A New Level of Architectural Complexity in the Human Pyruvate Dehydrogenase Complex*†

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Mammalian pyruvate dehydrogenase multienzyme complex (PDC) is a key metabolic assembly comprising a 60-meric pentagonal dodecahedral E2 (dihydrolipoamide acetyltransferase) core attached to which are 30 pyruvate decarboxylase E1 heterotetramers and 6 dihydrolipoamide dehydrogenase E3 homodimers at maximal occupancy. Stable E3 integration is mediated by an accessory E3-binding protein (E3BP) located on each of the 12 E2 icosahedral faces. Here, we present evidence for a novel subunit organization in which E3 and E3BP form subcomplexes with a 1:2 stoichiometry implying the existence of a network of E3 “cross-bridges” linking pairs of E3BPs across the surface of the E2 core assembly. We have also determined a low resolution structure for a truncated E3BP/E3 subcomplex using small angle x-ray scattering showing one of the E3BP lipoic domains docked into the E3 active site. This new level of architectural complexity in mammalian PDC contrasts with the recently published crystal structure of human E3 complexed with its cognate subunit binding domain and provides important new insights into subunit organization, its catalytic mechanism, and regulation by the intrinsic PDC kinase.

Many enzymes associate permanently or transiently into large, multifunctional complexes. The evolution of these multienzyme assemblies allows for substrate channeling and active site coupling: sequential catalytic reactions proceed through the rapid transfer of intermediates between individual components and without diffusion into the bulk medium. The mitochondrial 2-oxoacid dehydrogenase complexes are such a family of high molecular mass (4–10 MDa) multienzyme assemblies that serve as models for the study of protein-protein interactions and molecular recognition phenomena. They catalyze the oxidative decarboxylation of a vital group of 2-oxoacid intermediates in carbohydrate and amino acid metabolism. Principal members include the pyruvate dehydrogenase (PDC), 2-oxoglutarate dehydrogenase, and branched-chain 2-oxoacid dehydrogenase complexes. PDC is responsible for overall glucose homeostasis in mammals since it controls the key committed step in carbohydrate utilization, namely the conversion of pyruvate to acetyl-CoA and NADH, linking glycolysis to the citric acid cycle. In recent years, genetic and physiological defects in PDC have been implicated in a wide range of diseases including various types of metabolic acidosis and mitochondrial myopathies (1); as major autoantigens in the autoimmune disease, primary biliary cirrhosis (2–4); in neurodegenerative disorders, e.g. Alzheimer disease (5, 6); and as primary targets for modification by environmental toxins and oxidative damage (7).

PDC consists of multiple copies of three different enzymes: pyruvate decarboxylase (E1), dihydrolipoamide acetyltransferase (E2), and dihydrolipoamide dehydrogenase (E3). A fourth protein, E3-binding protein (E3BP, protein X) is present in many eukaryotic complexes but has no known enzymatic function. PDC is regulated by a tightly bound kinase (PDC kinase (PDK)) and a loosely bound phosphatase (PDP). These act in concert to exercise acute control of its activity state (phosphorylated E1 is inactive (8)), thereby precisely regulating the flux of 2-carbon units entering the citric acid cycle. PDK is a major drug target since activation of PDC by limiting its phosphorylation has considerable potential for alleviating symptoms associated with impaired carbohydrate metabolism in severe diabetes.

The icosahedral core of mammalian PDC comprises 60 E2 enzymes and 12 copies of an E2-related protein, E3BP (9). Basic trimeric units of E2 form the 20 vertices of the icosahedron with E3BP bound in each of the 12 faces (10). Both E2 and E3BP have a similar, modular domain structure: human (h) E2 and E3BP consist of two (E2) or one (E3BP) N-terminal lipoyl domain(s) (LD) of ~80 amino acids. Each lipoyl domain carries a lipoic acid moiety covalently linked to a lysine residue situated at the tip of a type I β-turn. The lipoyl domain is followed by a subunit binding domain (SBD) of ~35 residues and a C-terminal domain of ~250 residues that is essential for core formation. In

