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Mechanistic implications from structures of yeast alcohol dehydrogenase complexed with coenzyme and an alcohol☆

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ABSTRACT

Yeast alcohol dehydrogenase I is a homotetramer of subunits with 347 amino acid residues, catalyzing the oxidation of alcohols using NAD^+ as coenzyme. A new X-ray structure was determined at 3.0 Å where both subunits of an asymmetric dimer bind coenzyme and trifluoroethanol. The tetramer is a pair of back-to-back dimers. Subunit A has a closed conformation and can represent a Michaelis complex with an appropriate geometry for hydride transfer between coenzyme and alcohol, with the oxygen of 2,2,2-trifluoroethanol ligated at 2.1 Å to the catalytic zinc in the classical tetrahedral coordination with Cys-43, Cys-153, and His-66. Subunit B has an open conformation, and the coenzyme interacts with amino acid residues from the coenzyme binding domain, but not with residues from the catalytic domain. Coenzyme appears to bind to and dissociate from the open conformation. The catalytic zinc in subunit B has an alternative, inverted coordination with Cys-43, Cys-153, His-66 and the carboxylate of Glu-67, while the oxygen of trifluoroethanol is 3.5 Å from the zinc. Subunit B may represent an intermediate in the mechanism after coenzyme and alcohol bind and before the conformation changes to the closed form and the alcohol oxygen binds to the zinc and displaces Glu-67.

Structures for yeast (*Saccharomyces cerevisiae*) alcohol dehydrogenase 1 (ADH1) determined at 2.4 Å (PDB ID, 4W6Z) show that the homotetramer is formed from back-to-back asymmetric dimers of subunits [1]. One subunit, in a "closed" conformation, binds the NAD coenzyme, and the substrate analog, trifluoroethanol, in an apparently "productive" mode that resembles an expected Michaelis complex, with the *pro-R* hydrogen of the methylene carbon of the alcohol directed toward C4N of the nicotinamide ring and the alcohol oxygen binding to the catalytic zinc in the classical tetrahedral coordination with Cys-43, Cys-153, and His-66. The other subunit, in an "open" conformation, has no bound coenzyme, and the catalytic zinc has an alternative, inverted, coordination where Glu-67 displaces the trifluoroethanol. (The open-to-closed conformational change involves a rotation of about 13° of the catalytic domain toward the coenzyme binding domain.) We suggested that the enzyme crystallized in the asymmetric form and that any coenzyme that might have been bound to the open subunit dissociated from the complex when the crystals were soaked for some days (for transport to the synchrotron) in a solution that did not contain the coenzyme. The asymmetry is puzzling because titrations of the active sites of the enzyme have suggested that all four subunits in a tetramer can bind coenzyme, and there is no good evidence for cooperativity in binding of NAD or in the kinetics of catalysis [1]. (See also Supporting Information in Ref. [1] for results on the stoichiometry of coenzyme binding.) Thus, it was necessary to determine if the crystallized enzyme would be asymmetric and would have coenzyme bound to both subunits if the concentration of coenzyme were maintained until the moment when the crystals were flash vitrified for data collection at 100 K.

Therefore, we examined data again for other crystals that were kept in crystallization media containing coenzyme, even if the crystals diffracted less well. From tens of data sets, from some thousands of crystallization attempts under different conditions by nine researchers, most crystals appeared to have the *P*622 space

Abbreviations: ADH, alcohol dehydrogenase; TFE, 2,2,2-trifluoroethanol ("ETF" in the PDB file, with the methylene carbon labeled as "C2"); rmsd, root mean square deviation.

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group [2], but were twinned. However, two data sets processed in the *P*321 space group, with a unit cell half as large as the one solved previously. The crystals in the *P*321 space group were partially twinned, and structures could not be determined earlier. Now, using molecular replacement with a dimer from the structure determined at 2.4 Å (4W6Z.pdb) as the search model, we found that these crystals have a dimer in the asymmetric unit, that the subunits have different conformations, and that both subunits bind coenzyme and alcohol. The dimer forms the biologically relevant tetramer via a crystallographic axis. The structures of the enzymes in the two different crystal forms are very similar, but the subunit with the open conformation in the new structure has small, local alterations that accommodate the coenzyme binding. The open conformation retains the alternative coordination of the catalytic zinc, and it appears that this subunit could represent an intermediate on the pathway to forming a productive ternary complex.

1. Materials and methods

1.1. Protein preparation

The gene for *Saccharomyces cerevisiae* ADH1 (*adc*1, YOL086c; UniProtKB, P00330), the constitutive, fermentative enzyme from the laboratory strain of baker's yeast was expressed from plasmid YEp13 in host yeast that did not express any of the three medium-chain, zinc-containing alcohol dehydrogenases [3–5], and the protein was purified to homogeneity as described [6].

1.2. Crystallography

The crystals were prepared by the hanging drop method at 4 °C with 10 mg/ml yeast ADH1, 100 mM sodium *N*-tris(hydroxymethyl) methyl-3-aminopropanesulfonate buffer, pH 8.4 (measured at 25 °C), 0.25 mM EDTA, 2 mM LiNAD$^+$, 0.2 M 2,2,2-trifluoroethanol (TFE), 1 mM $YbCl_3$, and 7% MPEG5000 (Fluka polyethylene glycol 5000 monomethyl ether) over reservoirs containing ~16% (15–19%) MPEG 5000 and 0.2 M TFE. For shipping to the synchrotron, the crystals were transferred to a solution with the same buffer and 2 mM NAD$^+$ as in the original drop, except that the concentration of TFE was raised to 0.287 M and MPEG-5000 was 25%.

