Three-dimensional structure of flavocytochrome b_2 from baker's yeast at 3.0-Å resolution

(x-ray diffraction/structure homology/flavoenzyme/cytochrome/electron transfer)

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ABSTRACT The structure of flavocytochrome b_2 from baker's yeast was solved at 3.0-Å resolution by the multiple isomorphous replacement method combined with solvent leveling procedures, using data collected from an area detector. The tetramer of M_r 230,000 has 4-fold symmetry. Each subunit contains a cytochrome domain consisting of the first 100 residues, a flavin-binding domain containing the next 386 residues, and an extended C-terminal tail of 25 residues. The cytochrome domain closely resembles microsomal cytochrome b_5 , whereas the flavin-binding domain contains a parallel β_8/α_8 barrel motif similar to glycolate oxidase and trimethylamine dehydrogenase. Two of the four cytochrome domains are disordered in the crystals. The flavin ring and heme group are separated by about 16 Å between their centers, and their planes are inclined by about 17° to each other.

Flavocytochrome b_2 [L-lactate:ferricytochrome c oxidoreductase, EC 1.1.2.3] from baker's yeast (*Saccharomyces cerevisiae*) is a tetrameric enzyme of M_r 230,000 (1) and is located in the intermembrane space of mitochondria (2). Each subunit of M_r 57,500 (3) contains one heme and one FMN, both bound noncovalently (4). The enzyme catalyzes the two-electron oxidation of lactate to form pyruvate, with subsequent electron transfer to cytochrome c via the flavin and heme groups. This process occurs in two major steps. First, the substrate, lactate, is oxidized and two electrons are passed to the flavin, which becomes fully reduced to the flavin hydroquinone (5–7). Then, electrons are transferred one at a time, with the formation of a semiquinone intermediate (7), to the heme moiety and then to cytochrome c . The rate-limiting step in lactate oxidation is the initial abstraction of the β proton (6, 8).

The amino acid (9) and genomic DNA (10) sequences of flavocytochrome b_2 are known. Approximately the first 100 residues comprise a heme-binding domain, cytochrome b_2 core, which can be released as a stable, intact fragment by tryptic digestion of the enzyme (11). This core is homologous in sequence to microsomal cytochrome b_5 (12) and shares with it a number of spectroscopic and chemical properties. These similarities had suggested that the conformation of cytochrome b_2 core is similar to that of cytochrome b_5 and that the two heme-containing fragments share a common fold, the "cytochrome b_5 fold" (13).

The remaining 411 residues contain the flavin-binding domain. Included in this piece is a short region, near residue 310, that is susceptible to digestion by yeast or other proteases in the absence of protease inhibitors such as phenylmethylsulfonyl fluoride (14). Cleavage does not result

in disruption of the quaternary structure of the enzyme but does lead to an alteration of its kinetic properties (3).

METHODS

Crystals of intact flavocytochrome b_2 were prepared by dialysis against 2-methyl-2,4-pentanediol as described (15) and diffract to beyond 3.0-Å resolution. They are trigonal, space group $P3_221$, with cell parameters $a = 165.51$ Å and $c = 113.71$ Å. The densities of four crystals were determined by flotation in a bromobenzene/xylene density gradient and used to calculate (16) an average molecular weight of 117,000 in the asymmetric unit and a solvent content of 68%.

Two heavy-atom compounds, *cis*-Pt(NH₃)₂Cl₂ and Na₂-IrCl₆, gave suitable intensity changes and maintained crystalline order to high resolution. These only occurred after the crystals had been transferred to an artificial mother liquor containing 35% 2,5-hexanediol. In this medium $a = 166.18$ Å and $c = 112.61$ Å.

Four sets of x-ray intensity data were collected to 3.0-Å resolution at the University of California, San Diego, area-detector facility (17). One was from a native crystal (also transferred to the artificial mother liquor), one from a crystal of the platinum derivative, and two from crystals of the iridium derivative. Because of increased crystal decay, the two iridium data sets are incomplete but complement each other. The crystals, mounted in thin glass capillaries, were enveloped in the artificial mother liquor or soaking solution, held in place by pipe-cleaner fibers, and maintained at 0–5°C by a cold nitrogen gas stream. The data collection statistics are summarized in Table 1.

Each of the three derivative data sets was locally scaled (18) to the native data using the ROCKS (19) crystallographic computing program as adapted for the VAX computer (20). The two iridium data sets were treated separately throughout the analysis to avoid errors caused by differences in substitution.

