J. Biochem. 123, 668-674 (1998)

Crystal Structure of Eucaryotic E3, Lipoamide Dehydrogenase from Yeast[1]

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Received for publication, October 27, 1997

The crystal structure of eucaryotic lipoamide dehydrogenase from yeast has been determined by an X-ray analysis at 2.7 (partially at 2.4) Å resolution. The enzyme has two identical subunits related by a pseudo twofold symmetry. The tertiary structure is similar to those of other procaryotic enzymes. The active site, consisting of FAD, Cys44, and Cys49 from one subunit and His457′ from the other subunit, is highly conserved. This enzyme is directly bound to the core protein E2 of the 2-oxoglutarate dehydrogenase complex, whereas it is bound to the pyruvate dehydrogenase complex through a protein X. The calculated electrostatic potential suggests two characteristic regions for binding with these two proteins.

Key words: crystal structure, lipoamide dehydrogenase, 2-oxoglutarate dehydrogenase complex, pyruvate dehydrogenase complex, X-ray analysis.

The dihydrolipoamide (lipoamide) dehydrogenases are widely known as the E3 component of the pyruvate (PDC), 2-oxoglutarate (OGDC), and branched-chain 2-oxoacid (BCDC) dehydrogenase multienzymatic complexes (*1-3*). The architecture of the complexes is composed of multicopies of E1, E2 and E3.[3] The former two enzymes, E1 and E2, are different among the three complexes corresponding to their respective substrates mentioned above, but the E3 enzyme is a common component among them, which catalyzes the oxidation reaction of dihydrolipoyl group attached to the lysine residue of E2 (*1*). The E2 components form the structural core of the complex. OGDCs in all organisms and PDCs in Gram-negative bacteria have a similar core with twenty-four copies of E2 packed with the 432 symmetry. The cores of PDCs in procaryotic Gram-positive bacteria (*4*) and eucaryotes exhibit 532 symmetry and contain sixty copies of E2. In other words, E3s in the latter organisms are incorporated into both cores with 432 and 532 symmetries.

More strictly speaking, in procaryotes, E3 binds the E3 binding domain (E3BD) of E2 directly even in different architectures. In eucaryotes, however, E3 binds E2 directly in OGDC in a similar way (*1-3*), but for PDC another component X (*5-7*) is required to incorporate E3 into the E2 core.[4] E3 binds E3BD of X (*8*), instead of E2, and then X binds E2 (*8*). This difference suggests that the procaryotic E3 has one face to bind to E2 only, while the eucaryotic E3 has regions for binding the two kinds of proteins, E2 and X.

The tertiary structures of various components have been elucidated: E3BD of OGDC from *Escherichia coli* (*9*), the lipoyl domains of E2 of PDC from *Bacillus stearothermophilus* (*10*) and from *E. coli* (*11*), E3:E3BD complex of PDC from *B. stearothermophilus* (*12*), the cubic E2 core of PDC from *Azotobacter vinelandii* (*13*), and E3s from *A. vinelandii* (*14*), from *Pseudomonas putida* (*15*), and from *Pseudomonas fluorescens* (*16*). These are all from procaryotic systems. On the other hand, the three-dimensional structures of eucaryotic E3s have not yet been reported, though a low-resolution structure of yeast E3 from *Saccharomyces oviformis* has been reported (*17*). To elucidate the reason for the different structural properties mentioned

[1] This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas (No. 08214203, 07230224, and 07250205) from the Ministry of Education, Science, Sports and Culture of Japan, and by the Sakabe project of TARA (Tsukuba Advanced Research Alliance), University of Tsukuba.

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Abbreviations: OGDC, 2-oxoglutarate dehydrogenase complex; PDC, pyruvate dehydrogenase complex; BCDC, branched-chain 2-oxoacid dehydrogenase complex; E1, pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, or branched-chain 2-oxoacid dehydrogenase; E2, acetyltransferase, succinyltransferase, or acyltransferase; E3, dihydrolipoamide(lipoamide) dehydrogenase; FAD, flavin adenine dinucleotide; E1BD, E1 binding domain; E3BD, E3 binding domain.

[3] Each complex consists of the following components, PDC (E1, pyruvate dehydrogenase; E2, acetyltransferase; E3, lipoamide dehydrogenase), OGDC (E1, 2-oxoglutarate dehydrogenase; E2, succinyltransferase; E3, lipoamide dehydrogenase), and BCDC (E1, branched-chain 2-oxo acid dehydrogenase; E2, acyltransferase; E3, lipoamide dehydrogenase).

