The subunits of the dihydrolipoyl acetyltransferase (E2) component of mammalian pyruvate dehydrogenase complex can form a 60-mer via association of the C-terminal I domain of E2 at the vertices of a dodecahedron. Exterior to this inner core structure, E2 has a pyruvate dehydrogenase component (E1)-binding domain followed by two lipoyl domains, all connected by mobile linker regions. The assembled core structure of mammalian pyruvate dehydrogenase complex also includes the dihydrolipoyl dehydrogenase (E3)-binding protein (E3BP) that binds the I domain of E2 by its C-terminal Y domain. E3BP similarly has linker regions connecting an E3-binding domain and a lipoyl domain. The composition of E2-E3BP was thought to be 60 E2 plus ~12 E3BP. We have prepared homogenous human components. E2 and E2-E3BP have $\theta_{20,W}$ values of 36 S and 31.8 S, respectively. Equilibrium sedimentation and small angle x-ray scattering studies indicate that E2-E3BP has lower total mass than E2, and small angle x-ray scattering showed that E3 binds to E2-E3BP outside the central dodecahedron. In the presence of saturating levels of E1, E2 bound ~60 E1 and maximally sedimented 64.4 ± 1.5 S faster than E2, whereas E1-saturated E2-E3BP maximally sedimented 49.5 ± 1.4 S faster than E2-E3BP. Based on the impact on sedimentation rates by bound E1, we estimate fewer E1 (~12) were bound by E2-E3BP than by E2. The findings of a smaller E2-E3BP mass and a lower capacity to bind E1 support the smaller E3BP substituting for E2 subunits rather than adding to the 60-mer. We describe a substitution model in which 12 Y domains of E3BP replace 12 I domains of E2 by forming 6 dimer edges that are symmetrically located in the dodecahedron structure. Twelve E3 dimers were bound per E2$_{60}$-E3BP$_{12}$ mass, which is consistent with this model.

The mitochondrial pyruvate dehydrogenase complex (PDC) catalyzes the irreversible conversion of pyruvate to acetyl-CoA along with the reduction of NAD$^+$. PDCs from all known sources contain the pyruvate dehydrogenase (E1), the dihydrolipoyl acetyltransferase (E2), and the dihydrolipoyl dehydrogenase (E3) components. Mammalian PDC has a highly organized structure in which the E2 component plays a central role in the organization, integrated chemical reactions, and regulation of the complex (1–5). Besides those universal components, the mammalian and subsequently some other eukaryotic PDCs were shown to contain another component, called E3-binding protein (E3BP); this protein was originally designated protein X (6, 7). Mammalian E3BP was first characterized as a component with a reactive lipoyl group on a single lipoyl domain that, alone, could support the overall reaction (12). E3BP was tightly bound to E2 (7) by its C-terminal region (10). The E3BP component was then shown to contribute to the organization of the complex by binding the E3 component (13–18). This work provides new insights into the integration of E3BP into the central framework of the mammalian complex.

Although first characterized in the bovine complex, the most thoroughly characterized E3BP is that associated with the yeast PDC (19–23). Both the yeast (19, 20, 22) and mammalian E3BP (2, 7, 10, 13, 18) consist of three globular domains connected by two linker (or hinge) regions (Fig. 1). At the N terminus is a lipoyl domain (designated L3) which is connected by linker region to the E3-binding domain (B’) which, in turn, is connected to a C-terminal domain (I’). The E2 component of the mammalian complex has a similar structure with two lipoyl domains (L1 and L2), an E1-binding domain (B), and a C-terminal inner (I) domain (Fig. 1) (1–4, 12, 13, 24–26). With E2 alone, the I domain assembles via 20 trimers (27) connecting as the corners of a pentagonal dodecahedron (28–31).

The C-terminal inner (I’) domain of E3BP associates with this inner core dodecahedron formed by the I domain of E2s (10, 20, 22). In the yeast PDC, convincing evidence has been presented that the E3BP component is bound by its I domain binding inside the dodecahedron formed completely by the I domain of E2 (21–23). The free yeast E3BP could be added to the E2 60-mer and was bound in this manner. A 60/12 addition

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\( \sum \) The on-line version of this article (available at http://www.jbc.org) contains additional text, Figs. 1S and 2S, and Refs. 1–9.

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\[ \text{The abbreviations used are: PDC, pyruvate dehydrogenase complex; E1, pyruvate dehydrogenase component; E2, dihydrolipoyl acetyltransferase component; L1 domain, NH$_2$-lipoyl domain of E2; L2 domain, interior lipoyl domain of E2; B, binding domain in E2 for E1; H1, H2, and H3 are the linker (hinge) regions of E2 (Fig. 1A); scE2, E2 with PreScission protease site introduced in H3 domain of E2; t-E2, truncated form of E2 with I domain and small part of H3 linker region; E3, dihydrolipoyl dehydrogenase; E3BP, E3-binding protein; L3, lipoyl domain of E3BP; B’, binding domain of E3BP that binds E3; I’ domain, inner domain of E3BP; H1’ and H2’ linker (hinge) regions in E3BP (Fig. 1A); AUC, analytical ultracentrifugation; SAXS, small angle x-ray scattering; $R_e$, $R_z$, and $R_d$ are, respectively, the unhydrated spherical Stokes radius, Stokes radius, radius of gyration, and particle excluded volume radius.} \]
model has been proposed for mammalian E2-E3BP (16). In part, this model was favored based on the results with the yeast system, and the fact that the smallest symmetry element in the dodecahedron has 12 pentagonal faces. In contrast to the yeast system, resolved bovine E3BP, which retained the capacity to bind E3 and had a functional lipoyl domain, failed to bind back to assembled E2 under non-chaotropic conditions or after a rapid transition to less chaotropic conditions (8, 14, 27, 32, 33) (see “Discussion”).

The sequence alignments of the human and yeast E2 and E3BP components (see Supplemental Material Fig. 1S) show that mammalian E2-E3BP is closely related to mammalian E2 (18). The alignment shows that both lipoyl domains in human E2 are equally related to the L3 domain of human E3BP, that there is little basis for aligning linker regions, and that the linker regions are very different in yeast components. Of particular importance for assembly of the E2-E3BP complex, the I and I’ domains of mammalian E2 and E3BP, respectively, are closely related in sequence and size. In marked contrast, the inner domain of yeast E3BP has no clear relationship to the inner domain of human or yeast E2 or to human E3BP based on its amino acid sequence and size (Supplemental Material, Fig. 1S). Although it is clear that the I domain of human E2 and I’ domain of human E3BP are homologs, it is interesting to note that there is a large increase in the number of charged residues in the I’ domain of E3BP and a decrease in histidine residues (Fig. 1), including one required for catalysis of the transacetylase reaction (18). Differences in the Stokes radii, apparent density, and non-ideality in equilibrium sedimentation between human E2 and E2-E3BP, uncovered in this work, may be related to these differences in the electrostatic properties of E2 and E3BP (Fig. 1) and amino acid composition of the linker regions (Supplemental Material Fig. 1S) (see “Discussion”).

