The 1.3 Å Crystal Structure of the Flavoprotein YqjM Reveals a Novel Class of Old Yellow Enzymes*

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Here we report the crystal structure of YqjM, a homolog of Old Yellow Enzyme (OYE) that is involved in the oxidative stress response of Bacillus subtilis. In addition to the oxidized and reduced enzyme form, the structures of complexes with p-hydroxybenzaldehyde and p-nitrophenol, respectively, were solved. As for other OYE family members, YqjM folds into a (α/β)8-barrel and has one molecule of flavin mononucleotide bound non-covalently at the COOH terminus of the β-sheet. Most of the interactions that control the electronic properties of the flavin mononucleotide cofactor are conserved within the OYE family. However, in contrast to all members of the OYE family characterized to date, YqjM exhibits several unique structural features. For example, the enzyme exists as a homotetramer that is assembled as a dimer of catalytically dependent dimers. Moreover, the protein displays a shared active site architecture where an arginine finger (Arg336) at the COOH terminus of one monomer extends into the active site of the adjacent monomer and is directly involved in substrate recognition. Another remarkable difference in the binding of the ligand in YqjM is represented by the contribution of the NH2-terminal Tyr28 instead of a COOH-terminal tyrosine...
structured compounds such as TNT induces the expression of YqjM, an OYE homolog, from *B. subtilis*. Data base searches have indicated that YqjM displays a high degree of sequence similarity to OYE1 from *Saccharomyces carlsbergensis* and its homologs (1). *Pseudomonas putida* XenA, a xenobiotic reductase, appears to be the closest homolog exhibiting 40% identity. Biochemical analysis of YqjM has shown that the enzyme shares some important common features with members of the OYE family (1). For example, YqjM binds the FMN cofactor non-covalently and reduces the flavin in the reductive half-reaction at the expense of NADPH. Like other members of the family, YqjM transfers electrons from the reduced flavin to the double bond of a variety of α,β-unsaturated carbonyl compounds and accepts also electrophilic xenobiotics such as nitroglycerin, N-ethylmaleimide, and trinitrotoluene as substrates for the oxidative half-reaction. Finally, phenolic compounds such as *p*-hydroxybenzaldehyde (pHBA) bind tightly to this enzyme and form charge transfer complexes due to the overlapping π-electrons of the electron-rich phenolate ligand and the electron-deficient flavin isalloxazine ring (1).

However, on comparing YqjM with OYE, several important differences are apparent. For example, photooxidation of YqjM leads directly to the fully reduced state; whereas in the yeast and plant homologs the red flavin semiquinone is kinetically stabilized (1). A further unique feature of YqjM is attributed to its oligomerization state. While all other OYE homologs are either monomeric or dimeric enzymes, YqjM is the only known family member that functions as a homotetramer (1) suggesting the evolution of particular residues involved in the interface formation between the protomers. This implies that the different modes of self-assembly within the OYE family may be correlated with a functional diversity. Finally, sequence comparisons have suggested significant changes in the active site of the enzyme. For example, Thr27 of OYE, which regulates the redox potential of the FMN cofactor (12), is replaced by Cys26 in YqjM, whereas the OYE-Tyr375, which is essential for substrate binding, has no conserved counterpart in YqjM.

The three-dimensional structures from OYE (13) and several of its homologs (14–17) have been elucidated and point to the fundamental fold of YqjM. However, to determine the molecular basis of the unique properties of YqjM, we solved the structures of the oxidized and reduced holoenzyme and of complexes with the inhibitors pHBA and *p*-nitrophenol (pNP), respectively. The data presented here confirm that YqjM shares the overall fold of the OYE family. Also it demonstrates unequivocally that YqjM is in fact the first characterized representative of a new class of OYE homologs showing fundamental differences to the classical OYE enzymes.

**EXPERIMENTAL PROCEDURES**

**Materials**—All materials used were of analytical grade. All chemicals were purchased from Sigma. Chromatographic columns were from Amersham Biosciences.

**Expression and Purification of the Native YqjM**—The construct of pET21a (Novagen) with the open reading frame of YqjM was expressed and purified as described in Ref. 1 with the following modifications: purification was performed with an AKTA-FLASH system from Amersham Biosciences using a HiPrep 16/10 DEAE FF column in the anion exchange step and a HiPrep 16/10 Phenyl FF column for the hydrophobic interaction chromatography. Additionally, a final gel filtration step was introduced using a HiLoad Superdex 200 gel filtration column equilibrated with 10 mM HEPES, pH 7.5, 150 mM potassium chloride, and 4 mM dithiothreitol. Fractions containing YqjM were pooled and concentrated to 10 mg/ml by ultrafiltration using Centriprep-30 (Amicon). Selenium-methionine-labeled YqjM (SeMet-YqjM) was expressed from the met’ *Escherichia coli* strain B834(DE3). The purification of SeMet-YqjM followed the same protocol as for the native enzyme.

