Biochimica et Biophysica Acta, 481 (1977) 364—376

BBA 68107

STRUCTURE OF PYRUVATE DEHYDROGENASE COMPLEX

COMPARISON BETWEEN FREEZE-ETCHING AND NEGATIVE STAINING

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(Received October 18th, 1976)

Summary

Pyruvate dehydrogenase complex (pyruvate : lipoate oxidoreductase (decarboxylating and acceptor-acetylating), EC 1.2.4.1), from pig heart, was studied by spray freeze etching and negative staining.

From freeze etching experiments an average particle weight of $7 \cdot 10^6$ was estimated.

Negative staining after glutaraldehyde fixation and freeze etching of unfixed and prefixed enzyme solutions yielded no significant difference in particle dimensions: the majority of the isometric complex molecules measured approximately 400 Å in diameter.

Tantalum tungsten shadowed freeze etch replicas indicated that the surface of the complex is built up of globular units. The relative positions of these units are in good agreement with the model still under discussion.

Introduction

Negative staining has become the most successful technique for the investigation of macromolecules by electron microscopy. However, in the case of the mammalian pyruvate dehydrogenase multienzyme complex, (pyruvate : lipoate oxidoreductase (decarboxylating and acceptor-acetylating), EC 1.2.4.1), which is one of the largest multienzyme systems yet known, negative staining causes a progressive decay into the component enzymes [1,2,4—6].

The instability of the multienzyme complex is probably the reason for the inconsistency of data concerning the molecular dimensions [1—6]. Fixation with glutaraldehyde prior to staining prevents this dissociation and yields particles which are of almost uniform size [6]. Approximately 90% range between 400 and 450 Å, with the majority, about 40%, being 400 Å in diameter. The

application of the divalent aldehyde however may create an artificial cross-linking of dissociated enzymes. Thus, the actual existence of such large complex particles in dilute aqueous solution prior to fixation has not yet been verified by electron microscopy.

Recently a technique has been reported which combines spray freezing with freeze etching [7,8]. In the course of spray freeze etching no fixatives, cryprotectant agents or any other potentially deleterious reagents are used. The technique therefore, provides a means of handling unstable macromolecular structures [9]. By applying this method to the study of the mammalian pyruvate dehydrogenase complex information on the molecular weight, the over all dimensions and to some extent on the fine structure of the hydrated but unfixed complex could be obtained.

Material and Methods

Reagents were obtained from the following companies: [1-^{14}C]pyruvic acid, sodium salt (5—20 Ci/mol), D,L-[1-^{14}C]glutamic acid (20—50 Ci/mol) for the preparation of 2-oxo-[1-^{14}C]glutaric acid according to [11] Amersham Buchler GmbH (Braunschweig, G.F.R.). Coenzyme A, lactate dehydrogenase, EC 1.1.2.7, dehydrolipoamide dehydrogenase, EC 1.6.4.3 (from pig heart muscle), NAD^+, NADH, phosphotransacetylase EC 2.3.1.8 (clostridium kluyveri), Boehringer (Mannheim, G.F.R.). Glutathion (reduced), sodium pyruvate, sodium oxoglutarate, sodium phosphotungstate, thiamine pyrophosphate, Merck (Darmstadt, G.F.R.). Glutaraldehyde 25%, DL-α-lipoamide, ferritin (horse spleen) 2 × crist., 10% aqueous solution, bovine serum albumin, crist. puriss., Serva (Heidelberg, G.F.R.). Scintillation dyes: Dimethyl-POPOP, Hyamine 10-X®, POPOP, PPO and Soluene-100®, Packard (Frankfurt/M., G.F.R.).

Enzyme preparation. The pyruvate dehydrogenase complex was isolated and purified from pig heart muscle as previously described [6] following the basic procedure given by Ishikawa et al. [1].

Protein assay. Protein concentrations were determined from the absorbance at 280 nm or by the method of Lowry et al. [12]. Both methods were calibrated by the dry weight of the pyruvate dehydrogenase complex and varied within ±20%.

Assay of enzyme activities. The activities of the pyruvate dehydrogenase complex and of the 2-oxoglutarate dehydrogenase complex were measured according to Reinauer et al. [10,11] using [1-^{14}C]pyruvate and [1-^{14}C]oxoglutarate as substrates.

Negative staining. Holey carbon films, thickened by the deposition of gold, several hundred Å thick, were coated with a 30—50 Å thick carbon film, which had been evaporated on freshly cleaved mica. The enzyme containing fraction of the sucrose gradient was diluted with 0.02 M phosphate buffer or with distilled water to give a final protein concentration of about 0.01%. A drop of this solution was brought onto the supports and dried in glutaraldehyde vapor. The specimen was then rinsed with water and transferred onto a drop of 2% sodium phosphotungstate (pH 6.8). After 30 s the residual fluid was removed with filter paper.

Cryofixation and freeze etching. The enzyme containing fractions (conc.