Given these structural relationships, the previously proposed 60/12 addition model (16, 18, 19) should not be considered an expected outcome for the organization of mammalian E2-E3BP. The E2-binding I’ domain of mammalian E3BP might take advantage of structural properties that derive from its close structural relationship to the I domain of E2. Here we provide evidence from a combination of studies that association of E3BP in the mammalian complex involves a substitution process rather than addition of E3BP to the E2 60-mer.

EXPERIMENTAL PROCEDURES

Materials and Methods—The construction of vectors for preparation of His tag free E1 (tag removed by PreScission protease) and the vectors for expressing E2, E2 plus E3BP, sc-E2 that contains a PreScission site in the third linker region and a series of modified constructs will be described elsewhere. A critical feature for removal of a truncated form of E2 (26) involved introducing silent mutations in the coding region for the L1 domain that removed a Shine-Dalgarno sequence, thereby preventing the codon for Met-59 serving as an internal start site. t-E2 is prepared from sc-E2 that is prepared as described for E2 below. Human E3 was prepared as described previously (34, 35). E1 from which the His tag is removed by PreScission protease is prepared as will be described elsewhere.2 We have developed extensively revised conditions for the preparation of human E2 and E2-E3BP (see Supplemental Material, Experimental Procedures).

Slab Gel Electrophoresis and Densitometric Analysis—SDS-PAGE was performed with the standard Laemmli system (36) with 10% acrylamide in the separating gel. Electrophoresis was performed at 14 mA and 4 °C. Gels were stained with Coomassie Brilliant Blue R-250. Band intensities were quantified with a Hewlett-Packard scanner, and the area density was analyzed by ImageQuant version 5.2 (Amersham Biosciences).

Analytical Ultracentrifugation (AUC) Studies of E2 and E2-E3BP and Their Complexes—Sedimentation velocity experiments were conducted at 20 °C by using an Optima XL-I ultracentrifuge using the 4-hole An-60 Ti rotor with loading of 370–400 μl of sample and 400–450 μl of matching buffer into 12-mm charcoal-filled double sector cells. Following an initial scan at 3,000 rpm to check the total absorbance and detect very large aggregates, sedimentation velocity runs were conducted at the desired speed with scans continuously collected at a 2–7-min interval with absorption optics (usually at 280 nm but also at 450 nm) and/or interference optics that are analyzed by time derivative procedures (37–41).

Sedimentation profiles, including a consecutive set of at least four scans, were analyzed to obtain the apparent distribution of sedimentation coefficient g(s) using by using DCDT software version 1.16 provided by J. S. Philo (www.jupiter.mailway.com) (39). The sedimentation coefficient was calculated by using g(s) fitting function in DCDT + software (39, 40). Buffer density and viscosity were calculated by Sedinter version 1.08 (www.jphilo.mailway.com). Partial specific volumes for E1, E2, E2, E2-E3BP, and E2-E3BP, calculated based on their amino acid compositions using Sedinter, are 0.739, 0.7414, 0.7462, 0.7485, 0.7459, and 0.7459 at 20 °C, respectively. In the calculation of the translational fractional coefficient ratios, f,f, and Stokes radii, R, of protein components and their complexes, masses based on the amino acid sequence of constructs (below) and the above partial specific volumes of protein components were used to calculate R, i.e. Stokes radii for the unhydrated sphere of the same mass and density.

Two different procedures (42) were used to analyze sedimentation velocity results in order to estimate the fraction of E1 or E3 component bound to the E2 or E2-E3BP. When a significant portion of a component was both bound and free, the level of free component was estimated by determining the change in concentration of trailing component in the presence versus the absence of E2 oligomer using absorption optics. The base line was determined by over-speed methods at the end of the run. The bound component was also estimated from the increase in the rapidly sedimenting complex due to bound component (fringe ratio method). The g(s) analysis in DCDT + software gave the g(s) distribution and extrapolated to the initial fringe. The initial fringe of complex fraction (i.e. E1-E2, E1-E2-E3BP) was calculated above the 20 S region of the g(s) distribution. The initial fringe of the complex fraction was then compared with that of E2 or E2-E3BP alone determined in the same manner. Refractive increments (fringe) are nearly constant for proteins (43, 44, and 59). The fringe ratio between E2 or E2-E3BP complex to E1 or E3 and E2 or E2-E3BP alone provides a weight concentration ratio. The number of bound E1 per E2 60-mer is as follows: (fringe of E1-E2/
fringe of $E2) = \frac{1}{1} \times M$ of $E2$ 50-mer of $M$ of tetramer, where $M$ is the calculated molecular mass. In the case of $E1$ or $E2$ binding to $E2$-3BP, a model-dependent molecular mass for $E2$-3BP is substituted for the $M$ of $E2$ in this equation.

Sedimentation equilibrium studies on $E2$ and $E2$-3BP were conducted using three charcoal-filled Epon centerpieces with 6-sector cells. Normally, 50-µl samples were overlaid over 25 µl of Fluorinert FC-43 silicon oil (3M Industrial Chemical Products Division) and 100 µl of matched solvent was introduced in the reference position. Each protein was evaluated at three concentrations, and equilibrium was attained at six rotor speeds. Following equilibration at the lowest speed during the 30-h period, at least an 8-h equilibration time was used for the transition to a higher speed. The stability of the concentration gradient established was confirmed over at least a 4-h period before making final measurements. The equilibrated protein gradients and their solvent controls were scanned 10 times at 0.001-cm intervals; the difference between the averaged sample and solvent boundaries yielded the equilibrium boundary of the solute protein.

Sedimentation equilibrium data were evaluated with Beckman software (version 4) that was provided with the Optima XL-I ultracentrifuge. Additional analyses were required because there was a decrease in mass with increasing concentration of sample. This indicates a non-ideality (molecular crowding) that is characteristic of a large solvent-filled or asymmetric molecule with substantial electrostatic repulsion (see “Discussion”). For the different speeds and concentrations, the apparent molecular weight, $M_{app}$, was plotted against the concentration ($A_{280}$ nm); these $M_{app}$ values were based on 20 points (0.02 cm) and were recalculated at each 0.001-cm interval. $M_{app}$ estimates within a 0.01 $A_{280}$ nm interval were averaged for all the above. From plots of these average $M_{app}$ and of $I_{app}$ versus concentration in mg/ml, the molecular weight, $M$, was estimated by extrapolation to zero concentration (45–48). For a given $M_{app}$ values to mg/ml, 1.34 mg/ml has a $A_{280}$ nm of 1 for 1-cm light path (or 0.75 A$_{280}$ nm per mg/ml). With 1.2-cm light path in the centrifuge, this was 1.154 mg/ml $E2$ per lg A at 280 nm. The concentration dependence of $M_{app}$, for a single solute is described by Equation 1,

$$M_{app} = M/(1 + c d \text{ln}y)$$

where $c$ is the concentration of protein solute (mg/ml), $y$ is its activity coefficient (45). The estimated d ln $y$ vs. the slope of the $I_{app}$ versus $c$ plot, was evaluated by using a simple model in which the excluded volume of the solute was estimated using the first virial coefficient ($B_2$) for an equivalent hard sphere (45), so that $M c \text{dln}y = B_2 = 8 V_r$ where $V_r$, like $B_2$, has units of liter/g and is an approximation of the effective excluded volume occupied by 1 g of solute. We applied a linear fit to the change in $I_{app}$ with concentration, so that we did not fit our results with higher order virial coefficients that have a higher order dependence on concentration. We used $V_r$ to calculate the excluded volume radius $R$.

