How Dihydrolipoamide Dehydrogenase-binding Protein Binds Dihydrolipoamide Dehydrogenase in the Human Pyruvate Dehydrogenase Complex*

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The dihydrolipoamide dehydrogenase-binding protein (E3BP) and the dihydrolipoamide acetyltransferase (E2) component enzyme form the structural core of the human pyruvate dehydrogenase complex by providing the binding sites for two other component proteins, dihydrolipoamide dehydrogenase (E3) and pyruvate dehydrogenase (E1), as well as pyruvate dehydrogenase kinases and phosphatases. Despite a high similarity between the primary structures of E3BP and E2, the E3-binding domain of human E3BP is highly specific to human E3, whereas the E1-binding domain of human E2 is highly specific to human E1. In this study, we characterized binding of human E3 to the E3-binding domain of E3BP by x-ray crystallography at 2.6-Å resolution, and we used this structural information to interpret the specificity for selective binding. Two subunits of E3 form a single recognition site for the E3-binding domain of E3BP through their hydrophobic interface. The hydrophobic residues Pro133, Pro154, and Ile157 in the E3-binding domain of E3BP insert themselves into the surface of both E3 polypeptide chains. Numerous ionic and hydrogen bonds between the residues of three interacting polypeptide chains adjacent to the central hydrophobic patch add to the stability of the subcomplex. The specificity of pairing for human E3BP with E3 is interpreted from its subcomplex structure to be most likely due to conformational rigidity of the binding fragment of the E3-binding domain of E3BP and its exquisite amino acid match with the E3 target interface.

The human pyruvate dehydrogenase complex (PDC)‡ with an approximate molecular mass of 8 × 106 Da consists of multiple copies of three catalytic enzymes known as pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2), and dihydrolipoamide dehydrogenase (E3) as well as the dedicated E3-binding protein (E3BP) (1). In addition, pyruvate dehydrogenase kinase and phosphatase interacting with the complex are responsible for regulation of PDC activity by a reversible phosphorylation/dephosphorylation mechanism that involves covalent modification of E1. PDC plays a key role in regulation of the flux of two-carbon units from pyruvate through acetyl-CoA into the Krebs cycle, yielding CO2, NADH, and H+.

The E1 component catalyzes the decarboxylation of pyruvate and the reductive acetylation of the lipoyl moieties of E2. The E2 component transfers the acetyl group to CoA. The E3 component oxidizes the reduced lipoyl moieties through reduction of NAD+ to NADH, thus preparing the lipoyl moiety for another cycle of catalytic reaction. Along with E3BP, the human E2 component forms the structural core of PDC and provides the binding site for E1 (2). E3BP (previously known as protein X) provides primarily the binding site for E3 (3, 4). In the absence of E3BP, PDC catalysis is supported at a rate of 4% only (5, 6). Both E2 and E3BP share considerable sequence identity (37%) as revealed in pairwise sequence alignment (7). The principal differences are that mammalian E3BP has a single lipoyl domain compared with two lipoyl domains in E2 and lacks the conserved active-site histidine residue that is essential for acetyltransferase activity (5, 8). These two proteins are of particular importance for selective binding of E1 and E3 to higher eukaryotic PDC core structures.

The tertiary structures of various components of prokaryotic PDC have been elucidated, including E1 from Escherichia coli (9); E1 with the lipoyl and E1/E3-binding domains of E2 from Bacillus stearothermophilus (10), the cubic E2 core from Azotobacter vinelandii (11), the pentagonal dodecahedron core from B. stearothermophilus (12), the lipoyl domain from E. coli (13), the lipoyl domain from A. vinelandii (14), the lipoyl domain from B. stearothermophilus (15), and the E1/E3-binding domain of E2 from B. stearothermophilus (16); E3 with the E1/E3-binding domain of the E2 subcomplex from B. stearothermophilus (17, 18); and E3 enzymes from A. vinelandii (19), Pseudomonas putida (20), and Pseudomonas fluorescens (21). On the other hand, structural reports of eukaryotic components have been limited to human E1 (22), an inner lipoyl domain of human E2 (23), E3 from Saccharomyces cerevisiae (24), recently reported human E3 (25), and an 8-Å resolution polyalanine chain structure apparently derived from pig E3 (26).