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3 The abbreviations used are: PDC, pyruvate dehydrogenase; E1, pyruvate decarboxylase; E2, dihydrolipoamide acetyltransferase; E3, dihydrolipoamide dehydrogenase; PDK, PDC kinase; PDP, PDC phosphatase; h, human; LD, lipoyl domain; SBD, subunit binding domain; AUC, analytical ultracentrifugation; ITC, isothermal titration calorimetry; SAXS, small angle x-ray scattering; SV, sedimentation velocity; SE, sedimentation equilbrium.
E2 the C-terminal domain is also the catalytic domain, while the active site is absent from E3BP rendering it incapable of catalyzing the acetyltransferase reaction. All domains are interconnected by Ala- and Pro-rich linker regions of ≈30 amino acids in length that impart the flexibility necessary for the lipoyl domains to visit all three active sites during catalysis.

In eukaryotes the E2:E3BP core of PDC provides the structural and mechanistic framework for the tight but non-covalent association of the heterotetrameric E1 and homodimeric E3 enzymes. The stability and overall subunit organization of the complex are governed by specific and tight protein-protein interactions between E2-SBD and E1 and E3BP-SBD and E3, respectively. However, PDCs from Gram-positive bacteria also have icosahedral cores but lack E3BP. Consequently, E1 and E3 have to compete for overlapping binding sites on E2; interaction of E2 with either E1 or E3 prevents complex formation with the other (11). Interestingly, PDC-deficient patients who are totally lacking the E3BP subunit possess partial complex activity (10–20% of controls) (8), apparently because the SBD of E2 has retained a limited ability to mediate low affinity E3 binding.

In Bacillus stearothermophilus PDC, E1 and E3 both bind to the SBD of E2 with 1:1 ratios (11–15). B. stearothermophilus E3/E2-SBD and E1/E2-SBD crystal structures show unequivocally that association of a second E2-SBD to E1 or E3 is impossible. In the case of E1 the binding site for E2-SBD is located across the 2-fold axis (16), whereas the binding site on E3 is close to the 2-fold axis of symmetry (17). Occupation of both binding sites on E3 would result in steric clashes in one of the loop regions (17). A similar arrangement in the crystal structure of human (h) E3 bound to E3BP-SBD has been reported very recently (18, 19).

Interestingly, however, the reported ratios for each of the constituent enzymes of mammalian PDC suggest the possibility of formation of 2:1 stoichiometric subcomplexes in this case. Thus, at maximal occupancy, 30 E1 molecules can associate with the 60-meric E2 core while 12 E3BP molecules associate with 6 E3 dimers (9).

Overexpressed hE3BP forms large aggregates and is unsuited to biophysical studies. Instead, we investigated the interaction of E3 with two different E3BP constructs consisting of the LD and/or SBD(s) (X di-domain (XDD) and X subunit binding domain (XSBD), respectively). A range of biochemical and biophysical approaches including native PAGE, analytical ultracentrifugation (AUC) and isothermal titration calorimetry (ITC) were used to show cross-bridge formation of hE3 with its cognate SBD in solution. A low resolution structure for this subcomplex was obtained by small angle x-ray scattering (SAXS).

EXPERIMENTAL PROCEDURES

Plasmid Construction—The construction of vectors used for the overexpression of N-terminally His-tagged human E3 and truncated versions of E3BP will be described elsewhere. Briefly, full-length hE3 and residues 59–216 of mature hE3BP were cloned into pET14b (Novagen). Residues 166–230 of hE3BP were cloned into pET30a (Novagen). The E3BP constructs comprise either both the lipoyl and subunit binding domains or the SBD on its own and are referred to as the XDD or XSBD, respectively, throughout.

Recombinant Proteins—E3, XSBD, and XDD were overexpressed in Escherichia coli BL21 DE3 pLysS (Stratagene). Cells were grown in LB to an $A_{600}$ of 0.6–0.8 and induced with 1 mM IPTG for 3–5 h at 30 °C. At induction, lipoic acid to a final concentration of 50 μg/ml was added to XDD cultures to maximize lipoylation. Cells were harvested by centrifugation at 15,300 × $g$ for 10 min and lysed in 20 mM Hepes buffer, pH 8.0, supplemented with Complete® EDTA-free protease inhibitors (Roche Applied Science) using the Bugbuster® kit (Novagen) according to manufacturer’s instructions. Insoluble cell debris was pelleted by centrifugation at 15,300 × $g$ for 15 min.

