Direct Evidence for the Size and Conformational Variability of the Pyruvate Dehydrogenase Complex Revealed by Three-dimensional Electron Microscopy

THE "BREATHING" CORE AND ITS FUNCTIONAL RELATIONSHIP TO PROTEIN DYNAMICS*§

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Structural studies by three-dimensional electron microscopy of the Saccharomyces cerevisiae truncated dihydrolipoamide acetyltransferase (tE2) component of the pyruvate dehydrogenase complex reveal an extraordinary example of protein dynamics. The tE2 forms a 60-subunit core with the morphology of a pentagonal dodecahedron and consists of 20 cone-shaped trimers interconnected by 30 bridges. Frozen-hydrated and stained molecules of tE2 in the same field vary in size ~20%. Analyses of the data show that the size distribution is bell-shaped, and there is an approximately 40-Å difference in the diameter of the smallest and largest structures that corresponds to ~14 Å of variation in the length of the bridge between interconnected trimers. Companion studies of mature E2 show that the complex of the intact subunit exhibits a similar size variation. The x-ray structure of Bacillus stearothermophilus tE2 shows that there is an ~10-Å gap between adjacent trimers and that the trimers are interconnected by the potentially flexible C-terminal ends of two adjacent subunits. We propose that this springlike feature is involved in a thermally driven expansion and contraction of the core and, since it appears to be a common feature in the phylogeny of pyruvate dehydrogenase complexes, protein dynamics is an integral component of the function of these multienzyme complexes.

The pyruvate dehydrogenase complexes (PDCs)¹ are among the largest (Mr ~10⁶ to 10⁷) and most complex multienzyme structures known. A central feature of these complexes is a 24-mer (Escherichia coli) or 60-mer (eukaryotes and some Gram-positive bacteria) dihydrolipoamide acetyltransferase (E2) core with the morphologies of a cube or a pentagonal dodecahedron, respectively (1–4). The cores have both functional and structural roles in organizing the multienzyme complex; the E2 activity is associated with the scaffold to which the other components are attached. These include the pyruvate dehydrogenase (E1) and dihydrolipoamide dehydrogenase (E3), which requires a binding protein (BP) to anchor it to the core of the yeast and mammalian PDCs, although, in E. coli and Bacillus stearothermophilus PDCs, BP is not required (1–4).

The E2 subunits have multidomain structures consisting of one, two, or three amino-terminal lipoyl domains, followed by an E1 and/or E2 binding domain, and a carboxyl-terminal catalytic domain (1–4). X-ray crystallography (5–9) and three-dimensional electron microscopy (10, 11) show that the E2 catalytic domains are arranged in cone-shaped trimers at each of the 8 or 20 vertices of the cubic or dodecahedral structures, respectively (7, 8, 10, 11). The trimers are interconnected by bridges to form an empty cage-like complex with the tip of the trimer directed toward the center of the structure.

Examination of the 4-Å resolution crystal structures of dodecahedral truncated E2 (tE2) cores from Enterococcus faecalis and B. stearothermophilus and the 2-Å resolution crystal structure of a cubic tE2 core from Azotobacter vinelandii show that an anchor residue in the C terminus of the 2-fold related trimers resides in a hydrophobic pocket formed by the adjacent subunits (8). Two such “ball-and-socket” joints on the 2-fold axis maintain the connections between adjacent trimers. It was proposed that the different spatial arrangements of the trimers in the cubic and pentagonal dodecahedron cores are the result of small differences in the nature and position of these interface residues (8).

Our three-dimensional electron microscopy studies of the Saccharomyces cerevisiae tE2 and mature E2 (mE2) reveal flexibility in the arrangement of the E2 cores that is extraordinary for a macromolecular protein complex. Cryoelectron microscopy affords a snapshot of the molecular composition of the preparation at room temperature in the absence of constraints imposed by a crystal lattice with a shutter speed of ~10⁻⁵ s (12), i.e. the time required to reduce the temperature of the specimen from room temperature to approximately ~170 °C, at

¹ The abbreviations used are: PDC, pyruvate dehydrogenase complex; E1, pyruvate dehydrogenase; E2, dihydrolipoamide acetyltransferase; E3, dihydrolipoamide dehydrogenase; E2, truncated dihydrolipoamide acetyltransferase; mE2, mature E2; EM, electron microscopy; BP, binding protein.