[4] In PDC of procaryotic Gram-positive bacteria, E3 binds E3BD of E2 even in the core with 532 symmetry.

above in eucaryotic E3s, the X-ray structure of E3, lipoamide dehydrogenase from *Saccharomyces cerevisiae* has been determined at 2.7 Å (partially at 2.4 Å) resolution. In this paper we discuss the regions of E3 involved in protein-protein interaction on the basis of the highly conserved tertiary structure.

STRUCTURE DETERMINATION

As described in the previous paper (*18*), crystals of the newly purified enzyme exhibited X-ray diffraction beyond 2.5 Å resolution. For structure determination, all the observed data were used, but their coverage decreased in the outer shell over 2.68 Å resolution from 83.5% to less than 50% (see Table I). The initial phases (*18*) derived preliminarily from molecular replacement (*18, 19*) were further improved by non-crystallographic symmetry averaging, solvent flattening and histogram matching techniques with the program SQUASH (*20*). The molecular structure was constructed by replacing, inserting, and deleting amino acid residues in the initial polyalanine model for molecular replacement, their conformations being accommodated into electron density maps using the program O (*21*). After four rounds of density modification, all the inserted amino acid residues and FAD molecules were clearly located. The atomic coordinates were refined in reciprocal space using the program X-PLOR (*22*) and the structure model was adjusted in real space with the program O (*21*), alternately. For the first several rounds of positional refinements, the two subunits of the dimeric enzyme were constrained with a non-crystallographic two-fold symmetry. After stimulated annealing refinement with the temperature decreasing from 3,000 to 300 K in steps of 25 K, the structure was further refined positionally without the non-crystallographic symmetry constraint, and then the individual temperature factors of all atoms were adjusted isotropically. Seventy-three peaks in total with greater than 3σ level in a difference electron density map and with a distance less than 3.4 Å away from the protein molecule were assigned as water molecules by taking hydrogen bond geometries into consideration, and they were added in the refinement. All residues and FADs were confirmed visually by their omit maps. During the course of refinements, the protein structure was verified using the program PROCHECK (*23*). The progress of refinement was monitored in terms of the free R value (*24*).

TABLE I. **Crystal data and refinement statistics for yeast lipoamide dehydrogenase.**

Space group	$P2_12_12_1$
Cell constants (Å)	
a	97.1
b	158.7
c	67.9
Z[a]	2
Limiting resolution (Å)	2.4
Observed reflections	97,677
Independent reflections[b]	33,372
R_{merge} (%)	6.7
Completeness (%)	97.4 (100-2.87 Å resolution shell)
	83.5 (2.87-2.68 Å resolution shell)
	42.7 (2.68-2.40 Å resolution shell)
Non-hydrogen protein atoms	7,356
Solvent molecules	73
Reflections used for refinement	25,458
Resolution range (Å)	10-2.4
R-factor (%)	20.2
R_{free}[c] (%)	26.2
R.m.s. standard deviation	
bond length (Å)	0.009
bond angles (°)	2.53
improper angles (°)	0.763
Average coordinate error (Å)	0.35

[a]Number of subunits in the asymmetric unit. [b]$I > 4\sigma$. [c]Calculated using the 10% fraction of the whole reflection data which were not used for refinement.

RESULTS

The final structure showed an R-factor of 0.202 for 25,458 reflections with $|F_o| > 4\sigma$ in the resolution range of 10–2.4 Å ($R_{free} = 0.26$ for 10% of the data). The quality of the final $2F_o - F_c$ map is shown in Fig. 1. The final structure has a small r.m.s. deviation of 0.009 Å in bond lengths and of 2.5° in bond angles from the ideal values. No residues lie in disallowed Ramachandran regions, as can be seen in Fig. 2. Crystal data, data collection and structure determination statistics are summarized in Table I.

As shown in Fig. 3, the yeast E3 is a dimeric enzyme composed of two identical subunits each with a molecular weight of 52,300 (478 amino acid residues). The two

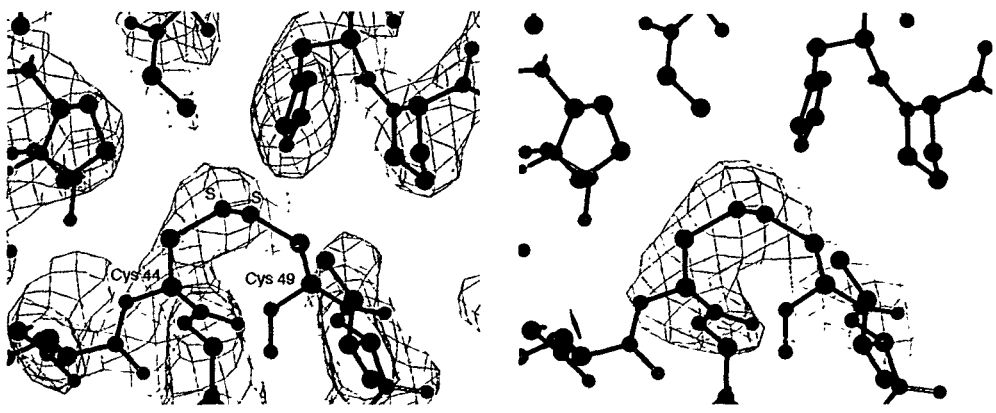

Fig. 1. **The final $2F_o - F_c$ electron density map (a) around Cys44 and Cys49, contoured at the 1.0σ level, and its $F_o - F_c$ omit map (b) calculated without them, contoured at the 2.5σ level.**