The human E3 component (474 amino acids) of PDC and a component of the branched-chain α-ketoacid dehydrogenase complex, the α-ketoglutarate dehydrogenase complex, and the glycine cleavage system are flavoenzyme oxidoreductases that contain a reactive disulfide bridge and a FAD cofactor that are directly involved in catalysis (27). Similar to bacterial E3, human E3 associates into tightly bound dimers...
required for enzymatic activity. In contrast, human E3BP (448 amino acids) is a monomeric and highly segmented structure consisting of three domains, viz. lipoyl (residues 1–80), E3-binding (residues 125–180), and inner (residues 215–448) domains connected by long flexible linkers (8). Although the three-dimensional structure of human E3BP has not been determined as yet, it has been suggested to be similar to that of the human E2 component (1, 8), which is also characterized by independent domains separated by long and flexible linkers.

We have determined the structure of human E3 and the E3-binding domain of E3BP to shed light on the molecular mechanism of the association of human E3 with E3BP. We found that overexpressed recombinant human E3BP aggregated into non-homogeneous assemblies, which prevented us from performing detailed crystallographic studies of its binding interactions with E3. Therefore, a construct of the didomain containing the lipoyl and E3-binding domains of E3BP (E3BPdd) without the inner domain was used to allow for mapping of interactions with E3. We have delineated the key contacts and structural elements that contribute to the specificity and stability of the E3-E3BPdd subcomplex and evaluated these results for features that appear to be responsible for the specific association of human E3 with E3BP.

EXPERIMENTAL PROCEDURES

Construction of Human E3, E3BP, and E3BPdd Expression Vectors—The coding sequence of human E3 (mature form) was subcloned into the pPROEX-1 vector for expression of human E3 protein with a His6 tag at its N terminus and then transformed into E. coli XL1-Blue cells. The coding sequence of human E3BP (mature form) was amplified from pPDHE2/E3BP by PCR with two primers of 5’- and 3’-ends including NotI preceding the first codon and XhoI following the last codon. The amplified DNA was ligated between the NotI and XhoI sites of expression vector pET-28b and then transformed into E. coli BL21(DE3) cells for expression. The expression vector for E3BPdd (amino acids 1–221) was constructed in the same way as that for E3BP. The DNA fragment coding for the His6 tag was added to the 3’-end of the E3BPdd coding sequence, and the complete coding sequence was verified by DNA sequencing. The resulting expression vector, pET-28b/E3BPdd, was transformed into BL21(DE3) cells for expression.

Expression and Purification of the E3-E3BPdd Subcomplex—Purification of E3 was performed as described previously (28). Purified E3 was then digested with recombinant tobacco etch virus protease to remove the His6 tag at the N terminus of the protein sequence, dialyzed, and concentrated to ~10 mg/ml.

For expression of E3BPdd, BL21(DE3) cells were grown in LB medium containing 30 μg/ml kanamycin at 37 °C until the absorbance at 600 nm reached 0.6–0.7 units. At this point, the cultures were supplemented with 0.2 mM lipoic acid and 1 mM isopropyl β-D-thiogalactopyranoside for overnight growth at 25 °C. Cells were harvested; resuspended in 50 mM potassium phosphate buffer (pH 7.5), 300 mM KCl, and 5 mM β-mercaptoethanol in the presence of protease inhibitors (0.2 mM phenylmethanesulfonyl fluoride and 0.2 mM benzamidine); and incubated with 1 mg/ml lysozyme for 30 min. The cells were then passed through a French press for disruption, and cell debris was removed. The supernatant was loaded onto a nickel-nitritolactacid acid affinity column. The column was washed with buffer A (50 mM potassium phosphate buffer (pH 7.5), 300 mM KCl, 5 mM β-mercaptoethanol, and 5% glycerol) and eluted with a linear gradient of 25–250 mM imidazole in buffer A. Fractions containing E3BPdd were dialyzed against 50 mM potassium phosphate buffer (pH 7.5) and 0.5 mM diethiothreitol and concentrated to ~10 mg/ml. The resulting E3BPdd preparation was ~95% pure as judged by densitometry of SDS-polyacrylamide gels.