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which point molecular motion is frozen. The tE2 and mE2 molecules exhibit ~20% size variability with a bell-shaped distribution that is related to conformational changes in the trimers and the bridges that connect adjacent trimers. Remarkably, the flexibility of tE2 core is also documented by stain electron microscopy. We propose that the expansion/contraction phenomenon is thermally driven and is related to a rotationally flexible ball and socket that forms the bridge-like contacts between adjacent trimers. A cantilever-like feature containing a proline residue may serve to augment and transmit the flexibility of an upstream loop to the ball-and-socket connection of the bridge. Sixty of these springlike features, two at each of the 30 bridges, are a common motif of the dodecahedral cores of \textit{B. stearothermophilus}, \textit{E. faecalis}, and presumably \textit{S. cerevisiae} and the human pyruvate dehydrogenase complexes. The “breathing” of the core structures affects the disposition of the bound constituents and may be an important component of the function of these multienzyme complexes by promoting the shuttling of the intermediates of catalysis associated with the lipoamide arm to the numerous reaction centers and by augmenting the catalysis through the movement of amino acid residues at the catalytic sites.

**EXPERIMENTAL PROCEDURES**

**Enzyme Preparations**—The \textit{S. cerevisiae} tE2 subunit, comprising either residues 206–454 or 181–454, was overexpressed in \textit{E. coli}, and the assembled cores were purified to near homogeneity as described (13, 14). The expression vector for mE2 was pYES m-32TX. The protein has 44 extra amino acid residues at the N terminus, including a His6 tag and the enterokinase cleavage site. Both tE2 and mE2 exhibited catalytic activity (acetyl transfer from [1-14C]acetyl-CoA to dihydrolipoamide) similar to wild-type E2.

**Electron Microscopy**—The tE2 preparation was diluted to 25 µg/ml in 0.25% methylamine tungstate stain containing 10 µg/ml bacitracin and immediately sprayed onto Butvar 76 film with a carbon backing to minimize shrinkage and to obtain randomly oriented molecules (15, 16). Application of the specimen to the Butvar film alone resulted in molecules that were smaller than their frozen-hydrated counterpart (10). Their application to a carbon film gave molecules primarily oriented with their pentagonal face bound to the carbon film and, consequently, were not useful for image processing. The images of the stained molecules were recorded at 0.2–0.4 µm under focus at a nominal magnification of × 50,000 under conventional irradiation procedures using a JEOL JEM 1200 electron microscope operated at 100 kV. For cryoelectron microscopy, 3 µl of the tE2 and mE2 preparations at 0.1 and 0.4 mM, respectively, were deposited, blotted, and quick-frozen in liquid ethane using a high pressure freezer (80,000 psi) with a carbon-coated holey grid. The vitrified specimens were recorded at 2–4 µm under focus with a dose of ~9 e⁻/Å² at ×50,000 magnification. Micrographs were digitized with a Zeiss SCAI microdensitometer (Carl Zeiss, Inc., Englewood, CO) using a step size of 2.8 Å/pixel at the specimen scale.

**Data Processing**—All data processing steps were carried out on SGI Octane dual processor workstations (Silicon Graphics, Inc.). First, individual tE2 and mE2 particles were boxed out manually using an x-window-based program Emtool (17). Subsequently, data processing steps included particle size variation determination and three-dimensional reconstruction of particles in various size groups, using our programs based on the principles of Fourier common lines (18, 19). The orientation determination and three-dimensional reconstruction were carried out using parallel programs for refinement (20) and for refinement of the match between the image and the projections computed from a three-dimensional model. The three-dimensional model was constructed from these refinements was used as a template for the next round of particle selection and refinement, resulting in a further improved model. This process was iterated for two cycles utilizing the entire data set until no more particles with correct orientation parameters could be obtained and no improvement was evident in the cross common line phase residual images and the computed projections.

The final reconstruction was calculated by merging data to 25-Å resolution (for tE2) or 30-Å resolution (for mE2) from particle images with defocus values around 2–3 µm, which were determined from the incoherently averaged Fourier transforms of particle images in each micrograph (23). The final reconstructions were corrected for the contrast transfer function of the microscope (24).

**Assessment of the Particle Size Variation**—We have developed procedures to analyze the size variability of the molecules. The relative size of the tE2 and mE2 molecules were determined using the program sizeDiff (David Zuckerman and Z. H. Zhou), which is a Unix and Windows NT/2000 program written in C++ based on Fourier common lines. SizeDiff uses an iterative method that minimizes the Fourier common line phase residual between the particle and a model template derived from the data set as described below. After the orientation and center parameters of each particle were determined and the structure was refined to ~30-Å resolution (see above), a preliminary three-dimensional reconstruction was calculated by combining all of the refined particles with a phase residual of less than 40°. Approximately 10–15% of the particles were eliminated in this step, and these were visually investigated in order to avoid discarding those particles that may have greater phase residual values because of a large size variation. However, the subset of discarded particles was found to be of poor quality. This “average” reconstruction was then used as the template for the first round of size determination. Initially, 20 projection images were generated at regularly spaced orientation intervals, and these were used as the template set. Each particle image was then isotropically scaled to best match the template projection by minimizing the averaged cross common line phase residual between the particle and the corresponding projection. The isotropic scaling does not accurately reflect the size variation of the single particle images, because the size variability of the molecule is not isotropic (see “Results”) and, consequently, may introduce errors in their alignment, especially when the discrepancy between the particle and the model is large. After obtaining the structure representative of the 1.0 size groups, it was used as a model after scaling for the 0.95 and 1.05 size groups, and these structures were finally used as models after scaling to align the 0.9 and 1.10 size groups, respectively. This bootstrap approach was required for the appropriate alignment of smallest and largest images. Subsequently, the models obtained from the alignment were refined as described below.