**Protein Crystallization**—Crystallization trials were performed at 19 °C using the sitting drop vapor diffusion method. After intensive screening, well diffracting crystals were obtained by mixing 3 μl of protein with 1.5 μl of a crystallization solution containing 0.1 M Tris/HCl, pH 8.5, 25% polyethylene glycol 3500, and 0.2 M sodium chloride. Crystal trials were set up in cryschem plates with a reservoir volume of 400 μl. After 5–7 days, crystal plates appeared that had the characteristic yellow color of the oxidized, enzyme-bound FMN cofactor. They belonged to the orthorhombic space group C2221, with unit cell dimensions of *a* = 51.5 Å, *b* = 185.2 Å, and *c* = 169.8 Å and contained two molecules per asymmetric unit corresponding to a solvent content of 54%. However the above-mentioned condition did not yield crystals of the SeMet-YqjM derivative. After a new screening round, a similar crystallization condition was found containing 0.1 M Tris/HCl, pH 8.5, 25% polyethylene glycol 3500, 0.2 M lithium sulfate, and 0.01 M strontium chloride. YqjM-SeMet crystals complexed with pHBA and pNP were obtained by cocrystallization using a ligand concentration of 3 mM. The presence of either pHBA or pNP resulted in a color change of the yellow YqjM crystals toward red. Reduction of YqjM crystals was achieved by soaking crystals of the oxidized enzyme in 100 mM NADPH until they turned colorless (approximately after 10 min). For cryo-measurements, crystals were transferred from the crystallization drop to the mother liquor supplied with 15% 2-methyl-2,4-pentanediol as cryo-protectant and rapidly frozen in a 100 K stream of nitrogen gas.

**Data Collection and Structure Determination**—Initial attempts to overcome the crystallographic phase problem by molecular replacement using the search models derived from other members of the OYE family failed. Therefore experimental phase information was required to determine the structure of YqjM. Phase information could be obtained by collecting a single-wavelength anomalous diffraction (SAD) data set from a single SeMet-YqjM crystal. SAD data of SeMet-YqjM and diffraction data of the complexes with pHBA and pNP were collected at the BW6 beamline at the Deutsches Elektronen Synchrotron (Hamburg, Germany) using a MARCCD detector. Crystals of the oxidized and reduced YqjM were measured in house using a MarResearch imaging plate.

All diffraction data were processed and scaled with the programs DENZO and SCALEPACK from the CCP4 package (18). For the SAD experiment, an x-ray absorption spectrum was recorded in the vicinity of the selenium edge. Diffraction data up to 1.8 Å resolution were then collected at a wavelength corresponding to the peak of this spectrum (*λ* maximum, 0.9792 Å). Subsequently, difference Fourier analyses performed with SHELX (19, 20) enabled us to identify all 10 of the theoretical selenium sites. Refinement of heavy atom parameters and phase calculation were conducted with SHARP (21) leading to a figure of merit of 0.68 for data between 20 and 1.82 Å resolution. Solvent flattening was performed with SOLOMON (22) and resulted in an electron density map of excellent quality, in which the entire backbone model of YqjM was built in with ARP/WARP (23). Energy-restrained crystallographic refinement against the 1.3 Å resolution data set was carried out with maximum likelihood algorithms implemented in CNS (24) using the protein parameters of Engh and Huber (25). Refinement, model rebuilding with the program O (26), and water incorporation were done smoothly via rigid body, positional, and coordinate optimization. The entire structure was checked using simulated annealing composite omit maps. Finally, after the addition of the FMN cofactor molecules, the refinement converged at a R-factor of 18.9% (*R*iso = 20.8%). All residues of YqjM could be traced in the electron density map and exhibited good stereochemistry (Table 1). Also the FMN cofactor molecules were well defined by electron density as well as four sulfite ions, two of which were bound within the active sites and the other two stabilizing crystal lattice contacts. In the Ramachandran plot, 91% of the 674 residues were found in the most favorable, 8.6% in the favorable, 0.3% in the generously allowed, and no residue was found in the disallowed region. The data collection, phasing, and refinement statistics are summarized in Table 1. All parameter and topology files were created with the program XFLOR2D (27).

Graphical presentations were made using the programs MOLSCRIPT (28), RASTER3D (29), DINO (www.dino3d.org) and PYMOL (www.pymol.org).

**Sequence Alignment and Phylogenetic Reconstruction**—Amino acid sequences were retrieved by BLAST searches using the Swiss-Prot data base and aligned using the web-based program MULTALIN (available at www.toulouse.inra.fr/software/multalin.html). To perform a phylogenetic analysis, the amino acid sequences were aligned with ClustalW and the result saved in the NEXUS format. The program PAUP* 4.0b10 was used to analyze the phylogenetic relationships of the OYE/YqjM homologs.