subunits are related by a pseudo twofold symmetry with the r.m.s. displacement of 0.56 Å for C_α atoms. The individual C_α displacements larger than 3σ [σ is estimated to be 0.35 Å from a Luzzati plot (*26*)] are locally found in the loop regions with residues 70-71, 133-140, 267-268, and 351-353, which are all located on the molecular surface

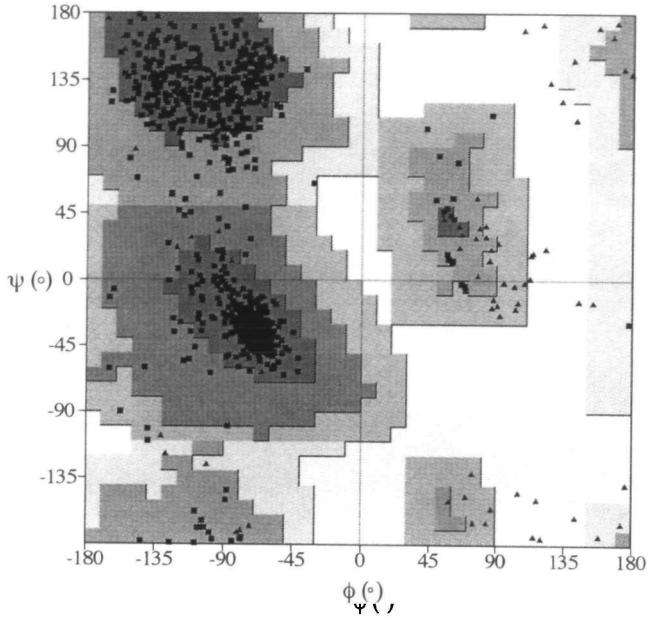

Fig. 2. **A Ramachandran plot of the final structure, produced by PROCHECK (*23*).** ▲ and ■ indicate glycine and non-glycine residues, respectively.

(see Fig. 4). Thus, the two subunits have essentially the same structure despite local differences due to crystal packing. Each subunit contains 13 α-helices and 24 β-strands which constitute 31 and 27% of the whole residues, respectively. The subunit is divided into the four domains as already defined by Thieme *et al.* (*27*) for gluthathione reductase: the FAD domain (residues 1 to 153, 3 α-helices, and 9 β-strands), the NADH domain (residues 154-287, 3 α-helices, and 9 β-strands), the central domain (residues 288-356, 2 α-helices, and 1 β-strand), and the interface domain (residues 357-478, 5 α-helices, and 5 β-strands). A disulfide bond is formed between Cys44 and Cys49, which are essential for the dehydrogenase activity.

The solvent accessible surface was calculated with the program SURFACE in CCP4 (*28*). About 3,900 Å^2 (16%) of the subunit surface is buried through dimerization. The region involved in the subunit-subunit interaction is constituted from the FAD domain and the interface domain. The residues 61-83 in the FAD domain come into contact with those related by a pseudo twofold symmetry, in which residues 74-82 form a bent anti-parallel β-sheet with the corresponding residues 74′-82′ of the counter subunit, as shown in the top and side views in Fig. 3. In addition, the remaining hydrophobic residues (Ile74, Val76, Ile80, and Ile82 in the β-strand and Leu61, Phe62, and Met65 in the adjacent α-helix) also interact with the corresponding residues of the other subunit. In the interface domain, α-helices and loops involving residues 397-477 interact with the other subunit through hydrogen bonds and hydrophobic interactions. The two active sites of the enzyme are located separately at the interface of two subunits and each consists of FAD, Cys44, and Cys49 from one subunit and