Further classification of the particles and the refinement of the reconstruction were accomplished by utilizing the particles ±1% of the designated size. After repeating a second cycle of size variation analysis using sizeDiff, ~80% of the particles remained in this 2% size bin. After eliminating outliers, the remaining particles were merged to generate a reconstruction to be used as the template in a third cycle of analysis. Over 96% of the particles were in this 2% size bin, thus demonstrating the convergence of the data set and that the final reconstruction had optimal resolution. The convergence of the data set and the optimiza-

**Three-dimensional Visualization and Comparison with Atomic Structure**—The atomic coordinates of the crystal structure of the dodecahedral form of \textit{B. stearothermophilus} tE2 (PDB identification number 1BSS) was also analyzed by the polar Fourier transform method (26, 27), and the size distribution was similar to that shown in Fig. 1 (data not shown). The three-dimensional visualization and comparison with atomic structure was carried out using the Iris Explorer (NAG, Inc., Downers Grove, IL), a software program designed for scientific visualization and comparison with atomic structure. The atomic coordinates of the crystal structure of the dodecahedral form of \textit{B. stearothermophilus} tE2 (PDB identification number 1BSS) were superimposed on the electron density map. The resulting image was then manipulated using the Iris Explorer software. The final image was then exported as a greyscale image and printed on a high-resolution inkjet printer.
RESULTS

Size Variation of the tE2 Molecules—This study reveals an ~20% size variation of the tE2 images (Fig. 1). This extraordinary size variation of a macromolecular complex raised considerable skepticism concerning the significance of these data and their interpretation. Indeed, our original observation of a size variation of tE2 and its subcomplexes was ascribed to magnification variations of the electron microscope, and consequently, only those particles with sizes that were within 3% of the average were used in the reconstructions (11). As a result, the structures of tE2, tE2.BP, tE2.BP, and tE2.BP.E3 were representative of 10–15% of the data sets (11). Below, we examine and discount three trivial possibilities for the size variation, which, if not discounted, could muddle the interpretation of the findings of this study.

Magnification Variation of the Electron Microscope—Electron microscopy studies of icosahedral virus capsids reveal a maximum variation of ~1% in their sizes in a given field. This variation in size was attributed to electron microscope magnification variation, although the possibility that this size variability was related to the molecules was not ruled out (31). In any event, a 20% size distribution of the images in the same field recorded in stain (10) and vitreous ice (10) (Fig. 2) in the present study is well outside the range of variation normally attributed to an electron microscope. Moreover, during the recording period of this study, numerous other macromolecular images were recorded without any noticeable size disparity. Therefore, we conclude that the large size variability of these images is not related to the electron microscope.

Distortion of the Molecules—A more plausible explanation for the size difference is that these flexible (“soft”) particles may be easily squashed to a variable degree between the two air-water interfaces in vitreous ice or that the particles may collapse in the stain. We have ruled out both possibilities. A tilt experiment of the stained molecules shows that they interconvert upon tilting the stage 33° (10). For example, a round particle with a larger profile that was not imbedded in stain was found to have an oval shape upon tilting the stage, demonstrating that the tilt experiment is a sensitive method of detecting molecules that are not spherical (10). Similarly, a tilt experiment employing vitreous ice did not reveal any particles that were flattened (Fig. 2). Furthermore, analysis of the size distribution of the particles in the same field showed that the small and larger particles are co-mingled and are not segre-
The center of the molecule.

The trimers along their 3-fold axes from related to a variation of the distance of B features (Fig. 1). The superimposed molecule across the bell-shaped distribution curve (Fig. 1). The superimposed reconstructions are rendered at the same threshold (contour level). The structures in A show that there is a continuous size change of the molecule across the bell-shaped distribution (Fig. 1). The superimposed semitransparent and wire frame structures (B) show that the size change is related to a variation of the distance of the trimers along their 3-fold axes from the center of the molecule.