All proteins were purified using immobilized Zn$^{2+}$-chelate chromatography on a BioCAD 700E work station (Applied Biosystems). A 20MC column was preloaded with 0.1 M ZnCl$_2$ and equilibrated using binding buffer (20 mM Hepes, 10 mM imidazole, pH 8.0). Bound protein was washed with binding buffer and eluted in a linear 10–500 mM gradient of imidazole in 100 mM NaCl, 20 mM Hepes, pH 7.0. E3 was further purified by gel filtration on a Sephacryl S-300 column (Amer sham Biosciences) in 2 mM EDTA, 50 mM Tris–HCl, pH 7.5. DNA contamination of XSBD and XDD preparations was removed using HQ anion exchange chromatography. XSBD and XDD were buffer exchanged into 20 mM Tris–HCl, pH 7.5, loaded onto the column and following an extensive wash step eluted in a linear 0–1 M NaCl gradient. The final step of XSBD and XDD purification was gel filtration on Sephacryl S-100 (XSBD) and S-300 (XDD) columns (Amer sham Biosciences), respectively, in 2 mM EDTA, 50 mM Tris–HCl, pH 7.5.

XDD/E3 complex was reconstituted from the purified proteins at a stoichiometric ratio of 3:1 (XDD:E3) using a final size exclusion step on Sephacryl-S300 in 2 mM EDTA, 50 mM Tris–HCl, pH 7.5, to remove unbound XDD. The protein concentrations of XSBD and XDD were determined using their calculated extinction coefficients at 280 nm of 9,800 and 11,380 M$^{-1}$ cm$^{-1}$, respectively. E3 was quantitated using FAD absorbance at 450 nm ($ε = 22,600$ M$^{-1}$ cm$^{-1}$).

Native Polyacrylamide Gel Electrophoresis—Mixtures of XSBD:E3 at ratios from 4:1 to 1:3, and XDD:E3 from 10:1 to 1:3, were prepared, keeping the amount of E3 used constant at 160 pmol. The samples were incubated at 25 °C for 15 min, and 5–10 μg of protein were loaded onto Tris-glycine gels (5% stacking, 8% resolving gel) and subjected to native PAGE. Gels were stained with Coomassie Brilliant Blue R-250.

Analytical Ultracentrifugation—Sedimentation velocity (SV) experiments were conducted at 4 °C in a Beckman Coulter Optima XL-I analytical ultracentrifuge (Palo Alto, CA) using an An-60 Ti rotor. A rotor speed of 45,000 rpm was selected, and interference optics scans were collected every minute until sedimentation was complete. The concentration of E3 in the XDD:E3 mixtures was fixed at 4.9 μM. The XDD concentrations were adjusted to achieve XDD:E3 ratios of 4:1 to 1:3. Samples (380 μl) were loaded into 12-mm charcoal-filled epon double sector centripieces. SV analysis of the purified XDD/E3 complex was conducted as described above, covering a concentration range of 6–44 μM. The data were analyzed using the program SEDFIT (20), which allows the subtraction of radial and
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time-independent noise and direct modeling of the sedimentation boundary as a continuous distribution of discrete, non-interacting species (c(s) analysis). Sedimentation coefficients were further evaluated using finite element analysis in SEDFIT, which finds the sedimentation coefficients that best fit the Lamm equation. Buffer density and viscosity as well as protein partial specific volumes were calculated using the program SEDNTERP (21).

Sedimentation equilibrium (SE) analysis of XDD:E3 stoichiometric mixtures was conducted using the same samples analyzed by SV at rotor speeds of 8,500, 12,000, and 16,000 rpm and the base line determined by overspeeding to 49,000 rpm. 20 replicate data sets were recorded using absorbance optics with a step size of 0.001 cm. Samples (30 μl) were loaded into 3-mm double sector charcoal-filled epon centerpieces. Attainment of equilibrium was ascertained with WinMATCH until no net movement of protein was observed in scans recorded 3 h apart.