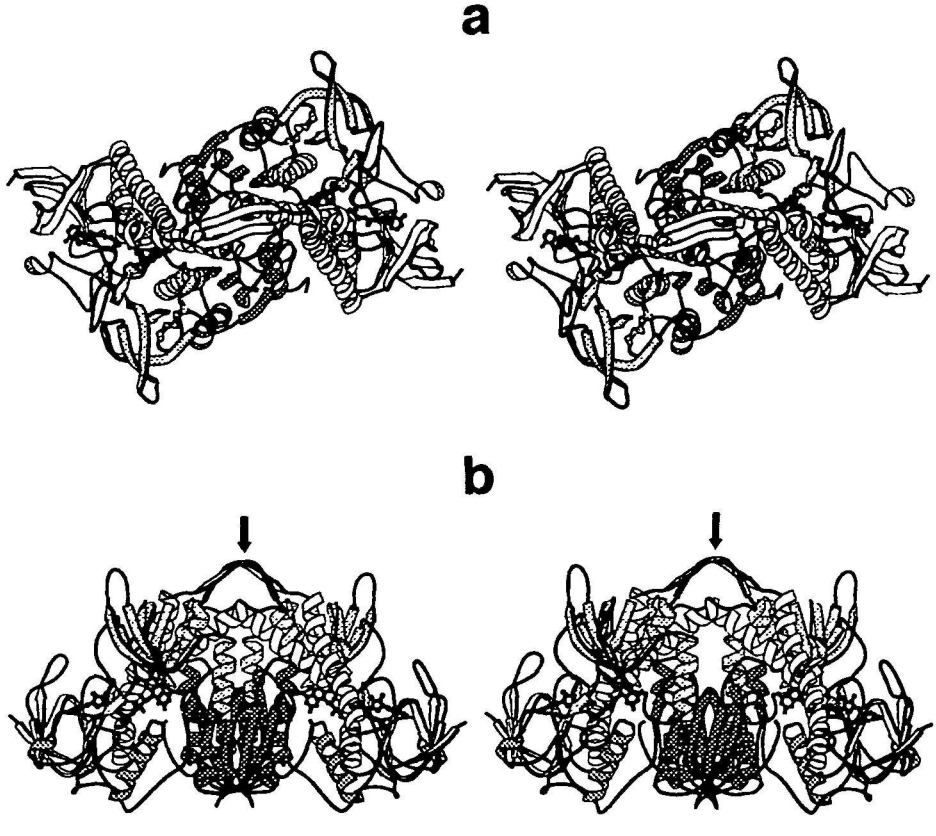

Fig. 3. **A stereo diagram of a ribbon drawing of the dimeric yeast lipoamide dehydrogenase (a) viewed down the molecular twofold axis and (b) viewed perpendicular to it.** The FAD molecules are drawn as ball-and-stick models. An arrow indicates the intersubunit β-sheet (residues 74-82). The four domains (FAD, NADH, central, and interface domains) are depicted with no shade and with shades of light gray, gray, and dark gray, respectively. The figures were produced with the program MOLSCRIPT (*25*).

