Quaternary Structure of Yeast Pyruvate Carboxylase: Biochemical and Electron Microscope Studies†

Nadine D. Cohen,* Merton F. Utter, Nicholas G. Wrigley, and Anthony N. Barrett

ABSTRACT: Electron microscope and cross-linking studies of pyruvate carboxylase from yeast show that this enzyme is a tetramer with a rhombic appearance. In agreement with earlier sedimentation equilibrium measurements [R. E. Barden et al. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4308–4312], cross-linking experiments with the diimido ester, dimethyl suberimidate, suggest an overall molecular weight of 480 000 for the tetramer. An investigation of the polypeptide composition of the enzyme by using sodium dodecyl sulfate ($NaDodSO_4$) gels and $NaDodSO_4$–urea gels and isoelectric focusing in 8 M urea shows that the individual polypeptides are similar or identical in size and charge. The biotin content of the enzyme (3.5 mol/mol of enzyme) also indicates that each protomer contains 1 mol of biotin. The overall electron microscope appearance of native enzyme and cross-linked enzyme is comparable; i.e., the tetramers are rhombic in shape and seem to have two elongated and two rounded subunits. Measurements of the individual tetramers confirm this rhombic shape for the molecule. By using the electron microscope evidence and biochemical data, an unusual model is proposed for the quaternary structure of yeast pyruvate carboxylase. The basic features of the model are (1) the protomers are very similar or identical; (2) the subunits are shaped roughly as prolate ellipsoids; (3) the subunits are arranged such that their centers are located on the corners of a rhombus; and (4) diagonally opposite pairs of subunits lie in orthogonal planes. Computer-generated rotations of the proposed model in three dimensions are similar in appearance to actual tetramers of yeast pyruvate carboxylase observed in the electron microscope. Computer simulation of several different negatively stained models shows that the proposed structure is consistent with the electron microscopic appearance of yeast pyruvate carboxylase and eliminates a number of alternative structures.

Pyruvate carboxylases isolated from chicken liver, rat liver, and other animal tissues are tetrameric enzymes of about 440 000–520 000 molecular weight (Utter et al., 1972). In the presence of denaturants such as $NaDodSO_4$,[1] these enzymes will dissociate to polypeptides of 110 000–130 000 molecular weight (Barden et al., 1975). Studies on the chicken liver enzyme indicate that its polypeptides are similar in both size and charge, and that each protomer contains one molecule of covalently bound biotin (Barden et al., 1975), suggesting that the tetramer is composed of four identical subunits.

As determined by sedimentation equilibrium, yeast pyruvate carboxylase is also a tetrameric enzyme of about 446 000 molecular weight (Barden et al., 1975) and its subunits are 120 000 molecular weight (Utter et al., 1972). Preliminary electron microscope studies suggested that this enzyme was rhombic in appearance (Valentine, 1968). This apparent structure could only arise if the enzyme subunits were located in different planes of orientation, or if the enzyme was composed of two different types of subunits. To further elucidate this problem, cross-linking and electron microscope studies of yeast pyruvate carboxylase were conducted.

The results of these studies further support the concept that yeast pyruvate carboxylase is a tetrameric enzyme composed of identical subunits. In addition, the four subunits of yeast pyruvate carboxylase appear elongated and do indeed lie with their centers on the corners of a rhombus plane, and not at the corners of a tetrahedron or a square, as might have been expected. Also, one pair of diagonally opposite protomers lies with its long axes in one plane, while the second pair appears to have its long axes in another plane orthogonal to the first. These last two features lead to such an unusual model of the tetramer as to call the electron microscope conclusions in question. Therefore, computer simulation studies of other more "likely" models were undertaken to see if they would fit the data better. They did not, thus giving support to the unusual model described in this paper.

Experimental Procedure

Enzyme Purification. *Saccharomyces cerevisiae* (Baker's yeast) was obtained from Red Star International. Pyruvate carboxylase was purified as described by Young et al. (1969). Electrophoretic analysis of the purified enzyme on $NaDodSO_4$–polyacrylamide gels revealed only one major band with contaminants amounting to less than 15%.

Protein Determination. Protein was determined in most cases by the biuret method (Layne, 1957); however, in some instances, the spectrophotometric method of Warburg & Christian (1941) was used. This method yields lower values and requires a correction factor of approximately 1.4 (Barden et al., 1975).

$NaDodSO_4$ Gels. Proteins were denatured by boiling for 5 min in 1% $NaDodSO_4$ and 1% β-mercaptoethanol before electrophoresis. The gels contained 3.5% acrylamide, 0.135% methylenebisacrylamide, and running buffer (0.1 M borate, 0.1 M sodium acetate, 0.1% $NaDodSO_4$, pH 8.5). Electrophoresis was done at 6 mA/tube. After electrophoresis, the gels were cut at the dye marker and stained with Coomassie Blue. Gels were diffusion destained in 33% methanol–10% acetic acid. The stained gels were scanned at 570 nm by using a Gilford Model 2000 spectrophotometer equipped with a linear transport.