5—10 mg/ml) of the sucrose gradient were pooled. For comparison with the unfixed complex 1 ml of the protein solution was rapidly mixed with 20 ml of 2.5% glutaraldehyde in 0.01 M phosphate buffer at pH 7.0. The unfixed and the fixed samples were then dialysed against 0.001 M phosphate buffer (pH 7) and concentrated by the hollow fibre technique. Finally the protein concentration was adjusted to ≈1 mg/ml with distilled water. To obtain optimal cryofixation 0.3—0.5 ml of the solutions were then sprayed into propane at liquid nitrogen temperature and further processed as previously described [7,8].

Ferritin was purified on Sepharose 6B and the main fraction was then desalted on Sephadex G 25. It was spray frozen at a concentration of approximately 0.2 mg/ml. Bovine serum albumin was purified on Sephadex G 100, desalted and sprayed at a final concentration of 0.07 mg/ml.

For freeze etching a Balzers unit BA 360 was used. The specimens were cleaved at −100° C in a vacuum around $1 \cdot 10^{-6}$ Torr and etched for 30—120 s. The specimens were then cooled to −140° C within 30 s and shadowed either with platinum-carbon or tantalum-tungsten [13,14]. The shadowing angle was 45° and the mean thickness of the tantalum-tungsten film was 5—10 Å as controlled by a quartz-crystal oscillator (Balzers QSG 201). Subsequently the specimens were backed with carbon, cleaned with 70% sulfuric acid, washed and picked up on grids.

Electron microscopy. Most micrographs were taken with a Siemens Elmiskop 102 equipped with a tilting stage at magnifications of 40 000, 100 000, 160 000 and 200 000×. These magnifications were carefully calibrated and were reproducible within ±5%. The objective lens current was monitored to correct for changes in magnification due to focusing. Micrographs for the molecular weight determination were taken at 20 000×. The microscope was operated at 100 kV with a 50 μm objective operture.

Results

Molecular weight of the enzyme complex

Freeze etching of spray-frozen specimens allows one to determine molecular weights via particle counting [15]. Two solutions, one containing the sample and the other containing a protein of known molecular weight ("standard") were spray frozen one after the other in the same vessel containing the quenching fluid. The concentrations of both solutions were known. As standards, either ferritin or bovine serum albumin were used. After freeze etching the sprayed droplets of the sample and of the standard were observed side by side within the same replica. Obviously the etching conditions were identical for both of them. Then the number of particles per area was determined in both kinds op droplets. The molecular weight of the enzyme complex (M_e) is then

$$M_e = \frac{n_{st}}{n_e} \cdot \frac{c_e}{c_{st}} \cdot M_{st}$$

(M_{st} molecular weight of standard, n_{st} and n_e number of particles per area of standard and of enzyme, c_{st} and c_e the concentrations of the solutions).

The accuracy of the method depends mainly on the protein assay of the enzyme preparation. In the case of the pyruvate dehydrogenase complex the