**Enzyme Assays**—The apparent steady state kinetic constants were determined as described in Ref. 1. Briefly, the assays were performed in 0.1 M Tris/HCl, pH 7.5, at 25 °C in the presence of an oxygen-consuming...
namely helices residues and harbor additional secondary structural elements, structure). The COOH-terminal loops range in length from 5 to 31 atoms and followed by the oxidation of YqjM was solved by SAD and refined at 1.3 Å resolution to an (see also Fig. 1). For nomenclature of secondary struc- strand 6 (see also Fig. 1B for nomenclature of secondary structure). The COOH-terminal loops range in length from 5 to 31 residues and harbor additional secondary structural elements, namely helices aB, aC, aD, and aE and strands bC and bD. In addition, YqjM has a 310-helix aA and two short antiparallel b-strands prior to b-strand 1 that close the barrel on its NH2- terminal side. A short 310-helix aF is present after the COOH-terminal helix 8.

The overall structure of YqjM strongly resembles the structures of other OYE homologs (13–17). The closest structural relative was found to be pentaerythritol tetrinate (PETN) reductase from Enterobacter cloacae (22) where 278 residues could be aligned with YqjM with an r.m.s.d. value of 1.29 Å, followed by morphinone reductase (MR) from P. putida (286 aligned Co with an r.m.s.d. of 1.4 Å), 12-oxophytodienoate reductase (OPR1) 1 from Lycopersicon esculentum (286 Ca, 1.46 Å) and OYE (282 Ca, 1.45 Å). A common feature among these oxidoreductases is the NH2-terminal b-hairpin structure that lids the b-barrel. Except for the loops L4 and L8, all COOH-terminal loops of the b-barrel adopt conformations that are distinct in the OYE flavoproteins. In general, these loops form the active site and provide substrate as well as reaction specificity. While other members of the OYE family show some similarities in the respective loop structures, YqjM exhibits unique structural features in the corresponding active site region. Especially the loops L3, L5, and L6 adopt different conformations and vary considerably in length. For example, the crystal structures of OPR1 (16), PETN reductase (15), and MR (14) have shown that the loop L3 forms a part of the hydrophobic active site tunnel and seems to play a role in substrate discrimination. In these enzymes loop L3 is extended and folds as an additional two stranded b-sheet, which covers half of the active site. In contrast, the corresponding loop of YqjM is folded as a short 310-helix (aC), which is packed on the wall of the b-barrel. As a result, the YqjM active site is hardly protected by any loop structure and thus is remarkably wide and accessible for potential substrate molecules.

### RESULTS AND DISCUSSION

**Overall Structure**—The x-ray structure of native oxidized YqjM was solved by SAD and refined at 1.3 Å resolution to an R-factor of 18.9% (Table I). The monomer of YqjM is comprised of one compact domain representing the frequently observed (a/b)8-barrel or TIM barrel fold, where a cylindrical core of eight twisted b-strands is surrounded by eight helices (Fig. 1). Similar to other (a/b)8-structures, all of the turns at the NH2-terminal end of the barrel are composed of only three or four residues, while the loops at the COOH-terminal end are much longer and build up the active site. The numbering of these loops refers to the preceding b-strands, e.g. loop L6 follows strand 6 (see also Fig. 1B for nomenclature of secondary structure). The oligo-meric state of YqjM observed in the crystal is the same as in solution (11). It is a tetrameric enzyme, in which the subunits are organized with 222 (D2) point group symmetry (Fig. 2). The tetramer has overall dimensions of 72.0 x 51.4 x 184.6 Å and resembles a four-petaled cloverleaf with a central hole. The subunits are arranged in such a way that their active sites, defined by the bound FMN molecule, open up in different directions to solvent but are connected with each other by the central hole. In the C2221 crystal form, two of the 2-fold rotation axes coincide with the crystallographic a and b axis, respectively. The two monomers, which are present in the asymmetric unit (named A and B, their crystallographic symmetry mates C and D), are essentially identical and are related by a non-crystallographic 2-fold axis. As discussed below, the YqjM tetramer is organized as a dimer of active dimers, i.e. AB and CD, respectively. The (a/b)8-domains of the corresponding monomers interact extensively around the dimer 2-fold axis such that their barrel axis are approximately parallel to each other but open up into opposite directions. The dimer axis passes between helix 1 of the two monomers, where both elements come close together. Additionally, interaction of helix aE and the entire COOH terminus including helix aF with the neighboring counterpart contribute considerably to the binding energy of both subunits. The interaction sites a1/a1a and the aE/aF are arranged in a perpendicular fashion and are stabilized by the formation of a large hydrophobic cluster involving residues Met27, Pro39, Phe40, Met42, Ala43, Ile46, Ile50, Pro314,
FIG. 1. **A**, ribbon diagram of the YqjM monomer complexed with pHBA in orthogonal mode. YqjM is colored according to its secondary structure: magenta, helices; violet, β-strands, and gray, coils. The flavin cofactor and the ligand pHBA are drawn in stick form with FMN in yellow and the ligand in green. **B**, topology of YqjM. Helices are displayed as rectangles and strands as arrows. The helices and strands of the barrel are numbered according to their order in the barrel. The extra barrel secondary elements are designated by letters. The numbers at the beginning and the end of each secondary element account for the amino acid number.