Isoelectric Focusing in Urea. Isoelectric focusing in polyacrylamide gels was carried out as described by MacGillivray & Rickwood (1974). Yeast pyruvate carboxylase was denatured by dialysis for 16 h against 0.3 M Tris-HCl buffer, pH 8.3, containing 8 M urea. The enzyme was mixed with 10% acrylamide solution, 40% ampholine solution (pH 3–10),

† From the Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106 (N.D.C. and M.F.U.), and the Laboratory of Biological Ultrastructure, National Institute for Medical Research, London, England (N.G.W. and A.N.B.). *Received May 24, 1978; revised manuscript received March 7, 1979.* This work was supported in part by National Institutes of Health Grants AM-12245 and GM 22920. N.G.W. acknowledges gratefully support of NATO Research Grant No. 1124 (1977).

[1] Abbreviations used: DMS, dimethyl suberimidate; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/79/0418-2197$01.00/0

and ammonium persulfate. Focusing was performed at 4 °C for 17 h. Protein was fixed by exposure to 5% trichloroacetic acid and visualized by staining with Coomassie Blue. The pH gradient within the gels was measured in corresponding blank gels.

Cross-Linking. Protein was cross-linked by using dimethyl suberimidate (Aldrich) as described by Davis & Stark (1970). Reactions were usually done in 50 mM Tris-HCl, pH 7.8, containing 0.2 M KCl. Dimethyl suberimidate was dissolved in buffer immediately prior to use and rapidly titrated to pH 7.8 with 1 M KOH. Protein concentrations were maintained at 1 mg/mL or less during the reaction, while diimido ester concentrations were approximately 20 mM. The reactions were terminated by addition of 1% $NaDodSO_4$ or dialysis against buffer to remove excess reagents. Ovalbumin cross-linked molecular weight standards were prepared as described by Carpenter & Harrington (1972).

Biotin Determinations. Biotin content was determined by using a radioactive assay developed by Rylatt et al. (1977), based on the binding of the labeled biotin to avidin.

Electron Microscopy. Enzyme preparations were diluted to 50–100 μg/mL in 50 mM Tris-HCl, pH 7.2, containing 0.2 M KCl at 23 °C. Samples were then applied to a film of carbon evaporated on a small piece of mica and then floated on negative stain (2% sodium silicotungstate, pH 7.0, 2% ammonium molybdate, pH 7.2, or 0.5% uranyl oxalate, pH 6.8). The film was then picked up on a copper electron microscope grid, blotted, and dried. The grids were examined in a JEM-100C microscope and plates were exposed to the image at a magnification of ×65 000. The microscope was calibrated by the use of the 87.5-Å spacing of catalase crystals (Wrigley, 1968), giving dimensional accuracy of the order of 2% for individual measurements. However, measurements of 100 pyruvate carboxylase molecules showed variations much greater than this due to distortions and variable stain penetration so that, in aggregate, measurements were subject to uncertainties of the order of 10–20%.

Computer Modeling. Models of the quaternary structure of yeast pyruvate carboxylase were generated by computer methods described by Barrett et al. (1977). In this method, the stain is modeled, leaving the supposed protein structure as cavities within the stain, so simulating faithfully the physical process of image formation in the electron microscope. Our computer images were compared directly with actual electron micrographs.

Results

Cross-Linking Studies. The molecular weight of yeast pyruvate carboxylase as determined by sedimentation equilibrium is 446 000, while the protomer molecular weight is 120 000 (Barden et al., 1975). Although the subunit multiplicity and stoichiometry indicate that the enzyme is a tetramer, an independent means was sought to assess its subunit structure. Thus, cross-linking studies with dimethyl suberimidate (DMS) were carried out. Cross-linking conditions were varied across time, protein concentration, and reagent condition. Figure 1 illustrates the results of a typical experiment in which the time was varied. $NaDodSO_4$ gels of native, partially cross-linked (20 min exposure) and fully cross-linked (90 min exposure) enzyme are presented. After 20 min of incubation with the cross-linking reagent, four protein bands could be observed corresponding to the monomer, dimer, trimer, and tetramer, while at 90 min approximately 90% of the enzyme was fully cross-linked to the tetramer. In other experiments in which DMS concentration was varied, less than 50% of the protein was fully cross-linked

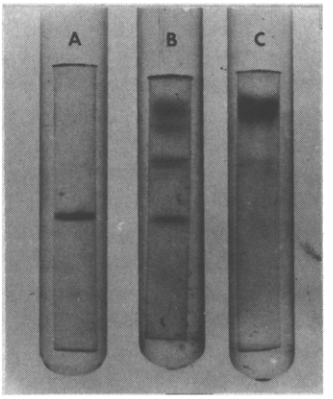

FIGURE 1: $NaDodSO_4$ gels of native, partially cross-linked, and fully cross-linked yeast pyruvate carboxylase. The enzyme was incubated with DMS for 0 (A), 20 (B), or 90 min (C). The samples were then treated with 1% $NaDodSO_4$ and 1% β-mercaptoethanol.

when reagent concentrations were maintained below 1.5 mg/mL, while, at DMS concentrations of greater than 4.0 mg/mL, better than 80% of the protein could be fully cross-linked to the tetrameric form. Protein concentrations, in most cases, were maintained at 1.0 mg/mL or less to minimize intermolecular cross-linking. However, no differences were noted in the cross-linking patterns produced when protein concentrations were increased from 0.75 to 1.4 mg/mL.