Following separate purification protocols, E3 and E3BPdd were combined in a 4-fold molar excess over E3BPdd and incubated for 30 min at room temperature. Approximately 200 μg of combined E3 and E3BPdd in a 100-μl volume was applied to a size-exclusion chromatography column (Superdex HR-200) and eluted with 50 mM potassium phosphate buffer (pH 7.5) and 150 mM NaCl. At this time, elution peaks were collected separately and analyzed by SDS-PAGE. Only material from the elution peak containing two protein bands corresponding to the E3-E3BPdd subcomplex was concentrated to ~15 mg/ml before being used in crystallization experiments.

Crystallization, Structure Determination, and Refinement—Crystals were grown from droplets of 2 μl of protein solution at 15 mg/ml and 2 μl of reservoir solution containing 10–20% polyethylene glycol 6000, 200 mM diammonium citrate, and 1 mM sodium azide. Droplets were equilibrated against 0.5 ml of reservoir solution at a temperature of 293 K. X-ray diffraction data were collected using a 1.1-Å wavelength at beamline X25 at the Brookhaven National Laboratory at a temperature of 100 K from crystals soaked prior to cryocooling in reservoir solution containing additional 15% (v/v) glycerol. Diffraction data were processed using HKL2000 (29).

Determination of the structure of the E3-E3BPdd subcomplex was achieved by molecular replacement for determination of the structure of E3 and by de novo building of the E3BPdd structure based on the phases obtained from the E3 content. The search model for molecular replacement was a dimer of E3 together with a FAD cofactor constructed from A. vinelandii and yeast E3 structures (Protein Data Bank codes 3LAD and 1JEH) (20, 24, 30), which have 49 and 57% sequence identities to the human sequence, respectively. The orientation of the first E3 dimer (polypeptide chains A and B) was found with program AMoRe within the CCP4 program suite (31) and subsequently refined with the CNS program (32) using a maximum-likelihood simulated annealing protocol with non-crystallographic symmetry restraints. After a first E3 dimer was established, the iterative process of addition and refinement of four E3 dimers using the data from the 5.00 to 3.0-Å resolution range led to an overall R = 41.4% and Rfree = 41.6%. At this point, the total of five E3 dimers provided the phase information sufficient for beginning the determination of the structure of E3BPdd. As shown in Fig. 1 (A and B), the difference maps generated with the five E3 dimers showed a pattern of two parallel helices located close to the surface of each E3 subunit with three strands in the middle. Initially, the polyalanine model of the E3-binding domain of E3BP (helices α1 and α2) at one of the E3 subunits was refined and sequentially replaced with the complete sequence. Refinement of two E3-binding domains of E3BP (Fig. 1, C and D) at each E3 dimer led to superposition of residues 152–159 of the loop regions of the E3-binding domains of E3BP, large average B-values exceeding 100 Å² for nearly all residues, and no improvement in the refinement statistics. Instead, the electron density pattern was interpreted as representing two alternative positions of E3BPdd, each of them with half-occupancy. Subsequent model building with the XtalView program (33) and cycles of refinement with the CNS program (32) led to further improvements of the model and R = 33.4% and Rfree = 35.6%. The average B-factor for the E3-binding domains of

