We have recently reported that *Shewanella oneidensis*, a Gram-negative \( \gamma \)-proteobacterium with a rich arsenal of redox proteins, possesses four old yellow enzyme (OYE) homologues. Here, we report a series of high resolution crystal structures for one of these OYEs, *Shewanella* yellow enzyme 1 (SYE1), in its oxidized form at 1.4 Å resolution, which binds a molecule of PEG 400 in the active site, and in its NADH-reduced and \( p \)-hydroxybenzaldehyde- and \( p \)-hydroxycetophenone-bound forms at 1.7 Å resolution. Although the overall structure of SYE1 reveals a monomeric enzyme based on the \( \alpha \beta_{8} \) barrel scaffold observed for other OYEs, the active site exhibits a unique combination of features: a strongly butterfly-bent FMN cofactor both in the oxidized and NADH-reduced forms, a collapsed and narrow active site tunnel, and a novel combination of conserved residues involved in the binding of phenolic ligands. Furthermore, we identify a second \( p \)-hydroxybenzaldehyde-binding site in a hydrophobic cleft next to the entry of the active site tunnel in the capping subdomain, formed by a restructuring of Loop 3 to an “open” conformation. This constitutes the first evidence to date for the entire family of OYEs that Loop 3 may indeed play a dynamic role in ligand binding and thus provides insights into the elusive NADH complex and into substrate binding in general. Structure-based sequence alignments indicate that the novelities we observe in SYE1 are supported by conserved residues in a number of structurally uncharacterized OYEs from the \( \beta \)- and \( \gamma \)-proteobacteria, suggesting that SYE1 represents a new subfamily of bacterial OYEs.

The OYE family has been studied since the 1930s when Warburg and Christian isolated a yellow enzyme from brewers’ bottom yeast (*Saccharomyces carlsbergensis*) (1). Theorell purified the enzyme in 1935 and showed that it was composed of a colorless apoprotein and a yellow dye. The dye turned out to be a vitamin \( B_{2} \)-derived molecule, FMN, and to be essential for catalysis. When a second yellow enzyme was isolated from yeast, the first enzyme was named old yellow enzyme (OYE), 3 a name that has persisted till this day. Since then, OYE has become synonymous with the study of flavoproteins, but despite decades of research culminating with a dissection of the catalytic mechanism (2–4), the physiological role of OYE has only recently begun to emerge (5–7).

The OYE family has grown steadily in recent years and now features the bacterial nitro-ester reductases PETN reductase (8), glycerol trinitrate reductase (9), XenA/B reductase (10), the bacterial morphinone reductase (11), the YqjM from *Bacillus subtilis* (41), the plant oxophytodienoic acid reductases (12, 13), several yeast OYEs (14–17), and an enzyme involved in prostaglandin synthesis in *Trypanosoma cruzi* (18). Although these enzymes originate from different organisms and catalyze different reactions, they share several common functional characteristics. They are able to reduce simple and complex unsaturated aldehydes and ketones (11, 19), nitro-esters (8–10, 20), and nitro-aromatic substrates (21–23), and yet clear preferences are observed. Furthermore, OYEs can form long wavelength charge transfer interactions with phenolic compounds (24), in turn exhibit a distinct binding mode in the active site involving hydrogen bonding of the phenolate oxygen to a strictly conserved histidine/asparagine or histidine/histidine pair (25). The reaction mechanism of OYE proceeds by a ping-pong mechanism consisting of an oxidative and reductive half-reaction. Consistent with the plethora of functional similarities among members of the OYE family, the first crystal structures reported revealed a common \( \alpha \beta_{8} \) barrel fold with significant variations in the capping subdomain (25–27). Nonetheless, interesting differences have begun to emerge in recent studies. For example, morphinone reductase lacks the general acid (tyrosine) that...
protonates the substrate (28). In another case, enzymes possessing two histidines (23) in the active site for substrate binding can catalyze the reduction of \( \text{TN}^{\cdot}\) to the H-Feisenheimer complex, whereas the enzymes with only one histidine cannot. Finally, YqjM has a flavin that is butterfly-bent in both the reduced and the oxidized state (29).