SE data were analyzed using global analysis in the Beckman XL-A/XL-1 software implemented in MicroCal ORIGIN. SE analysis of purified XDD/E3 complex was carried out as described above using rotor speeds of 10,500, 15,000, and 18,000 rpm. The data were analyzed as before but included fitting of the second virial coefficient, B. A starting estimate for B of XDD/E3 was calculated using the program COVOL (22) based on molecule dimensions and surface charge. The values of M_{w,app} and B at infinite dilution were determined by plotting 1/M_{w,app} against concentration (in g/ml), where M_{w,app} is the inverse of the y intercept and B can be calculated from the slope.

**Microcalorimetry**—ITC measurements were carried out in a VP-ITC microcalorimeter (Microcal Inc., Northampton, MA) at 25 °C as described by Jung et al. (13). All proteins were dialyzed overnight in 25 mM Tris-HCl, pH 7.0. E3 at a concentration of 40.7 μM was injected in 10-μl aliquots into the reaction cell containing 6.2 μM XDD. Data were analyzed using non-linear regression in the MicroCal ORIGIN software package, assuming a simple binding model.

**Small Angle X-ray Scattering**—SAXS experiments were performed on beamline X33 of the EMBL outstation Hamburg at storage ring DORIS III of the Deutsches Elektronen Synchrotron (DESY). The scattering curves of purified XDD/E3 complex at concentrations of 1.5, 6.3, 9.6, and 22.6 mg/ml were recorded at 10 °C using a sample-to-detector distance of 2.6 m, covering a range of momentum transfer of 0.01 < s < 0.42 Å^{-1} (s = 4π sin θ/λ, where 2θ is the scattering angle and λ = 1.5 Å, the wavelength of the x-rays). The data were normalized to the incident beam and corrected for the detector response. The scattering of the buffer was subtracted and the difference curves scaled for concentration using the program PRIMUS (23). The final scattering curve was obtained by merging the low angle region of the low concentration curve with the high angle region of the high concentration curve to eliminate possible interparticle interaction effects in the low angle region of the high concentration data. The maximum dimension, D_{max} and particle distance distribution function p(r) were obtained by indirect Fourier transformation using the program GNOM (24, 25). The radius of gyration, R_g was obtained by employing both the Guinier approximation and GNOM. Ab initio shape reconstruction was done using the programs DAMMIN (26) and GASBOR (27, 28) without imposing symmetry restraints. DAMMIN restores the structure as a collection of densely packed beads in a dummy atom model inside a search volume (a sphere with diameter D_{max}). The program starts from a random configuration and uses a simulated annealing algorithm to explore the landscape of acceptable low resolution structures. GASBOR represents particles as collections of dummy residues using one dummy residue per amino acid residue. The dummy residues are randomly distributed within the search volume and simulated annealing is used to obtain a chain-compatible spatial distribution. Ten GASBOR models were superimposed and averaged, and the resulting Protein Data Bank file used as search space for a final DAMMIN reconstruction.

**Homology, Hydrodynamic, and Rigid Body Modeling**—A homology model of the human XDD/E3 complex was built using SWISSMODEL (29, 30) based on the structures determined by protein crystallography or NMR for E3/E2-SBD from B. stearothermophilus (Protein Data Bank ID 1EBD) (17), E3 from Saccharomyces cerevisiae (Protein Data Bank ID 1EH) (31), and human E2-LD (Protein Data Bank ID 1FYC) (32). Models of XDD/E3 with lipoyl domains in different positions were generated (see “Results”) and their sedimentation coefficients calculated using the program HYDROPRO (33, 34). HYDROPRO calculates the hydrodynamic parameters of rigid particles from their atomic structures. For rigid body modeling with the program Rayuela (35), the complex was defined by five “domains”: E3, XSBD1, XSBD2, XLD1, and XLD2. Rayuela allows translation and rotation of each individual domain, calculates a SAXS curve as well as sedimentation coefficient for each structure, and compares it with the experimental data. Using a simulated annealing algorithm, it determines the lowest energy conformation for each domain. The best model obtained with Rayuela, as judged by its fit to the experimental data, was used for superimposition onto the ab initio model using the program SITUS (36, 37).