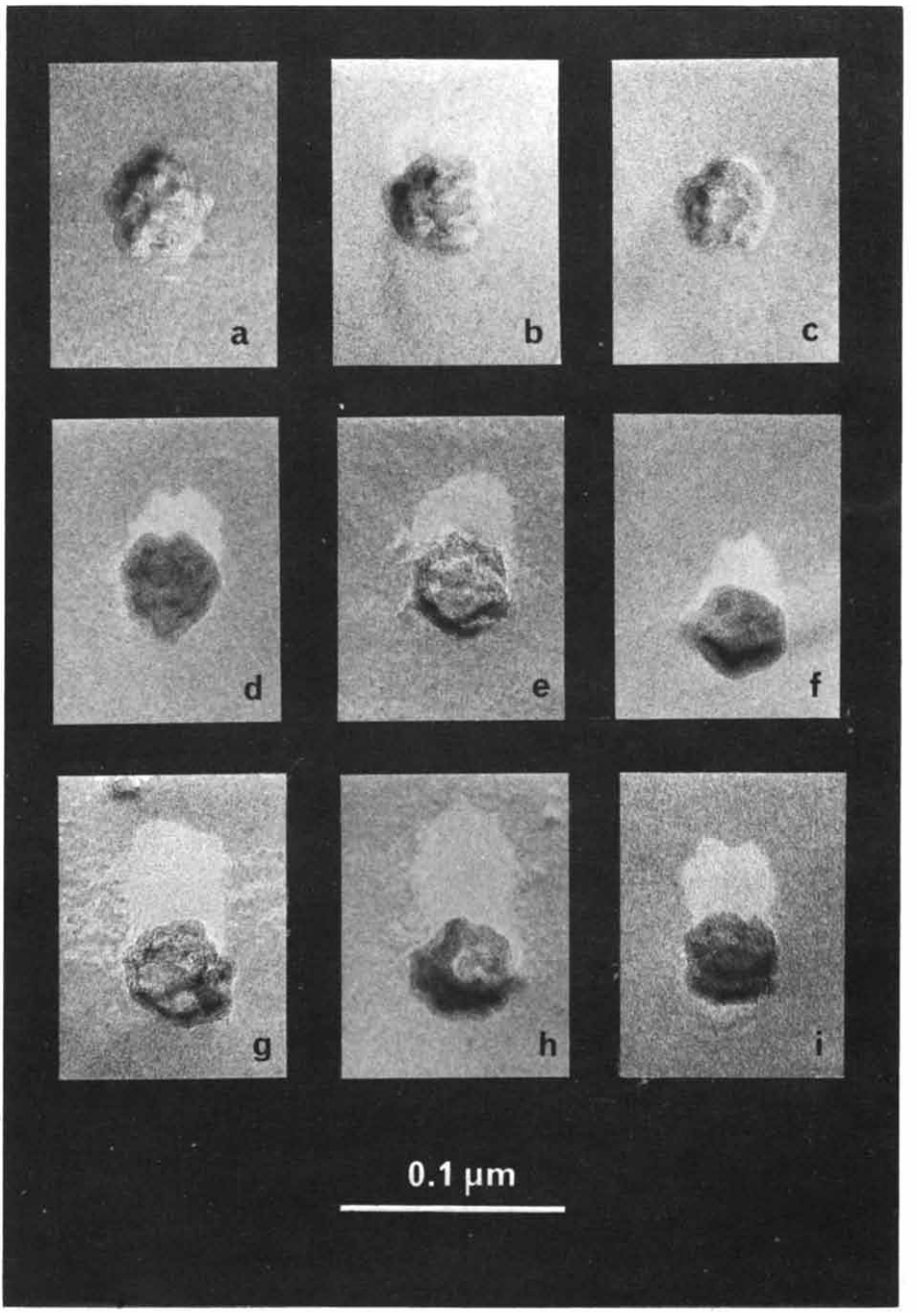

Fig. 1. Pyruvate dehydrogenase complex freeze etched without fixation. (a—c) The particles are still partially embedded in the surrounding ice. Globular "morphological units" are visible: (b), hexagonally and (c), pentagonally shaped particles. (d—i) The etching time was sufficient to fully expose the complex to the vacuum at the time of replication. For discussion of possible artifacts see text. (e) and (g) hexagonally shaped particles; and (f) pentagonally shaped particle. Electron microscopy magnification: 160 000X, final magnification: 300 000X.

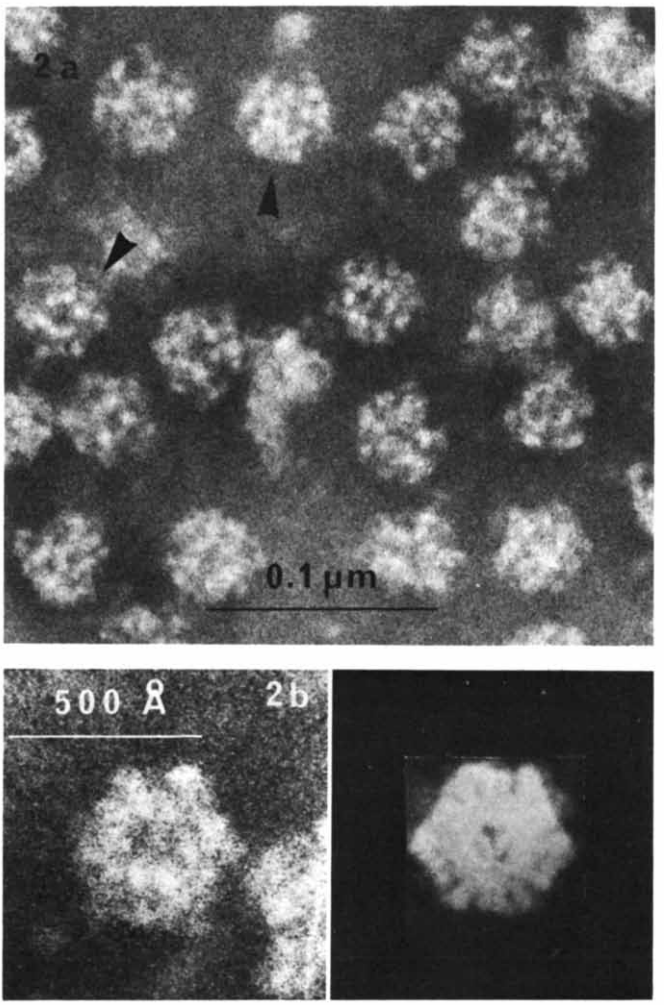

Fig. 2. (a) Pyruvate dehydrogenase complex, prefixed with glutaraldehyde vapor and negatively stained. Arrows indicate hexagonally shaped particles. Electron microscopy magnification: 160 000×, final magnification: 300 000×. (b) Correlation of a prefixed and negatively stained enzyme complex with an X-ray picture of the model projected close to the 3-fold rotational axes. Electron microscopy magnification: 160 000×, final magnification: 500 000×.

protein assay was calibrated by dry weight determination, a method which involves the usual problem of an exhaustive dialysis of a sucrose-containing protein solution and which introduces a possible error which cannot be accurately calculated.