Fig. 3. A, 2-fold views of the shaded surface representations of the intact and cut-away reconstructions from the images of the frozen-hydrated molecules of the selected size groups; B, the overlay structures representative of the 0.90 (yellow) and 1.10 (light blue) size groups. The structures were computed from the images corresponding to the size groups shown in Fig. 1. A, the circle surrounding the structures has a diameter corresponding to the 1.10 structure and is shown as an aid in determining the relative size of the structures. The reconstructions are rendered at the same threshold (contour level). The structures in A show that there is a continuous size change of the molecule across the bell-shaped distribution curve (Fig. 1). The superimposed semitransparent and wire frame structures (B) show that the size change is related to a variation of the distance of the trimers along their 3-fold axes from the center of the molecule.

gated (10). Finally, since a size increase due to flattening of the particle would severely disrupt the icosahedral symmetry of the molecule, the reconstruction representative of the largest particles should have the poorest resolution or not be amenable to the icosahedral reconstruction methods. In this regard, a sensitive gauge of the accuracy of the alignment of the particle images to their template is obtained by a comparison of the phase residual values. The average phase residuals for the 0.90, 0.95, 1.0, 1.05, and 1.1 size groups of the stain data (Fig. 1) are 19.8°, 17.1°, 18.0°, 18.7°, and 19.7°, respectively. This exceptionally narrow range and their low numerical values are strong evidence that the icosahedral symmetry of the molecules selected for the reconstructions has been well preserved over the 0.9–1.1 size variation. Moreover, the resolution values ~20–25 Å are comparable for all reconstructions, and most significantly, the larger reconstruction agrees very well with the comparable x-ray structure of B. stearothermophilus tE2 filtered to the resolution of the electron microscope structure (see below).

Incomplete Structures—The electron microscope fields show that there are a significant number of particles that lack the full complement of 20 trimers in stain and ice (see Ref. 10 and Fig. 2C). The E2 components of the α-keto acid dehydrogenase family of multienzyme complexes are known to form tightly associated trimers that are held together by weaker connections to form the cagelike scaffold (7–9). The connections between trimers involve C-terminal residue(s) of adjacent subunits. In this regard, the truncated E2 of the dihydrolipoamide succinyltransferase from E. coli is normally a 24-mer, but it formed stable trimers when expressed with a C-terminal His6 tag and failed to associate further (32). These broken and/or incompletely formed cages are probably related to the tenuous connections between trimers, and the incomplete structures are lacking, to a variable extent, some of the trimer building blocks. Although we can readily visualize fragments of the core (Fig. 2C) and avoid these images in the reconstruction, structures that lack a small number of trimers may not be possible to discern. Hence, it is possible that the size variation of the images is related to incomplete molecules with variable number of trimers. In this scenario, only the images near the 1.1 size classification are representative of complete structures, and consequently, over 90% of the data set consists of molecules with variable trimer content (incomplete structures, Fig. 1).

A priori, these incomplete cages may be expected to have the same radius of curvature and thus the same size of the complete structure, since the nature of the interaction between adjacent trimers determines the dodecahedral shape of the molecule (8). Shown in Fig. 2C are some images that are representative of incomplete structures based on visual inspection. Interestingly, the missing portion of the molecule does not appear to affect the radius of curvature of the remaining complex, since the size of the images is comparable with those in Fig. 2, A and B. Accordingly, any structures lacking a small and undetectable number of trimers that got included would not be expected to affect the size of the reconstructions.

In any event, we have determined the relationship between the size and mass of the structures by comparing the relative masses of reconstructions (which were normalized with respect to the number of particles in each set) from images in the 0.95 and 1.05 size groups recorded from one micrograph of frozen-hydrated molecules. Although this procedure restricts the number of particles in the reconstructions, it offers a more meaningful comparison of the relative masses, since such variables as defocus, exposure, ice thickness, and contrast are minimized or eliminated. It should be noted that the 10% difference in the diameter of the particle corresponds to over a 30% increase in the volume of the particle, which may be related to a 30% variation in its mass. A threshold of 2.40 S.D. of the reconstruction from the 1.05 size group gives a molecular volume that corresponds to the 1.6 × 10^6 M_r of the 60-subunit tE2. The same threshold applied to the reconstruction from the
0.95 size group (34% smaller structure) also gave a molecular volume that corresponds to \(1.6 \times 10^6\) M_r. This argument is further supported by a comparison of the structures representative of the 0.9 and 1.1 size groups, which vary in diameter 17% (see below). Again, if this size variation is related to a difference in their trimer composition, the smallest structure should have about eight fewer trimers than the 1.1 size group structure. We believe that images representative of such a disrupted structure could be detected by visual inspection (Fig. 2C). Moreover, since the icosahedral symmetry of these complexes would be significantly perturbed, the resolution of the smaller structures should be poorer than that of the larger structures. It is reassuring that the resolution of the reconstructions are comparable and independent of their sizes (see below). Accordingly, we conclude that the particles in these data sets have similar subunit composition, and therefore the size variation of the particle images seen in Fig. 1 is independent of any small subunit variation in the particles.