We have recently probed the genome of *Shewanella oneidensis*, a Gram-negative \( \gamma \)-proteobacterium with a remarkable respiratory versatility (30) (www.tigr.org), using yeast OYE1. We identified four OYE homologues (NP718044, NP718043, NP719682, and NP718946) that we named SYE1–4 for *Shewanella* yellow enzyme (31). SYE1 (NP718044) is the *Shewanella* OYE homologue with the greatest sequence identity to OYE1. Here, we report structural studies of SYE1 at high resolution and show that SYE1 exhibits a number of novel features that can be mapped to strongly conserved sequence fingerprints in several structurally uncharacterized OYE homologues from the \( \beta \)- and \( \gamma \)-proteobacteria. This suggests that SYE1 may be the prototype of a new subfamily of bacterial OYE homologues.

**EXPERIMENTAL PROCEDURES**

**Materials**—The Q-Sepharose FF and Source 30Q matrices and the MonoQ and Superdex 200 columns were purchased from Amersham Biosciences, and the pACYC-Duet-1 vector was from Novagen. Structure screens 1 and 2 were from Molecular Dimensions Ltd., and the additive screen was from Hampton Research. Trizma base, ammonium sulfate, FMN, MES, Na\(_2\)HPO\(_4\), and NaH\(_2\)PO\(_4\) were purchased from Sigma; HEPES and PEG 400 were from Fluka; NaCl and NADH were from Fisher; and 2-decenal, 2-dodecenal, and \( \text{D-thiogalactopyranoside} \) was from Duchefa.

**Cloning, Expression, and Purification**—The *syel* gene was excised from a pGEX-SYE1 construct using EcoRI and NotI and cloned in the first multiple cloning site of pACYC-Duet-1, generating pACYC-SYE1. This cloning strategy puts the FMN cofactor. The correctness of the structure solution was confirmed by molecular replacement using maximum-likelihood methods implemented in the program PHASER (33). The search model was generated from the coordinates of morphinone reductase (Protein Data Bank entry 1GWJ) which exhibited a 50% sequence identity with SYE1. Nonconserved residues were replaced by alanine (or glycine), and all of the insertions were (Protein Data Bank entry 1GWJ) which exhibited a 50% sequence identity with SYE1. Nonconserved residues were replaced by alanine (or glycine), and all of the insertions were
were subsequently incubated in 100 mM Tris, pH 8.0, 2 M ammonium sulfate, supplemented with 50 mM of the desired ligand. Complex formation in the crystals was monitored via the rapid change of color from yellow to lime-green, in the case of NADH 30 min to several hours. After incubation, the crystals were flash frozen in liquid nitrogen.

Kinetic Studies—All of the kinetic parameters were determined under strict anaerobic conditions, and all of the assays were performed in triplicate. The reaction mixtures consisting of 0.5× phosphate-buffered saline buffer, pH 7.3 (5.75 g of Na2HPO4, 1.49 g of NaH2PO4, and 2.92 g of NaCl/liter), 60–120 μM NADH, and varying concentrations of substrate were prepared within a glove box (Coy Laboratories, Grass Lake, MI) and transferred to sealable quartz cuvettes (Hellma).

The concentration of NADH was determined spectrophotometrically using a molar extinction coefficient of 6220 M⁻¹ cm⁻¹ at 340 nm. Enzyme solutions were prepared in sealed flasks and made anaerobic by bubbling with N2 gas. The reactions were initiated by the addition of enzyme to the reaction mixtures using a Hamilton needle. The enzyme concentration was kept constant at 100 nM. SYE activity was assayed by following the oxidation of NADH using a double beam Uvikon 943 spectrophotometer. Apparent steady state kinetic constants were determined by least squares fitting procedures to the standard Michaelis-Menten equation.

Protein Data Bank Accession Codes—The coordinates and structure factors for the four structures of SYE1 reported here are available via the Protein Data Bank (www.rcsb.org) with accession codes 2GOU, 2GQ8, 2GQA, and 2GQ9.