**Calculated Subunit, Component, and $E2$ Oligomer Masses**—Masses of components and complexes were calculated from the amino acid sequence of our constructs using the PeptideMass program at the ExPASy site (us.expasy.org/) with cofactors (lipoyl groups on $E2$ and $E3$BP and PAF on $E3$) added for masses used in evaluating AUC data. The protein molecular masses of $E1a$, $E1b$, $E2$, and $E3$ were 40,229, 35,904, 59,620, and 50,274 Da, respectively; these masses are all slightly larger than those reported originally for the same native amino acid sequence, probably due to the program used. The expression cDNAs for $E2$, $E3$BP, and $E1b$ subunits encoded the native sequences plus an N-terminal Met. Sequencing revealed the presence of the native N-terminal sequence (i.e. Met removed) for $E2$ and $E3$BP subunits. The subunit molecular masses for our constructs are as follows: $E2$, 59,620 Da (with 2 lipoyl groups 59,996 Da); $E3$BP, 48,040 Da (with lipoyl group 48,228 Da); $E1a$, $E1b$, tetramer, 153,718 Da (the $a$ subunit has non-native GSHG sequence at its N terminus, the $b$ subunit has non-native RGS sequence at its N terminus, again N-terminal Met was not included); $E3$ with lipoyl group (has non-native MWSSHHHHS sequence at its N terminus); $E3$, 25,797 Da. $E2$ has only native $E2$ sequence (residues 322–561); this includes the last 10 residues of the $E3$ linker region and the complete I domain of $E2$ (see Supplemental Material, Fig. 1S).

**Determination of Protein Concentration**—The absorbance at 280 nm and an interference fringe of proteins were measured by AUC with detection by absorbance and Rayleigh interference optics. Protein levels were measured based on the refractive index increment that is independent of the protein source (43, 44). AUC procedures were generally described (60). The reference (400 µl of matched buffer) and protein sample (150 µl) were placed in a synthetic boundary double sector cell with a 1.2-cm path. Dialyzed bovine serum albumin was used as a standard protein based on the extinction coefficient of 0.614 $A_{280}$ for 1 mg/ml (43). Absorbance and interference measurements were carried out simultaneously at a rotor speed of 10,000 rpm for 10 min at 20°C. Absorbance measurements were made between 0.5 and 1.2 OD, and interference measurements were up to 18.5 mg/ml.

**Single X-ray Scattering**—SAXS experiments were carried out using the RIKEN Structural Biology beam line 1 (BL45XU), which accepts x-ray at wavelength of 0.9 Å from an undulator source of the electron storage ring Spring-8 (49). Scattering was detected with an x-ray image intensifier and cooled CCD (XR-11 + CCD): each scattering profile was collected with a circularly averaged area and then analyzed into two-dimensional structure factors (50). Data were corrected for background at 4°C for 1 s. The sample to detector distance was determined by meridional reflection of collagen. This distance was 3270 mm to cover a very small angle region ($S < 1/100$ nm$^{-1}$) where $S$ is the scattering vector defined by $S = 2 \sin(\theta)/\lambda$, where $\lambda$ is the x-ray wavelength. Judging from the stability of intensity versus time, there was no radiation damage of protein sample during data collection. The “radius of gyration,” $R_g$ which equals the second moment of the electron density of protein, has nothing to do with a rotating particle size but is the root mean square of mass-weighted distances of all subvolumes in a particle estimated out from the center of the mass. $R_g$ values at different concentrations of oligomer were evaluated using the Guinier approximation (51), $I(0) = I(0) R_0^2$ where $I(0)$ is the scattering intensity at different S, and forward scattering; $I(0)$ is the scattering intensity at the origin ($S = 0$). From a Guinier plot of $\ln(I(S))$ versus $S^2$, $I(0)$ is obtained as the y intercept; $R_g$ is obtained from the slope, $(2\pi)^2/S^2$. These $I(0)_{app}$ and $R_g_{app}$ values are plotted against the concentration of the protein to obtain $I(0)$ and $R_g$ values independent of non-ideality effects of protein concentration. With homogenous, non-aggregated samples, the $I(0)$ estimated at zero concentration of the protein is proportional to the molecular weight of the protein. It is worth noting that for a solid sphere of radius $R$, $R_g = 0.775 R$, and that for asymmetric molecules with Stokes radius, $R_g > 0.775 R$, and that $R_g$ can be much smaller than $R$, if the density of the outer part of a structure is lightly populated as occurs with $E2$ and $E2$-3BP oligomers. The distance distribution function, $P(r)$, which is defined by $P(r) = 2 \pi r^2 S^2(I(0)sin(2\pi rS)/S)$, was calculated by the indirect Fourier transform method of GNOM (52). $P(r)$ is the relative probability of finding electron density at a distance $r$. The maximum dimension, $D_{max}$ was determined from the first zero cross-point of the $P(r)$ function (51). In SAXS experiments, absolute photon counting is measured, and $I(0)$, $\ln(I(0))$, and $P(r)$ have no units so that arbitrary units are shown in the figures.

RESULTS

**E2 and E2-3BP Preparations and SDS-PAGE Analysis**—For the AUC and SAXS studies, we needed to prepare highly purified $E2$ and $E2$-3BP. Removal of contaminating nucleic acid proved particularly challenging. In the sequence of steps (see Supplemental Material), treating the 8–16% PEG cut with nucleases and, after the first gel filtration step, treating the precipitation of residual nucleic acid-containing material with polyethyleneimine (not high molecular weight polymer) resulted in a product with an $A_{280}$ nm to $A_{260}$ nm ratio of 1.4 which is in good agreement with ratio expected based on the amino acid composition of these components.