FIG. 2. **A**, ribbon diagram of the YqjM tetramer represented in orthogonal mode. YqjM is a dimer of catalytically dependent dimers. Each subunit is colored differently. Functionally connected subunits are shown in similar colors. The cofactor FMN is presented in space filling form with carbon in yellow, oxygen in red, and nitrogen in blue.
and Phe315. This cluster is enclosed by a pronounced hydrogen bonding network formed by Ser29, His44, Arg48, Leu311, Arg312, Gln333, Tyr334, Arg336, Gly337, and Trp338. Most notably, the COOH-terminal end is directed by the latter interactions toward the active site of the neighboring subunit. One of the COOH-terminal residues Arg336* (the asterisk denotes the neighboring subunit) is even protruding as an arginine finger into the adjacent active site, where it forms a part of the substrate binding pocket (near the flavin dimethyl benzene ring). Thus the shared active site strongly suggests that the dimer is the active unit of YqjM. Its formation shields 1241 Å² per monomer (9% of the entire monomer surface), while the other monomer-monomer contacts are considerably smaller. The contact area between A and C comprises 5% of the accessible monomer surface and the monomers A and D do not interact at all with each other. The contacts between the dimers AB and CD are mediated by the helix-loop-helix motifs of helices 6 and 7 and comprise mainly hydrophobic interactions involving Val260, Phe261, Pro262, Tyr264, Val266, Met285, and Met291. The few polar interactions are established by Gly263, Glu270, Asn298, Arg280, and their symmetry mates.

As mentioned earlier, YqjM is the only known tetrameric enzyme of the OYE family identified so far. While OPR and MR exist as monomers, OYE (13) as well as MR (14) were found to be dimeric enzymes. An important difference in YqjM compared with OYE and MR is the shared active site with the contribution of the COOH-terminal Arg336* of the neighboring subunit. In the related MR, the COOH terminus forms similar secondary structural elements as in YqjM, which however, fold back onto their own subunit. Also the observed dimers of OYE and MR are formed by entirely different interfaces involving helices 4, 5, and 6 in OYE and helices 2, 8, and the NH2-terminal β-strands in MR. In both cases, the dimer subunits function as independent entities.

The structural data also explain earlier observations that a COOH-terminal hexahistidine tag disrupts the quaternary assembly leading to the formation of monomeric and dimeric enzyme forms (11). Since the structural data show that the COOH terminus of native YqjM contributes considerably to the close interaction with the neighboring subunit, a COOH-terminal extension impairs the formation of the AB dimer and also critically interferes with substrate binding and subsequently catalysis (11).

The Flavin Binding Site—YqjM binds the FMN cofactor non-covalently at the carboxyl-terminal end of the β-barrel, particularly above the β-strands 1 and 8. The isoalloxazine ring
occupies a similar orientation as in other OYE homologs. Noteworthy is that the bound pHBA and pNP compounds in YqjM are Located in site within 1.2 a. Amino acids and FMN are presented as in Fig. 3.

In contrast to OPR and MR, where the isoalloxazine ring is planar (14, 16), the electron density for the oxidized FMN cofactor in YqjM revealed unequivocally the so-called butterfly bending at the hypothetical N(5)-N(10) hinge. This result is surprising since the oxidized flavin ring system is widely described to be planar (30–32). It was assumed that only upon reduction the isoalloxazine molecule bends along the N(5)-N(10) axis. However, reduction of YqjM does not lead to any further bending of the isoalloxazine ring. The observed deviation from planarity for the YqjM flavin might be a result of interactions with the apoprotein in such a way that could modulate its chemical behavior.

In fact, the redox chemistry of the flavin is controlled and precisely tuned by several interactions with the apoprotein as illustrated in Fig. 3. Many of these interactions are conserved within the OYE family. In the following we will describe similarities and differences in binding the FMN cofactor starting with the isoalloxazine ring and moving then to the ribityl side chain and the terminal phosphate group.

Similarly as in OYE, the specific electronic contribution of the pyrimidine ring is determined by interactions of a glutamine (Gln125) with N(3) and O(2) as well as of an arginine (Arg215) with O(2) and N(1). Furthermore, N(1) is hydrogen bonded to His167 and N(3) to His164. The FMN O(4) is in hydrogen bonding distance to the main chain carbonyl oxygen of Ala260 and the Cys26 sulfhydryl group. Also comparable with OYE, in YqjM, the dimethyl benzene ring of FMN is bound to the protein by hydrophobic interactions with the side chains of Met25, Leu311, and Arg308. In contrast, the whole lateral part of the flavin binding pocket is strikingly differently organized (Fig. 3A). Notably, instead of a bulky aromatic phenylalanine as in all other OYE homologs, an arginine (Arg336) protrudes from the neighboring monomer and forms part of the flavin binding pocket (Fig. 3A).