RESULTS AND DISCUSSION

Overall Structure of SYE1—The structure of oxidized SYE1 was determined by molecular replacement methods using morpholine reductase as the search model and was refined at 1.4 Å resolution (Table 1). SYE1, like all OYE homologues characterized to date (26, 27, 29, 37), folds into an α8β8 barrel containing one noncovalently bound FMN molecule located at the top of
the β barrel (Fig. 1A, A and B). Like morphinone reductase (37) and PETN reductase (27), SYE1 has four extra barrel elements: (a) the N-terminal β-hairpin that closes the bottom of the barrel (Fig. 1A, bottom arrow); (b) a capping subdomain formed by secondary structure elements between β3 and α3; (c) an α-helix linking β4 and α4, which in OPR1 (26) is thought to aid in substrate recognition; and (d) the α-helix between β8 and α8 that contributes to the binding of FMN.

In the course of refinement difference electron density maps showed clear additional density for three nonprotein elements: a PEG 400 molecule, a sulfate ion, and a β-octyl glucoside molecule (Fig. 1A). PEG 400 was an essential component of the crystallization condition and could be traced in the active site of SYE1 (Fig. 1C). OPR1 also binds PEG in the active site, but in that case the PEG molecule is doubly bent and resembles the substrate 12-oxophytodienoic acid. In SYE1 the PEG 400 molecule is extended, and only the first six atoms (corresponding to two ethylene glycol units) enter the active site tunnel, whereas the rest of the molecule bends around the hydrophobic active site entrance and extends outwards like the tail of a pig (Fig. 1C). Interactions between the PEG molecule and SYE1 are largely based on van der Waals’ interactions with the hydrophobic lining of the active site. The sulfate ion and the β-octyl glucoside molecule participate in intermolecular contacts between two adjacent SYE1 molecules in the crystal lattice. The β-octyl glucoside molecule lies on one side parallel to His39 and is coordinated on the other side by Lys361 from a symmetry-related molecule. In contrast to the sugar ring, the tail of the β-octyl glucoside molecule is not well defined in the electron density maps. The sulfate ion is coordinated by His337 and Arg336 and by His344, Arg301, and Arg323 from a symmetry-related SYE1. The last two amino acids are involved in the coordination of the flavin phosphate oxygens.

The overall structure of SYE1 superimposes well with a number of OYE homologues: morphinone reductase (1.2 Å r.m.s.d. over 344 Ca atoms), PETN reductase (1.3 Å r.m.s.d. over 347 Ca atoms), OPR1 (1.4 Å r.m.s.d. over 317 Ca atoms), OYE (1.6 Å...
Insights into Substrate Binding from a Bacterial OYE Homologue

The active site and FMN environment of SYE1. A, a bird’s eye view of the FMN and its interactions. The atoms in the isoalloxazine ring of FMN are numbered according to standard nomenclature. Red spheres represent water molecules, and the dashed lines indicate hydrogen bonds. B, side view of the FMN to illustrate the butterfly bending. Leu13 from the structure of morphine reductase from Pseudomonas putida is shown together with Met25 of SYE1 as reference. Red spheres represent water molecules, and the dashed lines indicate hydrogen bonds. C, sequence alignment of several OYE homologues from the proteobacteria we have identified via a BLAST search using SYE1 as reference. Red spheres represent water molecules, and the dashed lines indicate hydrogen bonds. Indicated in red are the equivalents of Met25. Presented from top to bottom are: Shew_on1, S. oneidensis, SYE1; Shew_fri, Shewanella frigidimarina, ZP00639696; Burk_cen, Burkholderia cenocepacia, ZP00462570; Rals_met, R. metallidurans, ZP00596228; Pseu_aer, Pseudomonas aeruginosa, AAC04723; Vib_cho, Vibrio cholerae, ZP00754593; Shew_on3, S. oneidensis, SYE3 (NP719662); Pho_pro, Photobacterium profundum, CAG22470; Chro_vi, Chromatium violaceum, NP091915; Ps_MorB, P. putida, morphine reductase; Ec_OnR, Enterobacter cloacae, PETN reductase; Le_OPR, Lycopersicon esculentum, oxophytodienoic acid reductase; Sc_OYE, S. carlsbergensis, OYE1; Bs_YqjM, B. subtilis, YqjM.