The portion of this work concerned with the determination of the structure of this subcomplex has been presented previously (Makal, A., Hong, Y. S., Potter, R., Vet-talkoromakankauv, A. K., Korotchkina, L. G., Patel, M. S., and Ciszak, E. M. (2004) Poster presented at the American Crystallographic Meeting, Chicago, IL (July 17–22, 2004), Collected Abstracts, p. 93).
E3BP dropped to 36 Å², a value close to the average $B$-value for the E3 structures (26 Å²) and reasonable for a small and loosely bound element of the subcomplex structure. Apart from the E3-binding domain of E3BPdd, there was no traceable electron density for the lipoyl domains due to disorder. Therefore, the process of protein structure determination was completed with the elucidation of polypeptide chains (A–J) of five dimers of E3, each of them binding two FAD cofactor molecules, and with the elucidation of nine polypeptide chains (K–O) corresponding to the E3-binding domains of E3BP. In four of five E3/E3BPdd subcomplexes, the E3-binding domains of E3BP were refined with 0.5 occupancy factors. The E3-binding domain of E3BP (chain O) bound to chains I and J of the E3 dimer was refined with a partial occupancy of 0.7. The final crystal structure of each E3/E3BPdd subcomplex contains coordinates for two complete E3 subunits (474 amino acids each) and 41 or 42 residues of E3BPdd for a total of 5256 amino acids, 10 FAD cofactor molecules, and 491 water molecules in the unit cell. The final steps of the refinement were conducted using REFMAC combined with the translation-libration-screw protocol within the CCP4 program suite (31), in which each subunit was assigned a separate anisotropic tensor. The final cycles of refinement resulted in $R = 20.8\%$ and $R_{\text{free}} = 27.5\%$. The Pro $^{453}$ residues in all E3 molecules were refined in cis-conformation. The side chains of Cys $^{50}$ in all E3 molecules were refined in two orientations representing the disulfide bridge at only half of the molecules. The break of the disulfide bridge likely resulted from radiation-induced damage to the disulfide bond incurred during the exposure. Analysis of the geometry of subunits with PROCHECK within the CCP4 program (31) showed that 86.1% of the residues are in the most favorable regions of the Ramachandran plot, with an additional 13.9% located in additional allowable regions. The cumulative statistics of data collection and refinement are shown in Table 1.
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The values shown in parentheses correspond to the highest resolution range extending from 2.68 to 2.60 Å. R.m.s.d. root mean square deviation.

| Data collection statistics | Resolution range (Å) | 50.00 to 2.60 (2.68 to 2.60) |
|----------------------------|----------------------|-------------------------------|
| Wavelength (Å)             | 1.1000               |                               |
| Space group                | P2₁2₁2₁              |                               |
| Cell dimensions (Å, °)     |                       |                               |
| a                          | 168.8                | 90.0                          |
| b                          | 186.9                |                               |
| c                          | 217.5                |                               |
| α = β = γ                  | 0.275                |                               |
| No. unique reflections     | 188,229 (12,561)     |                               |
| Completeness (%)           | 88.4 (59.5)          |                               |
| Redundancy                 | 3.3 (2.0)            |                               |
| Mosaicity                  | 0.035                |                               |
| R<sub>free</sub>           | 0.164 (0.357)        |                               |

Summary of data collection, reduction, and refinement

The E3BP subcomplex of PDC, recombinant human E3 and a 221-residue didomain fragment of human E3BP that contains the lipoyl and E3-binding domains of E3BP were separately expressed, purified, and incubated for crystallization experiments. The crystalline subcomplex was obtained from the solution containing both proteins at a subunit molar ratio of 2:1, thus corresponding to one E3 dimer/E3BPdd molecule. The same stoichiometry of E3 binding to E3BP was determined previously (2). The three-dimensional structure of the subcomplex was determined by x-ray crystallography using a combination of molecular replacement for the elucidation of the E3 structure and de novo building of the E3BPdd structure based on the phases obtained from the E3 content. The unit cell was composed of five E3-E3BPdd subcomplexes, each consisting of two subunits of E3 and one E3BPdd. Because no residues in the lipoyl domain of E3BPdd could be located in the electron density maps, we focused this study on the analysis of the E3 component and the E3-binding domain of E3BP of the subcomplex. Fig. 2 shows the structure of one of these subcomplexes (subunits G, H, and N as deposited in the Protein Data Bank (30) and Protein Data Bank code 1ZY8). The overall root mean square deviations in the family of subcomplexes are small and range between 0.38 and 0.46 Å for C<sup>a</sup> atoms.