Comparisons of the Three-dimensional Structures of Truncated E2—We have previously proposed that cryoelectron microscopy affords a reliable means of determining the size variability and the molecular architecture of macromolecules at room temperature, since the specimen is cooled rapidly to a temperature that freezes their flexibility. The good agreement between the frozen-hydrated and stain (recorded at room temperature) data sets of the size variation and their single particle images (Fig. 1) and their reconstructions (Figs. 2 and 3) strongly support this proposal.

The images of frozen-hydrated and stained molecules (Fig. 1B) and their reconstructions (Figs. 3 and 4) clearly show the continuous size variability across the distribution curve, and the intact and cut-away structures demonstrate that they...
Analyses of frozen-hydrated recombinant mE₂ molecules. A, the size variation of the data (331 images); B, radial density plot of the reconstruction representative of the 1.0 size group; C, shaded surface view of the reconstructions representative of the size groups indicated; D, superimposed reconstructions of the largest (green) and smallest (red, semitransparent) reconstruction and its cut-away inside view. The resolution of these reconstructions between 30 and 34 Å is ~10 Å different from those from the tE₂ data set because of the fewer particles used in the E₂ data set. The E₂ images exhibit a similar bell-shaped size distribution of the tE₂ (Fig. 1), and their reconstructions also show a continual variation in size of the structure (B) across the size distribution. The radial density plot of the 1.0 structure shows a significant peak of density inside the core that is attributed to the insertion of the flexible N-terminal lipoic domains, presumably through the pentagonal opening of the core, since the tE₂ reconstruction lacks this peak (11). The shaded surface views show that the largest (1.05) structure accommodates more of the N-terminal domain presumably because of the increased size of its internal cavity. Its radial density plot shows that the area under the internal peak is more than double that corresponding to the 0.95 structure (data not shown). The superimposed structures (D) support the previous proposal that the expansion of the core is related to an increase in the distance of the trimers on their 3-fold axes to the center of the molecule.
maintain a very similar dodecahedral architecture with its 20 triangle-based trimers at its corners interconnected by 30 bridges (Figs. 3 and 4).

The architecture of the reconstructions from the ice and stained molecules of corresponding size are very similar, as are their size variations (Figs. 3 and 4). The ice and stain reconstructions exhibit a diameter variation of 17% (232–272 Å) and their size variations (Figs. 3 and 4). The ice and stain reconstructions were obtained by converting the atomic x-ray coordinates to electron densities at 25 Å using a Gaussian filter (30). A, the diameter of the molecule measured between the outside edge of opposing trimers in the 2-fold orientation is ~256 Å. The ribbon representation of the bridge on its 2-fold axis shows that there is an ~10-Å gap between adjacent trimers and that the trimers are interconnected by two C-terminal extensions of the polypeptide chain from the opposing subunits (red and blue) that form the bridge. The companion image shows the orthogonal view of the bridge as denoted by the arrow. B, the 3-fold view of the trimers in the ribbon presentation shows the extensive interactions of the three identical subunits, denoted in different colors, which comprise the building blocks of the complex. Its orthogonal view denoted by the arrow further delineates the subunit-subunit interactions in the trimer. The similarity between the slices from the largest S. cerevisiae structure (Fig. 5) and the x-ray structure suggests that the x-ray structure is more representative of the largest EM structure that also has a pronounced gap between adjacent trimers.

The overlay comparison of the smallest and largest structures computed from ice (Fig. 3) and stain (Fig. 4) data provides clues as to the manner by which their sizes are related. The outer surface of the largest structure encompasses all of the surface of the smaller one, whereas both of the cut-away inside views exhibit only the inner surface of the smallest structure. In other words, the largest structure is on the outside of the overlays, whereas the smallest structure is on its inside. Consequently, the largest and the smallest structures are related by a change in the orientation and separation of the trimers (see below) in contrast to a global (isotropic) swelling of the trimers, which would result in the largest structure totally encompassing the smallest one on both its inside and outside surfaces. Recall that the smallest and the largest structures have the same mass, further supporting the proposed relationship between the sizes of these structures.

