Vol. 90, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 27, 1979 Pages 431-438

CRYSTALLIZATION OF A DIHYDROLIPOYL TRANSACETYLASE - DIHYDROLIPOYL DEHYDROGENASE SUBCOMPLEX AND ITS IMPLICATIONS REGARDING THE SUBUNIT STRUCTURE OF THE PYRUVATE DEHYDROGENASE COMPLEX FROM *ESCHERICHIA COLI*

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Received August 2,1979

Summary: A subcomplex consisting of dihydrolipoyl transacetylase and dihydrolipoyl dehydrogenase, two of the three enzymes comprising the *Escherichia coli* pyruvate dehydrogenase complex, has been crystallized. X-ray diffraction data establish that the space group is $P2_13$ with unit cell dimension a=211.5Å. The unit cell contains four molecules of the subcomplex, each possessing 3-fold crystallographic and molecular symmetry. This finding, together with biochemical and electron microscopic data reported elsewhere, establish unequivocally that dihydrolipoyl transacetylase, the core enzyme of the pyruvate dehydrogenase complex, consists of 24 identical subunits with octahedral (432) symmetry. In the case presented here, the 432 symmetry of the transacetylase is reduced to 3-fold symmetry in the subcomplex by the addition of dihydrolipoyl dehydrogenase subunits. Crystal density measurements indicate that the dihydrolipoyl transacetylase present in these crystals is considerably smaller than the core mass generally reported for intact transacetylase. The implications of these findings are discussed with respect to the subunit stoichiometry and structure of the *E. coli* pyruvate dehydrogenase complex.

Multienzyme complexes are heterologous protein systems that catalyze two or more sequential steps in a metabolic pathway. These supramolecular assemblages normally consist of stoichiometric amounts of the constituent enzymes which in turn are usually organized in a specific and highly symmetrical way. Large, ordered aggregates catalyzing a CoA- and NAD^+-linked oxidative decarboxylation of α-keto acids have been isolated from microorganisms and from animal tissues (1). The pyruvate dehydrogenase complex and the α-ketoglutarate dehydrogenase complex from *Escherichia coli* have been separated into three component enzymes capable of reassembling into the native complexes. Each complex is organized about a core enzyme (E2) consisting of dihydrolipoyl transacetylase or dihydrolipoyl transsuccinylase to which pyruvate dehydrogenase or α-ketoglutarate dehydrogenase (E1) and dihydrolipoyl dehydrogenase (E3) are joined by noncovalent bonds (2).

The appearance of dihydrolipoyl transacetylase in electron micrographs is that of a cube (2) very similar in size and appearance to the corresponding dihydrolipoyl transsuccinylase which possesses octahedral (432) symmetry as shown by X-ray crystallography (3). On the basis of these electron micro-

0006-291X/79/180431-08$01.00/0

graphs, symmetry considerations, reconstitution experiments, flavin content, and sedimentation equilibrium molecular weights, it was concluded (4, 5) that dihydrolipoyl transacetylase consists of 24 apparently identical polypeptide chains arranged with octahedral (432) point group symmetry and that the polypeptide chain stoichiometry of the pyruvate dehydrogenase complex is 2:2:1 (E1:E2:E3). This subunit stoichiometry is supported by the findings of Frey and coworkers (6) and of Hammes and coworkers (7). On the other hand, Vogel *et al.* (8) and Perham and coworkers (9, 10) propose a polypeptide chain ratio of about 1-2:1:1 (E1:E2:E3). Vogel ***et al.*** suggest that the intact "unique" complex consists of 16 copies of each of the three component polypeptide chains. Perham and coworkers (10) have reported data consistent with octahedral (432) symmetry for the E2 core of the complex. However, their subunit stoichiometry and that of Vogel *et al.* (8) require a higher flavin content in the complex than that found by other investigators (4, 11).

Because establishing exact stoichiometry is crucial to an understanding of the structure, subunit-subunit interactions, and reaction mechanisms of the complex, crystallization of the *E. coli* dihydrolipoyl transacetylase in a form suitable for X-ray diffraction analysis was undertaken. In this communication we report the crystallization of a subcomplex consisting of dihydrolipoyl transacetylase and dihydrolipoyl dehydrogenase derived from the *E. coli* pyruvate dehydrogenase complex. A low resolution data set has been collected, and the structure has been solved using model calculations and real space molecular replacement methods (12-16). The results unequivocally support the conclusion that the transacetylase has octahedral (432) symmetry and rule out the 16 subunit (D_4 symmetry) model.