Overall Structure of Human E3 in the E3-E3BPdd Subcomplex—The biologically active form of E3 is a dimer in which the subunits are associated by a 2-fold symmetry axis. Human E3 in this study presents an α/β-type structure (34) that is similar to those of unbound E3 with and without NAD<sup>+</sup> substrate (22); bacterial and yeast E3 enzymes (16–18); and fragments of other homodimeric flavoenzymes, including thioredoxin reductase (35), glutathione reductase (36), trypanothione reductase (37), p64k protein (38), and E3 from the glycine decarboxylase multienzyme complex (39). The overall root mean square deviations between the structures of known E3 enzymes (C<sup>a</sup> atoms) are in the range of 0.65–1.2 Å. The major differences are between the human and bacterial structures in the loop regions following the Lys<sup>37</sup> and Ala<sup>186</sup> using human E3 numbering. In these regions, E3 sequences from A. vinelandii, P. fluorescens, and the glycine cleavage system contain additional inserts of six to eight amino acids that extend the loops on the surface.

Like all E3 structures, the human E3 subunit folds into four domains, viz. the FAD-binding domain (residues 1–150), the NAD-binding domain (residues 151–280), the central domain (residues 281–350), and the interface (C-terminal) domain (residues 351–474). The FAD-binding domain interacts with the other three domains; together with the similar NAD-binding domain, the FAD-binding domain comprises nearly two-thirds of the residues, and both present the layered β/α/β-type fold characteristic of nucleotide-binding proteins. Their secondary structures have the characteristic nucleotide-binding motifs consisting of a β-strand, a short linker, and a helix with a glycine-rich amino acid sequence (Gly-X-Gly-X-Gly) (40). In human E3, the nucleotide sequence motifs start at residue 13 (Gly<sup>13</sup>-Ser-Gly<sup>15</sup>-Pro-Gly-Gly<sup>18</sup>) in the FAD-binding domain and at residue 186 (Gly<sup>186</sup>-Ala-Gly<sup>189</sup>-Val-Ile-Gly<sup>192</sup>) in the NAD-binding domain. A cavity between the FAD- and NAD-binding domains, which normally hosts the NAD<sup>+</sup> substrate, leads straight to the α-side of the isoalloxazine ring of the FAD molecule. When entering the cavity, the NAD<sup>+</sup> moiety is guided by the glycine-rich NAD-binding motif, which likely facilitates the positioning of the nicotinate amide planar ring parallel to the FAD planar aromatic ring providing for the π-π interactions. This similarity among all known E3 structures allows us to expect the same type of NAD<sup>+</sup> binding to occur in human E3 bound to the PDC core.

The central domain connects with the interface (C-terminal) domain through a eight-residue loop (residues 343–350) (Fig. 2). Notably, this loop forms contacts with the E3-binding domain of E3BP of the E3-E3BPdd subcomplex. The interface domain of E3 itself presents an

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| Mosaicity                  | 0.035                |                               |
| R<sub>free</sub>           | 0.164 (0.357)        |                               |