The size variability of the core is related to conformational changes in the trimers and their interconnecting bridges (Fig. 5). The views of 1-pixel-thick slices along the 2-fold axis from the center of the core show small variations in the protein density distribution and a significant increase in the indentation between adjacent trimers that is part of the outside surface of the bridge structure. The views of slices down the 3-fold axis also indicate that the protein density shifts somewhat from the corners of the triangular shaped image toward its center. In both cases, the protein density distribution in the 1.1 structure appears to best match that seen in the x-ray structure.

The 20–25-Å resolution of the reconstructions would appear to prohibit a visual detection of the change in the separation of the trimers, since the 0.90 and 1.10 structures differ in diameter by ~40 Å, corresponding to a 14-Å change in the trimer separation. This is calculated based on the geometric relationship of the diameter of a dodecahedron and its bridges. Nonetheless, the visualization of features smaller than the resolution of the reconstruction has been documented for numerous three-dimensional EM structures (33, 34). A comparison of the solid-shaded structures (Figs. 3B and 4B) and their 2-fold slices (Fig. 5) shows a significant change in the separation of adjacent trimers.

Recombinant mE$_2$ Structures—We have chosen to study extensively the structural variation of the tE$_2$ scaffold because the flexible N-terminal half of mE$_2$ may result in three-dimensional reconstructions that are more difficult to interpret. Also, the x-ray structure of B. stearothermophilus tE$_2$ more closely corresponds to the structure of S. cerevisiae tE$_2$. Even so, the relevance of the size variation of the tE$_2$ to the wild-type E$_2$ may be questioned, since nearly half of the protein of the E$_2$ subunit is lacking. Accordingly, we have investigated size variation of intact, recombinant mE$_2$, utilizing frozen-hydrated molecules.

**Fig. 7.** Two- and 3-fold views of the three-dimensional structures of B. stearothermophilus tE$_2$. The shaded surface representations were obtained by converting the atomic x-ray coordinates to electron densities at 25 Å using a Gaussian filter (30). A. The diameter of the molecule measured between the outside edge of opposing trimers in the 2-fold orientation is ~256 Å. The ribbon representation of the bridge on its 2-fold axis shows that there is an ~10-Å gap between adjacent trimers and that the trimers are interconnected by two C-terminal extensions of the polypeptide chain from the opposing subunits (red and blue) that form the bridge. The companion image shows the orthogonal view of the bridge as denoted by the arrow. B, the 3-fold view of the trimers in the ribbon presentation shows the extensive interactions of the three identical subunits, denoted in different colors, which comprise the building blocks of the complex. Its orthogonal view denoted by the arrow further delineates the subunit-subunit interactions in the trimer.

**Fig. 8.** Ribbon diagram of the residues from the C-terminal region of the x-ray structure of B. stearothermophilus tE$_2$ that comprise the putative spring that interconnects adjacent trimers. A loop region comprising residues 397–403 is anchored to a β-sheet. This is followed by a four-turn α-helix beginning with residue 403 and disrupted by a proline (residue 420) that directs residues 421–425 to the subunit of the adjacent trimer. The C-terminal Met (residue 425) resides in a hydrophobic pocket (HP) of the adjacent subunit to form the ball-and-socket connection. Residues 403–425 form a cantilever-like structure, which is attached to the loop at its N-terminal end. We propose that the assembly of residues 397–425 forms the springlike connection between adjacent trimers that can adjust to account for the size variability of the core.
A plot of the number of mE2 particles versus relative size gives the similar bell-shaped curve associated with the tE2 data sets, except the plot may reflect a skewed distribution of particle sizes, possibly as a consequence of the internalization of the N-terminal domains of mE2 (see below) (cf. Fig. 6A with Fig. 1A). The 0.95, 1.0, and 1.05 solid-shaded structures of mE2 are similar to the corresponding tE2 structures (cf. Fig. 6 with Fig. 3). This finding affords structural validation of numerous biochemical studies (1–3, 35) that showed that the N-terminal half of intact E2 is composed of flexible domains and, as a consequence, is not revealed in the images of the mE2 reconstructions.

The overlay structures of the 0.95 and 1.05 mE2 reconstructions are entirely consistent with those corresponding to tE2 (cf. Fig. 6D with Figs. 3 and 4B) and show that the size variations are similar. The good agreement between these overlay structures is further validation that the size variation of the core is related to the variable distance between adjacent trimers. We were unable to compare structures representative of the 0.90 and 1.1 data sets because of the more limited number of images in the data set. However, we have analyzed about 3000 particle images of recombinant human E2. SizeDiff gave a size distribution profile very similar to that of tE2 (Fig. 1),3 further supporting the proposal that the size variability is a fundamental property of the C-terminal domain of the E2 subunit.