The structure was analyzed initially at low resolution, using a 6.0-Å resolution subset of the data. Isomorphous difference Patterson maps of both derivatives were solved automatically by the Patterson superposition program HASSP (21), giving one platinum and two iridium binding sites. Three additional platinum sites and one additional iridium site were located by difference Fourier methods. The handedness of the space group was found to be $P3_221$ from anomalous difference Fourier maps (22). The heavy-atom positions were refined by least squares using the program

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Table 1. Data collection statistics

Data set	Total no. of observations	No. of unique reflections	R_{sym}^*	$\Delta F_{\text{iso}}^\dagger$ %
Native	467,057	43,625	0.079	
Pt	461,447	42,645	0.096	11.8
Ir-1	321,990	37,565	0.118	17.6
Ir-2	373,725	40,481	0.118	15.2

* $R_{\text{sym}} = \sum |I - \langle I \rangle| / \sum \langle I \rangle$ summed over all observations of all reflections.

$\dagger \Delta F_{\text{iso}}$ is the mean isomorphous structure factor difference.

HEAVY (21) based on the origin-removed difference Patterson function. Protein phases were computed by the multiple isomorphous replacement (MIR) method including anomalous scattering. The figure of merit at 6.0-Å resolution was 0.82.

Solvent leveling was carried out at 3.0-Å resolution using the procedure of Wang (23). After nine cycles, the figure of merit improved from an initial value of 0.29 to 0.71 and resulted in an accumulated phase shift of 53°. During this procedure, a total of three masks were computed based on a solvent content of 0.68 obtained by assuming two protein subunits per asymmetric unit.

RESULTS

Molecular Symmetry at Low Resolution. A native anomalous difference Fourier map at 6.0-Å resolution contained a single peak in the asymmetric unit. This peak corresponded to a region of high electron density in the 6.0-Å resolution native map and was interpreted to be the site of a heme iron atom. The peak was also found in anomalous difference maps for the two derivatives.

When sections of the 6.0-Å-resolution native map were viewed normal to a crystallographic 2-fold axis, the molecule was found to possess 4-fold symmetry. This can be clearly seen in Fig. 1*a*, which shows a slab of electron density from several superimposed sections of the 6.0-Å-resolution map viewed along the crystallographic 2-fold axis. The two major iridium binding sites (Fig. 1*a*) and two of the four platinum sites also obey the molecular 4-fold symmetry.

However, when sections passing through the single iron site were plotted, the 4-fold symmetry was partially lost. Fig. 1*b* shows a slab of electron density with the iron positions close to the middle section. In this slab, the 4-fold symmetry is well maintained within a 30-Å radius of the 4-fold axis. Outside that radius, only the crystallographic 2-fold symmetry remains. The electron density at the 4-fold-related iron position as well as the protein density surrounding it is completely absent, indicating that there is positional disorder for one of the two b_2 core domains per asymmetric unit.

Tracing the Chain. Two different versions of the solvent-leveled 3.0-Å-resolution electron density map were used to trace the course of the polypeptide chain. One of these was used without further modification to trace the b_2 core portion of the molecule in the subunit where it is ordered. The other version was averaged (24) about the molecular 4-fold axis and used to trace the rest of the structure. The non-solvent-leveled map was also examined at 3.0-Å resolution in the region expected for the disordered b_2 core domain and was found to contain no significant electron density in that region.

The polypeptide chain was traced manually in the 3.0-Å-resolution minimap, making use of the amino acid sequence to identify prominent side chains and keep the chain in register. The polypeptide chain was found to be segregated sequentially into an N-terminal cytochrome domain, a central flavin-binding domain, and an extended C-terminal tail. The main chain was very clearly defined throughout most of the flavin-binding domain and C-terminal tail and could be easily

FIG. 1. (a) A slab of five superimposed sections of the 6.0-Å electron density map perpendicular to the crystallographic 2-fold axis, showing the molecular 4-fold symmetry. Two pairs of Ir sites are indicated. (b) A slab of five superimposed sections passing through the iron sites, which are indicated. Two cytochrome domains are indicated by solid lines. The expected position of the 4-fold related cytochrome domains are indicated by dashed lines.

followed. In this portion, one short segment was disordered and another two segments were located in weak electron density, necessitating contouring at a lower level of density. The electron density of the ordered cytochrome domain was somewhat weaker than that of the flavin-binding domain and more difficult to follow. The N-terminal residue could not be located, and one region of the main-chain density was discontinuous. The heme and FMN groups could be easily identified as discrete pieces of high electron density, the heme being approximately centered on the iron position.

Description of the Molecule. Fig. 2*a* shows the flavocytochrome b_2 tetramer viewed parallel to the molecular 4-fold axis. The subunits of the tetramer are packed so that the four flavin-binding domains form an oblate ellipsoid about 100 Å in diameter and about 60 Å thick. The cytochrome domains protrude from the edge of the ellipsoid above its mid-plane.

Fig. 2*b* shows an isolated subunit of flavocytochrome b_2 .

