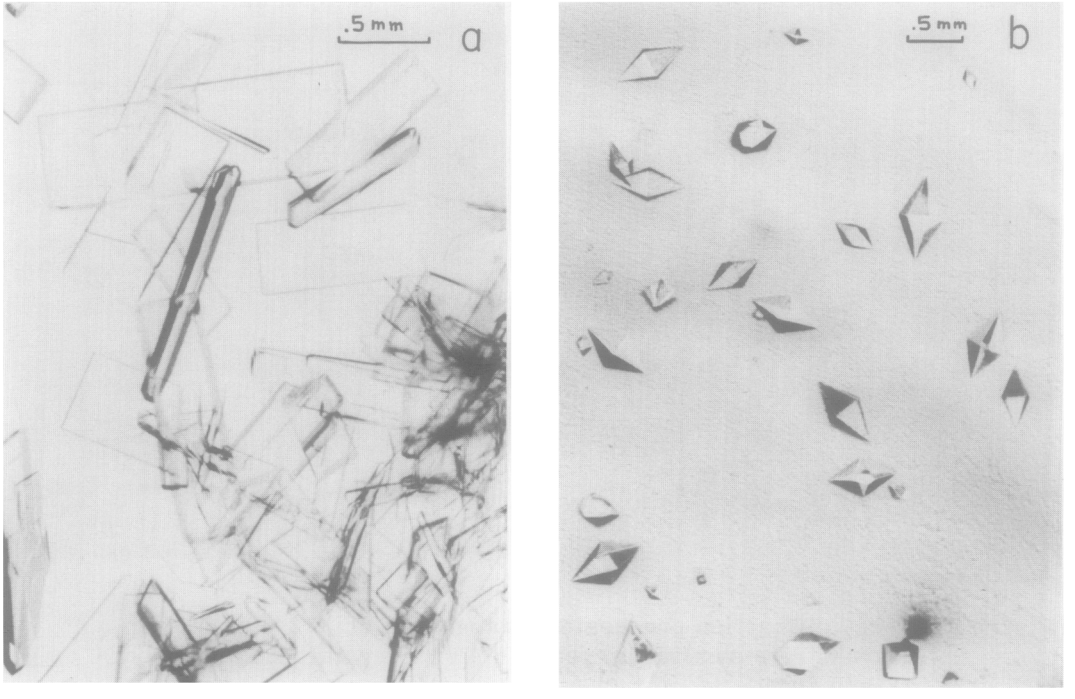

Figure 1: Photomicrograph of citrate synthase crystals.
a) From chicken heart muscle ("ATP" form). The crystals from chicken heart muscle purified by "Blue-dextran" Sepharose affinity chromatography showed similar morphology.
b) From pig heart muscle.

Table I. Crystal Forms of Citrate Synthase

	Chicken Heart ("Blue-Dextran" form)	Chicken Heart ("ATP" form)	Pig Heart
Space group	$P2_12_12$	$P2_12_12$	$P4_12_12$
a (Å)	171.8	154.9	76.9
b (Å)	107.1	106.9	76.9
c (Å)	63.4	64.0	200.2
Volume ($Å^3$)	1.17×10^6	1.06×10^6	1.18×10^6
Vm ($Å^3$ per dalton)	2.92	2.65	2.98
Molecule per asymmetric unit	1	1	1/2