Fig. 2 shows the SDS-PAGE profile of purified $E2$, $E2$-3BP, and $E1$; high purity is supported in each preparation. Coomassie-stained bands of a range of levels of $E2$, $E2$-3BP, and bovine heart PDC were analyzed by area scanning densitometry (data not shown). The relative staining intensity of the recombinant $E2$-3BP and bovine heart PDC indicated that in each case there was 2.8–3.5-fold greater staining of the $E2$ band than the $E3$BP band; the upper end of this range was observed for the staining of lower protein levels ($E2$, 1.2 µg). This indicates that the recombinant $E2$-3BP and heart PDC have a similar ratio of $E2$ to $E3$BP. Sanderson et al. (16) performed a detailed densitometric analysis of ratio of $E3$BP to $E2$ in heart PDC and estimated 13.4 $E3$BP adding to $E2$ 60-mer. Using the masses for the $E2$ and $E3$BP subunits described...
in their paper, this would indicate E3BP constituted 16.8% of E2-E3BP protein. By using the correct masses for E2 and E3BP (59.6 kDa rather than 52 kDa for E2 and 48 kDa rather 47 kDa for E3BP), this fraction of protein predicts ∼14.5 E3BP per complex if a model is used in which E3BP is added to E2 60-mer. If a substitution model is used, this fraction of E3BP protein fits 12 E3BP and 48 E2.

**AUC Analysis of E2 and E2-E3BP—Sedimentation velocity studies were conducted on E2 and E2-E3BP.** Fig. 3 shows g(s*) profiles for E2 and E2-E3BP from the center of the final gel filtration step. The majority of E2 sedimented with an s_{20, w} of 36 S. A small portion (5 ± 2%) of a faster moving species with an S value of 55 S was also observed. The faster sedimenting species is consistent with a dimer based on the n^2 rule. Fractions from the leading edge of the gel filtration column contained higher levels of this larger species. This larger species is present at the first gel filtration step, and subsequent concentrating of proteins by pelleting with the preparative ultracentrifuge gives a somewhat larger portion. The level was then decreased in all but the leading fractions in the final gel filtration step. It was also important for size homogeneity not to select fractions from the distal end of the trailing side of the peak. Even though these fractions contained highly purified E2 and E2-E3BP, sedimentation velocity analyses revealed a broader trailing side in the g(s*) pattern suggesting a small portion incomplete particles.

We have performed a large number of sedimentation studies on E2 and E2-E3BP and have repeatedly observed that E2 sediments faster than E2-E3BP. Most sedimentation studies were conducted in 50 mM potassium phosphate buffer, pH 7.2, containing 0.5 mM EDTA, 0.2 M ammonium chloride, 1 mM β-mercaptoethanol (buffer A). Some studies were conducted at a lower ionic strength in 50 mM potassium phosphate buffer, pH 7.5, and 0.5 mM EDTA (buffer B). Different E2 preparations sedimented at rates that were within 0.4 S of the s_{20, w} of 36.0 and 31.8 obtained with the E2 and E2-E3BP preparations, respectively, used to obtain the results shown in Fig. 3 and Table I. When these same preparations were transferred to the lower salt buffer B, the s_{20, w} values decreased to 34.4 S for E2 and 31 S for E2-E3BP. This decrease is consistent with the large excluded volume effects found in this buffer in sedimentation equilibrium studies below.

**Table I.** Structural parameters derived from analysis of sedimentation velocity studies on E2, t-E2, and E2-E3BP. Parameters for these calculations including partial specific volumes are described under “Experimental Procedures.”

| E2 oligomer | Molecular mass* | s_{20, w} | R_0 | R_s | D_s |
|-------------|-----------------|-----------|-----|-----|-----|
| E2 60-mer   | 3,599,760       | 36.0      | 2.195 | 10.2 | 22.4 | 448 |
| t-E2        | 1,547,820       | 31.8      | 2.75  | 10.75 | 29.5 | 590 |
| E2-E3BP     | 4,178,496       | 31.0      | 2.75  | 10.75 | 29.5 | 590 |
| 60/12 model | 3,458,544       | 24.4      | 2.42  | 10.1 | 24.4 | 488 |

* The molecular mass of the oligomers includes mass of lipoyl groups in E2 and E2-E3BP.
the frictional properties beyond those of the E2 60-mer (see under “Discussion”).

Also included in Table I is information on a recombinant inner core structure, t-E2, that was made from a recombinant E2 60-mer by cleavage at a PreScission protease site inserted just prior to the dodecahedron forming E2, domain (Fig. 1). As will be described elsewhere, t-E2 exhibits the normal pentagonal dodecahedron organization in cryo-EM studies.3 This spherical but open structure has a smaller \( f/0 \) of 1.55, which predicts an \( R_c \) of 11.95 nm. The \( D_0 \) of 23.9 nm is slightly larger than the average diameter of 23 nm observed in cryo-EM studies but is within the 21–25 nm observed due to the breathing of the inner core,3 a process described for yeast E2 (31) (see “Discussion”). At its N terminus, t-E2 includes an 11-amino acid segment (964.5 Da) of the C-terminal end of linker region preceding the I domain of E2; this extra structure is presumably located outside the dodecahedron and contributes modestly to the hydrodynamic diameter of t-E2. The appreciably larger \( f/0 \) and \( R_c \) of the E2 60-mer than t-E2 60-mer is consistent with the linker connected binding and lipoyl domains creating significant drag due to its propensity to extend out from the inner core (24, 53, 54).

SAXS of E2 and E2-E3BP—E2 and E2-E3BP were examined by SAXS analysis (Fig. 4). These studies used preparations that had very low levels of dimer based on AUC analyses. Guinier plots of the scattering intensities for E2 and E2-E3BP lacked significant upward curvature (Fig. 4A), indicating a minimal effect of larger aggregates in these gel-filtered preparations. \( R_g \) values of E2 and E2-E3BP (derived from Fig. 4A data) were estimated to be 152.5 ± 0.6 and 150.5 ± 2.0 Å, respectively. \( I(0) \) estimated at the zero concentration is 15,160 ± 0.6 and 150.5 ± 100, respectively. \( I(0) \) values estimated at the zero concentration were 15,040 for E2 and 13,910 for E2-E3BP. These values are in good agreement with those from the Guinier plot made with the full data sets, which is generally considered to provide more accurate values.

The smaller \( I(0) \) indicates that E2-E3BP has a 7.6% smaller mass than E2 (or at least 4.9% smaller, applying the maximum experimental error). This is definitely not consistent with E3BP mass adding to the mass of the E2 60-mer, but it is also a somewhat greater decrease in mass than the 4% predicted by the 48/12 substitution model. The \( R_g \) values reflect the radial distribution of density and are expected to be much smaller than \( R_c \), which reflects the hollow character of these structures. In contrast to \( I(0) \), the \( R_g \) values are generally smaller for E2-E3BP than E2 60-mer is about 1.3% smaller. The \( I(0) \) and \( R_g \) values indicate that E2-E3BP and E2 have a similar radial distribution of protein mass. The distance distribution function (see Supplemental Material Fig. 2S) shows similar mass distributions for E2 and E2-E3BP.