**RESULTS**

**Native Polyacrylamide Gel Electrophoresis**—Initial binding studies were carried out by mixing either XSBD or XDD with E3 at different molar ratios and subjecting them to native PAGE. The addition of XSBD or XDD to E3 results in a noticeable bandshift, which increases as more XSBD or XDD is added. At a stoichiometry of 2:1 all of the E3 present is shifted into the lower mobility band corresponding to the XSBD/E3 (Fig. 1A) or XDD/E3 (Fig. 1B) complex. At ratios lower than 2:1, excess E3 is seen (Fig. 1), while at stoichiometries higher than 2:1, excess E3 remains (Fig. 1B). At the pH used to conduct native PAGE (pH 8.8), XSBD moves toward the cathode and thereby out of the gel due to its high pl of 9.9. No intermediates in complex formation were observed.

**Analytical Ultracentrifugation**—SV experiments were conducted for uncomplexed E3 and XDD as well as for different XDD:E3 stoichiometries and the sedimentation profiles fitted using c(s) analysis in SEDFIT (20). The c(s) profiles of free XDD and E3 show single peaks with apparent sedimentation coefficients of 2.0 and 5.9 S, respectively (Fig. 2A). Fig. 2B clearly shows the complete disappearance of the XDD peak at a stoi-
chiometry of 2:1. The E3 and XDD/E3 peaks overlap completely, although the peak position is slightly reduced with an apparent sedimentation coefficient of 5.7 S. A globular protein of higher molecular weight is expected to sediment faster (and therefore exhibit a higher sedimentation coefficient) than a protein of lower molecular weight. Therefore, it can be inferred that the complex has an extended shape which offsets the gain in molecular weight with respect to free E3 and causes it to sediment at the same speed as E3. Similarly, the elongated shape and thus the large hydrodynamic radius of XDD/E3 can also be inferred from gel filtration chromatography (supplemental Fig. 1). Its apparent molecular mass, $M_{w,app}$, of 300 kDa on a standardized column is considerably higher than predicted from the amino acid sequence (144 kDa).

The minor broad peak around 7.5–8 S observed at all ratios corresponds to aggregate species and is greatly reduced if the complex is purified to uniformity by gel filtration prior to AUC analysis (Fig. 2C). c(s) analysis of SV profiles from the purified complex also shows that a single species is present in solution.
bar a small amount of aggregate that is present at high concent-
trations only, a result substantiated by native PAGE (results not
shown). No free protein or intermediate is observed at any con-
centration, in accordance with the expected tight interaction
between XDD and E3. Finite element analysis of SV data with a
single-species model yielded the true sedimentation coeffi-
cients for XDD/E3 at all experimental concentrations. These
were extrapolated to infinite dilution to give a sedimentation
coefficient for XDD/E3 independent of concentration of
$$s_{20,w}^0/ H_{11005} = 5.68 \pm 0.05 S$$ (Fig. 2).

The apparent average whole cell molecular weight, $$M_{w,app}$$,
was determined for each XDD:E3 stoichiometric mixture using
sedimentation equilibrium (SE). The $$M_{w,app}$$ peaks, as expected,
at a stoichiometry of 2:1 (Fig. 3) when all protein present is
associated into complex, due to the tight interaction between
XDD and E3. At all other molar ratios, free XDD or E3 is
observed, thus depressing $$M_{w,app}$$. The value of $$M_{w,app}$$ at a
molar ratio of 2:1 (XDD:E3) of 137 kDa is lower than expected
when compared with the calculated molecular mass of 144 kDa.
This is attributable to the non-ideal behavior of the complex. SE
data for XDD/E3 complex purified by gel filtration were also
analyzed: agreement with the expected molecular mass was
substantially improved when non-ideality arising from molecu-
lar shape and surface charge was accounted for by inclusion of
the second virial coefficient ($$B$$) in the data analysis. The values
obtained experimentally for $$B$$ (7.2 x 10^{-4}, 1.7 x 10^{-5}, and
2.9 x 10^{-2} ml g^{-2} for rotor speeds 10,500, 15,000 and
18,000 rpm, respectively) correspond very well to $$B$$ calculated
with COVOL (22) of 1.7 x 10^{-4} ml g^{-2}.