By applying this technique to several preparations of the unfixed enzyme complex an average particle weight of $7 \cdot 10^6$ was obtained.

Size of the enzyme complex

Figs. 1a—c show freeze etch replicas of some complexes which were still halfway burried in the ice matrix and were thus not fully exposed to the vacuum when the replicas were made. In these pictures the enzyme complex appears

polyhedral, almost spherical, just as in prefixed negative stained preparations (Fig. 2). Therefore, on these types of specimens the orientation of the particles with respect to their rotational axes need not to be considered when their size is determined.

Figs. 1d—i show examples of replicas where the etching time was sufficient to fully expose the enzyme complex to the vacuum. In this case the thermal

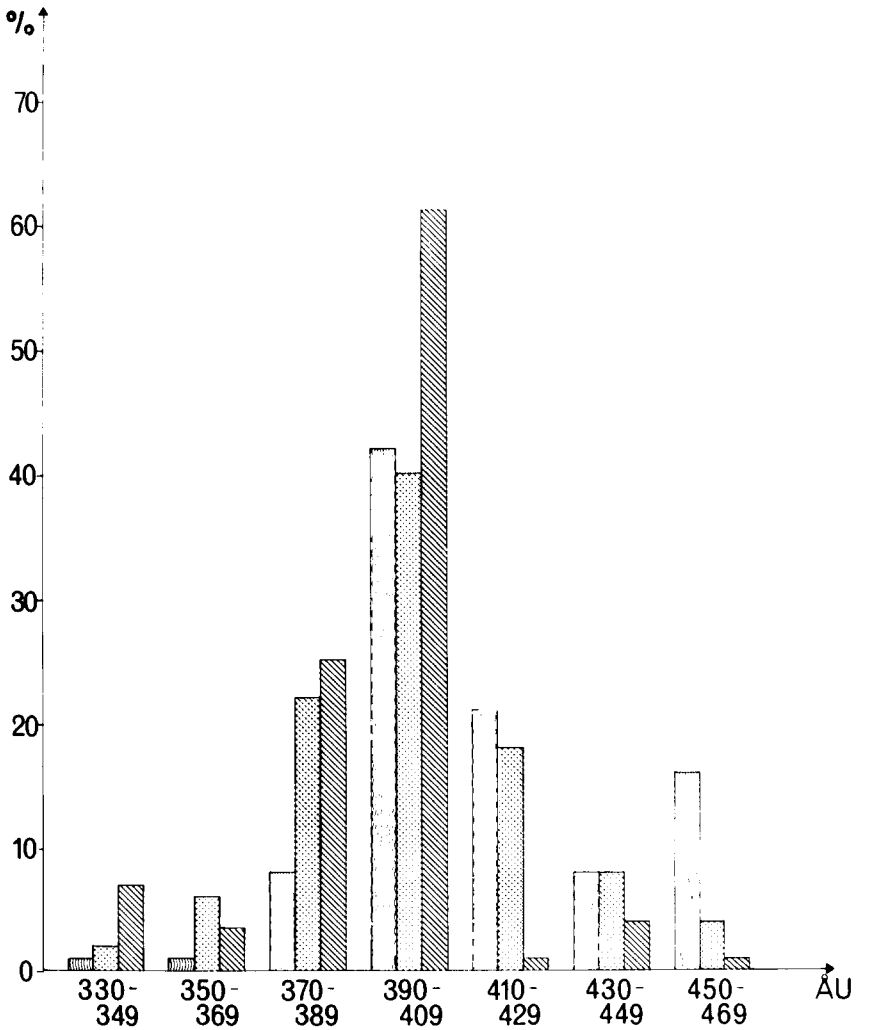

Fig. 3. Histogram of particle diameters observed on different preparations of the pyruvate dehydrogenase complex. The class interval is 20 Å.

contact of the particles with the ice was definitely poorer than when they were partially embedded in the cold matrix. The possibility, therefore, is increased that thermal artifacts occur during shadowing and carbon backing. Deformation of the particle replica during drying must also be considered. Indeed some of the particles appear to be distorted while their shadow on the ice still seems to depict the fine structure of the original particle surface (compare Fig. 5). This indicates that the deformations are not primarily caused by the metal shadowing but rather by the carbon backing with its inherent higher thermal load [16] or by the drying of the replica. In addition Figs. 6a and b demonstrate that the "distorted" appearance of particle "ghosts" can also be caused by an unfavorable orientation of the particular area of the replica to the normal plane of observation in the microscope.

In the case of fully etched particles the maximum shadow width was used as the measure of the particle size. Comparison of the prefixed negatively stained complex and of the freeze etched unfixed and prefixed preparations shows no significant difference in particle size. As shown in the Fig. 3 the variation is somewhat larger in freeze-etched specimens, but it has to be born in mind that the relative position of the individual particles with respect to the frozen matrix and to the shadowing direction is variable and not discernible. The histogram shows further that independent of the preparation, the majority of the particles were found in the 390—410 Å class.