The molecular weights of the products of partial cross-linking were calculated by comparing the relative mobilities of these species vs. the relative mobilities of cross-linked ovalbumin. The ovalbumin standards were produced by intermolecular cross-linking of this monomeric protein (Carpenter & Harrington, 1972). As calculated, the monomer, dimer, trimer, and tetramer of yeast pyruvate carboxylase had molecular weights of 127 000, 260 000, 390 000, and 485 000, respectively. These values are quite consistent with the predicted molecular weights of 120 000, 240 000, 360 000, and 480 000. These results thus are in reasonable agreement with the earlier sedimentation equilibrium studies (Barden et al., 1975) and indicate that yeast pyruvate carboxylase is a tetramer of approximately 480 000 molecular weight. Using this method of assessing molecular weights, Carpenter & Harrington have reported an average deviation of 6% among different proteins. Thus, the calculated molecular weights of an unknown may vary depending upon the protein selected as a molecular weight standard. For example, the monomer, dimer, trimer, and tetramer of yeast pyruvate carboxylase were found to have molecular weights of 140 000, 290 000, 435 000 and 535 000, respectively, when cross-linked chicken liver pyruvate carboxylase was used as a molecular weight standard.

Subunit Composition of Yeast Pyruvate Carboxylase. When yeast pyruvate carboxylase was subjected to electrophoresis in polyacrylamide gels containing either 0.1% $NaDodSO_4$ or 0.1% $NaDodSO_4$ and 8 M urea, only one protein band was observed (Figures 2A and 2B). These results suggest that the protomers of yeast pyruvate carboxylase are identical with respect to both size and charge. Further support for this argument was obtained when the enzyme was applied to an isoelectric focusing gradient containing 8 M urea (Figure 2C). Under these conditions, the enzyme dissociates to its subunits (Tolbert, 1970). Only one protein band was detected on the gels after the enzyme had been focused for 17 h in a gradient from pH 3 to 10. Thus, the subunits of yeast pyruvate carboxylase appear to be similar with respect to their overall size and their overall charge.

The biotin content of the enzyme was measured by using a radioisotopic assay (Rylatt et al., 1977). Approximately 3.5

Table I: Measurements of the Dimensions of Yeast Pyruvate Carboxylase Obtained from Electron Micrographs[a]

actual mm measurements	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0
equivalent in A ± 2%	47	56	65	75	84	93	103	112	123	131	140	150	159	168	178	187
A				13	38	44	5									
B					2	18	22	41	14	3						
C[b]	10	32	41	102	7	8										
D										8	37	54	1			
E												2	0	57	26	15

[a] As is shown in Figure 6, five dimensions were measured on 100 tetramers of yeast pyruvate carboxylase on enlarged prints at ×358 450 ± 2% magnification. The 0.5-mm measuring intervals and their A equivalents are shown at the top. The numbers in rows A-E refer to the number of measured tetramers (total, 100) with dimensions as indicated. [b] Two measurements were made on each tetramer.

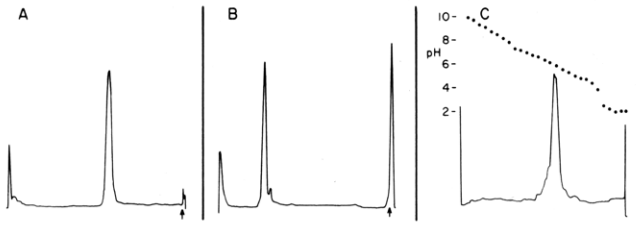

FIGURE 2: Spectrophotometric scans of yeast pyruvate carboxylase under denaturing conditions. (A) Enzyme was dissociated with 1.0% $NaDodSO_4$ and 1.0% β-mercaptoethanol and then applied to 3.5% polyacrylamide gels containing 0.1% $NaDodSO_4$. The arrow indicates the migration of the dye front. (B) Enzyme was dissociated with 1.0% $NaDodSO_4$ and 8 M urea and then applied to 8.0% polyacrylamide gels containing 0.1% $NaDodSO_4$ and 8 M urea. (C) Enzyme was dissociated by dialysis against buffer containing 8 M urea for 16 h. The enzyme was then applied to a polyacrylamide gel containing ampholytes (pH 3–10) and 8 M urea. After focusing for 17 h, the protein was fixed by immersing the gels in 5% trichloroacetic acid. The dots show the pH gradient measured on an accompanying blank gel.

mol of covalently bound biotin was bound per mol of enzyme. This implies that each protomer of the enzyme contains 1 mol of covalently bound biotin and is further evidence for the tetrameric structure of the molecule.

Electron Microscopy of Yeast Pyruvate Carboxylase. Preparations of native yeast pyruvate carboxylase were negatively stained with either 2.0% sodium silicotungstate, 2% ammonium molybdate, or 0.5% uranyl oxalate and examined in the electron microscope (Figure 3). Although the approximate size and shape of the molecules were comparable in all three negative stains, the relative numbers of intact tetramers varied considerably. Accordingly, further electron microscope studies were carried out on preparations of yeast pyruvate carboxylase which had been fully cross-linked with DMS. Figure 4 shows a comparison of the native and cross-linked enzymes as visualized by staining with 2% sodium silicotungstate. The native and cross-linked tetramers were similar in overall appearance, i.e., size and shape; however, the relative numbers of intact molecules were substantially greater in the cross-linked preparation.