When non-ideality is accounted for the molecular weight
estimates for XDD/E3 are 151, 142, and 141 kDa for rotor
speeds 10,500, 15,000 and 18,000 rpm, respectively (results not
shown). These values compare very well to the calculated
molecular mass of 144 kDa for a 2:1 complex of XDD:E3. When
non-ideality was included in the data analysis for the 2:1 stochi-
ometric XDD:E3 mixture, $$M_{w,app}$$ was increased to 147, 142,
and 141 kDa for rotor speeds 8,500, 12,000, and 16,000 rpm,
respectively, in excellent agreement with the results for the
purified complex and the calculated molecular weight. For all
other stoichiometric mixtures of XDD:E3, $$M_{w,app}$$ decreased
with increasing rotor speed which is indicative of the presence
or formation of high molecular weight aggregate, as shown also
by the SV results in Fig. 2B. Only for the 1:1 mixture was
$$M_{w,app}$$ consistently lower than the calculated molecular weight. This
was due to the choice of fitting limits: the aggregate was steeply
distributed and thus effectively excluded from the subsequent
analysis, resulting in an underestimate of $$M_{w,app}$$. Because sto-
chiometric mixtures other than 2:1 contained more than one
species (plus aggregate), the exponential describing the heavier
component was disproportionately affected by the inevitable
loss of data from the cell base, resulting in a corresponding
reduction in $$M_{w,app}$$.

Isothermal Titration Calorimetry—The heats of interaction
of XDD with E3 were determined using ITC. Complex forma-
tion is exothermic (i.e. negative peaks in the ITC output) (Fig.
4A). Differential thermal binding curves were obtained by integration of the data and analyzed using standard non-linear regression to obtain estimates of the binding stoichiometry, equilibrium association constant, and enthalpy of binding (Fig. 4B). The data confirm the tight binding of XDD to E3 ($K_a = 2.8 \times 10^7 \text{M}^{-1}$) with a stoichiometry of 2:1. Binding is characterized by a large, favorable enthalpy change ($\Delta H = -12.1 \text{kcal/mol}$) and a small negative entropy change ($T\Delta S = -1.7 \text{kcal/mol}$). This experiment was confirmed by similar ITC analysis of full-length E3BP-E3 interaction (data not shown), which shows that full-length E3BP binds E3 with similar affinity and identical stoichiometry.

Homology and Hydrodynamic Modeling—Homology modeling was used in combination with AUC and SAXS (see below) to gain more insight into the XDD/E3 structure. A model of the human XDD/E3 complex was built from homology models (generated with SWISSMODEL (29, 30)) for E3, E3BP lipoyl, and subunit binding domains. The crystal structure of hE3 was solved very recently (38) (Protein Data Bank ID 1ZMC). Superimposition of these coordinates with those of the homology model gave a very low rms deviation of 0.6 Å; the two structures are almost identical, differing primarily in the NAD domain. The structure of the B. steaerothermophilus E2-SBD/E3 complex (17) was used to position the human E3 and XSBD homology models with respect to each other. The second XSBD molecule was positioned exploiting the E3 2-fold symmetry axis. Several models with different positioning of the two lipoyl domains were generated, while taking into account available biochemical and structural data. In an extended conformation, both LD1 and LD2 were located ~65 Å from the SBDs, in accordance with previous electron micrograph data (10, 39, 40) and overall PDC dimensions. The resulting model XDD/E3$_{\text{extrn}}$ has a maximum dimension, $D_{\text{max}}$, of 227 Å. The lack of structural information for part of the linker region and the paucity of related sequences meant that it was not possible to model this section of the linker using either homology or secondary structure modeling. A second complex model with both lipoyl domains docked in the E3 active sites and a $D_{\text{max}}$ of 134 Å (XDD/E3$_{\text{dock}}$) was also generated. In the process Lys97 was positioned 14 Å from E3 active site residues Cys45 and Cys50, accommodating the lipoate cofactor linked to Lys97 (32), in agreement with data from mutation studies on E3 mechanism (41, 42). Although other E3 residues involved in the binding of the lipoyl domain are not known, access to the E3 active site is only possible from one side of the molecule. The FAD and NAD$^+$ cofactors otherwise obstruct the interaction between the lipoamide and the cysteine pair (31). An intermediate model was generated with one lipoyl domain present in the extended conformation and the second docked in the active site (XDD/E3$_{\text{int}}$, $D_{\text{max}} = 156$ Å). Sedimentation coefficients of 5.3, 5.9, and 6.5 S were calculated for XDD/E3$_{\text{extrn}}$, XDD/E3$_{\text{int}}$, and XDD/E3$_{\text{dock}}$, respectively, using the program HYDROPRO (33, 34). Accordingly, the XDD/E3$_{\text{int}}$ model with one docked and one extended lipoyl domain gave the best agreement with the experimentally determined sedimentation coefficient of 5.68 S and also agrees best with the experimentally determined $D_{\text{max}}$ of 158 Å (see below).