Equilibrium Sedimentation Studies on E2 and E2-E3BP—Equilibrium sedimentation studies also support a smaller mass for E2-E3BP than E2. The above sedimentation velocity and SAXS studies were conducted in buffer A. Because we observed somewhat less increase in dimer aggregate after extended incubation of E2 and E2-E3BP at 4°C in buffer B, equilibrium sedimentation was conducted in this lower ionic strength buffer. With both structures the \( M_{app} \) decreased with increasing concentration (Fig. 5A), which indicates a large non-ideality for these water-filled, negatively charged assemblages given the low concentrations used. Extrapolation to a concentration of zero of a plot of \( 1/M_{app} \) versus concentration (Fig. 5B) yielded an \( M \) for E2 of 3.5 ± 0.1 million (Table II), which agrees within experimental error with the calculated mass of the E2 60-mer based on the amino acid sequence (Table I). The zero concentration extrapolation (Fig. 5B) gave an \( M \) of 3.3 ± 0.07 million for E2-E3BP (Table II), which is slightly below the mass calculated for the substitution model in which 12 E3BP replace 12

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Footnote:

3 X. Yu, Y. Hiromasa, J. K. Stoops, T. Peng, L. Hu, T. E. Roche, and Z. H. Zhou, unpublished data.
Mated by extrapolation to zero concentration. From the slope of the very large increases considering excluded volume, derived and used in a hard sphere model to estimate the exponent in a model in which the excluded volume of 1 (see Discussion) the approximation that d ln $\gamma$/d$c$ was estimated. The value for $E2$-E3BP is within experimental error (assuming our model). Other steps were as described under “Experimental Procedures.”

![Summary of the analysis of the sedimentation equilibrium studies on E2 and E2-E3BP (Fig. 5)](image)

**TABLE II**
Summary of the analysis of the sedimentation equilibrium studies on E2 and E2-E3BP (Fig. 5)

| Oligomer | $M^*$ | $d \ln \gamma/dc$ | $V_e^b$ | $R_e^b$ | $D_e^b$ |
|----------|-------|-----------------|---------|---------|---------|
|          | $\times 10^6$ | (L/mol g$^3$) $\times 10^7$ | ml g$^{-1}$ | nm | Å |
| E2       | 3.5 ± 0.1 | 1.77 ± 0.3 | 77.4 ± 15 | 47.6 ± 4 | 952 |
| E2-E3BP  | 3.3 ± 0.07 | 2.51 ± 0.2 | 103.5 ± 9 | 51.3 ± 2 | 1026 |

$^a$ Estimated at a protein concentration equal to zero.
$^b$ The equilibrium sedimentation data in Fig. 5B were fit by Equation 1 (see “Experimental Procedures”); the approximation that $d \ln \gamma/dc = R_e$ was used to estimate the excluded volume radius ($R_e$), using the first virial coefficient in a model in which the excluded volume of E2 or E2-E3BP is represented by equivalent hard spheres (45).

E2 in a 60-mer (Table I). This mass is 22% below the mass predicted by the addition model. These sedimentation equilibrium results, like the SAXS results above, strongly indicate that the E2-E3BP mass is smaller than E2 and that E2-E3BP cannot be formed by E3BP adding to the E2 60-mer.

From the slope of the $1/M_{app}$ plot, $d \ln \gamma/dc$ (see Equation 1) is derived and used in a hard sphere model to estimate the excluded volume, $V_e$, of 77.4 ml/g and hard sphere radius, $R_e$ of 47.6 nm ($D_e = 952$ Å) for E2 (Table II). The $V_e$ and $R_e$ values estimated for E2-E3BP (Table II) are significantly larger than for E2. Indeed, the $V_e$ of 103.5 ml/g and $R_e$ of 51.3 nm constitute very large increases considering E2-E3BP has a smaller mass than E2. Considering the probable error, these values support E2-E3BP having a larger excluded volume than E2. For both E2 and E2-E3BP, the $D_e$ values estimated from the equilibrium sedimentation studies are much larger than the $D_{max}$ obtained from SAXS studies or the $D_e$ from sedimentation velocity studies. Because the latter studies were conducted in the higher ionic strength buffer A, charge repulsion probably makes a major contribution to the large $D_e$ values. Certainly, introduction of E3BP significantly increases the observed non-ideality (see “Discussion”).

**Binding of E1 to E2 and E2-E3BP**—Binding of E1 to E2 was evaluated using sedimentation velocity with 4.3 to 179 E1 per E2 60-mer. Fig. 6A shows that $S^*$ changed from 35 for E2 to 99.4 ± 1.2 with E1 added to E2 60-mer ratio. Fig. 6, B and C, shows bound E1 plotted against the ratio of E1 added per E2 60-mer and free E1, respectively. Half-maximal binding was observed at 0.25 ± 0.07 μM free E1 (Fig. 6C). Maximum binding was estimated to be 59.8 ± 1.71 E1 tetramers per E2 60-mer. This strongly indicates 1 E1 tetramer is bound per E2 subunit. The lower part of the binding curve appears near hyperbolic but there is considerable scatter for low concentrations when analyzed by a Klotz or a Scatchard plot (data not shown). The Klotz plot analysis gives an apparent binding affinity of 0.29 μM (error range 0.21 to 0.48 μM). The extrapolated maximal binding is 66 E1 per E2 60-mer, but this is clearly not supported by the sharp transition in the experimental data as after about 60 E1 are bound.

Fig. 7 shows similar analyses for the binding of E1 to E2-E3BP. A maximum $S^*$ value of 81 ± 1 S is reached which is 18.4 S below that with saturated binding of E1 to E2 (Table III).
and a 14.9 S smaller net increase in S (Fig. 7A and Table III). Because the estimated masses of the E1 complexes are very different using the $E_{240}$$E_{33}B_{12}$ model versus the $E_{248}$$E_{33}B_{12}$ model (below), Fig. 7B shows the estimated E1 bound per $E_{248}$$E_{33}B_{12}$ with the masses calculated for these two models. The data fit a maximum of either 60 ± 2.5 or 49.5 ± 2 E1 bound (Table III), and this does not allow the models to be distinguished. However, it is very unlikely that $E_{240}$$E_{33}B_{12}$ has a 4.5% larger mass than $E_{240}$$E_{33}B_{12}$, which is closer to the estimate for $E_{248}$$E_{33}B_{12}$ (Table III). Due to the increase in sedimentation rate with the level of bound component. Like the above decrease in frictional properties, the linear increase is unusual and suggests a fill-in process in which an extended and unorganized external framework becomes more organized due to E1 binding. In the case of E1 binding to E2, a constant increment for E1 binding to E2$E_{33}B_{12}$ when E2$E_{33}B_{12}$ are bound. By using the $E_{240}$$E_{33}B_{12}$ model, E1 binding is estimated to give ΔS = 0.89 ± 0.07, whereas fitting the observed change in S to the $E_{240}$$E_{33}B_{12}$ model yields a ΔS = 1.11 ± 0.08 which is closer to the estimate for E2 (Table III). Due to the difference in frictional properties (above), E1 binding to E2$E_{33}B_{12}$ may have a slightly smaller impact on S than with E2, so that the maximum increase in S of 49.5 probably reflects slightly more E1 being bound than predicted by a proportional change in S (49.5 - 60/64.4 = 46.1). However, there seems to be little reason for the binding of E1 to the E2 subunit of E2$E_{33}B_{12}$ having a 20% smaller impact on sedimentation rate than for E1 binding to E2. Again, an estimate near 48 is much more reasonable than 60.