FIGURE 2. The active site and FMN environment of SYE1. A, bird’s eye view of the FMN and its interactions. The atoms in the isoalloxazine ring of FMN are numbered according to standard nomenclature. Red spheres represent water molecules, and the dashed lines indicate hydrogen bonds. B, side view of the FMN to illustrate the butterfly bending. Leu13 from the structure of morphine reductase from Pseudomonas putida is shown together with Met25 of SYE1 as reference. Red spheres represent water molecules, and the dashed lines indicate hydrogen bonds. C, sequence alignment of several OYE homologues from the proteobacteria we have identified via a BLAST search using SYE1 as reference. Red spheres represent water molecules, and the dashed lines indicate hydrogen bonds. Indicated in red are the equivalents of Met25. Presented from top to bottom are: Shew_on1, S. oneidensis, SYE1; Shew_fri, Shewanella frigidimarina, ZP00639696; Burk_cen, Burkholderia cenocepacia, ZP00462570; Rals_met, R. metallidurans, ZP00596228; Pseu_aer, Pseudomonas aeruginosa, AAC04723; Vib_cho, Vibrio cholerae, ZP00754593; Shew_on3, S. oneidensis, SYE3 (NP719662); Pho_pro, Photobacterium profundum, CAG22470; Chro_vi, Chromatium violaceum, NP091915; Ps_MorB, P. putida, morphine reductase; Ec_OnR, Enterobacter cloacae, PETN reductase; Le_OPR, Lycopersicon esculentum, oxophytodienoic acid reductase; Sc_OYE, S. carlsbergensis, OYE1; Bs_YqjM, B. subtilis, YqjM.
Filters of SYE1 in Complex with p-HBA and p-ACE—Our choice of phenolic ligands was limited by the fact that previous experiments had shown that SYE1 is not capable of binding phenolic ligands with high pKₐ values (pKₐ > 9), such as p-cresol (31). We therefore decided to pursue complexes with p-hydroxybenzaldehyde (p-HBA) and p-hydroxyacetophenone (p-ACE). Upon incubation with the phenolic compounds, the crystals turned from yellow to lime-green. Structures of the complexes were determined by difference Fourier analysis and were refined with full occupancy of the ligands in the active site to 1.7 Å resolution (Table 1).

The two phenolic compounds were well defined in the difference electron density maps, and the orientation of the molecules could be readily determined in the active site. p-HBA and p-ACE are orientated in the same fashion (Fig. 3A). The phenolic oxygen is placed above the C-2 atom of the flavin, forming a hydrogen bond with His181 and Asn184. Although the aldehyde carbonyl of p-hydroxybenzaldehyde in OYE (25) is hydrogen-bonded to Tyr375 (OYE numbering) because of a mutation to phenylalanine in SYE1. Morphinone reductase has a Trp279 corresponding to Trp274, but it is located toward the outside of the barrel. Two evolutionary scenarios may explain the role of Loop 6; the restructuring of Loop 6 in SYE1 arose when the enzyme had to substitute for the lost Tyr375 interaction, or Loop 6 appeared first in the active site. Because Trp274 may substitute effectively for the Tyr375 interaction, a Tyr to Trp mutation was not harmful for catalysis. It has been shown for morphinone reductase that a Y356F mutation (equivalent of Tyr375 OYE numbering) because of a mutation to phenylalanine in SYE1. Morphinone reductase has a Trp279 corresponding to Trp274, but it is located toward the outside of the barrel. Two evolutionary scenarios may explain the role of Loop 6; the restructuring of Loop 6 in SYE1 arose when the enzyme had to substitute for the lost Tyr375 interaction, or Loop 6 appeared first in the active site. Because Trp274 may substitute effectively for the Tyr375 interaction, a Tyr to Trp mutation was not harmful for catalysis.

The importance of the butterfly-bent FMN residue, whereas the Phe350 is always preceded by a leucine residue as a probe (Fig. 2C). Interestingly, all of the other structurally characterized OYE to date have a leucine instead of methionine at position 25, and they all exhibit planar FMNs in the oxidized state. It has been proposed that a bent flavin would destabilize the formation of semiquinones and thus one-electron reactions of the flavin (39). It is interesting to note that semiquinone species were observed during the photoreduction of OPR1 (13) and OYE (40), but not of YqjM (41) and SYE1 (31).