The ribityl moiety of the cofactor is anchored to Arg215, Ser23, Ser249, Gin265, and the main-chain atom of Pro24. Furthermore, the proline main chain carbonyl oxygen is equidistant to N(1) and N(5) and may play a role in stabilizing the reduced flavin. Like the other OYE enzymes, the FMN phosphate group is embedded in an electrostatic groove of the apoprotein that is formed by loops L7 and L8. Here, the positively charged guanidino group of Arg308 and the main chain atoms of Gly284, Met285, Phe305, Gly307, Arg308, and Glu309 are involved in an extensive network of polar interactions with the phosphate group and thereby enable FAD and FMN to be discriminated between. The FMN phosphate is bound further by the positive end of the macro dipole of helix eA and by the guanidino group of Arg312 that is involved in a strong, water-mediated interaction. Remarkably, the phosphate recognition motif of YqjM seems to be unique and is not shared by any other member of the OYE family.

Another difference to other OYE structures is that O(4) is not hydrogen-bonded to the side chain of a threonine, which has been shown to control the redox potential of the FMN cofactor in OYE (12). Instead, it interacts with Cys26 (Fig. 3B). Moreover, the electron density map of the free enzyme shows clearly that this cysteine can adopt several conformations (Fig. 4). It either interacts with the flavin O(4) or alternatively forms a hydrogen bond with N(5), indicating that the redox properties of the flavin might be altered depending on the conformation of Cys26. Furthermore, the side chain of Cys26 has the ability to interact with the delocalized ring electrons of Tyr28 (Fig. 4).

Interestingly, in the presence of each of the ligands (pHBA or pNP), Cys26 points exclusively toward O(4) (Fig. 5). These observations may suggest that Cys26 might act as a redox sensor that controls the redox potential of flavin depending on the presence of substrates. Future studies will aim at testing this hypothesis.

The Substrate Binding Site—The major difference in YqjM compared with related OYE enzymes is manifested in the construction of the substrate binding pocket. Our structural analyses of the oxidized and the reduced enzyme, and of complexes with various ligands, provide valuable insight into how substrate selection might be achieved. The ligands chosen in this analysis were pHBA and pNP, which are phenolic aromatic compounds acting as competitive substrate inhibitors.

The Fo – Fc electron density map of the uncomplexed oxidized enzyme shows a strong positive peak above the flavin isoalloxazine ring, which is most probably caused by a bound sulfate ion, a component of the crystallization solution (Fig. 5). A similar observation was reported for OYE (13) and PETN reductase (15). In both cases, a chloride ion occupies the corresponding position in the active site. The sulfate ion was bound specifically by Tyr28, Lys109, His164, His167, Tyr169, and Arg336, and might point to some characteristics of the physiological substrate. In accordance with the structural data obtained for OYE and PETN reductase, the reduction of oxidized YqjM crystals with NADPH resulted in the replacement of the sulfate ion by two water molecules, thus reflecting the charged electronic state of the flavin dihydroquinone.

Fitzpatrick et al. (1) have shown that phenolic inhibitors bind in the anionic phenolate state, which is in agreement with the architecture of the active site. Similar to OYE, the ligand phenolic group is located within hydrogen bonding distance to His164 and His167, whereas the aldehyde group of pHBA is bound via the hydroxyl group of Tyr28 thereby aligning the compound parallel above the si-face of the flavin cofactor (Fig. 5). Proper positioning of pNP in the active site requires an additional hydrogen bond between the nitro group and Arg336 from the neighboring subunit above the flavin xylene ring (Fig. 5). The before mentioned Lys109 that is involved in the binding of the sulfate has no function in binding either of the ligands.

While the binding of the proximal anionic hydroxyl group of various ligands in YqjM via the two histidines His164 and His167 equals the binding motifs of other OYE homologs (His191 and Asn194 in OYE (13), His187 and His190 in OPR1 (16), His186 and Asn189 in MR (14), and His181 and His184 in PETN reductase (15)), there are striking differences in binding the distal functional group of the inhibitors. For example, a tyrosine residue generally serves as the binding partner for the aldehyde as well as the nitro group of the characterized inhibitors (Fig. 5). However, the OYE tyrosine originates from a COOH-terminal fragment (Tyr276), whereas in YqjM the NH2-terminal Tyr28 is involved. Apparently, to bind a substrate, a tyrosine

FIG. 4. Electron density map of Cys26 and Tyr28 in the active site. The \( F_o - 2F_c \) map is contoured at 1.2 a. Amino acids and FMN are presented as in Fig. 3.
seemed to be evolutionary favored in all so far known OYEs, including YqjM, but interestingly in YqjM this residue is provided by a completely different structural part of the enzyme namely the loop region after strand H9252 (L1). Intriguingly, this tyrosine in YqjM seems to be properly positioned by Cys26, one of the residues modulating the cofactor redox state.

As mentioned previously, a unique feature of YqjM is the shared active site that is partially formed by the COOH terminus of the neighboring subunit. The complex structure with pNP indicates that Arg190 is directly involved in substrate/inhibitor recognition as it develops a strong hydrogen bond with the nitro group. Since this feature has not been observed in any other OYE homologs, the distinct YqjM active site architecture might allow recruitment of a distinct substrate. Future studies are aimed to further characterize an optimal YqjM substrate, which should be instrumental in revealing the biological function of YqjM.