MATERIALS AND METHODS

The procedures for growing cells of *E. coli* (Crookes strain) and for the isolation and resolution of the pyruvate dehydrogenase complex are described elsewhere (4).

A variety of crystallization conditions were surveyed by using microdiffusion cells containing from 25 to 100 μl of either the whole complex, subcomplexes, or resolved dihydrolipoyl transacetylase core. Protein concentration, pH, and temperature were varied while employing ammonium sulfate, polyethylene glycol, and methyl pentanediol as precipitating agents. The larger crystals were obtained from solutions of ammonium sulfate or methyl pentanediol and were analyzed by the precession X-ray method.

To establish the composition of crystals obtained from the intact complex, previously X-irradiated crystals or small crystals having identical crystal habit and optical properties when viewed under a polarized microscope were rinsed repeatedly before use. Crystal density measurements were carried out essentially as described by Low and Richards (17) using a xylene-bromobenzene gradient and small droplets of salt solutions of known density as standards. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis was performed in 1 mm

capillaries for 3 h at 1 mA/tube. The gels were stained and destained according to the procedure of Weber and Osborne (18). For electron microscopy, a large crystal was rinsed and crushed in 10 µl of 2 mM phosphate buffer, pH 7. The crystal fragments were diluted with 0.2 ml of sodium phosphotungstate negative stain and subjected to repeated sonication before being sprayed onto support films and examined in a Siemens Elmiskop I (19).

Intensity data were collected using small angle precession camera techniques with an Elliott rotating anode X-ray generator equipped with a copper target and a graphite monochromator. A total of 7 zero layer precession photographs were collected at various orientations of the crystal. The individual films were densitometered on a modified Nikon profile projector (20). After correction for Lorentz-polarization effects, the intensity data were scaled together yielding an R-factor of 14%. The data set contained 87 independent reflections to a resolution of 20Å^{-1}.

The initial phasing was generated by model calculations employing solid sphere scattering curves. The general positions and radii of these spheres were determined by comparing the magnitudes of observed and calculated structure amplitudes. Rigid-body least squares structural refinement, electron density difference maps, and molecular replacement techniques (12-16) were used to improve the model.

RESULTS AND DISCUSSION

Pale-yellow, or sometimes red, crystals were obtained. Anaerobic photoirradiation of flavoprotein in the presence of EDTA is known to produce semiquinoid forms that are red or blue in color (21). Both crystal forms were optically inactive and displayed a 3-fold axis of symmetry in their crystal habit. Their X-ray diffraction patterns were indistinguishable at the resolution (≈8Å) observed. The dominant morphology of the pale-yellow crystals is tetrahedral (Figure 1A). Figure 1B shows a precession photograph taken down

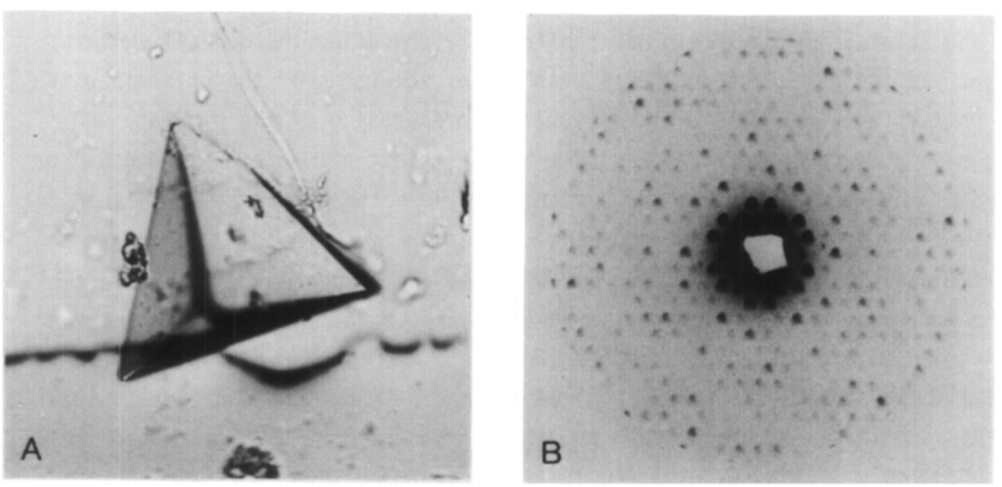

Figure 1. A) Tetrahedral morphology of E2'-E3 subcomplex crystals. B) Zero layer, 3.5° X-ray precession photograph down the [111] axis.