Small Angle X-ray Scattering and Rigid Body Modeling—SAXS curves for purified XDD/E3 complex (Fig. 5A) were acquired at three different protein concentrations to account for the effects of interparticle interference. The radius of gyration $R_g$ determined using the Guinier approximation was $38 \pm 1$ Å. The particle distribution function, $p(r)$ (Fig. 5B) gave a $D_{\text{max}}$ of 158 ± 5 Å and $R_g = 41 \pm 1$ Å, which agrees well with the value obtained using the Guinier approximation. Ab initio shape restoration produced an elongated, asymmetric structure (Fig. 6). Rigid body modeling was used in combination with ab initio modeling to analyze the structure of the XDD/E3 complex: using translations and rotations, the lowest energy conformation of each “domain” (E3, XSBD1, XSBD2, XLID1, XLID2) within the complex was determined using the program Rayuela (35) as judged by fits to the SAXS data. This model was superimposed upon the ab initio model (Fig. 6). While most of the complex model fits well into the SAXS envelope, it was not possible to fit residues 265–297, which form part of the E3 NAD domain. The differences in structure between human E3, its yeast counterpart, and our homology model are not significant enough to alter the fit of the high resolution structure within the SAXS envelope. However, E3 is thought to adopt a more
DISCUSSION

We have shown for the first time using a number of independent approaches that two E3BP-derived molecules interact with a single E3 homodimer, thus enabling the formation of 2:1 stoichiometric “cross-bridges” in human PDC. These findings are in direct contrast to the crystal structures obtained for the B. stearothermophilus E3/E2SB (17) and hE3/E3BP-SBD complexes (18, 19) where only one SBD is associated with E3 due to steric hindrance by a loop in E3BP which prevents binding of a second SBD. Comparison of the crystal structures determined for unbound and bound hE3 and yeast E3 (16, 31, 38) yields only very small differences in coordinates. The highly homologous (54% identical) hE3 and yeast E3 may crystallize similarly. As our results were determined in solution we propose that either crystal packing and/or crystallization buffer compositions affect the structures determined for eukaryotic E3 and E3/E3BP-SBD complex. Unfortunately, E3 is too large for structure determination via NMR spectroscopy. However, unpublished SAXS data from our laboratories suggest that the solution conformation of free hE3 is more elongated ($D_{\text{max}} = 130 \AA$) and flattened than that in the crystal structure. The solution conformations of other proteins, including yeast pyruvate decarboxylase (43), can differ significantly from their crystal structures (44–49). The highly homologous (54% identical) hE3 and yeast E3 may crystallize similarly.

Since 60 E2s can bind up to 30 E1 heterotetramers at maximum occupancy, we speculate that similar E2/E1 cross-bridges form at the edges of the icosahedral core. Indeed, recent AUC and ITC analyses support this hypothesis (data not shown).