Geometry of the complex.

In addition to the particle size and molecular weight the freeze etched specimen offers further information about structural details of the enzyme complex. Comparing the techniques of negative staining and freeze etching the different information obtainable by both methods is obvious. Whereas the image of a negatively stained particle is a parallel projection with all the protein units

TABLE I

MOLECULAR DIMENSIONS OF PYRUVATE DEHYDROGENASE COMPLEX

Comparison of experimentally observed dimensions of the pig heart pyruvate dehydrogenase complex with values calculated from the model. The model is based on a 230 Å sized pentagonal dodecahedral core. 30 morphological units are located along the edges, and have contact points to their next neighbours. A transfer of the acetyl residue by lipoic acid would be geometrically possible if the 12 morphological units which are located on the faces are approximately 70 Å in diameter. The limited resolution of electron microscopic preparation does not justify a more detailed model.

Diameter	Calculated from the model (Å)	Observed in micrographs (Å)	Preparation technique
Core (Lipoate acetyltransferase)	230	230 *—240	negative staining [6]
Subunits of the core	68	60— 70	negative staining
Pyruvate dehydrogenase complex	420	400—450	negative staining freeze etching
Subunits of the shell (a) located on the edges	95	80—100 (location undistinguishable)	freeze etching
(b) located on the faces	68		

contributing to a rather complicated superposition pattern, the replica of a freeze etched particle gives only information about its surface, which in the case of the pyruvate dehydrogenase complex is the outer shell.

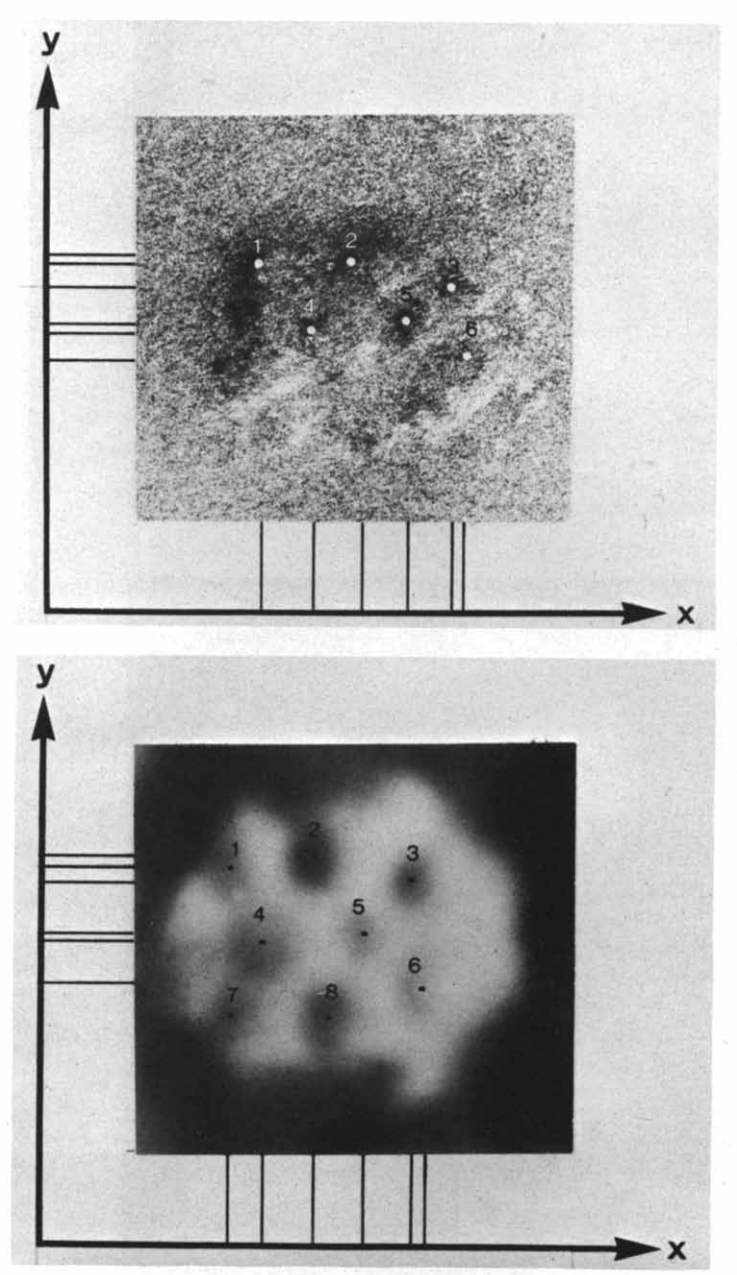

Fig. 4. Correlation of a well preserved replica of the complex with the model. The relative positions of the morphological units (see indices) are in fair agreement with those of the model as indicated by similar projection patterns for both, the model and the complex. The orientation of the complex within the coordinates was adjusted to correspond to the positions of two arbitrarily chosen morphological units 2 and 3 of the model. Deviations within the projection patterns are of the magnitude of 20 Å or less. Electron microscopy magnification: 160 000X, final magnification: 1 000 000X.

