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Crystallization of Eukaryotic E3, Lipoamide Dehydrogenase, from Yeast, for Exhibiting X-Ray Diffraction beyond 2.5 Å Resolution, and Preliminary Structure Analysis[1]

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Lipoamide dehydrogenase, which is a common component of α-keto acid dehydrogenase complexes, has been highly purified from yeast (*Saccharomyces cerevisiae*) to reveal its structure at higher resolution. New crystals obtained by a desalting method exhibited diffraction beyond 2.5 Å resolution. The cell dimensions are $a=97.1$, $b=158.7$, and $c=67.9$ Å, and the space group is $P2_12_12_1$. There is a dimeric enzyme in the asymmetric unit. The crystal structure was solved by means of the molecular-replacement technique and refined in a preliminary manner.

Key words: crystallization, lipoamide dehydrogenase, X-ray study.

α-Keto acid dehydrogenase complexes form a family of well-organized multienzyme systems which catalyze the oxidative decarboxylation of α-keto acids with NAD^+ as a coenzyme (*1-5*).

$$RCOCOOH + CoASH + NAD^+ \longrightarrow CO_2 + RCO\text{-}S\text{-}CoA + NADH + H^+$$

These complexes consist of multiple copies of, in general, three kinds of enzymes, E1, E2, and E3. E1s and E2s are different depending on the substrate, respectively. For example, in the pyruvate dehydrogenase complex (PDC), E1 is pyruvate dehydrogenase and E2 is acetyltransferase, and in the 2-oxoglutarate dehydrogenase complex (OGDC), E1 is 2-oxoglutarate dehydrogenase and E2 is succinyltransferase. These complexes, however, commonly contain the same lipoamide dehydrogenase as E3, which catalyzes the oxidation of the lipoic acid of E2 in the last step of the whole reaction.

$$E2\text{-}Lip(SH)_2 + NAD^+ \longrightarrow E2\text{-}LipS_2 + NADH + H^+$$

The architecture of these complexes differs with the organisms. The core structures are composed of 24 copies of E2 with a 432 symmetry in Gram-negative bacteria. In Gram-positive bacteria and eukaryotes, however, 60 copies of E2 form the core of PDC with a 532 symmetry, though the core of OGDC still exhibits a 432 symmetry. It is interesting that in the latter organisms, E3 is incorporated into the cores with 532 and 432 symmetries, respectively. More strictly speaking, protein X is required for binding with E3 in the icosahedral core of yeast PDC and pig heart PDC. Therefore, these eukaryotic E3s would have two faces to function in both architectures. The crystal structures of E3s from Gram-negative bacteria have been reported (*6, 7*), but the detailed structure of a eukaryotic E3 has not yet been revealed, though the 4.5 Å resolution structure of E3 from *Saccharomyces oviformis* has been reported (*8*). In order to clarify the reaction mechanism in the highly organized multi-enzyme systems, we have continued to solve this structure at higher resolution. Recently, the enzyme was newly isolated and purified from baker's yeast (*Saccharomyces cerevisiae*), and we have obtained new crystals which diffract beyond 2.5 Å resolution. In this paper, the purification procedure, crystallographic data and preliminary structure determination are described.

Five hundred grams of pressed baker's yeast (*Saccharomyces cerevisiae*) was disrupted with ethyl acetate according to a similar procedure to that reported by Kawahara Y. *et al.* (*9*) except that proteins were extracted at 30°C. Since yeast E3 is relatively thermostable, other proteins were removed to some extent by heat-treatment of the supernatant at 70°C for 10 min. After fractionation with ammonium sulfate, the precipitate of the active fractions was suspended in 50 mM sodium phosphate buffer (pH 7.6) and then dialyzed against the same buffer overnight. The dialysate was applied to a hydroxylapatite column (2.6×27 cm) equilibrated with the same buffer. The column was washed consecutively with about 200 ml of 50, 100, and 200 mM sodium phosphate buffer (pH 7.6), respectively. The enzyme was eluted with 200 mM sodium phosphate buffer (a linear gradient of 0-8% ammonium sulfate). The active fractions were dialyzed against 50 mM potassium phosphate buffer (pH 8.0) containing 100 mM NaCl overnight. The dialyzate was put on a nickel-chelating Sepharose FF column (2.6×16 cm). The column was washed with 50 mM phosphate buffer (pH 8.0) containing 100 mM NaCl to elute