Current concepts of PDC subunit architecture envisage the E2:E3BP core forming the structural and mechanistic framework for the assembly to which the E1 and E3 enzymes bind tightly but non-covalently. The peripherally bound E1 and E3 enzymes are precisely positioned and oriented around its surface to facilitate optimal interaction of their active sites with the lipoyl “swinging arms” of E2/E3BP during the catalytic cycle. In this model, E1 and E3 interactions with the “core” assembly are

\[ \text{model of E2 and E3BP within the human core has been proposed more recently by Hiromasa et al. (50): the authors suggested that E3BP replaces some of the E2 molecules, resulting in a core formed from 48 E2 molecules and 6 E3BP dimers. However, our model of cross-bridge formation is compatible with both models for the organization of the core assembly, although in the case of a substitution model for the core assembly the E3BP/E3 cross-bridges would obviously also be orientated along its edges. Studies to distinguish between the two different core models are currently underway in our laboratories.} \]

Since E3BP is thought to be located in the 12 faces of the icosahedral E2/E3BP core (10, 40), its 2:1 stoichiometric relationship with the dimeric E3 enzyme suggests the potential for the formation of cross-bridges across the core surface (Fig. 7). A substitution model of E2 and E3BP within the human core has been proposed more recently by Hiromasa et al. (50): the authors suggested that E3BP replaces some of the E2 molecules, resulting in a core formed from 48 E2 molecules and 6 E3BP dimers. However, our model of cross-bridge formation is compatible with both models for the organization of the core assembly, although in the case of a substitution model for the core assembly the E3BP/E3 cross-bridges would obviously also be orientated along its edges. Studies to distinguish between the two different core models are currently underway in our laboratories.

Since E3BP is thought to be located in the 12 faces of the icosahedral E2/E3BP core (10, 40), its 2:1 stoichiometric relationship with the dimeric E3 enzyme suggests the potential for the formation of cross-bridges across the core surface (Fig. 7). A substitution model of E2 and E3BP within the human core has been proposed more recently by Hiromasa et al. (50): the authors suggested that E3BP replaces some of the E2 molecules, resulting in a core formed from 48 E2 molecules and 6 E3BP dimers. However, our model of cross-bridge formation is compatible with both models for the organization of the core assembly, although in the case of a substitution model for the core assembly the E3BP/E3 cross-bridges would obviously also be orientated along its edges. Studies to distinguish between the two different core models are currently underway in our laboratories.

4 M. Smolle, O. Byron, and J. G. Lindsay, unpublished data.
Another important aspect of PDC function that is influenced by its subunit organization is regulation of its activity state by an intrinsic PDK and loosely associated phosphatase (PDP). It is reported that only 1–3 molecules of PDK are bound per complex (51). Both regulatory enzymes associate with the inner E2 lipo domain whereby the lipo domain of the lipo domain provides an important part of the recognition site. PDP and PDK both act by de/phosphorylation of E1 on any of three specific E1α serine residues. Phosphorylation of E1 renders the complex inactive. Since only one molecule of PDK is present per complex, the enzyme has to migrate around the surface of the complex to act on the entire population of bound E1 molecules. A hand-over-hand mechanism has been proposed where the dimeric PDK molecule migrates from one inner lipo domain to its nearest neighbor (52). Regular spacing of the E2 lipo domains linked by a system of E1 cross-bridges could greatly facilitate this type of movement and provide convenient access to all E1 molecules.

The low resolution x-ray scattering structure determined for the human XDD/E3 subcomplex conclusively verifies its 2:1 stoichiometry and high stability. Unexpectedly, the subcomplex proved to be highly asymmetric. These data are compatible with a model in which one lipo domain is peripherally extended away from the E3 dimer, whereas the second lipo domain is docked into one of the E3 active sites that are located at the monomer–monomer interface.

During XDD purification, the lipo group was maintained in its dithiol form by isolation in the presence of DTT. Reduced lipoate is the true substrate for the E3 enzyme, and its reoxidation by E3 was prevented by the absence of NAD+. The redox state of the lipoamide cofactor was confirmed by modification with PEG 5000 maleimide, a sulfydryl group reagent, that leads to a dramatic increase in the apparent molecular weight of XDD, provided it is present in its reduced form (data not shown). Current studies focus on (a) the influence of the redox state of the lipoamide prosthetic group on the overall conformation of the XDD/E3 subcomplex, (b) refining our biophysical and structural analysis of the E2DD/E1 subcomplex, and (c) assessing the implications of this previously unrecognized architectural feature on PDC function.

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