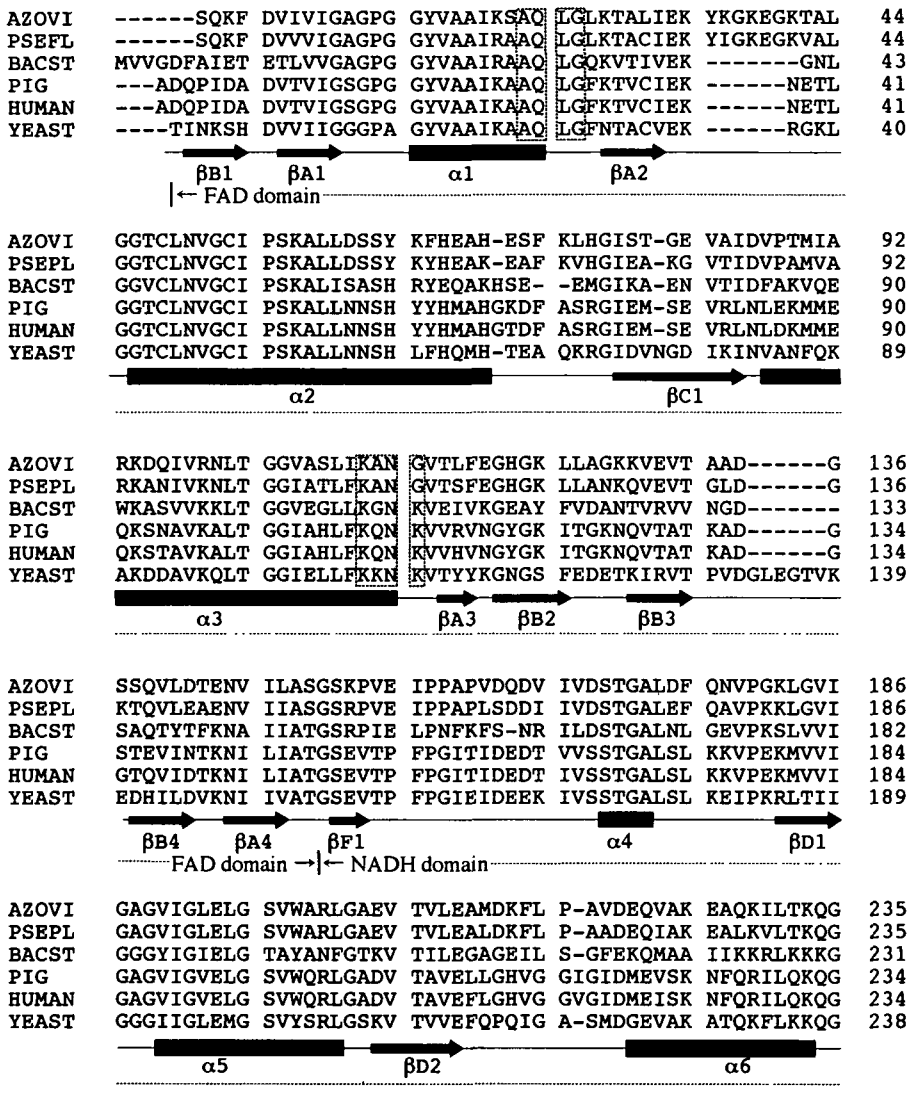

Fig. 4. (Continued on the next page)

His457′ from the other subunit (see Fig. 6).

DISCUSSION

It is interesting to compare the present eucaryotic E3 structure with those of procaryotes, *A. vinelandii* (*14*), *P. putida* (*15*), *P. fluorescens* (*16*), and *B. stearothermophilus* (*12*). The new β-sheet found in yeast E3 is missing in the other E3s, except *P. fluorescens* E3. Figure 5 shows their structures superimposed on the corresponding C_α atoms with the closest tertiary structure. Large differences occur only in the loop regions containing insertion or deletion of amino acids and in the new β-strand (βC1), as shown in Fig. 4. The r.m.s. deviation of C_α atoms without those residues is 0.9 Å among the molecules. It is thus concluded that the yeast E3 has the same basic tertiary structure as the procaryotic E3s, even though the amino acid sequence identity is rather low, namely 42-44%. Although the *P. putida* E3 (*15*) is a component of BCDC (of which the architecture is not yet known) and has an even lower sequence identity with the yeast E3 (35%), the tertiary structure is very similar to those of the other E3s.

All E3s have two catalytic sites, each of which is formed with the flavin moiety of FAD and two cysteine residues from one subunit and a histidine residue from the other subunit. Their r.m.s. displacement is only 0.3 Å, as shown in Fig. 6. The active site is highly conserved among the eucaryotic and the procaryotic E3s even though the amino acid identity is less than 50%. As pointed out in the structure of E3 from *A. vinelandii* (*14*), for the catalytic reaction, a lipoamide group of E2 must enter the active site toward the Cys44 and Cys49 side of the flavin plane, because the nicotine moiety of NAD^+ interacts on the other side (*15*). In this situation, the lipoyl domain of E2 will approach the active site formed between the FAD and the interface domains, from one side, *i.e.* from the front side toward the right flavin moiety in Fig. 3 (b).

In eucaryotic PDC, E3 binds E3BD of protein X but not E2(acetyltransferase) which has an E1BD, while, in eucaryotic OGDC, E3 binds E3BD of E2(succinyltransferase) directly. The E3 binding site of E2 has been proposed based on an X-ray analysis of E3BD of E2(acetyltransferase) from *B. stearothermophilus* (*12*) complexed with E3. The essential basic amino acids, arginine and lysine (in a box in

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AZOVI  LKILLGARVT GTEVK-NKQV -TVKFVDAEG EKSQAF--DK LIVAVGRRPV  281
PSEPL  LNIRLGARVT ASEVK-KKQV -TVTFTDANG EQKETF--DK LIVAVGRRPV  281
BACST  VEVVTNALAK GAEER-EDGV -TVTYEANGE TKTIDA--DY VLVTVGRRPN  277
PIG    FKFKLNTKVI GATKKSDGNI -DVSIEAASG GKAEVITCDV LLVCIGRRPF  283
HUMAN  FKFKLNTKVT GATKKSDGKI -DVSIEAASG GKAEVITCDV LLVCIGRRPF  283
YEAST  LDFKLSTKVI SAKRNDDKNV VEIVVEDTKT NKQENLEAEV LLVAVGRRPY  288
        βD3     βE1         βE2          βE3    βD4      βF2
                                               NADH domain →|←