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Abbreviations: $Lip(SH)_2$: reduced form of the lipoamide group attached to the enzyme; $LipS_2$: oxidized form of the lipoamide group attached to the enzyme.

unbound proteins. Then the adsorbed proteins were eluted with a linear 0-50 mM imidazole gradient in 50 mM potassium phosphate buffer (pH 8.0) containing 100 mM NaCl. The active fractions were pooled and then concentrated. The purity of the enzyme, E3, was confirmed by SDS-PAGE, as shown in Fig. 1.

The enzyme activity of yeast E3 was assayed spectrophotometrically by following the reduction of NAD^+ at 340 nm at 37°C with a Shimadzu UV-265 recording spectrophotometer. DL-Dihydrolipoamide was synthesized according to Reed *et al.* (*10*). The reaction mixture (1 ml) comprised 100 mM potassium phosphate buffer (pH 8.0), 3 mM DL-dihydrolipoamide, 1.5 mM NAD^+, 1.5 mM EDTA, and enzyme solution.

Several precipitants were examined as to crystallization of yeast E3, partly using a Crystal Screen kit (Hampton Research), but no crystalline materials appeared. Single crystals were obtained in a similar way to that previously reported (*8*). A 10-20 mg/ml protein solution in 25 mM potassium phosphate buffer (pH 7.0) was sealed in a micro dialysis cell and then dialyzed against water containing 5% (w/v) polyethylene glycol 6000 at 25°C. Needle crystals precipitated and grew up to $0.2\times0.2\times1.0$ mm in size in a few days. The crystallization conditions were almost the same as the previous ones.

X-ray diffraction data were collected with synchrotron

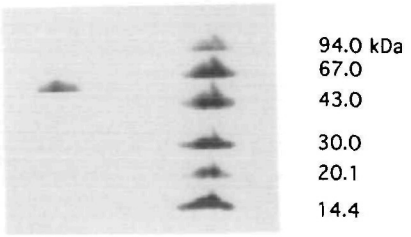

Fig. 1. **SDS-PAGE of the purified enzyme.** The molecular weights were calibrated with a LMW kit E (Pharmacia Biotech) in the right lane.

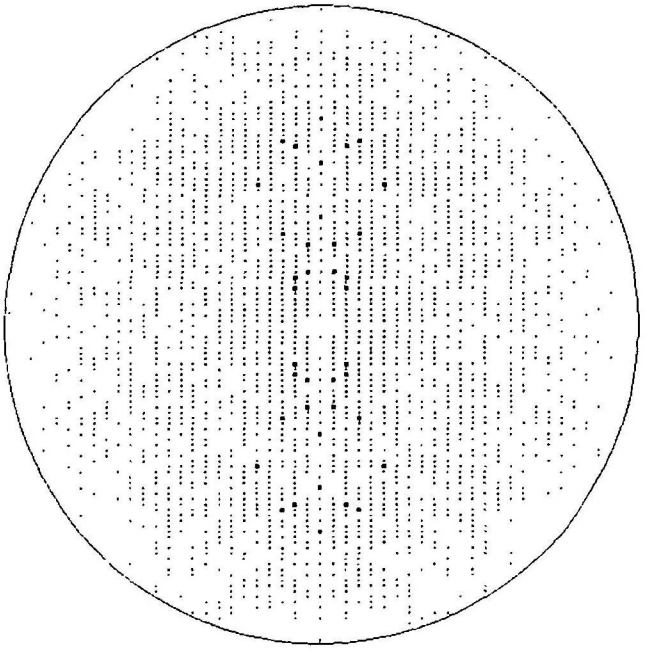

Fig. 2. **A pseudoprecession plot of the 0*kl* zone, calculated using the program, HKLVIEW, from the CCP4 suite** (Collaborative Computational Project Number 4, 1994). The outer edge of the pattern shows 2.4 Å resolution.