However, there is a difference between the 1.05 size group tE2 and mE2 structures in that the latter displays significant density inside the scaffold not seen in the former (cf. Figs. 3A and 6C). A comparison of the E2 0.95, 1.0, and 1.05 structures indicates that the larger scaffold accommodates more internal density. This result is confirmed by a comparison of the radial density plots (data not show). The observation that tE2 lacks this internal density (Figs. 3A and 4A) (11) shows that some of the 60 N-terminal lypoyl domains of E2 reside inside the scaffold and the larger scaffold may accommodate more of this portion of the subunit. In contrast, the outside of the tE2 and mE2 cores are similar (Figs. 3, 4, and 6), indicating that the N-terminal domains that reside outside of the core are not restricted and, hence, do not appear in the reconstruction. The internalization of the N-terminal flexible domain also occurs in the cubic core of the branched-chain α-keto acid dehydrogenase complex as determined by dark field electron microscopy studies (36). Thus, the large openings in these E2 scaffolds and their empty interiors readily accommodate a portion of their highly flexible N-terminal domains.

DISCUSSION

Visualization of Protein Dynamics by Electron Microscopy—That proteins exhibit conformational flexibility has been appreciated for many years. More recently, this subject has gained in interest because of its significance to the function of macromolecular complexes (37, 38). For example, recent reports show that the dynamics of enzymes in regions that are removed from the catalytic site may be crucial to the enzyme’s activity (37). Moreover, proteolysis studies of the γ-peptides inside flock house viral capsid show that they are in equilibrium with the capsid’s surface, and their dynamic domains may contribute to the initiation of RNA release and transllocation (39). Small angle x-ray scattering studies of the electron-transferring flavoprotein from Methylophilus methylotrophus indicate that the protein samples a range of conformations in solutions and, consequently, renders its structure complementary to its putative binding site on the trimethylamine dehydrogenase (40).

Three-dimensional electron microscopy is well suited to the study of the details of protein dynamics. The pH-sensitive size changes of cowpea chlorotic mottle virus (41) and HK97 virus (42) were documented by cryoelectron microscopy. Particle classification protocols made possible the identification of structural intermediates in the conversion pathway and, therefore, further insight into dynamics of the process (41, 42). In the present study, our sizeDiff program sorts particles based on their relative size (Figs. 1 and 6A) and subsequently documents structural (Figs. 3 and 4) and conformational (Fig. 5) changes that are related to the size variation of the structures.

Mechanisms for the Size Variability of the E2 Cores—Recall that the overlay of the smallest and largest structures shows that the surface of the larger structures completely resides on the outside, whereas the surface of the smaller structures is seen to predominate on the inside of the core (Figs. 3B, 4B, and 6D). Moreover, the morphology of the trimer building blocks and their interconnecting bridges appear independent of the size of the structure. Thus, we conclude that the size variability of the dodecahedral scaffold appears to be primarily related to a change in the variable distance of the trimers on their 3-fold axes to the center of the molecule. Consequently, the distance between adjacent trimers is variable.

The structure representative of the 1.0 size group in the bell-shaped distribution curve should be energetically most favorable (Fig. 1). We propose that the size change of the molecule involves a significant contribution from a synchro-
ous change in the length of the bridges with their springlike connections. A completely asynchronous or random change in bridge length would produce molecules of the same size, and consequently, the 20% size variation observed in this study would not be detected. The harmonious change in the length of the bridges is energetically more favorable than the asynchronous alternative, because the latter disrupts the symmetry of the molecule at an energy cost. Comparisons of comparable slices of the reconstruction representative of the different size groups show that the scaffold expansion and contraction is coupled to changes in the protein density distribution in the trimers and their bridges (Fig. 5). The different extent of the demarcation between adjacent trimers appears to be the most significant conformational change associated with the size variation.

The x-ray structure of B. stearothermophilus tE2 offers a plausible explanation for the flexible springlike connectivity between adjacent trimers (Fig. 7). Although the subunits of the trimers exhibit extensive connections between them (Fig. 7B), adjacent trimers are only held together by interactions of the C-terminal methionine of one subunit with a hydrophobic pocket afforded by the adjacent subunit (Fig. 7A) (8). This “ball-and-socket” connection would be expected to afford trimer-trimer interaction with rotational flexibility. The x-ray structure also suggests a springlike connection between adjacent trimers consisting of a C-terminal loop region (residues 397–402) that is anchored to a b sheet followed by an a-helix (residues 403–419) (Fig. 8). Proline 420 at the C-terminal end of the rod-shaped a-helix introduces an elbow bend that directs the C-terminal methionine (residue 425) into the hydrophobic pocket of the subunit of the adjacent trimer. The C-terminal residues (421–425) following the proline are part of the bridge-like feature of the complex. The a-helix/proline/C-terminal residues produce a cantilever-like structure that may serve to transmit and contribute to the movement of the flexible loop region. This study shows that the bridge length varies ~14 Å, which corresponds to an approximate deviation of ±7 Å in the length of the bridge from its position in the energetically most stable structure represented by the 1.0 size group.