AZOVI  TTDLLAADSG VTLDERGFIY VDDYCATSVP GVYAIGDVVR GAMLAHKASE  331
PSEPL  TTDLLAADSG VTLDERGFIY VDDHCKTSVP GVFAIGDVVR GAMLAHKASE  331
BACST  TDELGLEQIG IKMTNRGLIE VDQQCRTSVP NIFAIGDIVP GPALAHKASY  327
PIG    TQNLGLEELG IELDPRGRIP VNTRFQTKIP NIYAIGDVVA GPMLAHKAED  333
HUMAN  TKNLGLEELG IELDPRGRIP VNTRFQTKIP NIYAIGDVVA GPMLAHKAED  333
YEAST  IAGLGAEKIG LEVDKRGRLV IDDQFNSKFP HIKVVGDVTF GPMLAHKAEE  338
          α7                             βA5              α8
       Central domain

AZOVI  EGVVVAERIA GHKAQMNYDL IPAVIYTHPE IAGVGKTEQA LKAEGVAINV  381
PSEPL  EGVMVAERIA GHKAQMNYDL IPSVIYTHPE IAWVGKTEQT LKAEGVEVNV  381
BACST  EGKVAAEAIA GHPSAVDYVA IPAVVFSDPE CASVGYFEQQ AKDEGIDVIA  377
PIG    EGIICVEGMA GGAVHIDYNC VPSVIYTHPE VAWVGKSEEQ LKEEGIEYKV  383
HUMAN  EGIICVEGMA GGAVHIDYNC VPSVIYTHPE VAWVGKSEEQ LKEEGIEYKV  383
YEAST  EGIAAVEMLK TGHGHVNYNN IPSVMYSHPE VAWVGKTEEQ LKEAGIDYKI  388
                              βG1        βG2         α9         βG3
       Central domain →|← Interface domain

AZOVI  GVFPFAASGR AMAANDTAGF VKVIADAKTD RVLGVHVIGP SAAELVQQGA  431
PSEPL  GTFPFAASGR AMAANDTTGL VKVIADAKTD RVLGVHVIGP SAAELVQQGA  431
BACST  AKFPFAANGR ALALNDTDGF LKLVVRKEDG VIIGAQIIGP NASDMIAELG  427
PIG    GKFPFAANSR AKTNADTDGM VKILGQKSTD RVLGAHIIGP GAGEMINEAA  433
HUMAN  GKFPFAANSR AKTNADTDGM VKILGQKSTD RVLGAHILGP GAGEMVNEAA  433
YEAST  GKFPFAANSR AKTNQDTEGF VKILIDSKTE RILGAHIIGP NAGEMIAEAG  438
                   α10          βG4          βG5          α11

AZOVI  IAMEFGTSAE DLGMMVFAHP ALSEALHEAA LAVSGHAIHV ANRKK-----  476
PSEPL  IGMEFGTSAE DLGMMVFSHP TLSEALHEAA LAVNGHAIHI ANRKKR----  477
BACST  LAIEAGMTAE DIALTIHAHP TLGEIAMEAA EVALGTPIHI ITK-------  470
PIG    LALEYGASCE DIARVCHAHP TLSEAFREAN LAASFGKAIN F---------  474
HUMAN  LALEYGASCE DIARVCHAHP TLSEAFREAN LAASFGKSIN F---------  474
YEAST  LALEYGASAE DVARVCHAHP TLSEAFKEAN MAAYD-KAIH C---------  478
                α12          α13
                                       Interface domain →|
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Fig. 4. **Comparison of primary structures among procaryotic and eucaryotic E3s.** The sequence data were taken from GenBank (*29*). Key to sequence origins: AZOVI, *Azotobacter vinelandii*; PSEFL, *Pseudomonas fluorescens*; BACST, *Bacillus stearothermophilus*. The bottom line shows the four domains of the yeast E3, together with the secondary structures. Rectangles and arrows indicate α-helices and β-strands, respectively. Amino acids in boxes surrounded by solid lines are those located in the conserved negative potential region ① (see Fig. 8) around the 2-fold axis for E2 binding. The outlined letters indicate the essential acidic amino acids for binding with E3BD of E2. Amino acids, found in the region whose potentials characteristically differ between yeast E3 and procaryotic E3 (surrounded by dotted line in Fig. 8) are enclosed in the dot-lined boxes. Amino acids in shaded boxes are those found in region ② of Fig. 8. Bold letters indicate amino acids replaced in eucaryotic E3s with basic amino acids from neutral ones.

Fig. 5. **A stereo diagram of four lipoamide dehydrogenase structures.** They are superimposed between the corresponding C_α atoms of the dimers, but only the subunits are shown for clarity. The C_α atoms are traced with thick lines for yeast, thin lines for *Azotobacter vinelandii*, broken lines for *Pseudomonas fluorescens*, dotted lines for *Bacillus stearothermophilus*.

Fig. 7) pointed out by Mande *et al.*(*12*), are also found in the corresponding site of E3BD of yeast OGDC E2 (succinyltransferase), but they are replaced with neutral or hydrophobic ones in the E3BD of the yeast protein X.[5] Therefore these differences suggest that the yeast E3 is

[5] Recently the amino acid sequence of the human protein X has been reported (*6*), and in this molecule the positive charges in this region are reduced.