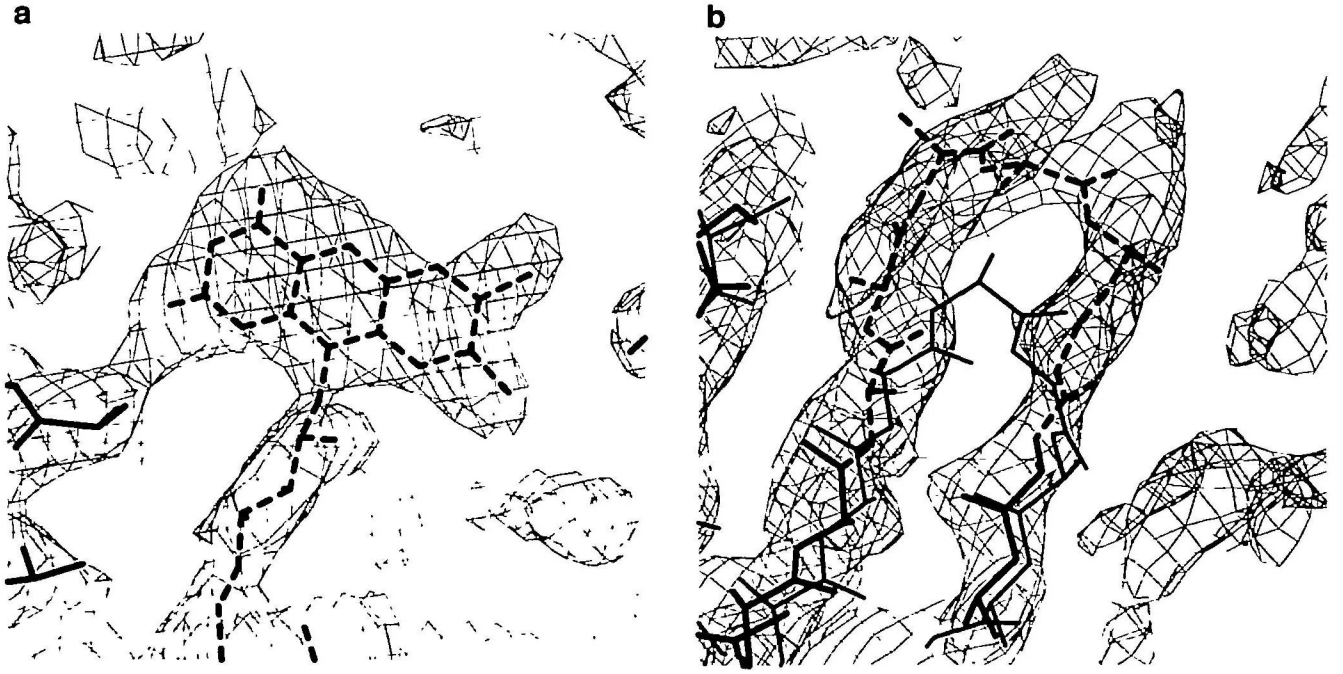

Fig. 3. **Omit maps of (a) FAD and (b) inserted residues, calculated with 17,765 reflexions of 30-3.0 Å resolution data.** The thin lines indicate the peptide backbone of the reference molecule and thick lines the polyalanine model of lipoamide dehydrogenase. FAD in (a) and amino acids in (b), indicated by broken lines, were omitted in the calculations.

TABLE I. **Results of molecular replacement.** Calculations were performed with the program, AMoRe (*16*), using the 30–5.0 Å resolution data. C_c is the correlation coefficient, α, β, and γ are the Eulerian angles, and (T_x, T_y, T_z) is the translation vector. A reasonable solution is indicated by bold letters.