In the micrographs of cross-linked yeast pyruvate carboxylase, approximately 90% of the intact molecules had a characteristic rhombic appearance. This appearance was confirmed in far more fields than we can show in Figure 4 by numerous lay observers and more objectively by direct measurement. The observers were less unanimous as to the elongated appearance of one opposite pair of protomers and the rounded appearance of the other pair, and our measurements were also more equivocal on this point.

Five dimensions were measured on each of 100 tetramers on enlarged prints of the electron micrographs. As defined in Figure 5, these included (A) the center-to-center distance between the subunits located at the right and left sides of the molecule; (B) the center-to-center distance between the subunits located at the top and bottom of the molecule; (C) the length of the subunits located along the right and left sides of the molecule; (D) the overall width of the molecule; and (E) the overall height of the molecule. As illustrated in Table I, these measurements show typical skewing (except dimension E) toward higher values. This phenomenon is consistent with a foreshortening of many molecules which would result if the tetramers were not lying perfectly flat on the electron microscope grid. The range of values in each case testifies to the difficulty of measuring single molecules in negative stain. However, looking at the modes of the distributions, there is no doubt that the overall height (E) of the tetramer (168 Å) is larger than the overall width (D) (150 Å). A difference is also noted in the center-to-center distances between opposing pairs of subunits, A vs. B. We were more reluctant to draw firm conclusions regarding individual subunit dimensions,

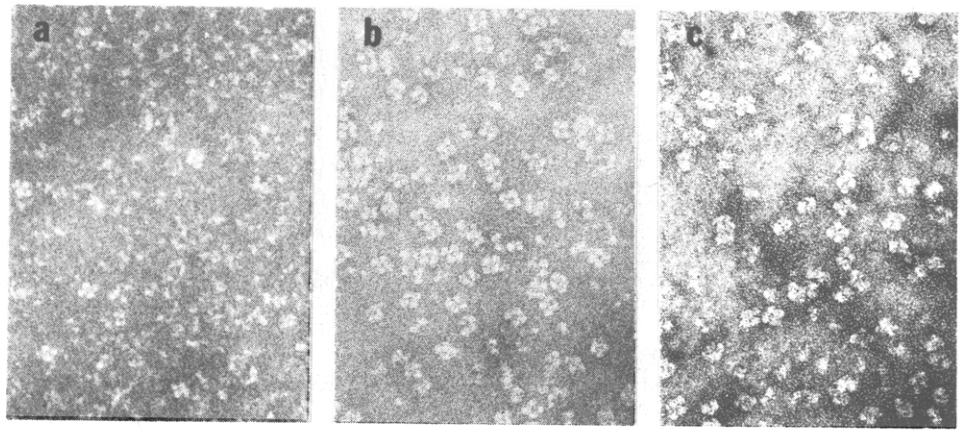

FIGURE 3: Electron micrographs of yeast pyruvate carboxylase. The enzyme was negatively stained with either (a) 2% sodium silicotungstate, (b) 2% ammonium molybdate, or (c) 0.5% uranyl oxalate. Magnification 160 000×.