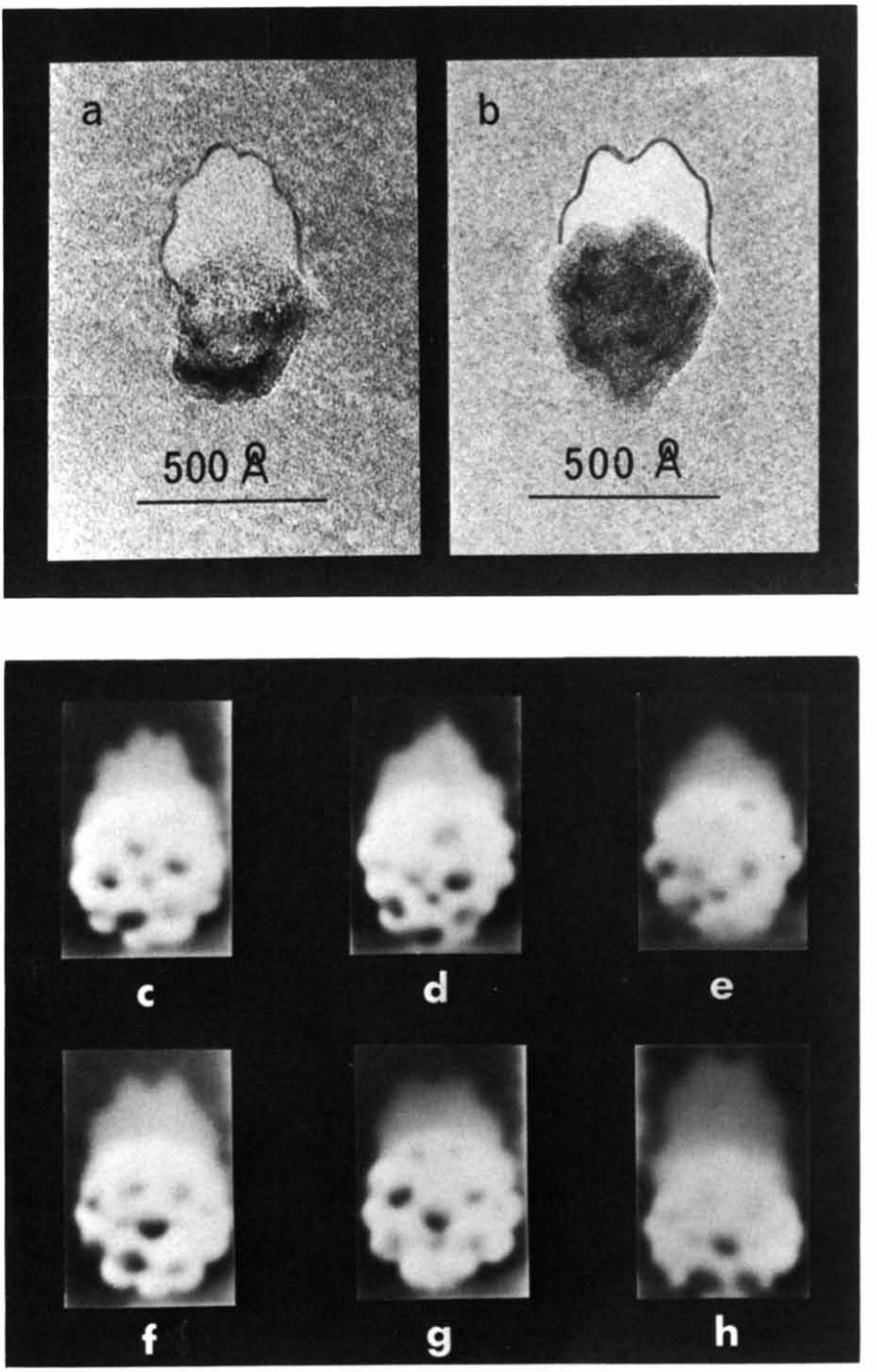

Fig. 5. Comparison of typical shadows as observed on freeze etched replicas and on the model illuminated by a point light source. Shapes of the shadows are characterized by two extremes. In a, c, d, and e, the shadow shows its maximum in the middle. In b, f, g, and h, the shadow exhibits a slight depression in the middle. Electron microscopy magnification: 160 000X, final magnification: 500 000X.

In order to check whether or not the patterns observed on the electron micrographs can be correlated with the subunit arrangement which has been proposed previously [2,3,6], the corresponding icosahedral models of the complex were photographed in various orientations. The models were constructed assuming spherical subunits, as described in ref. 6 and Table I.