Rotation function

Solution	α	β	γ	C_c
S1	**23.0**	**47.0**	**122.3**	**0.105**
S2	112.8	67.4	295.2	0.068

Translation function

Solution	T_x	T_y	T_z	C_c	R-factor
S1-1	**0.1253**	**0.3754**	**0.2788**	**0.450**	**0.497**
S1-2	0.2611	0.0533	0.3057	0.264	0.563

radiation ($\lambda = 1.00$ Å) at the Photon Factory using a Weissenberg camera for macromolecules (*11*). The intensities recorded on imaging plates (Fuji Film) were digitalized with a Fuji Film BAS2000 and integrated for each reflexion with the program, WEIS (*12*). The reflexion data obtained from different frames of Weissenberg photographs were scaled in one data set with the program, ROTAVATA, in CCP4 (*13*).

The space group was confirmed to be the same as the previous one (*8*). The cell dimensions were, however, slightly different, the new parameters being $a = 97.1$, $b = 158.7$, and $c = 67.9$ Å, with a dimeric enzyme in the asymmetric unit. Among 97,677 observed reflexions beyond 2.5 Å resolution, 33,372 independent ones were obtained with $R_{merge} = 6.7\%$. A precession image generated from the observed data is shown in Fig. 2. The completeness of the data was 94.0% at 2.7 Å and 80.2% at 2.4 Å resolution. The present crystal is expected to allow solving of the structure at higher resolution to determine the details of the three-dimensional structure of eukaryotic E3, which is very impotant for revealing the structural principle of the highly organized multienzyme complex.

As compared with the previous crystal (*8*), the unit cell is smaller by 6% in volume. The R factor between the two data sets, *i.e.*, the present and previous ones, was 15%, suggesting the possibility that the crystal structures are different from each other. As for different crystal packing, we have applied the molecular replacement method to solve the structure *de novo*. Another reason for molecular replacement was that it was difficult to prepare suitable crystals of heavy atom derivatives, because the crystals were obtained by a desalting method. As it has been found that the tertiary structures of E3 and glutathione reductase (GR) are similar, a polyalanine structure for the dimeric enzyme, which was constructed from the atomic coordinates of human erythrocyte GR (*14*) deposited in the Brookhaven Protein Data Bank (3GRS) (*15*), was used as a probe molecule. Molecular replacement calculations were performed with the four programs, MERLOT (*16*), MOLREP (Takenaka, A., unpublished work), X-PLOR (*17*), and AMoRe (*18*), to confirm their consistency, and they gave a unique solution (Table I) with reasonable molecular packing. The correctness was also easily judged by R-omit profile analysis (*19*). The crystal structure was refined by the least-squares method with rigid-body approximation [program X-PLOR (*17*)]. The R value was 48.7% for 30–5.0 Å resolution data. There are no abnormal contacts between molecules. Based on the structure, the initial phases were calculated and were improved by means of density modification techniques with the programs, DENAF (Takenaka, A., unpublished work) and SQUASH (*20*). The electron densities for FAD, and for inserted and deleted amino acids appeared on their omitted maps (see Fig. 3). Adjustment of main chain conformations and addition of the FAD molecule decreased the R value further to 37.3% for 17,765 reflexions in the case of 30–3.0 Å resolution data. As compared with the previous structure (*8*), the dimeric molecule is rotated by 2.6° and shifted by 2.3 Å in the unit cell. These small differences will be related to the decrease in the unit cell volume.

As a means to solve the structure of yeast E3 at higher resolution has become available, model building and its refinement are in progress. This would be a promising approach for revealing the structure of eukaryotic E3. For comprehensive understanding of the structure of multienzyme complexes, it is necessary to X-ray analyse the structures of the other components, E1 and E2.