In this respect, the behavior of YqjM and SYE1 is similar to other bacterial OYE homologues such as PETN reductase (42), morphinone reductase (43), XenA reductase, and XenB reductase (10), all of which have planar flavin groups. Clearly then, a mechanism other than the bending of the flavin is responsible for the lack of semiquinone formation in these enzymes. Although the importance of a butterfly-bent FMN is not yet known, our analysis suggests that a methionine residue at position 25 may be a general prerequisite for a butterfly-bent flavin group across all OYEs.

**TABLE 2**

| Enzyme               | Reference | pKₐ (µM)                  |
|----------------------|-----------|---------------------------|
| SYE1                 | This study| 9.3 ± 1.2                 |
| Morphinone reductase | 28        | 8.9 ± 0.6                 |
| Morphinone reductase Y356F | 28        | 61.2 ± 3.2               |
| OYE                  | 28        | 7.5                       |
| XenB reductase       | 51        | 5 ± 1                     |
| XenA reductase       | 51        | 28 ± 2                    |
| Glycerol trinitrate reductase | 52 | 8 ± 1                     |

**FIGURE 3.** Binding of p-HBA and p-ACE in the active site of SYE1. A, stereo view of p-HBA and p-ACE bound in the active site of SYE1. The FMN is indicated in orange, p-ACE is in magenta, and p-HBA is in green. The dashed lines represent hydrogen bonds. B, sequence alignment illustrating the conservation of Trp274 and Phe350 in several OYE homologues. For the abbreviations of the OYE homologues used in the alignment please refer to the legend of Fig. 2.
We propose that these sequence fingerprints, combined with the structural features in the active sites of SYE1, indicate that OYE homologues possessing these features may constitute a new subfamily of OYEs in /H9252- and /H9253-proteobacteria.

NADH-reduced Structure—Upon incubation of SYE1 crystals with NADH, the yellow color of the crystals fades away, indicating a reduction of the flavin. However, the occupancy of NADH in the active site was too low to allow its modeling in the active site. This appears to be a recurring theme in the OYE superfamily, because attempts to obtain such complexes in morphinone reductase (37), OPR1 (26), and OYE (25) also failed. It has been noted for other homologues that reduced flavin harbors ordered water molecules above the ring that replace bound ions (Cl\(^{-}\)) (25, 27). In native SYE1, there are no such water molecules because of the presence of PEG 400 in the active site, but upon reduction with NADH, PEG 400 no longer occupies the active site, and instead three ordered water molecules move into its place.

**The “Open” Conformation of Loop3 Provides Insights into NADH Binding**—The NADH-reduced and the p-ACE bound structures differ little from the PEG bound SYE1 structure, the most notable feature being the disappearance of the \(\beta\)-octyl glucoside molecule in the NADH-reduced structure, which brings about a 90° flipping of His\(^{39}\) around its hinge. The structure of the p-HBA complex, on the other hand, was not straightforward to interpret. Incubation with p-HBA increased the internal disorder of the crystals, resulting in the disappearance of the electron density of many side chains. As in the NADH-reduced structure, the \(\beta\)-octyl glucoside molecule was washed away in the soak, whereas the sulfate ion became highly disordered.

At first glance, a p-HBA molecule could be unambiguously modeled in the active site of SYE1 stacked against the flavin ring in an orientation similar to what we observed for p-ACE. Quite unexpectedly, a second p-HBA molecule (p-HBA-B) was found sandwiched between two Phe residues (Phe\(^{132}\) and Phe\(^{350}\)) at the entrance to the active site at the capping subdomain (Fig. 4, A and B). The occupancy of this second site has been estimated to 0.6 based on a comparison of the refined B-factors of p-HBA-A and p-HBA-B. The occupancy of p-HBA-B has been estimated to 0.6. C, comparison of surface representations of SYE1 bound by p-HBA (left panel) and with p-ACE (right panel) illustrating the dramatic structural rearrangement of Loop 3 in the capping subdomain upon binding of a second molecule of p-HBA. D, sequence alignment of OYE homologues revealing the presence of either a Phe or Trp at position 132.
observed in the p-ACE complex. In that case, the methyl group of p-ACE would approach only 2.4 Å from Trp70 and would cause a steric clash. Trp70 is part of Loop 2, which is structurally conserved in SYE1, morphinone reductase, and PETN reductase.