Comparison of the ligand complexes of YqjM and OYE (13) with pHBA demonstrates that the aromatic ring of the inhibitor is positioned in a similar parallel fashion to the flavin isoalloxazine ring, but in YqjM the aldehyde oxygen is rotated by 180° pointing in the opposite direction. This difference can be attributed to significant structural rearrangements in this part of the YqjM active center, in particular in the recruitment of Tyr28 for the ligand binding.

However, in each case, pHBA as well as pNP is perfectly...
aligned for the hydride transfer from the flavin N(5), although neither are substrates of YqjM. Furthermore, Tyr169, which is located 3.4 Å above the C(1) atom of pHBA and pNP (Fig. 5), can be considered as an acid catalyst protonating the substrate at the C(1) position in the oxidative half-reaction. This tyrosine residue superimposes perfectly with Tyr196 in OYE (13), Tyr192 in OPR (16), and Tyr186 in PETN reductase (15). It has been shown that replacing Tyr196 in OYE by a phenylalanine resulted in a dramatic slowing of the oxidative half-reaction with 2-cyclohexenone (33). Thus, a common catalytic mechanism can be anticipated for OYE, OPR, PETN reductase, and YqjM.

The backbone r.m.s.d values between free YqjM and the two complex structures are rather low (674 Ca, 0.141 Å for pHBA, 674 Ca, 0.163 Å for pNP) indicating that ligand binding does not induce pronounced structural rearrangements, not even in the active site. Only subtle but significant differences could be observed that are necessary to accommodate the ligands. In particular, the side chains of Tyr28 and Arg336* reorient to form proper hydrogen bonds. However, while Tyr28 shifts only slightly away after binding of both ligands, Arg336* rearranges slightly away after binding of both ligands, Arg336* rearranges markedly. Arg336e can obviously adopt various conformations depending on the presence/absence of substrates/inhibitors (Fig. 5). In the uncomplexed, reduced enzyme, the side chain of Arg336e, which was not involved in any specific contacts, was poorly defined by electron density. This indicates that it is highly flexible. In the uncomplexed, oxidized enzyme, a sulfate ion was bound in the active site with Arg236e contributing to its coordination via interaction with an ordered water molecule resulting in one distinct, well defined conformation. In the presence of pNP, the side chain of Arg336e adopts two distinct conformations, both of which support binding of the nitro group of the ligand. These findings illustrate how a flexible, single residue can support binding of different ligand molecules.

Evidence for a New Class of OYE Oxidoreductases—Similar sequences to those of S. cerevisiae OYE3 and B. subtilis YqjM were searched for in the Swiss-Prot data base. From each search, a total of 12 sequences were selected and aligned using MULTALIN. Inspection of the alignment revealed that the structural differences between OYE and YqjM noted before are also reflected by the conservation of different residues in these two groups of proteins. Most notably, Cys26 and Tyr28 are highly conserved in all YqjM family members, whereas all classical OYE homologs feature a highly conserved threonine in the equivalent position of Cys26 (Fig. 6A). The conserved Tyr28 in the YqjM family members has no equivalent in the classical OYE family, as this residue is replaced by Tyr375 (OYE) in the active site of the enzyme. Hence, this residue is invariant in this family of enzymes. Another salient distinction is the highly conserved COOH terminus in the YqjM family, in particular the invariant Arg312, Gln333, Tyr334, and Arg336e (Fig. 6C). The latter amino acid is involved in setting up the active site of the other monomer in the dimer (see also Fig. 5), and thus we predict that this contribution to the active site in the dimer is a conserved feature in the YqjM family. On the other hand, Arg312 is involved in the flavin phosphate binding. Additionally, it participates together with Gln333 and Tyr334 in the formation of the characteristic dimer interface. Therefore these residues are only conserved in the YqjM but not in the OYE family. Likewise, other residues contributing to the dimer interface, such as His44 and Arg48, are only conserved in the YqjM family (Fig. 6A). Based on this sequence analysis, it is evident that the structural characteristics of YqjM must also be present in other homologous proteins. Clearly, this family of YqjM homologs is distinct from the classical OYE homologs.

This conclusion is also supported by a phylogenetic analysis using sequences of both the OYE and YqjM family. As shown in Fig. 7, the proteins form two distinct clades, one comprising all of the classical OYE homologs (upper clade) and the other all of the YqjM homologs (bottom clade). The support for this tree, as evaluated by the bootstrap procedure implemented in PAUP, is very high, especially for the branch separating the two clades. Therefore, it can be concluded that YqjM is the first member of a subfamily of OYE homologs that so far only comprises bacterial proteins, whereas the classical OYE family possesses members of both prokaryotic and eukaryotic (yeast and plants) species.