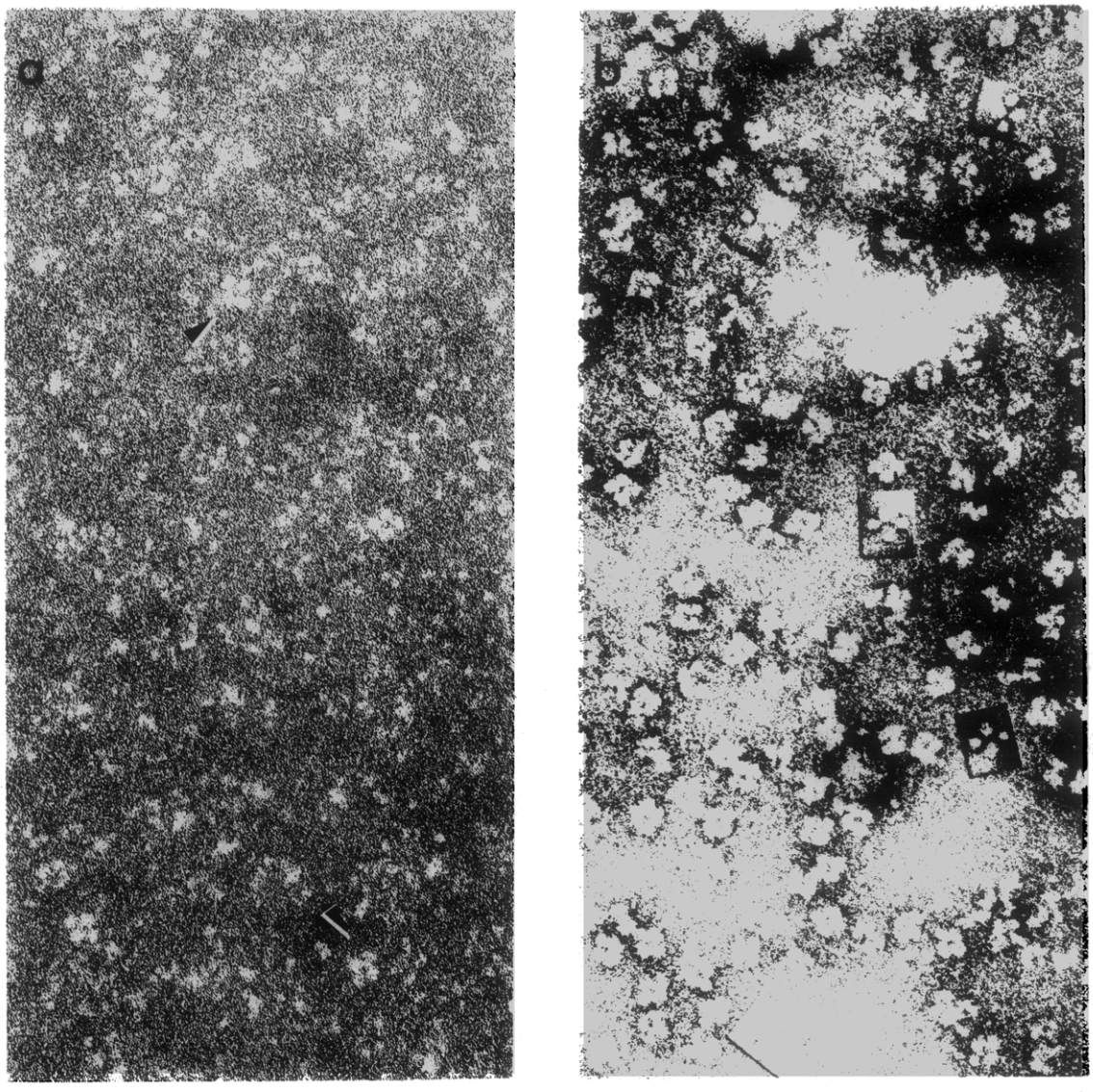

FIGURE 4: Electron micrographs of native (a) and cross-linked (b) yeast pyruvate carboxylase. In each case, the protein was negatively stained with 2.0% sodium silicotungstate. Five computer-generated tetramers are superimposed on the electron micrograph in b. These negative stain simulations were matched in size to the molecules of pyruvate carboxylase to illustrate their similarity in appearance. Magnification 200 000×.

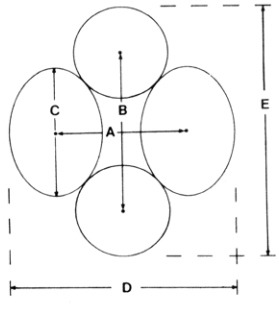

FIGURE 5: Measurements were made of the dimensions of yeast pyruvate carboxylase from electron micrographs. The dimensions measured on each tetramer were (A) the center-to-center distance between the subunits located at the right and left sides of the molecules; (B) the center-to-center distance between the subunits located at the top and bottom of the molecule; (C) the length of the subunits located along the right and left sides of the molecule; (D) the overall width of the molecule; and (E) the overall height of the molecule.

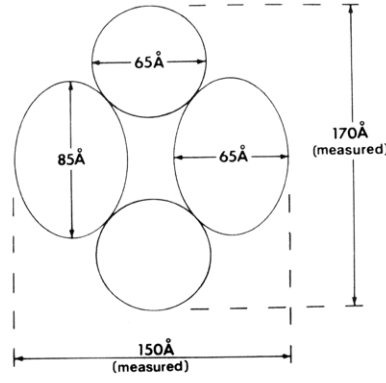

FIGURE 6: Model for the quaternary structure of yeast pyruvate carboxylase. The subunits are prolate ellipsoids of approximately 85 Å long and 65 Å wide. The two subunits on the left and right sides of the model lie with their long axes in the plane of the paper. The two subunits on the top and bottom of the tetramer lie with their long axes perpendicular to the plane of the paper.

noting only that, in general, the left and right subunits appear elongated and top and bottom subunits rounded (Figure 4).

From these observations and our knowledge of the subunit composition of yeast pyruvate carboxylase, a model for the quaternary structure of this enzyme was developed (Figure 6). This incorporates a number of features. (a) The subunits within the model are identical with respect to size and shape, a notion which is strongly supported by our biochemical studies. (b) The overall size of the model tetramer, 170 Å by

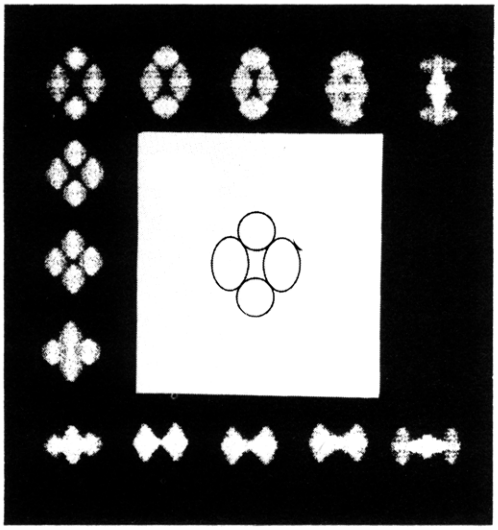

FIGURE 7: Computer model of yeast pyruvate carboxylase. The views along the outside of the figure were generated by rotations of the basic model, located in the center of the diagram, about the horizontal and vertical axis. The untilted projection is shown in the top left hand corner.