FIG. 2. Stereo diagrams. (a) Tetramer of flavocytochrome b_2 viewed along the 4-fold axis. The ordered cytochrome domains and flavin-binding domains are represented by thin solid lines. The disordered cytochrome domains are shown as thin dashed lines. The C-terminal tails (residues 487–511), the heme groups, and the FMN groups are shown as thick lines. (b) Isolated cytochrome and flavin-binding domains, residues 2–486, of flavocytochrome b_2 . The cytochrome domain (residues 2–100), the β_8/α_8 -barrel (residues 192–465), and the prosthetic groups are represented by thick lines. The disordered segment (residues 301–310) is indicated by a dashed line. The remainder of the chain is shown in thin lines.

The ordered cytochrome domain, containing the first 100 residues, is folded similarly to cytochrome b_5 (25) and is oriented with the heme crevice facing the flavin-binding domain. The interface region between them appears to be inaccessible to solvent. This arrangement of cytochrome and flavin-binding domains is similar to that of the cytochrome and flavoprotein subunits of *p*-cresol methylhydroxylase with respect to each other as found at 6-Å resolution (26).

The flavin-binding domain is shown schematically in Fig. 3a. A central segment of peptide chain, residues 192–465, forms an eight-stranded β/α -barrel (Fig. 4). A flanking segment on the N-terminal side, residues 100–191, contains four helices, A–D. A flanking segment on the C-terminal side, residues 466–486, contains three short helices, J–L.

The β/α -barrel motif is interrupted by an excursion of the chain by about 40 residues, between secondary structural elements β_4 and α_4 (Fig. 3a). This excursion contains two helices, E and F, and a disordered segment, from residues 301–310, corresponding approximately to the region sensitive to yeast proteases (14). About 75% of this excursion extends into the solvent and appears to possess considerable flexibility, as judged by the generally weaker electron density compared to the rest of the flavin-binding domain.

The C-terminal tail, residues 487–511, extends from the flavin-binding domain and contains one short helix. The tail makes contact successively with each of the other three subunits, passing close to the molecular 4-fold axis where it forms a short β annulus with symmetry-related segments (Fig. 2a).

Binding of Flavin and Heme. The FMN is located at the C-terminal end of the β -barrel as shown in Fig. 4. The plane of the flavin ring is adjacent to strand β_1 and is inclined to the barrel axis by about 45° . The position of O2 is nearly coincident with the barrel axis and the 7 α - and 8 α -methyl groups are near the edge of the barrel. The ribityl chain is extended and is approximately perpendicular to the barrel axis. The phosphate group is located between the C-termini

of strands β_7 and β_8 and at the N-terminus of helix I (Fig. 3a) before α_8 .

The heme group is located in a crevice in the cytochrome domain. The heme ligands are His-43 and His-66, as expected from the sequence comparison with cytochrome b_5 (12). Both propionates are extended away from the heme crevice toward the flavin-binding domain and are shielded from solvent.

The plane of the flavin ring is inclined to that of the heme group by about 17° . The distance from the heme iron to the center of the isoalloxazine ring is about 16 Å and about 10 Å from the closest edge of the pyrrole ring to N-5 of the flavin. There are no intervening side chains between the two prosthetic groups.

Comparison with Other Proteins. The conformation of the cytochrome domain of flavocytochrome b_2 is very similar to that of the heme-binding fragment of cytochrome b_5 , as expected from the sequence homology (12), except for a 12-residue segment starting after His-66 of b_2 core. In this segment the cytochrome b_2 sequence contains two insertions when compared with the cytochrome b_5 sequence, which probably account for the localized difference in structure.

The conformations and orientations of the heme groups in the two proteins are somewhat different. In flavocytochrome b_2 both propionates are fully extended, whereas in cytochrome b_5 one of them is bent back toward the molecule and is partially shielded from solvent. When the C α coordinates of the two proteins are aligned, the two porphyrin rings lie nearly in the same plane but are rotated with respect to each other by $\approx 30^\circ$ about an axis through the iron and normal to the heme plane.

The flavin-binding domain is folded in a manner similar to the other two FMN-containing flavoenzymes of known structure, glycolate oxidase (27) and trimethylamine dehydrogenase (28). The three flavin-binding domains are compared schematically in Fig. 3. All three domains contain eight-stranded β/α -barrel motifs and differ completely from other classes of flavoproteins such as glutathione reductase

FIG. 4. Stereo diagram showing the β_8/α_8 -barrel of the flavin-binding domain, residues 192–465, viewed parallel to the barrel axis. The eight central β -strands, the FMN, and helix I, between β_8 and α_8 , are drawn in thick lines.

etry of one heme *c* per heme *b*₂ (37). If the mode of binding is physiological, then subunits with the ordered as well as the disordered *b*₂ core domains can form a productive complex with cytochrome *c*, and large displacements are not required. The above observation also suggests that a direct crystallographic study of the complex may be possible in the future.

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