The conservation pattern of the newly discovered YqjM family and the classical OYE family was plotted onto the surface of both enzymes (Fig. 8, A and B) and manifested once more the so far described differences of both families. As expected, residues that are involved in the dimer or tetramer formation are highly conserved in YqjM and differ from OYE. While residues forming the flavin binding pocket are in the OYE and the YqjM family similarly highly conserved, residues determining the probable substrate binding site exhibit considerable different patterns. Moreover, in addition to the previously described residues in YqjM (Tyr28, His164, His167, and Arg160), Lys109, which is situated at the distant entrance of the active site, is conserved within the whole YqjM family and, therefore, is suggested to function in substrate binding. The neighboring Arg108 as well as His105 are also conserved. However, they seem to play a role in adjusting the active site rather than direct substrate binding. Another
residue, whose strict conservation and proximity to the flavin cofactor indicates a role in substrate binding, is Thr\textsuperscript{70}. The nearby conserved Asp\textsuperscript{73} is situated within hydrogen bonding distance to Tyr\textsuperscript{28}. The active site access above the flavin is determined by hydrophobic residues namely Ile\textsuperscript{69} and Phe\textsuperscript{124}, both of which are conserved within the family. Apart from His\textsuperscript{164}, His\textsuperscript{167}, and Tyr\textsuperscript{169}, none of the residues mentioned here show conservation in the OYE family, thus indicating significant differences in the substrate specificity. Assuming that OYE as well as YqjM derives from a common ancestor, a divergent evolution must have occurred that adapted YqjM to its specific function and a specific substrate.

**Implications toward the Natural Substrate**—The architecture of the active site of YqjM in combination with steady state kinetic investigations provides information toward the true nature of the substrate of this enzyme. Activity assays were performed with duroquinone, menadione, prednisone, and 1,4-androstadiene 3,17-dione and compared with the kinetic data obtained for cyclohex-2-enone and trans-hex-2-enal published in Ref. 1 (Table II). The turnover rates with cyclohex-2-enone, duroquinone, menadione, and trans-hex-2-enal are very similar, while no NADPH oxidation could be observed with 1,4-androstadiene 3,17-dione or prednisone. This indicates that the active site can accommodate at least two ring systems but not

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**Fig. 8. Surface properties of YqjM.** Conserved residues obtained from the sequence alignment of the YqjM and the OYE families (see Figs. 6 and 7) are mapped on the respective surfaces (A, YqjM and OYE dimer; B, YqjM monomer). Red indicates the strictly conserved residues. The degree of conservation is decreasing from red (more than 90%) over orange (more than 75%) to yellow (more than 50%). The electrostatic potential in the active site was mapped onto the surface of YqjM (C). Red indicates negatively charged and blue positively charged areas.
Arg336*. This group has to be partially electronegative and functional group that is defined by the residues Tyr28 and structural prediction that the unique construction of the YqjM group opposite to the first. Thus, the kinetic data confirm our that the enzyme prefers substrates with a second functional erence for a single ring system and could even be taken to imply (has two carbonyl groups showing the highest specificity erence of the carbonyl functional group, with duroquinone, which small molecular weight compound.

substrate(s) of YqjM and clarify whether it is a protein or a Future work is anticipated to determine the physiological substrate(s) of YqjM and clarify whether it is a protein or a physiological substrate could incorporate one or more aliphatic trans-hex-2-enal, it would also be conceivable that the case for several quinones, should be located opposite to the carbonyl function as is the active site is wide (Fig. 8C) and based on the activity (ubiquinone. Since the active site pocket is very hydrophobic (Fig. 8C) and based on the activity with trans-hex-2-enal, it would also be conceivable that the physiological substrate could incorporate one or more aliphatic side chains containing functional groups. Further anchoring points seem to be represented by Lys109 and Thr70. Fig. 8 clearly illustrates that the YqjM active site is wide open, easily accessible, and hydrophobic. Together with the present kinetic data, this construction argues against the adaptation toward a specific substrate molecule. It rather appears that the huge substrate binding pocket guarantees binding of a large spectrum of different substrate molecules allowing the transfer of reduct equivalents from NADPH to a bunch of suitable oxidants, as proposed previously (1). Such an unspecificity would be essential to control the net redox state of the cell which might be disturbed by oxidative stress. Alternatively, the large hydrophobic flavin pocket of YqjM might be required to accept another protein as substrate. Future work is anticipated to determine the physiological substrate(s) of YqjM and clarify whether it is a protein or a small molecular weight compound.

### TABLE II

| Substrate          | $k_{cat}$ | $K_m$  | $k_{cat}/K_m$ |
|--------------------|-----------|--------|--------------|
| Cyclohex-2-enone$^a$ | 4.38      | 293 ± 29 | 0.0150       |
| trans-Hex-2-enal$^a$| 4.68      | 2602 ± 186 | 0.0018      |
| Duroquinone         | 2.67      | 19 ± 0.8 | 0.141        |
| Menadione           | 3.04      | ≥541 ± 139 | ≈0.0036      |
| Prednisone          | ND$^b$    | ND$^a$ | ND$^b$      |
| 1,4-Androstadiene 3,17-dione | ND$^b$ | ND$^a$ | ND$^b$     |

$^a$ Taken from Ref. 1.
$^b$ ND, not detectable.