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RESULTS AND DISCUSSION

Structure Determination—To characterize the molecular structure of the binding in the human E3-E3BP subcomplex of PDC, recombinant human E3 and a 221-residue didomain fragment of human E3BP that contains the lipoyl and E3-binding domains of E3BP were separately expressed, purified, and incubated for crystallization experiments. The crystalline subcomplex was obtained from the solution containing both proteins at a subunit molar ratio of 2:1, thus corresponding to one E3 dimer/E3BPdd molecule. The same stoichiometry of E3 binding to E3BP was determined previously (2). The three-dimensional structure of the subcomplex was determined by x-ray crystallography using a combination of molecular replacement for the elucidation of the E3 structure and de novo building of the E3BPdd structure based on the phases obtained from the E3 content. The unit cell was composed of five E3-E3BPdd subcomplexes, each consisting of two subunits of E3 and one E3BPdd. Because no residues in the lipoyl domain of E3BPdd could be located in the electron density maps, we focused this study on the analysis of the E3 component and the E3-binding domain of E3BP of the subcomplex. Fig. 2 shows the structure of one of these subcomplexes.
α/β-type fold consisting of the central five-stranded antiparallel β-sheet encircled by a single helix (residues 424–438) on one side and by four helices on the other. That single helix and its symmetry-related counterpart provide the central hydrophobic contacts between two E3 subunits that contribute to a functional dimer. The contacts between helices are secured by the side chains of Glu431 at the N-terminal ends of those helices and by Tyr438 at the C-terminal ends. The next fragments of the polypeptide chain, i.e., the loop and helix (positions 457–468), add hydrogen bonds that further support contacts between the subunits. Two residues of that loop (His452 and Pro453) of one subunit complete the assembly of the active site built mainly by the residues of the other subunit.

Comparison of the structures of human and bacterial E3 dimers in the unbound state shows that they remain very similar to that of human E3 bound to the E3-binding domain of E3BP and to that of E3 from B. stearothermophilus bound to the cognate E1/E2-binding domain of E2, also from PDC. The similarity in the structures of unbound and bound human E3 indicates that the binding of E3BP to E3 does not cause any detectable conformational changes either in the binding region or farther away in the E3 dimer structure. This observation implies that recognition occurs by shape and electrostatics rather than by an induced fit mechanism.

Tertiary Structure of the E3-binding Domain of Human E3BP—A single 4.5-kDa E3-binding domain of E3BP folds into two parallel helices, α1 (residues 133–140) and α2 (residues 160–171) (Fig. 2), connected by a 19-residue-long linker. These two helices remain in contact via mainly hydrophobic contacts. The linker runs partially along the first helix and then turns back to form a broad nine-amino acid loop (residues 151–159) before folding into the second α-helix (Fig. 2).

The fold of the E3-binding domain of human E3BP revealed here is similar to the other known E1/E3-binding domain of B. stearothermophilus E2, which has been determined multiple times: first by NMR spectroscopy without a protein binding partner (16), by crystallography in subcomplex with E3 (17), and then by NMR spectroscopy with the E1 component of PDC (18). It is noteworthy that the similarity of the E3-binding domain of E3BP and the E1/E3-binding domain of E2 in the free and bound forms includes not only the presence of two parallel helices, but also the fold of the connecting linker in both the crystal and solute environments. Overall, the root mean square deviations between the structure of the E3-binding domain of human E3BP and the three
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TABLE 2
Distance range and mean between the E3-binding domain of E3BPdd and two subunits of E3 in all five E3-E3BPdd subcomplexes of human PDC observed in a crystallographic unit cell

| Atom of E3BPdd | Atom of E3 | Distance range | Distance mean |
|----------------|------------|----------------|---------------|
| Ser438 O      | Asp447 O   | 2.62–3.07      | 2.84          |
| Pro137 (ring) | Asp444–Asp17α | 3.25–3.60†     | 3.45          |
| Ala134 N     | Asp444 O   | 2.60–3.36      | 2.95          |
| Ala134 N     | Asp444 O   | 2.72–3.20      | 2.95          |
| Asn137 O     | Arg447 Nα | 2.40–3.56      | 3.06          |
| Pro136 C      | Gly437 O  | 2.83–3.51      | 3.16          |
| Pro136 C      | Tyr438 O  | 2.74–3.60      | 3.32          |
| Pro136 C      | Tyr438 N  | 2.92–3.55      | 3.25          |
| Arg135 Nα    | Tyr438 Oα | 2.72–3.60      | 3.05          |
| Ile135 Cβ    | Tyr438 Cβ | 3.10–3.24      | 3.16          |
| Ile137 Cβ    | Tyr438 Cβ | 3.16–3.60      | 3.44          |
| Lys138 Nα    | Asp442 Oα | 2.41–3.24      | 2.85          |
| Lys138 Nα    | Gly443 Oα | 2.64–3.37      | 3.06          |