To simulate negative staining, parallel projections were produced either by photographing models made of transparent material immersed in a colored liquid, or by X-raying wooden models [6] (Fig. 2b). To imitate freeze etching, photographs were taken from wooden models, which were embedded to different degrees in a fine grained material and illuminated with a point light source at 45° C (Figs. 4 and 5). All photographs were adapted to the electron microscopic magnification and were blurred in order to simulate an assumed resolution of about 20 Å.

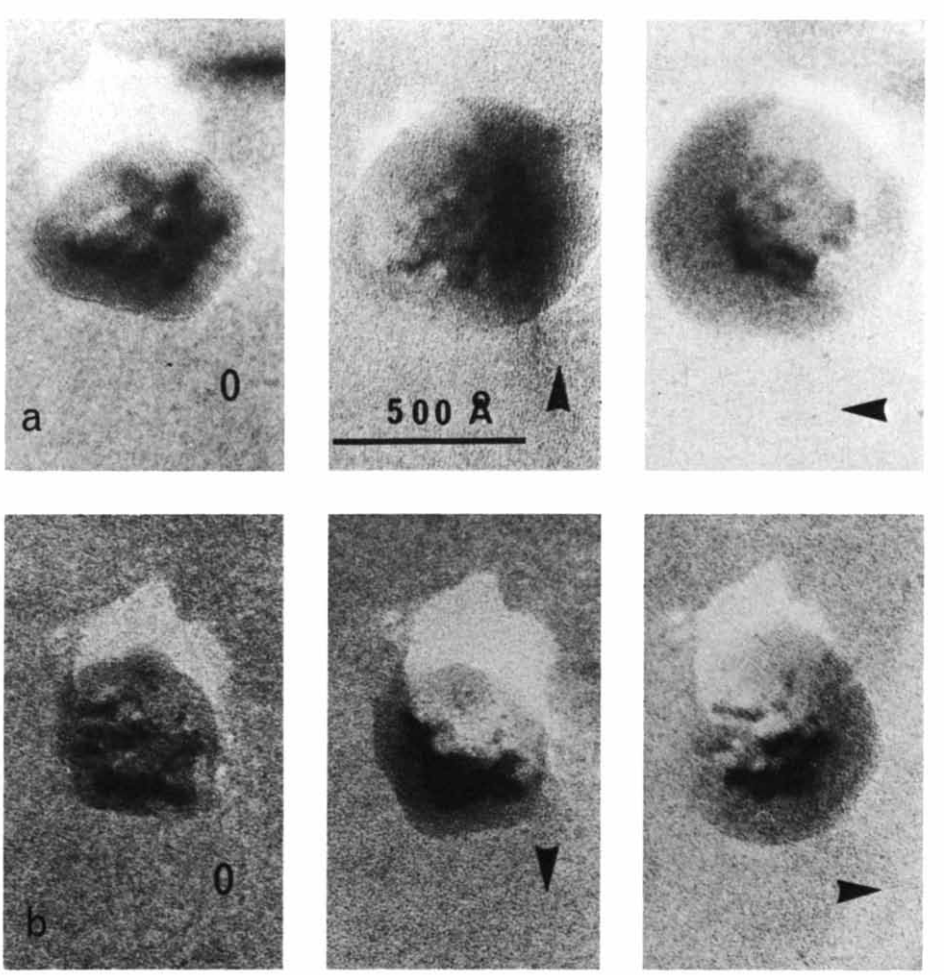

Fig. 6. Electron micrographs of two replicas of fully etched enzyme complexes. The overall appearance can be drastically changed by tilting the replica. The specimens were tilted at 200 000X magnification, the image rotating angle was close to zero. The tilting directions are indicated in the micrographs, and the tilting angles were approximately 30°. Final magnification: 500 000X.

In both types of specimens, the unfixed freeze etched and the prefixed negatively stained ones, the orientation of the complex to the plane of observation is random; subunits are clearly visible. In both specimens hexagonally shaped particles are noticed frequently (Figs. 1b, e and g, and Figs. 2a and b): possibly these are projections parallel to the 3-fold axes of an icosahedral structure since hexagonal shapes can also be observed on the model (Fig. 5e and Fig. 2b). Fig. 5c shows further that the complex should appear almost spherical when looked at parallel to the 5-fold axes. Occasional observations of pentagonally shaped particles in freeze etched preparations (Figs. 1c and f and Fig. 5a) can therefore not be positively correlated with any orientation of the model and are possibly due to distorted particle replicas or an unfavorable tilt.

As already mentioned the shadow of fully exposed freeze etched particles on the ice seems to be better preserved than the "ghosts" of the particles themself. The shape of the shadow can be characterized by two extremes: in one type the length of the shadow reaches its maximum approximately in the middle (Fig. 5a, also Figs. 1f and h), while the second type displays a depression in the middle of the shadow (Fig. 5b, also Figs. 1d and i). Corresponding shadows produced by varying the orientation of the model are shown in Figs. 5c—e and f—h. Fig. 5a shows at high magnification a freeze etched complex which was partially embedded in the ice during replication and is therefore well preserved. This picture demonstrates that the position of the individual morphological units can be correlated with the model if properly oriented.