The fortuitous discovery of a second binding site for p-HBA at the capping subdomain provides the first direct evidence to date for any OYE homologue that Loop 3 of the capping subdomain can indeed participate in ligand binding. This provides important new insights into the elusive NADH complex and into ligand binding in general. It is tempting to propose that the two binding platforms for p-HBA reflect the binding sites for the nicotinamide and adenine moieties of NADH, respectively. However, careful modeling of NADH, with the nicotinamide ring occupying the site of p-HBA-A at the flavin ring, shows that the aromatic platform presented by Phe132 and Phe350 can only serve as the binding site for the ribose group preceding the adenine moiety of NADH. Such a stacking interaction is frequently observed in protein carbohydrate interactions. The adenine moiety of NADH would therefore have to extend toward the exit of the active site channel, but in the absence of an obvious nearby binding cleft, it is difficult to speculate whether the adenine will bind to SYE1 at all. Interestingly, we find that all structurally uncharacterized SYE1 homologues we have identified in our sequence alignments exhibiting a tryptophan residue and a phenylalanine or tyrosine at positions 274 and 350, respectively (Fig. 3B, also have a phenylalanine or a tryptophan at position 132 (Fig. 4D). The strong conservation of such sequence features suggests that this group of OYE homologues likely binds NADH and other ligands in a similar fashion and that SYE1 can serve as a structural prototype for this subfamily of OYEs.

Our observation of a second binding site for p-HBA is consistent with the recent proposal that the distal part of the NADPH moiety interacts with Loop 3 in PETN (27). It has also been proposed that the preference for NADPH in PETN reductase is brought about by the presence of two arginine residues (Arg142 and Arg130) in Loop 3 (27, 44). In morphinone reductase (which prefers NADH), these arginines are replaced by a glutamate residue (Glu135), whereas in SYE1 we observe a lysine (Lys130) and a

| Kinetic constants for the binding of t-2-ketanals to SYE1 |
|---------------------------------------------------------|
| **Apparent kinetic constants were derived from steady state kinetic analyses using the software Graphpad Prism4. Assays were performed under strict anaerobic conditions in 1 ml of 0.5× phosphate-buffered saline buffer, pH 7.3, and 60–100 μM NADH. Enzyme concentration was 100 nM. Also given, is the structure of each t-2-ketanal and of PEG 400 for comparison purposes.** |
| **| **| **| **|
| **t-2-hexenal** | **2320 ± 510** | **0.67 ± 0.07** | **6.72** | **0.00289** |
| **t-2-decanal** | **2350 ± 730** | **0.29 ± 0.03** | **2.92** | **0.00124** |
| **t-2-dodecanal** | **2090 ± 360** | **0.31 ± 0.06** | **3.10** | **0.00148** |
| **PEG-400** | **-** | **-** | **-** | **-** |
glutamate (Glu\textsuperscript{139}). Because SYE1 exhibits a preference for NADH, this theory cannot be extended to all OYE homologues. When we compare the utilization of reducing power among OYE homologues, we find that the ones preferring NADPH accepted both NADPH and NADH, but with a preference for the former (18, 31, 37, 41, 45). On the other hand, OYEs that use NADH cannot accept NADPH. We therefore conclude that one should not search for a mechanism that discriminates between NADH and NADPH, but for one that excludes NADPH.

Further Insights into Substrate Selection and Binding—Thus far, activity of SYE1 has only been shown with relatively small molecules: N-ethylemaleimide, acrolein, and 2-cyclohexenone (31), whereas bulkier molecules, such as nitroglycerine and androstadiene/progesterone, exhibit no activity with SYE1. The shallow and narrow landscape of the active site of SYE1 inevitably imposes size restraints on candidate substrates. The identification of a PEG 400 molecule in the active site tunnel of SYE1 prompted us to explore whether SYE1 accepted long chain unbranched \( \alpha, \beta \)-unsaturated aldehydes as a substrate. Indeed, a steady state kinetic analysis showed that SYE1 showed reactivity toward trans-2-hexenal, trans-2-decenal, and trans-2-dodecenal, three naturally occurring components of plant oils with bactericidal properties against Salmonella (46, 47) (Table 3). The \( k_{cat}/K_m \) values of SYE1 for these substrates are rather similar, differing only by a factor of \( \sim 20 \) (13). This is probably due to the much narrower active site of SYE1, which likely can only accommodate substrates with short chains. OPR1, on the other hand, binds a complete PEG 400 molecule in its active site (26) and can thus accommodate larger substrates. Furthermore, SYE1 only binds the first six atoms of PEG 400 in the active site tunnel with the rest of the molecule extending outwards. A preference can be seen for the shorter chain of t-2-hexenal. A similar observation was made for OPR1 (13), which has the highest catalytic efficiency with the small molecule t-2-hexenal.