Contacts between E3-binding domain of E3BPdd and E3 subunit located closer to E3-binding domain

| Pro136 Nα | Tyr438 Cβ | 3.30–3.60 | 3.43 |
| Arg136 Nα | Glu437 Oα | 2.84–3.54 | 3.16 |
| Arg136 Nα | Glu437 Oα | 2.68–3.68 | 3.23 |
| Arg136 Nα | Glu437 Oα | 2.44–3.68 | 3.20 |
| Arg136 Nα | His434 Nα | 2.94–3.72 | 3.32 |
| Glu136 Oα | His434 Oα | 2.86–3.55 | 3.25 |
| Glu136 Oα | Val437 Nα | 2.80–3.58 | 3.21 |
| Arg135 Nα | Asp444 Oα | 2.44–3.55 | 3.02 |
| Arg135 Nα | Gly439 O  | 2.76–3.43 | 3.12 |
| Arg135 Nα | Tyr438 O  | 2.51–3.42 | 2.83 |

Denotes contacts between various atoms of these amino acids.

structures of the E1/E3-binding domain of B. stearothermophilus E2 calculated for Cα atoms are 1.03–1.34 Å.

E3-E3BPdd Binding Analysis—As shown in details in Fig. 3, the E3-binding domain of E3BP binds the interface of two subunits of E3 utilizing the hydrophobic patch formed by the end Tyr438 residues of the central helices (residues 424–438). The side chains of the Tyr438 residues are parallel to each other and only 3.6 Å apart. Ile157 in the E3-binding domain of E3BP inserts itself between both of them, thus solidifying the hydrophobic interface between the E3 subunits (Fig. 3B). Pro135 of helix α1 makes hydrophobic contacts between the central helix (residues 424–438) of one subunit and the helix (residues 442–447) of the other subunit. Pro154 forms similar contacts, although on the opposite side of Ile157. The other omnipresent contacts between E3 and the E3-binding domain of E3BP include Ser132, which precedes helix α1; Ala134, Arg136, Asn137, and Glu140 of helix α1; Arg155 of the loop preceding helix α2; and Lys160 of helix α2 (Table 2). These residues take part in the hydrogen bonds adjacent to the central hydrophobic patch (Fig. 3C). Helix α1 makes contacts (Arg136–Glu140) with the central domains of both subunits and the interface (C-terminal) domain of only one of them. Additional ionic type interactions between E3 and the E3-binding domain of E3BP derive from Arg155 of the loop of the linker before it turns into the head residue, Lys160 of helix α2. These interactions are similar to those referred to as the “electrostatic zipper” in the subcomplex of E3 with the E1/E3-binding domain of E2 from B. stearothermophilus PDC (17). Helix α2 is farther away from the E3 subunits and does not interact with the E3 dimer beyond its head Lys160 residue. Based on our findings, the electrostatic interactions between the E3-binding domain of E3BP and E3 alone in the human subcomplex are likely to be insufficient, but rather hydrophobic and hydrophilic interactions complement each other to form the stable subcomplex. The binding at the interface connecting two subunits of E3 excludes the possibility that two E3-binding domains of E3BP bind simultaneously. This conclusion is in accordance with the binding ratio reported also for the bacterial E2/E3 subcomplex (17). We deduce from the structure that the binding may promote closer association of E3 subunits for improved catalytic efficiency directed by the arrival of dihydrolipoamide and NAD+ substrates.