Discussion

In the oxidative decarboxylation of pyruvate five different enzymes are involved: the pyruvate dehydrogenase, EC 1.2.4.1, and its regulating enzymes, pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphatase, the lipoate acetyl transferase, EC 2.3.1.12, and the dehydrolipoamide dehydrogenase, EC 1.6.4.3. It has been proposed that all these enzymes are organized in a multienzyme complex where the subunits are arranged in a way to form an optimal functional structure (for a recent review see ref. 17). However, a complex which still contains the pyruvate dehydrogenase phosphatase has never been isolated. This enzyme is therefore thought to be almost completely stripped off during isolation [18]. Furthermore, two of the other constituent enzymes (dehydrolipoamide dehydrogenase and pyruvate dehydrogenase-kinase) are also partially lost during preparation. Thus the stoichiometry of the complex is still uncertain, and does not provide a quantitative basis for the construction of a precise model. Reed, L.R. and a number of coworkers [2,3, 22] have proposed a structural model which is based on electron microscopy. This requires, however, a definite number of subunits in order to satisfy physical principles for the construction of a symmetrical structure. The structure determining core, according to this model, is the lipoate acetyltransferase consisting of 20 morphological units * which are arranged in a pentagonal dodecahedron. This is verified by the excellent correlation between the model and the negatively stained lipoate acetyltransferase molecules [6].

* the term "morphological units" is used according to considerations of Caspar and Klug [21].

In the model of the core each one of the 20 morphological units has contact with three neighbours. This is consistent with the biochemical finding [19] that the core is built up by 60 identical protein chains. Thus each of the morphological units must be a trimer which however could not be resolved by electron microscopy. Recently the core has been crystallized. The preliminary analysis of the crystals also indicates that the pentagonal dodecahedron is the building unit [20].

According to the model the other constituent enzymes are assembled on the edges and faces of the preformed core to give a shell of definite size with icosahedral symmetry. It is apparent that the diameter of the total complex is determined by those morphological units which are located on the edges. If it is required that these units are in contact with each other, their diameter must be 90—100 Å, as the core diameter is 230 Å [6]. The diameter of the resulting complex would then be 420 Å, the volume of the circumscribed sphere being $4 \cdot 10^7$ Å^3 (Table I). There have been several experimental approaches to establish the dimension of the complex, but probably due to its instability, the data are in rather poor agreement. Sedimentation coefficients vary between $S_{20,w}$ 60—80 [4,6,18]. Electron microscopic measurements are equally differing. Hayakawa et al. [4] reported particle diameters of 210 Å for negatively stained and of 289 Å for shadowed specimens. The values given by Wieland and Siess [5] are in the same range. Reed has observed diameters between 300 Å [2] and 400—450 Å [3] respectively. Diameters close to the values calculated from the model have been consistently observed on negatively stained specimens after prefixation with glutaraldehyde [6]. The same particle diameters have now been obtained by freeze etching the fixed and the unfixed complex verifying the existence of such large particles in dilute aqueous solutions and in the absence of cross-linking fixatives. Independent of the size measurements the molecular weight of the unfixed complex was also determined by freeze etching. The value of $7 \cdot 10^6$, within the experimental error, is also compatible with the model.

A precise description of the shell which is built up by two, probably three different enzymes is still not possible by electron microscopy. The image interpretation of the randomly oriented negative stained complex is difficult. Even when the problems of stain distribution are neglected, the number of units which contribute to the contrast is too large to permit an exact reconstruction of the complex. Contrary to negative staining freeze etching reveals only the surface of the particle shell. For the reasons mentioned above, especially the particles which are still partially embedded in the ice matrix show the best preservation of the relative position of the morphological units. Unfortunately, these particles usually reveal too small a number of morphological units to positively prove icosahedral symmetry [21]. The packing of the visible units, however, when indexed and projected onto rectangular coordinates is comparable to that of the model (Fig. 4).

In summary it should be kept in mind that biochemical analysis gives an average over the total population of the complex which might be heterogeneous because of the instability of the individual particles. The fact that the molar ratio of the constituent enzymes is still uncertain is therefore not a priori evidence against the existence of a stoichiometric complex. The molecular

weight and the overall dimensions of the complex as obtained by electron microscopy were similarly based on an averaging of the total particle population. Observations and measurements concerning the fine structure, however, were performed on individual particles which were selected for their well preserved appearance and their compatibility with the model. This must be remembered when arguing that the partial dissociation of the complex has less influence on the electron microscopic than on the biochemical data.

Nevertheless, it can be concluded that the electron microscopic findings reported in this paper give further support to the model of the pyruvate dehydrogenase complex as proposed by Reed [2,3].

Acknowledgements

This investigation was supported by grants of the Deutsche Forschungsgemeinschaft. We thank Prof. Dr. H. Reinauer for this support and for stimulating discussions. We are indebted to Mrs. A. Bernitt, M. Marzi and I. Sprenger for their skilful technical assistance.

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