Data were collected May 18, 1997, on synchrotron beamline BW7A at the EMBL/DESY unit in Hamburg at 100 K, at a wavelength of 1.39 Å and a crystal to detector distance of 180 mm, with a MAR CCD 130 mm detector, and processed with Denzo, Scalepack, and Truncate [7,8]. A molecular replacement solution was found with the AB dimer of the tetrameric structure of ADH1 (4W6Z.pdb) using MolRep [8]. Further refinement used REFMAC5 and model building with the program O [8,9]. The refinement was accomplished with the twin operator (*h*, *k*, *l*, and −*h*, −*k*, *l*, with final refined twin fractions of 0.714 and 0.286) and by using 4 TLS elements, for the 2 catalytic domains, residues 1–154 and 294–347, with the associated 2 zincs and TFE, and the 2 coenzyme binding domains, residues 155–293, with the associated NAD. The structure was analyzed with PROCHECK, SFCHECK [8], MolProbity [10], and PARVATI [11]. The estimated solvent content is 55% and the Matthews coefficient is 2.75 Å^3/Da. The data and refinement statistics are given in Table 1. The X-ray coordinates and structure factors have been deposited in the RCSB Protein Data Bank as entry 5ENV.pdb.

Table 1
X-ray data collection and refinement statistics.

PDB entry	5ENV
Space group	*P*321
Subunits in asymmetric unit	2
Cell dimensions, Å	146.3, 146.3, 66.0
Cell angles, deg	90, 90, 120
Resolution range, Å (outer shell)	20.0–3.0 (3.00–3.05)
Measured reflections: total, unique	74,630, 15,427
Completeness, % (outer shell)	98.1 (75.1)
Twin fraction, H, K, L; −H, −K, L	0.714, 0.286
R_{symm}, (outer shell)[a]	0.064 (0.326)
mean $<I>/\sigma<I>$, (outer shell)	18.0 (2.7)
R_{value}, R_{free}, test %[b]	0.14, 0.20, 4.5
rmsd for bond dist. (Å), angles (deg)[c]	0.013, 1.76
mean *B* value, (Å^2), Wilson, REFMAC	60, 57[d]
Non-H atoms, protein, mean *B*	5164, 58
4 Zn, 2 NAD, 2 TFE heteroatoms, *B*	104, 58
Waters, *B*	20, 30
Estimated coordinate error, Å	0.28

[a] $R_{symm} = \Sigma(|I - <I>|)/\Sigma I$.
[b] $R_{value} = (\Sigma|F_o - kF_c|)/\Sigma|F_o|$, where *k* is a scale factor. R_{free} was calculated with the indicated percentage of reflections not used in the refinement [38].
[c] Root-mean square deviations from ideal geometry.
[d] Refinement with 4 TLS groups gave a residual mean *B* value of 18 for all non-H atoms.

2. Results

2.1. Overall structure

Subunit A of the present structure is very similar to subunits A and C in the previous structures (4W6Z.pdb) in having the closed conformation, bound coenzyme, and the oxygen of trifluoroethanol ligated to the catalytic zinc. In contrast, the B subunit is more open and similar to the B and D subunits in the previous structures, but, significantly, the B subunit has bound coenzyme. (The AB and CD dimers in the previous structures are the asymmetric units, related by a translation in the unit cell, producing two similar AB:AB and CD:CD tetramers.) The α-carbons of the AB dimer in the present structure are superpositioned on the α-carbons of the AB dimer from the previous structure (4W6Z.pdb) with an rmsd of 0.58 Å, the A subunits are superpositioned with an rmsd of 0.34 Å, and the B subunits with an rmsd of 0.66 Å. The A and B subunits of the present structure superposition on one another with an rmsd of 1.8 Å, indicating conformational differences in the subunits. After residues from the coenzyme binding domains (155–293) are superpositioned (rmsd 0.56 Å), the catalytic domains superposition with an rmsd of 0.84 Å, with a relative rotation of ~12°, which is similar to the rotation angle that describes the closure of the A subunit relative to the B subunit in the previous structure (Fig. 1). The conformational change closes up the active site cleft about an axis perpendicular to the plane indicated by the "X". Several secondary structural elements in the B subunit with bound coenzyme of the present structure show small movements as compared to the previous B subunit without coenzyme, including residues 201–208, 224–226, 245–250, 269–275, and 325–341, apparently due to the binding of coenzyme, but the overall "open" states are similar.

The biologically relevant molecule is a tetramer, formed from two back-to-back AB dimers about a crystallographic axis, as discussed and shown previously [1].

The average *B* factor for residues B6–B24, B41–B63 and B317–B341 are higher than those residues in the A subunit. These regions form loops of the catalytic domain that show the largest movement when the conformation changes from open to closed (Fig. 1). The overall *B* factor of 57 Å^2 for the whole structure is decreased to 18 Å^2 when the *B* factors from the TLS refinement are not added to the individual atomic *B* factors, suggesting that the four TLS elements have considerable mobility as units.

Electron density for Yb^{3+} was not observed, and an anomalous difference map showed no substantial peaks of density above background. Data for another crystal in the same space group