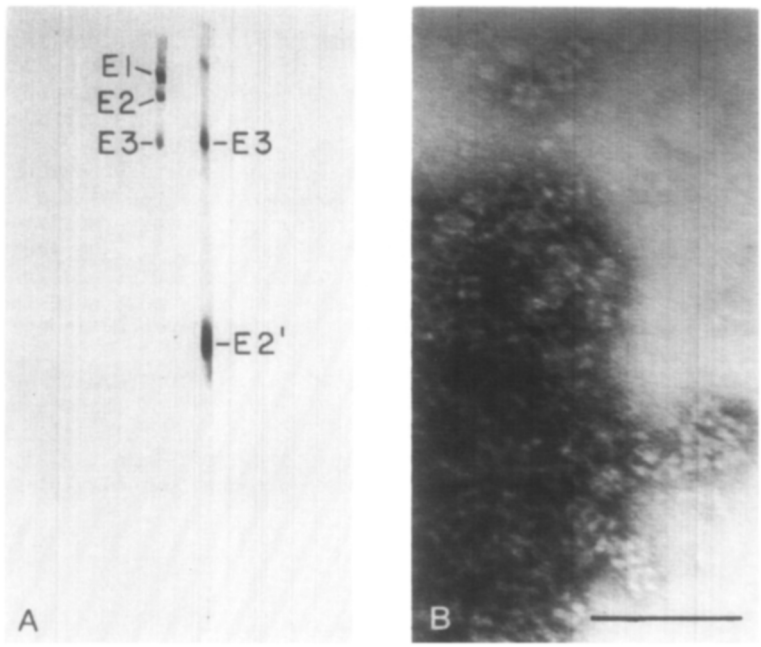

Figure 2. A) Sodium dodecyl sulfate/polyacrylamide gel electrophoresis patterns of purified pyruvate dehydrogenase complex (left) and dissolved crystals (right). B) Electron micrograph of negatively stained redissolved crystal fragments. The bar equals 500A.

the 3-fold symmetry axis; no 4-fold crystallographic axis was detected. The space group is $P2_1 3$. The unit cell dimension is a=211.5Å and its volume is $9.46 \times 10^6 Å^3$.

The presence of dihydrolipoyl dehydrogenase (a flavoprotein) in both the red and faint-yellow crystals was confirmed by comparing the dodecyl sulfate/polyacrylamide gel electrophoresis patterns of solutions of the crystals with the pattern obtained with a highly purified preparation of the pyruvate dehydrogenase complex (Figure 2A). Little if any intact pyruvate dehydrogenase or dihydrolipoyl transacetylase was observed in the patterns of either the red or yellow crystals. However, a component with estimated molecular weight of approximately 29,000 was observed on the gels. We suspected that this latter component came from dihydrolipoyl transacetylase chains that had been "nicked" by endogenous protease(s), since the transacetylase is reported to be very sensitive to proteolysis (22-24). The presence of cube-like entities typical of transacetylase was established by electron microscopy. The micrograph of the negatively stained crystal fragments in Figure 2B shows the same characteristic views of the transacetylase that are observed in isolated transacetylase particles (2). Apparently, the transacetylase chains are nicked by

endogenous protease(s) during the long period required for crystallization. This nicking apparently does not affect the state of aggregation of the transacetylase, but it is not known whether or not this limited proteolysis is a prerequisite for crystallization.