* D. B. McCarthy, X. Yu, Y. Hiromasa, T. E. Roche, and H. Zhou, and J. K. Stoops, unpublished data.
**FIG. 7.** Sedimentation velocity analyses of binding of E1 to E2-E3BP. A shows the E2-E3BP model-dependent change in S with the ratio of E1 to E2-E3BP. B shows the change in E1 bound. In all cases open symbols are for an analysis assuming a 60/12 model, and closed symbols are based on the 48/12 model for E2-E3BP. Data are combined from two experiments conducted with proteins equilibrated in buffer B. In the first experiment, E2-E3BP was maintained at 0.114 mg/ml, and sedimentation was monitored by A$_{280}$nm (circles, all panels). In the second experiment, E2-E3BP was held at 0.208 mg/ml, and sedimentation was monitored by absorbance and interference (squares, A) and bound E1 estimated by the decrease in trailing E1 (squares, B) or fringe ratio procedure (triangles, B) as described under "Experimental Procedures."

**TABLE III**

| E1-saturated E2 oligomer | $s_{20,w}$ | $\Delta S$ increase | Bound E1/E2 oligomer | $ff_0$ $^c$ | $D_1$ $^c$ |
|--------------------------|------------|---------------------|----------------------|------------|-----------|
|                           |            | Average/E1 bound $^b$ | Maximum change $^a$ |            |           |
| E2 60-mer                 | 99.4 ± 1.2 | 1.175 ± 0.04         | 64.4 ± 1.5           | 60.2 ± 0.5 | 57.8 ± 1.0 | 1.91      | 595       |
| E2-E3BP                   | 81.0 ± 1.0 | 0.89 ± 0.07          | 49.5 ± 1.4           | 60.0 ± 1.5 | 59.3 ± 2.5 | 2.43      | 768       |
| 60/60 E1                  |            | 1.11 ± 0.08          |                      | 49.7 ± 1.3 | 49.1 ± 2.1 | 2.17      | 640       |
| 48/48 E1                  |            |                      |                      |            |           |           |           |

$^a$ These studies were conducted in buffer B. The $s_{20,w}$ values of these E2 and E2-E3BP preparations were 35 and 31.5 S; the higher values in Fig. 2 and Table I were obtained in buffer A. The lower value, here for E2, fits an $ff_0$ of 2.26 and $R_s$ of 23.1 nm.

$^b$ This is an average of data in the range with $>7$ E1 to $<24$ E1 bound per oligomer (also slope of line in Fig. 6D for E2); the upper limit for near linear increase is raised to $28$ for 60/12 addition model.

$^c$ For calculation of $ff_0$ and $D_s$, we used the masses shown in Table I for E2 and E2-E3BP models; the mass of 60 E1 was added to E2. For E2-E3BP, the bound E1 level used is that estimated by change in trailing E1; if 48 E1 bound is used for E2-E3BP then $ff_0$ is 2.11 and $D_s$ is 620 Å.
Binding of the E3 Component to E2 and E2-E3BP Evaluated by AUC and SAXS—With 77.5 nm E2 alone and with 4.7 μm E3 (60 E3 per E2 60-mer), binding of E3 to E2 was evaluated using sedimentation velocity. An increase from 36.1 S for E2 alone to 36.2 S for E2 plus E3 was observed. E3 uses the lipoyl groups of E2 as a substrate; the small increase is consistent with the known weak interaction of E3 with these lipoyl groups. In contrast, E2-E3BP tightly binds E3. Fig. 8A shows the change in sedimentation velocity with the ratio of E3 added to E2-E3BP according to the E2a2-E3BP12 model (●) and the E2a0-E3BP12 model (○). Below 41 S, there was a linear increase in S and free E3 was not detected (i.e. <1.5%). A maximum s20,w of 41.9 ± 0.2 S was observed. E3 bound was estimated most accurately by extrapolation back from this maximum value to the cross point with the line extrapolated from the extended linear increase in S as when lower levels of E3 were bound. This extrapolation indicates that the number E3 dimers bound was 11.4 per E2a2-E3BP12 or 13.8 E3 per E2a0-E3BP12. Thus, the results are consistent with about 12 E3 dimers being bound, and there is a somewhat better fit by the E2a0-E3BP12 model. The maximal s20,w of 41.9 predicts f20,w of 2.26 (Rg = 25.2 nm) for this smaller E2-E3BP structure but requires an increase to 2.57 (Rg = 30.5 nm) for E2a0-E3BP12 model.

Fig. 9 shows a study in which E3 sedimentation is monitored at 450 nm, and the sedimentation of all protein was evaluated by interference optics. The level of bound E3 is estimated both from the increase in 450 nm absorbance (corrected for that from E2-E3BP) for the rapidly sedimenting E2-E3BP-E3 complex as compared with E2-E3BP (Fig. 9A) and from the change in area of g(s*) plot (Fig. 9B) for the rapidly sedimenting oligomers (monomer and dimer) without (32.2 S) and with a saturating level of bound E3 (43.75 S); free E3 had s20,w of 5.66 S. Based on the 450 nm measurements, the increase of E3 protein in the rapidly sedimenting complex versus the total protein in this complex (measured via interference optics) was estimated to be 12.25 ± 0.75 per E2-E3BP based on the 48/12 model and 14.8 ± 0.92 based on the 60/12 model. At low speeds in the presence or absence of E2-E3BP, the absorbance at 450 nm (corrected for E2-E3BP) was unchanged, indicating that binding of E3 to E2-E3BP does not affect the FAD extinction coefficient. The increase in protein in the rapidly sedimenting complex based on the areas in g(s*) plot (Fig. 9B) indicated that 12.0 or 14.5 E3 were bound to E2-E3BP by using these two models. Because the g(s*) profiles for these large oligomers are well resolved, it seems likely that the relative level of protein is accurately estimated with no more than 3% error. The estimated level of E3 binding corresponds to the 20.7% greater increase rapidly sedimenting protein predicted for saturated binding by the 48/12 model as compared with 60/12 model.

A titration study using SAXS also demonstrated saturation in E3 binding in a similar range (Fig. 8B). In the case of this mixture, the apparent Rg is determined from the innermost part of the scattering intensity. With addition of E3, Rg reached a maximum at ~16.15 nm (Fig. 8B) which corresponds to E2-E3BP-E3. Similarly, I(0) increased by a factor of nearly 2. Based on these results, E2-E3BP-E3 is expected to be 41% larger than E2-E3BP (square root of 2). This is more than predicted for binding of 12 E3 dimers to a E2a0-E3BP12 structure (27%) and much more than expected for this level binding to a E2a0-E3BP12 structure.