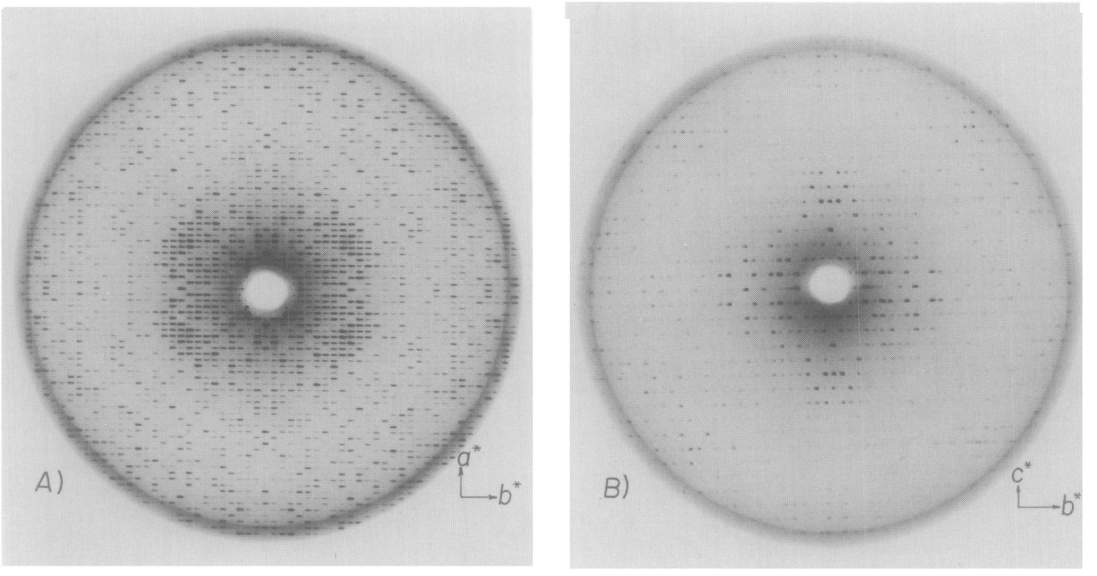

Figure 2: X-ray diffraction precession photographs (μ = 12°) of chicken heart citrate synthase crystals ("ATP" crystal form).
a) hk0 zone
b) 0kl zone

The chicken heart citrate synthase crystals obtained from "ATP"-Sepharose affinity chromatography are elongated rectangular plates. They tend to grow in clusters and are somewhat fragile. Precession photography (Figs. 2a and 2b) indicates that the space group is also $P2_12_12$. These crystals also diffract well to at least 3 Å resolution. Thus, they appear to be suitable for high resolution structure analysis. The cell dimensions and volume of this crystal form are shown in Table I. Assuming one molecule per asymmetric unit, the value for V_m was found to be 2.92 and 2.65 $Å^3$/dalton for "Blue-dextran" and "ATP"-Sepharose crystal forms, respectively. They are both within the range found by Matthews (14). It is interesting to note that the a-axis in "ATP" crystal form is about 17 Å shorter than that of "Blue-dextran" crystal form, while b- and c-axes

are virtually the same. The overall diffraction patterns of these two crystal forms remain similar, suggesting no gross rearrangement of the molecules within the crystal lattice. In view of the fact that we used the micro-crystals of "Blue-dextran" crystal form as seeds to promote the crystallization of "ATP" crystal form, it is difficult to understand why the a-axis in two crystal forms should differ by as much as 10% ($\sim$17 Å). One possible explanation could be that the "Blue-dextran" seed crystals have stood in the PEG solution for an extended period of time of two weeks or longer. The crystals could thus have formed a new stable "dehydrated" crystal packing. A similar phenomenon has been observed in the orthorhombic form of yeast phenylalanine transfer RNA (15). We have also observed this phenomenon in other protein crystals crystallized from PEG solutions, though the degree of shrinkage was somewhat less (unpublished results).

The space group of the tetragonal bipyramid crystals of citrate synthase from pig heart muscle was established with screened precession photographs as $P4_12_12$. The dimensions and volume of the unit cell are listed in Table I. Assuming one subunit of citrate synthase (MW of subunit = 50,000) in the crystallographic asymmetric unit, the volume per unit protein mass, V_m, is found to be 2.98 A/dalton, which is in close agreement to the average value for proteins found by Matthews (14). A similar crystal form of commercially available pig heart citrate synthase was obtained by Wiegand (7) from a potassium-sodium phosphate buffer solution. These crystals are also in space group $P4_12_12$ with cell dimensions a = b = 77.1 Å and C = 196.8 Å. It is likely that the two crystal forms are identical.

There are thus three suitable crystal forms of citrate synthase which can be used to carry out a high resolution X-ray diffraction study.

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