The density, D_c^w, of a water-soaked, cross-linked crystal was measured as 1.21 g/cm^3 by the flotation technique described in METHODS. Using the relationship

$$V_m = \frac{0.43}{D_c^w - 1}$$

which assumes a value of 1.35 g/cm^3 for the density of protein, a packing parameter (25), V_m=2.0Å^3/dalton, is obtained. Space group $P2_13$ has 12 general positions that can be visualized as four 2-fold screw-related molecules, each possessing a 3-fold axis of molecular symmetry. Each of these molecular units would then occupy a volume of 2.36 x 10^{6}Å^3, corresponding to a mass of ≈1.2 x 10^6 daltons.

We therefore employed model studies to test the proposal that the unit cell contains 4 complexes of $M_r \approx 1.2 \times 10^6$, with each complex possessing a 3-fold axis of symmetry. Having established the presence of both modified dihydrolipoyl transacetylase and dihydrolipoyl dehydrogenase in the crystal, and assuming a minimum of three flavoprotein dimers (6 chains) per complex, we calculate a mass of about 864,000 daltons for the modified transacetylase (E2') core. This value corresponds to a $M_r \approx 36{,}000$ for the modified transacetylase subunit if octahedral (432) symmetry is assumed, which is much smaller than the values of 65,000 to 80,000 reported for E2 subunits (4, 10, 24). However, it is in reasonable agreement with the value (≈29,600) reported by Bleile *et al.* (24) for the trypsin-stable subunit binding domain of the native transacetylase subunit. These investigators reported that the native transacetylase subunit consists of a compact domain of $M_r \approx 29{,}600$ and a trypsin-sensitive extended fragment of $M_r \approx 31{,}600$. The extended fragment contains the covalently bound lipoyl moieties, whereas the compact domain retains the intersubunit binding sites of the transacetylase and the other component enzymes of the pyruvate dehydrogenase complex and is responsible for the cube-like appearance of transacetylase in electron micrographs. The difference in molecular weights attributed to the compact domain and the modified transacetylase subunit found in the crystal could easily arise from errors in the crystal density or in estimation of the flavoprotein content.

The crystallographic modeling studies of the modified transacetylase-dihydrolipoyl dehydrogenase subcomplex confirmed our hypothesis concerning the size and shape of the modified transacetylase. This result provides unequivocal evidence that the dihydrolipoyl transacetylase has 3-fold molecular