150 Å, is consistent with the height and width measurements obtained from electron microscopy (Figure 5). (c) The appearance of two rounded subunits and two elongated subunits in the model is consistent with the electron microscopic appearance of yeast pyruvate carboxylase (Figure 4), but is neither proved nor disproved by measurements. This observation, in combination with the subunit identity, led to a proposal of an ellipsoidal shape for the subunits. (d) This assumption of an ellipsoidal shape requires that the diameter of the rounded subunits (end views) be equal to the width of the elongated subunits (side views). These features, plus the fact that the adjacent subunits must be touching, require the subunit dimensions to be 75–85 Å long and 60–65 Å in diameter. These dimensions, with an assumed partial specific volume of 0.75 mL/g, give a subunit molecular weight in the range of 120 000 to 150 000. This is acceptably close to the measured value of 120 000 daltons since the assumption of ellipsoidal shape is no more than a convenient approximation. Alternative subunit shapes, such as kidney shape, could be postulated which would still be consistent with this model.

Because of continuing doubts about subunit shape and layout, we reconstructed a model of Figure 6 in the computer and by rotating it in three dimensions generated different appearances (Figure 7). The model in the untilted position is shown at the top left. Rotation of the tetramer about the horizontal axis produces a symmetrical or square like projection (center left). Additional rotation of the molecule about this axis (90°) causes the tetramer to appear like a trimer (bottom left). As the tetramer is then rotated about the vertical axis, it assumes a dimer-like appearance (bottom row). The top row in this figure shows rotation from the untilted position about the vertical axis.

To provide evidence in support of the proposed model, comparisons were made between these tilted computer models and actual tetramers selected from the electron micrographs (Figure 8). Although a multitude of different projections was predicted, aside from the majority view (rhombic projection), only two of them were found consistently (the square and dimer) due to change tilting of a few molecules in our untilted pictures. In these cross-linked preparations of yeast pyruvate carboxylase, approximately 5–10% of the molecules are only cross-linked to a dimeric form. Therefore, it is conceivable that the dimeric structures observed in the electron micrographs are, in fact, true dimers rather than side-view tetramers. However, the marked brilliance of these dimers suggests a depth which is more consistent with a tetramer embedded on its side in the negative stain.

These computer simulations are actually positive images of the model. In electron microscopy, however, the appearance of a structure is a negative image, produced by electron scattering in the negative stain. It was important to determine whether the stain distribution itself could affect the appearance of the molecule. Specifically, is it possible that negative staining caused subunits which are really spherical to assume the appearance of elongated protomers, i.e., prolate ellipsoids? To investigate this problem, negative stain simulations of three different models were generated by using the computer (Figure 9). The first model (frames A, B, and C) consisted of four identical spherical subunits arranged in a tetrahedral fashion, a configuration originally thought to be most likely; three different stain distributions are shown, none of which resembles the actual micrographs. The second model (frame D) consists of the same four spheres but in a coplanar arrangement with their centers on the corners of a rhombus; this resembles the actual micrographs much more closely but still shows no apparent elongation of the right and left subunits. The third model (frame E) is that of Figures 6 and 7 with ellipsoidal subunits in rhombic arrangement and looks very similar to the tetramers observed in the electron microscope. This similarity in appearance is more clearly illustrated in the electron micrograph of Figure 5B, showing a typical field of molecules upon which the computer "tetramer" of Figure 9E has been superimposed. It is thus apparent that yeast pyruvate carboxylase is not composed of spherical subunits in a tetrahedral arrangement. In fact, spherical subunits could not be arranged in any fashion (e.g., with their centers at the corners of a rhombus) to produce subunit elongation comparable to that observed for yeast pyruvate carboxylase in the electron microscope.

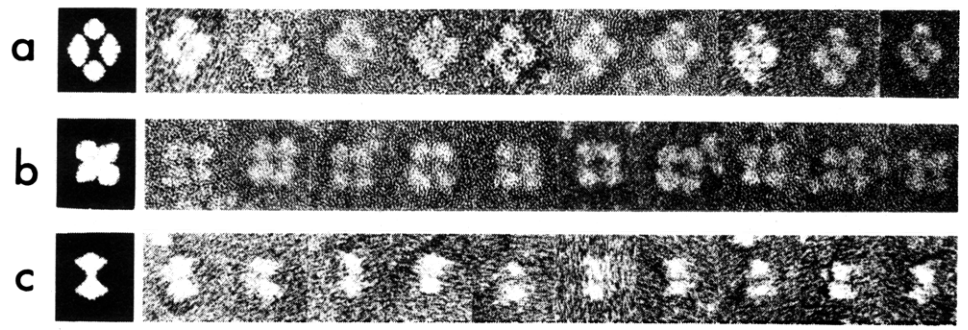

FIGURE 8: Computer models selected from Figure 7 were compared with actual electron micrographs of yeast pyruvate carboxylase. Examples of the (a) rhomboid presentation, (b) square-like presentation, and (c) dimer-like presentation were observed in electron micrographs of cross-linked yeast pyruvate carboxylase.

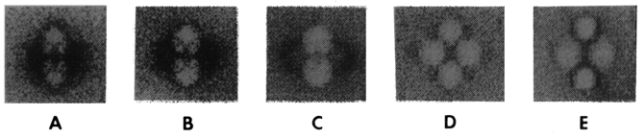

FIGURE 9: Negative stain simulations of three different models of pyruvate carboxylase were generated. A, B, and C are examples of a tetrahedral model. In this model the tetramer is composed of four spherical subunits which are arranged in a tetrahedral fashion. These three versions have different stain distributions. D shows the rhombic model by using spherical subunits, and in E the tetramer is composed of four coplanar prolate ellipsoids arranged with their centers at the corners of a rhombus as described in Figure 6. These computer simulations illustrating negative stain distributions were generated as described by Barrett et al. (1977).