Considering the fact that PEG 400 was absolutely necessary for protein crystallization and that it binds in the active site, one can argue for a physiological substrate similar to t-2-alkenal. trans-2-Alkenals are breakdown products in lipid peroxidation reactions (46), produced from polyunsaturated fatty acids. Substances such as t-2-hexenal and t-2-dodecenal are volatile compounds of plant oils, produced upon bacterial injury to the plant. S. oneidensis, however, is not a plant pathogen, and polyunsaturated fatty acids are only present in psychrophilic bacteria (48, 49). An involvement of SYE1 in detoxification of these compounds is thus unlikely. Nonetheless, the identification of a second ligand-binding site at a hydrophobic cleft near the mouth of the active site entrance, created by a restructuring of Loop 3 in the capping subdomain, indicates that the physiological substrate of SYE1 may be a molecule consisting of a bulky (possibly aromatic) head connected to a thin tail. The “tail” of the substrate could then intercalate to the active site, whereas the bulky head could be stabilized at the entrance of the active site.

Acknowledgment—We thank the EMBL Hamburg Outstation at the DORIS storage ring DESY, for synchrotron facilities and help with data collection.

REFERENCES

1. Williams, R. E., and Bruce, N. C. (2002) Microbiology 148, 1607–1614
2. Brown, B. J., Deng, Z., Karpus, P. A., and Massey, V. (1998) J. Biol. Chem. 273, 32753–32762
3. Kohli, R. M., and Massey, V. (1998) J. Biol. Chem. 273, 32763–32770
4. Xu, D., Kohli, R. M., and Massey, V. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3556–3561
5. Lee, J., Godon, C., Lagniel, G., Spector, D., Garin, J., Labarre, J., and Toledano, M. B. (1999) J. Biol. Chem. 274, 6040–16046
6. Haarer, B. K., and Amberg, D. C. (2004) Mol. Biol. Cell 15, 4522–4531
7. Reekmans, R., De Smet, K., Chen, C., Van Hummelen, P., and Contreras, R. (2005) FEMS Yeast Res. 5, 711–725
8. French, C. E., Nicklin, S., and Bruce, N. C. (1996) J. Bacteriol. 178, 6623–6627
9. Snape, J. R., Walkley, N. A., Morby, A. P., Nicklin, S., and White G. F. (1997) J. Bacteriol. 179, 7796–7802
10. Blehert, D. S., Fox, B. G., and Chambliss, G. H. (1999) J. Biol. Chem. 281, 6254–6263
11. French, C. E., and Bruce, N. C. (1994) Biochem. J. 301, 97–103
12. Schaller, F., and Weiler, E. W. (1997) J. Biol. Chem. 272, 28066–28072
13. Strassner, J., Fürholz, A., Machereux, P., Amrhein, N., and Schaller, A. (1999) J. Biol. Chem. 49, 35067–35073
14. Miranda, M., Ramirez, J., Guevara, S., Ongay-Larios, L., Pena, A., and Coria, R. (1995) Yeast 11, 459–465
15. Niino, Y. S., Chakraborty, S., Brown, B. J., and Massey, V. (1995) J. Biol. Chem. 270, 1983–1991
16. Stott, K., Saito, K., Thiele, D. J., and Massey, V. (1993) J. Biol. Chem. 268, 6097–60106
17. Komduur, J. A., Leao, A. N., Monastyrskya, I., Veenhuis, M., and Kiel, J. A. (2002) Curr. Genet. 41, 401–406
18. Kubata, B. K., Kabututu, Z., Nozaki, T., Munday, C. J., Fukuzumi, S., Ohkubo, K., Lazarus, M., Maruyama, T., Martin, S. K., Duszenko, M., and Urale, Y. (2002) J. Exp. Med. 196, 1241–1251