Fig. 10 shows the change with addition of E3 to E2-E3BP in the radial Patterson function. Binding of E3 to E2-E3BP increased Dmax from 390 ± 10 to 440 ± 10 Å. Additionally, the bell-shaped function and peak position of P(r) shifted to a larger r. This indicates a shift in the radial distribution of mass, wherein the bulk of the E3 mass binds to the exterior of the E2-E3BP dodecahedron. This result is very different from the mass distribution of E3 found in the yeast complex (see “Discussion”).

<sup>5</sup> Rg(app) is described as \( R_{g(app)}^2 = (p_1p_2^2R_g^2 + p_3p_4^2R_g^2 + p_5p_6^2R_g^2)^{1/2} \) where the subscripts, \( x = 1, 2, 3 \), correspond to free E3 dimer, E2-E3BP, and E2-E3BP-E3, respectively; for these three species, \( p_i \) indicates the relative proportions, \( n_i \) indicates the number of electrons, and \( R_g \) are the radii of gyration. Due to its smaller molecular mass of free E3 (low \( n_i \)), the smaller size (\( R_g \) of free E3 = 31 Å, data not shown), and the squaring of these terms, the contribution of free E3 to \( R_{g(app)} \) is negligible.
to an interparticle interference effect. The areas of the peaks for E2-E3BP and E2-E3BP-E3 complexes were calculated by DCDT+ program.

FIG. 9. Sedimentation velocity analysis of E3-saturated E2-E3BP by monitoring at 450 nm and interference optics. A shows a set of absorbance scans at 450 nm exhibiting the decrease in trailing E3 due to binding by E2-E3BP. E2-E3BP was added at 1.03 mg/ml and E3 at 0.675 mg/ml of buffer B. For the scans shown in A, sedimentation at 25,000 rpm had proceeded for 45 min. Appropriate base-line corrections were made after the components were cleared from the outer radius which included sedimentation for 2 h at 25,000 rpm and then 1.5 h at 40,000 rpm. B shows a g(s*) analysis of the data collected by monitoring this same run by interference optics. The areas of the peaks for E2-E3BP and E2-E3BP-E3 complexes were calculated by DCDT+ program.

Three laboratories developed chaotropic conditions for resolving E3BP from bovine E2 (8, 14, 17, 27); E2 subunits could re-assemble as a large oligomer without binding E3BP unless slow removal of chaotropic agent was used (17). The production of E3BP-free E2 allowed the loss of E3 binding to be demonstrated (14). We have confirmed that E3 does not bind to recombinant E2 that lacks E3BP. Even when E3BP was removed from E2 under mild conditions (8) with domain functions and E3BP solubility maintained (see Supplemental Material, Discussion 1) E3BP could not bind back to E2, which is in marked contrast to the capacity of yeast E3BP to bind to the yeast E2 60-mer under non-chaotropic conditions (22).

We have found that the E2 60-mer sediments significantly faster than E2-E3BP complex. By using the calculated mass of the 60/12 addition model (16), sedimentation velocity results predict a Ds of 590 Å, which is not only 30% larger than estimated for the E2 60-mer but larger than the diameter observed for the entire bovine PDC by cryo-EM (29, 31) and quasielastic light scattering (52). This model would then require an extraordinary increase in the frictional contribution due to the addition of E3BP. Because it is known that the I domain of E3BP associates with the I domain of E2 (10) and that E3BP has one less linker region and lipoyl domain than E2 (Fig. 1), this large increase required for E2-E3BP12 model seems very unlikely. The slow sedimentation of E2-E3BP still requires a larger Ds based on 48/12 model than the Ds estimated for E2. If, as occurs with the yeast E2-E3BP, the I domain were to reside inside the dodecahedron (21–23), that location should work against a significantly increased Ds.

We also find that E2-E3BP has a somewhat smaller molecular weight than the E2 60-mer based on equilibrium sedimentation studies and the I(0) from SAXS experiments. The molecular weight estimated for E2 in the equilibrium sedimentation study precisely fits the calculated mass of the E2 60-mer. Similarly, but with an even larger correction for non-ideality (see below), the molecular weight estimated for E2-E3BP matches that predicted for a composition of 48 E2 and 12 E3BP. Besides the AUC and SAXS studies reported here, cryo-EM studies show equivalent-appearing inner core pentagonal dodecahedron structures for our recombinant human E2 and human E2-E3BP; no fixed mass interior or exterior to the cores upon binding other components is not supported. These observations support E2-E3BP being a highly organized assemblage in which E3BP is specifically (i.e. symmetrically) placed.

Experimental conditions were as described for Fig. 9B symbols for the different E2-E3BP, to E3 ratios shown on the right side. The calculation procedure was the same as Fig. 5. P(r) values were not calculated from zero-extrapolated intensity curves. The Dmax, in the absence of E3, is smaller than 420 Å obtained for E2-E3BP in Fig. 5, due to an interparticle interference effect.

FIG. 10. Pair distribution function, P(r), of E2-E3BP with increasing E3. Experimental conditions were as described for Fig. 9B symbols for the different E2-E3BP, to E3 ratios shown on the right side. The calculation procedure was the same as Fig. 5. P(r) values were not calculated from zero-extrapolated intensity curves. The Dmax, in the absence of E3, is smaller than 420 Å obtained for E2-E3BP in Fig. 5, due to an interparticle interference effect.

DISCUSSION

Cumulatively, our laboratory has compared in a routine manner the staining patterns in SDS-polyacrylamide gels for hundreds of preparations of bovine PDC (heart and kidney), resolved E2-E3BP derived from bovine PDC, and human E2-E3BP made recombinantly by co-expression in Escherichia coli. From visual inspection, including side-by-side comparisons of different preparations, and based on densitometry analyses, these preparations contain a similar and nearly constant ratio of E2 to E3BP. This outcome occurs with the recombinant human E2-E3BP despite both proteins being expressed under the control of equivalent independent promoter regions that presumably supported the synthesis of at least as much E3BP mRNA as E2 mRNA and, therefore, translated protein. The fixed level of E3BP in the assembled E2-E3BP product suggests that there is a specific limit to how much E3BP can be incorporated. Because the finding of a fixed ratio is independent of whether E1 or E3 were bound, a process of reorganization of the

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dodecahedron is detected as would occur if the I’ domain of E3BP added to the inner or outer surface of this inner core structure. Again, this differs fundamentally from results with yeast E2-E3BP in which I’ domain of E3BP adds density inside the dodecahedron (23). The smaller mass for E2-E3BP than E2 strongly supports substitution of E3BP in the dodecahedron structure and fits a $E_{2\alpha}E_{3\beta}P_{12}$ model.