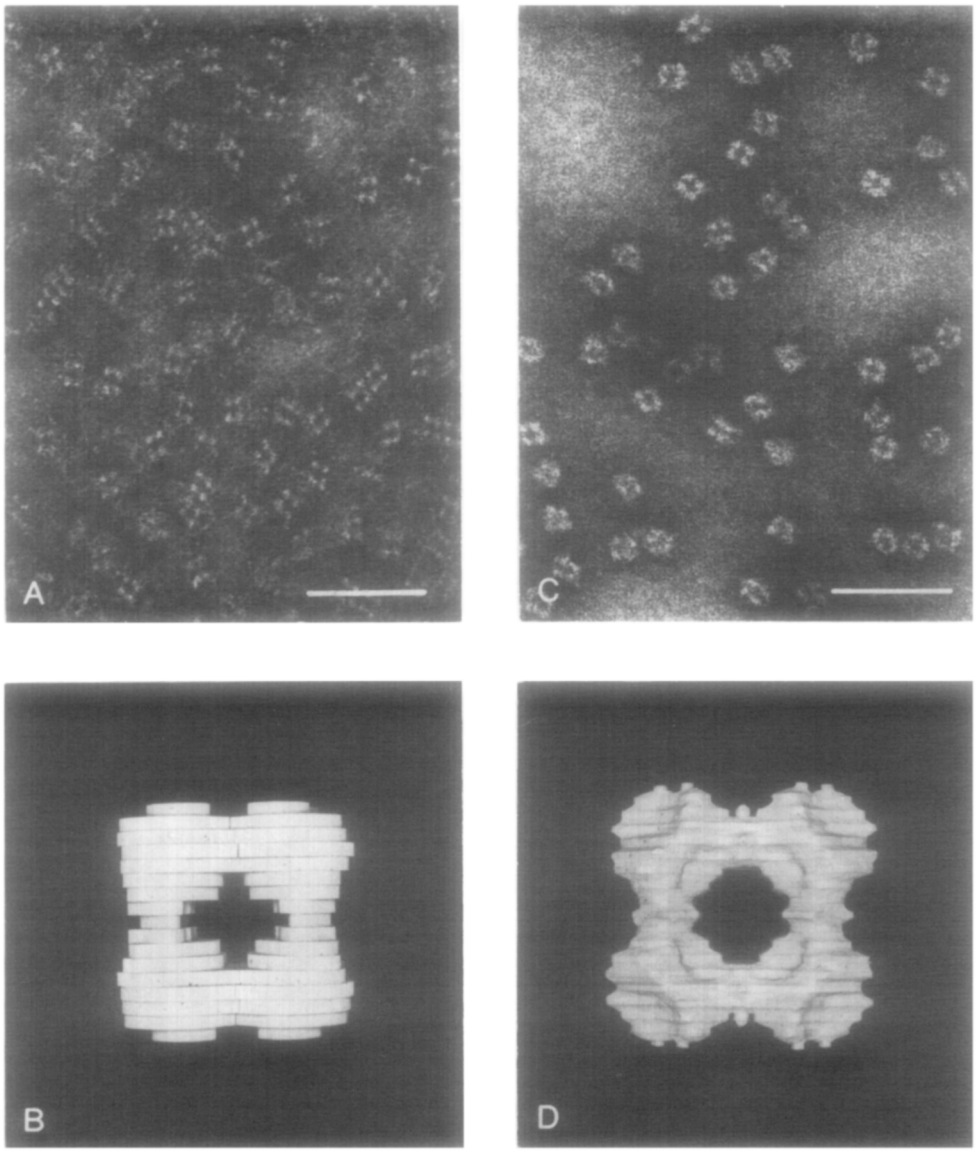

Figure 3. Electron micrograph images and models derived from three-dimensional electron density maps obtained by X-ray analysis. A) *E. coli* dihydrolipoyl transacetylase. The bar equals 500Å. B) 432 symmetry averaged model of E2' at 20 Å resolution. C) *E. coli* dihydrolipoyl transsuccinylase. (Scale same as in A.) D) 18Å resolution model of transsuccinylase (ref. 26).

symmetry which rules out any possibility that this enzyme is composed of 16 subunits (8). Furthermore, least-squares refinement results support the proposal that the modified transacetylase exhibits local (noncrystallographic) octahedral (432) symmetry. The symmetry-averaged model derived from our X-ray analysis is shown in Figure 3B with the electron micrograph images shown for comparison in Figure 3A.

It is interesting to note that the cube-like electron micrograph images of dihydrolipoyl transacetylase (Figure 3A) are very similar in size to the corresponding images of the modified transacetylase (Figure 2B) and of the dihydrolipoyl transsuccinylase (Figure 3C). Furthermore, the symmetry-averaged model of the modified transacetylase (Figure 3B) is strikingly similar to a model of dihydrolipoyl transsuccinylase (Figure 3D), derived from a similar X-ray analysis (26). Our results indicate that the cube-like core represents about one-half the mass of the intact transacetylase. These findings, in conjunction with those reported by Bleile *et al.* (24), indicate that dihydrolipoyl transacetylase consists of two dissimilar folding domains rather than two homologous, if not identical, folding domains as suggested by Hale and Perham (23) and Gebhardt *et al.* (22). Our findings also suggest that dihydrolipoyl transacetylase and dihydrolipoyl transsuccinylase subunits contain morphologically similar compact (subunit binding) domains, but differ in the size of the extended fragment (lipoyl domain). The transacetylase subunit possesses a higher molecular weight (≈65,000) than the transsuccinylase subunit (≈42,000), and the lipoic acid content of the transacetylase is about twice that of the transsuccinylase (27). These differences are consistent with the apparent presence of a larger lipoyl domain in the transacetylase than in the transuccinylase.

A more thorough description of the crystallographic methods employed, including the use of real space molecular replacement techniques to improve the phasing and extend the resolution of these results to identify potential flavoprotein (E3) binding sites, will be reported elsewhere.

ACKNOWLEDGMENTS

We thank Karen Hobson, Fred Hoffmann, Lynda Lindsay, and Penny Ruhmann for skilled technical assistance. This work was supported in part by USPHS Grants GM06590 and GM23306.

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