Generality of the Putative Flexible Bridge—We believe that E2 cores are exceptional examples of what has been designated “soft protein” (38). The x-ray structure of B. stearothermophilus tE2 shows that adjacent trimers are joined through two hydrophobic contacts between adjacent subunits (Figs. 7 and 8). The association energy due to hydrophobic interactions is similar to thermal energy at room temperature (38).

A common structural feature of the E2s is the trimer building blocks that reside at the 8 or 20 vertices of a cubic or dodecahedral structure, respectively. The A. vinelandii truncated E2 cubic core (7) also shares the ball-and-socket connection between adjacent trimers found in the E. faecalis and B. stearothermophilus dodecahedral E2s (8) and, presumably, the S. cerevisiae and human E2s. Although the cubic tE2 from A. vinelandii does not have the proline residue near the C-terminal connection found in the subunit of the dodecahedral cores, it readily dissociates into trimers upon binding E1 or E3 components. This indicates that the interaction between trimers is weak (43), which is confirmed by the x-ray structure (7). In contrast, E. coli truncated dihydrolipoamide succinyltransferase, which shares the cubic core arrangement with the A. vinelandii truncated acetyltransferase and its ball-and-socket connection, exhibits additional interactions between adjacent trimers that add significant stability to this complex (9). Interestingly, the C-terminal region of the succinyltransferase has all of the features of the springlike connection of the acetyltransferase (i.e. loop/cantilever with its associated proline) (9).

It is not known if the cubic 24-mer E2s exhibit the breathing core flexibility to the extent of the dodecahedral E2s. However, they both have a similar gap size between adjacent trimers that appears to accommodate the size variability of the yeast and human E2 cores. It is noteworthy that the B. stearothermophilus, E. faecalis, S. cerevisiae, and human dodecahedral E2s have a proline moiety 5–8 residues from their C termini, and this residue is often associated with a flexible region in polypeptide chains (44). Furthermore, sequence analyses using ClustalW (45) show a 49% sequence homology between these truncated E2s but a 55% homology between the last 29 residues of their C termini, indicating that they all share the loop region, cantilever, and ball-and-socket feature seen in the x-ray structure of tE2 from B. stearothermophilus and E. faecalis and thus similar flexibility.

Functional Consequences of the Protein Dynamics of the E2 Cores—The wild-type S. cerevisiae PDC comprises the 60-subunit E2 core, with BP, E3, and E1 attached. Our initial analyses of the S. cerevisiae PDC, its subcomplexes, and the recombinant tE2 subcomplexes indicate that the disposition and extent of binding of the constituents is related to the size of the core (data not shown). It is apparent then from these observations and the present study that the structural organization of PDC cannot be completely represented by a single reconstruction and that multiple reconstructions are required to gain an appreciation of the influence of protein dynamics on its architecture, organization, and function. In this regard, the E2 core of the wild-type PDC (Fig. 9B) also exhibits size variability. Thus, the complete complex exhibits a similar flexibility to that of the tE2 and mE2. Most interesting, the E1 on the outside of the core mimics the expansion/contraction of the underlying core (Fig. 9). The aforementioned conservation of the sequence homology of the dodecahedral cores of the S. cerevisiae, human, E. faecalis, and B. stearothermophilus through all phylogeny indicates that the flexibility associated with the yeast PDC has an important role in its function. We propose that the breathing core augments the movements of the lipoamide swinging arms between the catalytic centers and that the apparent movement of the entire complex may augment substrate channeling and promote catalysis by methods that are just beginning to be understood (46, 47). The rapidity of the expansion and contraction of PDC is unknown but may be investigated by dynamic light scattering and atomic force microscopy.

Although the PDC is an example without precedence of thermally induced global protein dynamics, this phenomenon may be more generally applicable than presently appreciated. For example, the photosynthetic reaction center of the bacterium Rhodobacter sphaeroides appears to be augmented by the thermal motion of the protein matrix (46, 48). Moreover, the studies of the flock house viral capsid indicate that its complex exhibits global protein dynamics that are also thermally driven (39). The present study shows that single particle analysis by three-dimensional electron microscopy is an appropriate and powerful method of examining protein dynamics, and as this technology achieves higher resolution structures, the generality of this phenomenon and its details may emerge. If so, the complexity of three-dimensional EM analyses will increase significantly. Nevertheless, these studies should significantly enhance the understanding of the relationship of protein dynamics to the structure and function of macromolecular complexes.

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