The non-ideality in the equilibrium sedimentation and high excluded volume (Table II) indicate potent molecular crowding effects for both E2 and E2-E3BP. The large $V_e$ values (i.e. volume from which the center of mass of one E2-E3BP is excluded by a second E2-E3BP) are difficult to interpret because of the soft nature and electrostatic character of the outer surface of these oligomers. There are several structural differences between E2 and E3BP that may contribute to the larger $V_e$ (see Supplemental Material, Discussion 2). Another possible contributor to an expanded structure for E2-E3BP as compared with E2 is described below and is directly related to the proposed substitution model.

Substantial support for a substitution model (48/12 proposed, below) came from evaluating maximal E1 binding by E2 and E2-E3BP. Saturation of E2 with E1 led to an increase in sedimentation that was consistent with E2 binding about 60 E1. Assuming the binding of E1 to E2 subunits in the E2-E3BP complex produces a similar increase in sedimentation rate; E1-saturated E2-E3BP appeared to bind about a dozen fewer E1. Fitting these E1 binding data with the 60/12 addition model requires an enormous increase in $f_{c0}$ and Stokes radius for E2-E3BP-E1 as compared with E2-E1. Cryo-EM studies detect similar outer diameters for E2-E3BP-E1 and E2-E1 (most about 500 Å with $<30$ E1 bound) and an increasing portion of larger structures (up to 550–560 Å) as more E1 are bound to E2.4 The latter size is in fairly good agreement with the hydrodynamic diameter of 594 Å for E1-saturated E2 considering all the effects that contribute to this parameter. The lower mass of E2-E3BP than E2, and the reduced capacity for binding E1 are consistent with the conclusion that there are about 12 E3BP in the E2-E3BP complex.

Based on the calculated mass of $E_{2\alpha}E_{3\beta}P_{12}$, we find from sedimentation velocity studies that the maximum capacity of E2-E3BP is to bind about 12 E3 dimers. Although numbers in this range have been suggested previously, those were arrived at by using the $E_{2\alpha}E_{3\beta}P_{12}$ model that predicts binding of 20% less E3 per mg of complex, an outcome not found in the present study. With E3-saturated E2-E3BP, we measured what portion of E3 was bound and trailing by specifically monitoring E3 sedimentation at 450 nm and measuring protein by using interference optics. This study clearly fit 12 E3 being bound based on the 48/12 model, but required nearly 15 E3 to be bound when fit to the 60/12 model for E2-E3BP. The accuracy of our estimates is enhanced because all our protein measurements were normalized by simultaneous interference and absorbance measurements in AUC studies. The procedures used previously (7,16) were both less quantitative and measured component ratios in conjunction with assuming that there were 60 E2 subunits per core. The saturated amount of binding of E3 dimers provides the best estimate of the number of E3BP per E2-E3BP. A value of 12 for $E_{2\alpha}E_{3\beta}P_{12}$ model is not only internally consistent but fits the smaller mass of E2-E3BP and reduced E1 binding capacity as compared with E2.

Cryo-EM studies on yeast E2-E3BP indicate that E3 dimers are bound in the pentagonal openings of the dodecahedral E2 core (23). The current SAXS study supports E3 mass adding exterior to the dodecahedron. The increase in $D_{max}$ of E2-E3BP-E3 was 50 Å. Even though $D_{max}$ of E3 alone is 90 Å (data not shown), this is a significant change because, in this extended region, E3 is buried in a cloud of lipoyl domains. E1 is definitely bound with gaps between E1 and the dodecahedron (29,55). Limited cryo-EM on bovine E2-E3BP suggested that E3 is located exterior to the dodecahedron inner core (29). Our results indicate that the structure for mammalian PDC should not be presented with E3 located within the 5-fold faces of the dodecahedron (55) as determined for the yeast complex (23). Cryo-EM studies are underway to detect the precise location of E3 and E3BP in these complexes.

We have analyzed potential models for substitution with a goal of maintaining symmetry to fit the nearly constant level of E3BP observed in various preparations (above). We concluded that there is only one model that allows symmetric substitution while simultaneously fitting 20 or less E3BP being incorporated. We would first emphasize that one E3BP cannot be added per trimer at all 20 corners of the dodecahedron to give a symmetric positioning of E3BP. The minimal number of pure E3BP trimers that could be symmetrically introduced is 8, which would require 24 E3BP subunits. The model shown in Fig. 11 introduces 12 E3BP in a completely equivalent and symmetric fashion. The first rule for assembly to fit this model is that the C-terminal domains of E3BP must self-associate as dimers that therefore connect two E3BP-containing trimers. One can easily justify that constraint. Based on high resolution three-dimensional structures of assembled bacterial E2 (30,56,57), the part of the E2 structure that associates along the 2-fold axis is known to be conserved (30). Recent cryo-EM and modeling results point to flexible “ball and socket” connections along the 2-fold axes between E2 subunits (31,58). In E2, this cantilever linkage along the 2-fold axis allows flexible move-
ment (extension and contraction) as the length of the 2-fold axis. This includes the critical C-terminal residue (Leu or Met in E2s) which forms the “ball” in a hydrophobic pocket in H2 helix. Human E3BP also has a deletion of 3 residues (8 symbols, Fig. 1S) in a region of the H2 helix that contributes a part of the hydrophobic “socket pocket.” It seems unlikely that E3BP could form an interaction with E2 subunits along the 2-fold axis. However, a distinct and probably less flexible interaction with itself is likely because there is still substantial sequence similarity between E3BP to E2 in the regions of E2 that associate along this axis.

The second requirement is that the I domains of E2 that are located in trimers containing an I domain of E3BP only associate with trimers containing I domains of E3. The resulting model has 12 trimers containing one E3BP that associates to form 6 E3BP dimer connections and 8 trimers containing only E2 that only associate via E2-E2 connections along the 2-fold axis. The above ball and socket connections along the 2-fold axis between 2 subunits expand and contract to cause substantial variation (up to 36 Å) in the diameter of the inner core of an E2 oligomer (31, 58). This symmetric arrangement of the 6 subunits and the full flexibility of the 8 trimers would maximize the capacity of the inner core to “breathe” in a coordinated fashion. Specifically, the flexibility might allow the structure to compensate for differences in the length and flexibility along the 2-fold axis formed by two E3BP. Given the conserved nature of the E2 structure supporting breathing, it seems likely that it makes a fundamental contribution to the function of the complex (see Supplemental Material, Discussion 4). This viable model fits the need for symmetry that is required to explain the fixed stoichiometry of 3BP has a reduced mass and decreased capacity for binding with itself is likely because there is still substantial sequence similarity between E3BP to E2 in the regions of E2 that associate along this axis.

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Organization of the Cores of the Mammalian Pyruvate Dehydrogenase Complex Formed by $E_2$ and $E_2$ Plus the $E_3$-binding Protein and Their Capacities to Bind the $E_1$ and $E_3$ Components

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