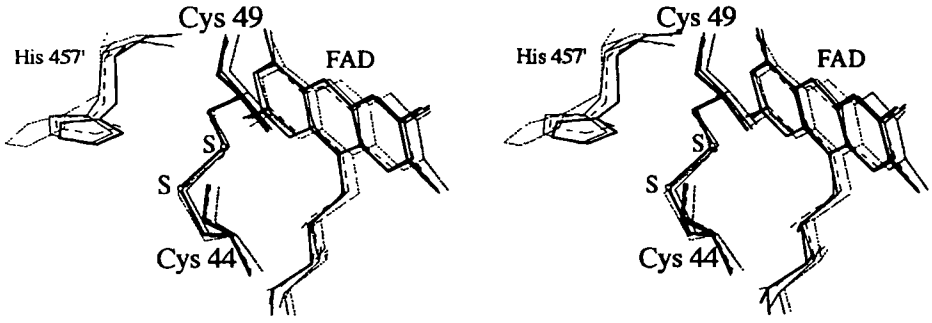

Fig. 6. **A stereo view of the active sites superimposed among four lipoamide dehydrogenases from yeast with thick lines, *Azotobacter vinelandii* with thin lines, *Pseudomonas fluorescens* with broken lines, and *Bacillus stearothermophilus* with dotted lines.** The residue numbers are for yeast E3.

Fig. 7. **Amino acid sequences of the E3 binding region of E2 in procaryotic PDC and in procaryotic and eucaryotic OGDC, taken from GenBank (*29*).** Key to sequence origins: AZOVI, *Azotobacter vinelandii*; ECOLI, *Escherichia coli*; BACSU, *Bacillus subtilis*; BACST, *Bacillus stearothermophilus*. The basic amino acids proposed to be the E3 binding site (*28*) are in a box. The bottom line shows the amino acid sequence in the corresponding region of yeast protein X. The basic amino acids are replaced with neutral or hydrophobic ones. (Although the amino acid sequence in the corresponding region of PDC E2 is similar to that of E3BD of OGDC E2 (*12*), it is omitted in the figure due to the fact that PDC E3 binds protein X but not E2.)

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AZOVI   E2(OGDC)   -LSPAARKIAE E-N---AIAA DSITGTGKGG RVTKEDAVA-
ECOLI   E2(OGDC)   -LSPAIRRLLA E-H---NLDA SAIKGTGVGG RLTREDVEK-
BACSU   E2(OGDC)   -ASPSARKLAR E-K--GIDLS QVPTG-DPLG RVRKQDVEA-
HUMAN   E2(OGDC)   -QPPSGKPVSA V-K---PTVA ---------- ----------
RAT     E2(OGDC)   -QPPSSKPVSA I-K---PTAA ---------- ----------
YEAST   E2(OGDC)   -KKPLQRKKLQ NQK---RTDQ PKK------- ----------

AZOVI   E2(PDC)    -AGPAVRQLAR E-F---GVEL AAINSTGPRG RILKEDVQA-
ECOLI   E2(PDC)    -ATPLIRRLAR E-F---GVNL AKVKGTGRKG RILREDVQA-
BACST   E2(PDC)    -AMPSVRKYAR E-K---GVDI RLVQGTGKNG RVLKEDIDA-
BACSU   E2(PDC)    -AMPSVRKYAR E-K---GVDI RKVTGSGNNG RVVKEDIDS-

YEAST   X (PDC)    -LLPSVSLLLA ENNISKQKAL KEIAPSGSNG RLLKGDVLA-
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bound to two different E3BDs of protein X and E2. On the other hand, the procaryotic E3 is bound to only the similar E3BDs of E2s in PDC and OGDC.

To investigate the structural properties of the yeast E3, an electrostatic potential surface was calculated and compared with those of the procaryotic E3s. The most remarkable feature is the large negative region ① localized at the bottom around the twofold axis of the dimeric enzyme, as shown in Fig. 8. This negative region is commonly found in all the E3s, and is just the same as the proposed binding site of E2 (*12*). In Fig. 4, the amino acid residues involved in this region are localized in four boxes. In particular, Glu442, Glu448, and Asp449 participate to create the negative potential which can facilitate binding with E3BD of E2 in yeast OGDC, because, as seen in Fig. 7, the E3 binding site has basic amino acids similar to those of *B. stearothermophilus* PDC. Therefore, this region is electrostatically conserved between the procaryotic and the eucaryotic yeast E3s as regards the binding site of E2.