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REFERENCES
1. Fitzpatrick, T. B., Amrhein, N., and Macheroux, P. (2003) *J. Biol. Chem.* 278, 19891–19897
2. Warburg, O., and Christian, W. (1932) *Naturwissenschaften* 20, 688
3. Theorell, H. (1935) *Biochem. Z.* 275, 344–346
4. Theorell, H., and Nygaard, A. P. (1954) *Arkiv Kem.* 7, 205–209
5. Theorell, H., and Nygaard, A. P. (1954) *Acta Chem. Scand.* 8, 1104–1105
6. Abramovitz, A. S., and Massey, V. (1976) *J. Biol. Chem.* 251, 5327–5336
7. Massey, V., and Schopfer, L. M. (1986) *J. Biol. Chem.* 261, 1215–1222
8. Stott, K., Saito, K., Thiele, D. J., and Massey, V. (1993) *J. Biol. Chem.* 268, 6997–6106
9. Vaz, A. D. N., Chakraborty, S., and Massey, V. (1995) *Biochemistry* 34, 4246–4256
10. Haarer, B. K., and Amberg, D. C. (2004) *Mol. Biol. Cell* 15, 4522–4531
11. Fitzpatrick, T. B., Auweter, S., Kitzing, K., Clausen, T., Amrhein, N., and Macheroux, P. (2004) *Protein Expression Purif.* 36, 280–291
12. Xu, D., Kohli, R. M., and Massey, V. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 3556–3561
13. Fox, K. M., and Karplus, P. A. (1994) *Structure* 2, 1089–1105
14. Barna, T., Messina, H. L., Petosa, C., Bruce, N. C., Scrutton, N. S., and Mootry, P. C. E. (2002) *J. Biol. Chem.* 277, 39976–39983
15. Barna, T. M., Khan, H., Bruce, N. C., Barsukov, I., Scrutton, N. S., and Mootry, P. C. E. (2001) *J. Mol. Biol.* 310, 433–447
16. Breithaupt, C., Strassner, J., Breitinger, U., Huber, R., Macheroux, P., Schaller, A., and Clausen, T. (2001) *Structure* 9, 419–429
17. Malone, T. E., Madson, S. E., Wrobel, L. R., Jeon, W. B., Rosenberg, N. S., Johnson, K. A., Bingman, C. A., Smith, D. W., Phillips, G. N., Jr., Markley, J. L., and Fox, B. G. (2005) *Proteins* 58, 243–245
18. Bailey, S. (1994) *Acta Crystallogr. Sect. D Biol. Crystallogr.* 50, 760–763
19. Sheldrick, G. M., and Schneider, T. R. (1997) in *Macromolecular Crystallography*, Part B, Vol. 277, pp. 319–343
20. Sheldrick, G. M. (2002) *Z. Kristallogr.* 217, 644–650
21. de La Fortelle, E., and Bricogne, G. (1997) in *Macromolecular Crystallography*, Part A (Carter, Jr., C. W., and Sweet, R. M., eds) Vol. 276, pp. 472–494, Academic Press, New York
22. Abrahams, J. P., and Leslie, A. G. W. (1996) *Acta Crystallogr. Sect. D Biol. Crystallogr.* 52, 30–42
23. Ferrakis, A., Morris, R., and Lamzin, V. S. (1999) *Nat. Struct. Biol.* 6, 458–463
24. Brünger, A. T., Adams, P. D., Clore, G. M., L., D. W., Gros, P., Graser-Kunsteve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) *Acta Crystallogr. Sect. D Biol. Crystallogr.* 54, 905–921
25. Englh, R. A., and Huber, R. (1993) *Acta Crystallogr. Sect. A* 47, 392–400
26. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) *Acta Crystallogr. Sect. A* 47, 110–119
27. Kleywegt, G., and Jones, T. (1998) *Acta Crystallogr. Sect. D Biol. Crystallogr.* 54, 1119–1131
28. Krasilnikov, P. I. (1991) *J. Appl. Crystallogr.* 24, 946–950
29. Merrit, E. A., and Murphy, M. E. P. (1994) *Acta Crystallogr. Sect. D Biol. Crystallogr.* 50, 869–873
30. Hasford, J. J., Kemnitzer, W., and Rizzo, C. J. (1997) *J. Org. Chem.* 62, 5224–5225
31. Simonson, R. P., and Tellin, G. (1980) *Mol. Cell. Biochem.* 33, 13–24
32. Massey, V., Muller, F., Feldberg, R., Schuman, M., Sullivan, P. A., Howell, L. G., Mayhew, S. G., Matthews, R. G., and Foust, G. P. (1989) *J. Biol. Chem.* 264, 3999–4006
33. Kohli, R. M., and Massey, V. (1998) *J. Biol. Chem.* 273, 32763–32770

 Crystal Structure of the Flavoprotein YqjM 27913

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