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REFERENCES

1. Reed, L.J. (1974) Multienzyme complexes. *Acc. Chem. Res.* **7**, 701-729
2. Reed, L.J. (1990) Structure-function relationships in dehydrolipoamide acyltransferases. *J. Biol. Chem.* **265**, 8971-8974
3. Perpham, R.N. (1991) Domains, motifs, and linkers in 2-oxo acid dehydrogenese multienzyme complexes: A paradigm in the design of a multifunctional protein. *Biochemistry* **30**, 8501-8512
4. Yeaman, S.J. (1989) The 2-oxo acid dehydrogenase complexes: recent advances. *Biochem. J.* **257**, 625-632
5. Patel, M.S. (1990) Molecular biology and biochemistry of pyruvate dehydrogenase complexes. *FASEB J.* **4**, 3224-3233
6. Mattevi, A., Schierbeek, A.J., and Hol, W.G.J. (1991) Refined crystal structure of lipoamide dehydrogenase from *Azotobacter vinelandii* at 2.2 Å resolution. *J. Mol. Biol.* **220**, 975-994
7. Mattevi, A., Obmolova, G., Kalk, K.H., van Berkel, W.J.H., and Hol, W.G.J. (1993) Three-dimensional structure of lipoamide dehydrogenase from *Pseudomonas fluorescence* at 2.8 Å resolution. *J. Mol. Biol.* **230**, 1200-1215
8. Takenaka, A., Kizawa, K., Hata, T., Sato, S., Misaka, E., Tamura, C., and Sasada, Y. (1988) X-ray study of Baker's yeast lipoamide dehydrogenase at 4.5 Å resolution by molecular replacement method. *J. Biochem.* **103**, 463-469
9. Kawahara, Y., Misaka, E., and Nakanishi, K. (1968) Purification and properties of lipoamide dehydrogenase from yeast, *Candida krusei*. *J. Biochem.* **63**, 77-82
10. Reed, L.J., Koike, M., Levitch, M.E., and Leach, F.R. (1958) Studies on the nature and reactions of protein-bound lipoic acid. *J. Biol. Chem.* **232**, 143-158
11. Sakabe, N. (1991) X-ray diffraction data collection system for moderm protein crystallography with a Weissenberg camera and an imaging plate using synchrotron radiation. *Nucl. Instrum. Methods* **A303**, 448-463
12. Higashi, T. (1989) The processing of diffraction data taken on a screenless Weissenberg camera for macromolecular crystallography. *J. Appl. Cryst.* **22**, 9-18
13. A suite of programs for protein crystallography (SERC Collaborative Computing Project No. 4, Daresbury Laboratory, Warrington UK, 1979)
14. Karplus, P.A. and Shultz, G.E. (1987) Refined structure of glutathione reductase at 1.54 Å resolution. *J. Mol. Biol.* **195**, 701-729
15. Bernstein, F.C., Koetzle, T.F., Williams, G.J.B., Meyer, E.F., Jr., Brice, M.D., Rodgers, J.R., Kennard, O., Shimanouchi, T., and Tasumi, M. (1977) The protein data bank: A computer-based

archival file for macromolecular structures. *J. Mol. Biol.* **112**, 535-542
16. Fitzgerald, P.M.D. (1988) *MERLOT*, an integrated package of computer programs for the determination of crystal structures by molecular replacement. *J. Appl. Cryst.* **21**, 273-278
17. Brünger, A.T. (1992) X-PLOR manual version 3.1, Yale University, New Haven, CT
18. Navaza, J. (1994) AMoRe: an automated package for molecular replacement. *Acta Cryst.* **A50**, 157-163
19. Tsuchiya, D., Sekiguchi, T., and Takenaka, A. (1995) A new evaluation of structure model in molecular replacements. *Abstracts of the Annual Meeting of the Crystallographic Society of Japan*, 45
20. Zhang, K.Y.J. (1993) SQUASH-combining constraints for macromolecular phase refinement and extension. *Acta Cryst.* **D49**, 213-222