Further comparison of electrostatic potentials showed an adjacent region surrounded with dotted lines in Fig. 8, whose potentials characteristically differ between the yeast E3 and the procaryotic E3s. The amino acids in this region are shown in dot-lined boxes in Fig. 4. This region is still negative in the procaryotic E3s as an extension from region ①. The lower part of the dot-lined region (surrounded with a full line as region ② in Fig. 8; the amino acids are shown in shaded boxes in Fig. 4) belongs to the interface domain distant from the active site. Several residues in region ② of yeast E3 definitely differ in charge or in polarity from those of the procaryotic E3s. In particular, the four basic residues, Lys387, Arg452, Lys465, and Lys474, in the yeast E3 (bold letters in Fig. 4) neutralize the negative field caused by Glu462 and Glu466. These changes are reflected in the molecular surface potential as if to differentiate it from the negative potential of the procaryotic E3. In other eucaryotic E3s, the same or similar amino acid changes occur (Fig. 4), suggesting a common feature of their electrostatic potentials conserved in eucaryotes. On the other hand, the amino acids of protein X, which correspond to those of E3BD on E2, are completely different, having a rather hydrophobic characters. If they participate in E3 binding, the binding site on E3 must also be hydrophobic. Such a region can be found adjacent to ②. Therefore region ② may be neutralized to facilitate the binding. It might thus be concluded that the region adjacent to ② is a possible binding site of protein X, because it is near the E2 binding site but distant from the other binding sites for the substrate and the coenzyme. As a result, the yeast E3 will have two binding regions for E2 of OGDC and for protein X of PDC. As discussed above, the tertiary structures of E3s are highly conserved between eucaryotes and procaryotes. In the evolutional process, the molecular surface of eucaryotic E3 might have changed to adapt to the architecture of PDC after introduction of protein X.

We are grateful to N. Sakabe and N. Watanabe for facilities and help during data collection at the Photon Factory (Tsukuba). We thank J.C. Thierry for helpful discussions and comments on this study.

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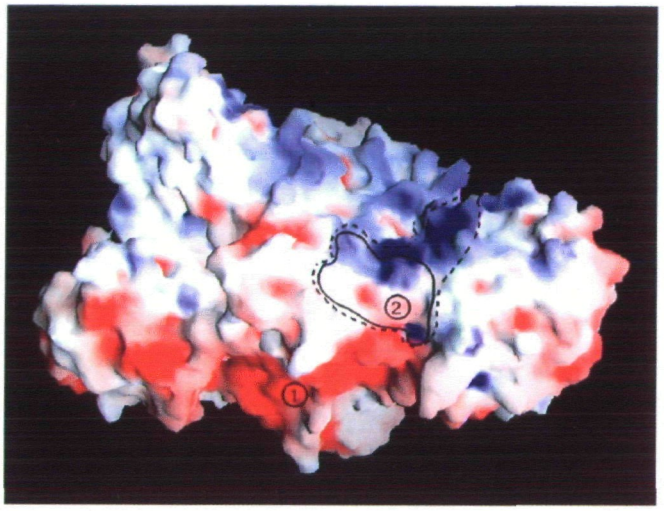
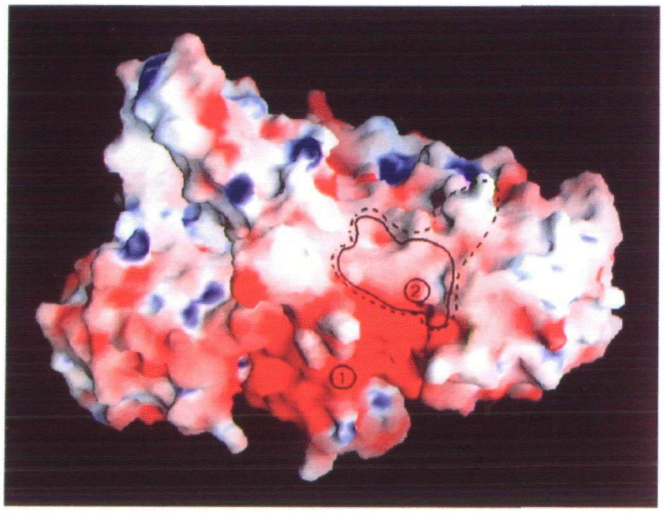


(a)



(b)

Fig. 8. **Electrostatic potential surfaces (positive in blue and negative in red) on the bottom side around the pseudo twofold axis, (a) for yeast E3 and (b) for *Azotobacter vinelandii* E3.** The central negative region ① has been proposed as the E3BD of E2 (*12*). A region, definitely changed in electrostatic potential between the yeast E3 (a) and the procaryotic E3 (b), is surrounded with dotted line. The surrounded region ② is proposed as a candidate for facilitating the protein X binding (see the text).

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