Structural Implications of E3-E3BP Binding for the Assembly and Functionality of PDC—To identify the amino acids that are responsible for specific binding to E3, it would be useful to compare the structure of human E3 in complex with the E3-binding domain of E3BP and the structure of the E1-binding domain of E2 from human PDC. The latter structure of the human subcomplex is not available, but the B. stearothermophilus subcomplex has been reported (10). Fig. 4 shows the superposition of those two available structures and the structure-guided alignment of exemplary sequences of E3BP and E2 subunit-binding domains from three α-ketoacid dehydrogenase complexes from various species. It appears that the length of the linker that connects helices α1 and α2 in the structures of the E3-binding domains of E3BP and the E1/E3-binding domains of E2 remains the same. Pro154 is conserved in E3BP and E2 from human PDC, but not in E2 from the human branched-chain α-ketoacid dehydrogenase complex (BCKDC), allowing us to propose that conformational rigidity of that loop is unique to human PDC component proteins and not to human BCKDC. Furthermore, the structure-guided alignment of sequence of the E3-binding domain of human E3BP with those of the E1-binding domain of E2 from human PDC and BCKDC and the E1/E3-binding domains of yeast and several bacterial E2 enzymes reveals that Pro154 is present in the proteins that evolved to bind selectively E3 (but not E1) in the complexes. It is noteworthy that, unlike human PDC, BCKDC is composed of E2 that can bind both E1 and E3 components.

The structure-based sequence alignment (Fig. 4B) also reveals strict conservation of the head amino acids of helices α1 (Pro135 and α2 (Lys160) and the glycine-rich nine-amino acid loop (residues 151–159) within the linker. The replacement of Ile157 with Arg in E2 from human PDC is minor in terms of the overall conformational change in the loop, but it sufficiently alters the charge of the loop, allowing for loss of an exquisite match of amino acid side chains involved in binding to the central hydrophobic patch of the E3 dimer. Therefore, we postulate that this single replacement leads to increased specific-
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**FIGURE 4. Comparison of the E3-binding domain of human E3BP and the E1/E3-binding domains of E2 enzymes from α-ketoacid dehydrogenase complexes.**

A, superposition (in stereo view) of amino acids from the binding sites of the E3-E3BPdd subcomplex from human PDC and the E1/E3-binding domain of the E2 subcomplex from *B. stearothermophilus* PDC. The polypeptide chains from the human subcomplex are shown in taupe and yellow (E3 subunits) and blue (E3-binding domain of E3BP), and those from the bacterial complex are all shown in orange (E1/E3-binding domains of E2). The significant ionic hydrogen bonds between Arg<sup>654</sup> and Phe<sup>324</sup> in the bacterial E1/E2 subcomplex are shown as dashed lines. B, sequence alignment guided by the structure of the E3-binding domain of human E3BP (huE3BP) (row a) with the selected sequences of the following: E1-binding domain of E2 from human PDC (huE2p) (row b), the E1-binding domain of E2 from *S. cerevisiae* (scE2p) (row c), the E1/E3-binding domain of E2 from human BCKDC (bsE2p) (row d), the E1/E3-binding domain of E2 from *B. stearothermophilus* PDC (bsE2p) (row e), and the E1/E3-binding domain of E2 from *E. coli* α-ketoglutarate dehydrogenase complex (ecE2p) (row f), the E1/E3-binding domains of E2 from *E. coli* PDC (ecE2p) (row g), the E1/E3-binding domains of E2 from the *E. coli* α-ketoglutarate dehydrogenase complex (ecE2o) (row h), and the E1/E3-binding domains of E2 from the *A. vinelandii* α-ketoglutarate dehydrogenase complex (avE2o) (i). Rows d–i represent the E1/E3-binding domains of E2 that non-selectively bind E1 and E3 component enzymes in respective complexes, including E2 from human BCKDC. The residues of helices α1 and α2 in the E3-binding domain of E3BP are shown beneath the yellow cylinders. The invariant residues marking the beginnings of these helices are shown in red. Other invariant amino acids of these helices are also highlighted in red. The amino acids of the glycine-rich loop are shown in green. The invariant proline and isoleucine residues in human E3BP and only proline in human and yeast E2 from PDC. Gray indicates amino acids in the place of those proline and isoleucine residues in other E2 enzymes. The residues of the E3-binding domain of E3BP that bind to E3 in the reported E3-E3BPdd crystal structure are underlined.

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