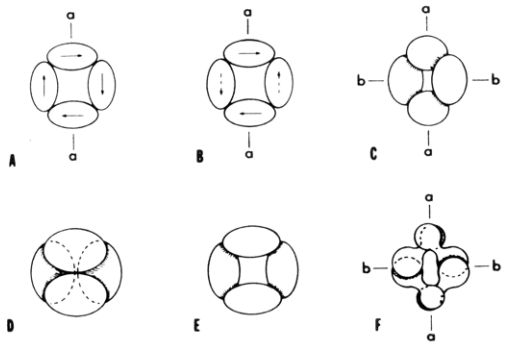

FIGURE 10: Alternative models for the tetrameric molecule of pyruvate carboxylase. (A) Square planar tetramer with elongated subunits illustrating C_4 symmetry. (B) Square planar tetramer with elongated subunits demonstrating D_2 symmetry. (C) A rhombic projection produced by tilting model A or B about axis a–a. (D) A tetrahedron with elongated subunits illustrating D_2 symmetry. (E) A noncoplanar D_2 structure with elongated subunits. (F) A noncoplanar D_2 structure composed of irregular dumbbell shaped subunits.

We wish to emphasize, however, that the important question is not whether the protomers are spherical or ellipsoidal, but what their mutual arrangement is in three dimensions. With our computer analogues, we have clearly shown that a rhombic arrangement is consistent with the electron microscopic appearance of pyruvate carboxylase. This has profound consequences for the mode of intersubunit binding, which is independent of their detailed shape as discussed in the next section.

Discussion

The results of the cross-linking studies presented here, in conjunction with sedimentation equilibrium studies reported earlier, clearly demonstrate that yeast pyruvate carboxylase is a tetrameric enzyme of approximately 480 000 molecular weight. At present, all indications are that the protomers of the enzyme are identical. They are similar in size and similar in charge, and appear to each contain one molecule of covalently bound biotin. Attempts to perform N-terminal analysis of this enzyme have been unsuccessful,[2] since the N terminal of the polypeptides of the enzyme appears to be blocked. Therefore, further information concerning the subunit composition of yeast pyruvate carboxylase must await more detailed analysis of primary structure.

Based on symmetry considerations alone, the most likely arrangement for the four (identical) subunits of pyruvate carboxylase would be structures showing either fourfold cyclic (C_4) or twofold dihedral (D_2) symmetry, as shown in Figure 10. A rhombic projection of the correct dimensions could be generated from a coplanar square tetramer with either

[2] R. E. Barden and M. F. Utter, unpublished observation.

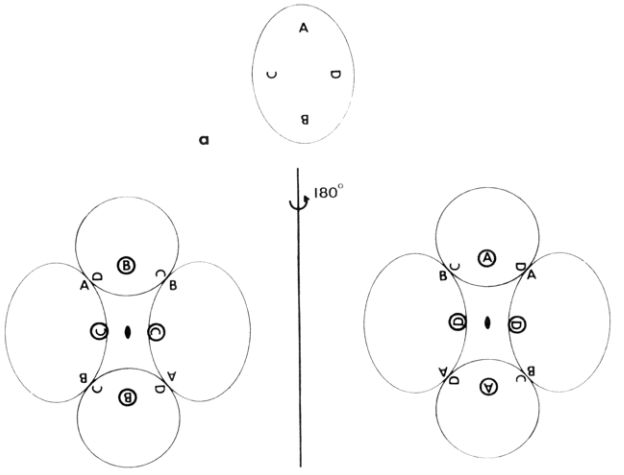

FIGURE 11: Model of yeast pyruvate carboxylase illustrating its binding sites. (a) The protomer of the enzyme has four separate binding sites A, B, C, and D. This model predicts the relative distance between opposing binding pairs designated here as A–B and C–D. For convenience the protomer is shown with all four binding sites on the same face. However, the model does not require that binding domains A and B lie on the same face of the protomer as C and D. (b) These two drawings show the binding sites on the tetramer. The two figures represent two separate views of the same molecule as it is rotated 180° about the vertical axis. The twofold axis of symmetry of this model is reflected by the presence of two sets of bonding pairs A–D and B–C. The circled letters show the open binding sites on each face of the tetramer.

symmetry (Figures 10A and 10B), provided the tetramers were uniformly tilted on the carbon film by some 36° about axis a–a (Figure 10C). The rhombic appearance would result from foreshortening along axis b–b, and if this projection had dimensions of 170 by 150 Å, as was observed, then the square tetramer must have measured 170 by 170 Å. The occasional square tetramers which were observed in our micrographs (Figure 8b) in fact had dimensions of about 150 by 150 Å, indicating that they resulted from a tilted view of the rhomboid rather than the converse situation. These measurements appear to rule out coplanar square structures such as Figures 10A and 10B.

Another D_2 arrangement—the tetrahedron of Figure 10A—has been shown by the computer models of Figures 9A–C to be inconsistent with electron microscope observations, and this is true whether the subunits are spherical or elongated, because of the stain distribution. The same applies to a similar D_2 noncoplanar structure in Figure 10E, which likewise cannot give a rhombic projection whether tilted or not. Yet another D_2 structure, shown in Figure 10F, could resemble the electron micrographs since the distribution of protein is essentially as we have shown in this paper. It closely parallels our model in Figure 6, except that the boundaries between subunits are drawn differently so that the twofold axes a–a and b–b pass *between* subunits, as they necessarily must in a truly D_2 structure. Thus the apparent "subunits" in our micrographs would actually be two half-subunits superimposed, each joined to its other half by a length of unwound polypeptide to create gaps as in Figure 10F. There is no evidence for such complex subunits in our micrographs, but we cannot rule out this type of structure with certainty.

For all these reasons, we propose our model of Figure 6 as the most likely structure of pyruvate carboxylase, even though it is not a D_2 structure and has only one true twofold axis as in Figure 11. An inescapable conclusion from this model is that each protomer contains four different bonding domains, denoted here as A, B, C, and D. This would be true with a rhombic arrangement even if the subunits were spherical. This

prediction of four binding sites would only be necessary, however, if one assumes that all four subunits are identical. If the protomers were similar in size and shape, but not actually identical, then each protomer need have only two binding sites. This present model also requires the tetramer to possess two different types of bonds designated here as A–D and B–C (Figure 11b). It is clear that a major problem with this model is that it allows for the presence of unoccupied or open binding sites. Under these conditions one might predict that the tetramer would polymerize to larger molecular weight forms, a phenomenon which has never been experimentally observed. However, it is conceivable that steric considerations would make such associations unlikely. Also, these arguments are based on the assumption that all of the binding sites on the monomer are actually functional. Instead, it is possible that during enzyme synthesis two of the sites on each protomer are somehow masked or "switched off". At this time we have no evidence along these lines.

Earlier studies from our laboratories carried out in conjunction with the late Dr. Robin Valentine had suggested that animal pyruvate carboxylases were composed of four rounded subunits arranged such that each protomer was located at the corners of a square (Valentine et al., 1966). However, more recent investigations in our laboratories (Cohen et al., 1979) as well as others (Goss et al., 1979) have shown that the animal forms of pyruvate carboxylase have an appearance similar to that of yeast pyruvate carboxylase; i.e., they are rhombic in shape. It is thus possible that the overall quaternary structures of yeast and animal pyruvate carboxylases are similar.

The proposed model for the quaternary structure of yeast pyruvate carboxylase is consistent with the electron microscope data and our present understanding of the subunit composition of this enzyme. To our knowledge no other tetrameric enzyme (other then the animal varieties of this enzyme) has been reported which has this quaternary structure. Further proof of the validity of this model must arise from additional chemical studies of the polypeptides of the enzyme, and from continued electron microscope studies. Specifically, electron microscope studies of the enzyme by using a tilting stage might be particularly useful. With this technique, electron micrographs of the same molecule at several orientations to the electron beam can be obtained. These rotations could then be compared with those predicted by the computer modeling. Studies along these lines are underway in our laboratories.

Acknowledgments

We are grateful to R. Chillingworth for his assistance in the computer studies and to H. Beegen for help in the preparation of this manuscript.

References

Barden, R. E., Taylor, B. L., Isohashi, F., Frey, W. H., II, Zander, G., & Utter, M. F. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4308–4312.

Barrett, A. N., Chillingworth, R. K., Perkins, W. J., & Wrigley, N. G. (1977) *Conf. Ser.—Inst. Phys. No. 36*, 131–134.

Carpenter, F. H., & Harrington, K. T. (1972) *J. Biol. Chem. 247*, 5580–5586.

Cohen, N. D., Beegen, H., Utter, M. F., & Wrigley, N. G. (1979) *J. Biol. Chem. 254*, 1740–1747.

Davies, G. E., & Stark, G. R. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 651–656.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.

Goss, N. H., Dyer, P. Y., Keech, D. B., & Wallace, J. C. (1979) *J. Biol. Chem. 254*, 1734–1739.

Layne, E. (1957) *Methods Enzymol. 3*, 450–454.

MacGillivray, A. J., & Rickwood, D. (1974) *Eur. J. Biochem. 41*, 181–190.

Rylatt, D. B., Keech, D. B., & Wallace, J. C. (1977) *Arch. Biochem. Biophys. 183*, 113–122.

Tolbert, B. (1970) Ph.D. Thesis, Case Western Reserve University.

Utter, M. F., Barden, R. A., & Taylor, B. L. (1972) *Adv. Enzymol. 42*, 1–72.

Valentine, R. C. (1968) *Electron Microsc., Proc. Eur. Reg. Conf., 4th, 2*, 3–10.

Valentine, R. C., Wrigley, N. G., Scrutton, M. C., Irias, J. J., & Utter, M. F. (1966) *Biochemistry 5*, 3111–3116.

Warburg, O., & Christian, W. (1941) *Biochem. Z. 310*, 384–421.

Wrigley, N. G. (1968) *J. Ultrastruct. Res. 24*, 454–464.

Young, M. R., Tolbert, B., & Utter, M. F. (1969) *Methods Enzymol. 13*, 250–257.