19. Vaz, A. D. N., Chakraborty, S., and Massey, V. (1995) Biochemistry 34, 4246–4256
20. Meah, Y., and Massey, V. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 10733–10738
21. French, C. E., Nicklin, S., and Bruce, N. C. (1998) Appl. Environ. Microbiol. 64, 2864–2868
22. Pak, J. W., Knoke, K. L., Noguera, D. R., Fox, B. G., and Chambliss, G. H. (2000) Appl. Environ. Microbiol. 66, 4742–4750
23. Williams, R. E., Rathbone, D. A., Scrutton, N. S, and Bruce, N. C. (2004) Appl. Environ. Microbiol. 70, 3566–3574
24. Abramovitz, A. S., and Massey, V. (1976) J. Biol. Chem. 251, 5327–5336
25. Fox, K. M., and Karplus, A. P. (1994) Structure 2, 1089–1105
26. Breithaupt, Jr., C., Straßner, Jr., Breitinger, U., Huber, R., Machereux, P., A., and Clausen, T. (2001) Structure 9, 419–429
27. Barna, T. M., Khan, H., Bruce, N. C., Barsukov, I., Scrutton, N. S., and Moody, P. C. E. (2001) J. Mol. Biol. 310, 433–447
28. Messina, H. L., Bruce, N. C., Satelle, B. M., Sutcliffe, M. J., Munro, A. W., and Scrutton, N. S. (2005) J. Biol. Chem. 280, 27103–27110
29. Kitzing, K., Fitzpatrick, T. B., Wilken, C., Sawa, J., Bourenkov, G. P., Machereux, P., and Clausen, T. (2005) J. Biol. Chem. 280, 27904–27913
30. Heidelberg, J. F. et al. (2002) Nat. Biotechnol. 20, 1118–1123
31. Brigó, A., Van den Hemel, D., De Smet, L., Carpentier, W., and Van Beeumen, J. (2006) Biochem J. 394, 335–344
32. Orwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
33. Storoni, L. C., McCoy, A. J., and Read, R. J. (2004) Acta Crystallogr. Sect. D Biol. Crystallogr. 60, 432–438
34. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gross, P., Grosse-Kunstleve, R. W., Jiang, S. J., Kuszewiski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D Biol. Crystallogr. 54, 905–921
35. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291
36. Bruns, C. M., Hubatsch, I., Riddershöft, M., Mannervik, B., and Tainer, J. L.
37. Barna, T., Messiha, H. L., Petosa, C., Bruce, N. C., Scrutton, N. S., and Moody, P. C. E. (2002) J. Biol. Chem. 277, 30976–30983
38. Haynes, C. A., Koder, R. L., Miller, A.-F., and Rodgers, D. W. (2002) J. Biol. Chem. 277, 11513–11520
39. Koder, R. L., Haynes, C. A., Rodgers, M. E., Rodgers, D. W., and Miller, A.-F. (2002) Biochemistry 41, 14197–14205
40. Stewart, R. C., and Massey, V. (1985) J. Biol. Chem. 25, 13639–13647
41. Fitzpatrick, T. B., Amrhein, N., and Macheroux, P. (2003) J. Biol. Chem. 278, 19891–19897
42. Khan, H., Harris, R. J., Barna, T., Craig, D. H., Bruce, N. C., Munro, A. W., Moody, P. C. E., and Scrutton, N. S. (2002) J. Biol. Chem. 277, 21906–21912
43. Craig, D. H., Barna, T., Moody, P. C. E., Bruce, N. C., Chapman, S. K., Munro, A. W., and Scrutton, N. C. (2001) Biochem. J. 359, 315–323
44. Duax, W. L., Pletnev, V., Addlagatta, A., Bruenn, J., and Weeks, C. M. (2003) Proteins 53, 931–943
45. Wada, M., Yoshizumi, A., Noda, Y., Kataoka, M., Shimizu, S., Takagi, H., and Nakamori, S. (2003) Appl. Environ. Microbiol. 69, 933–937
46. Croft, K. P. C., Jüttner, F., and Slusarenko, A. J. (1993) Plant Physiol. 101, 13–24
47. Kubo, I., Fujita, F., Nihei, K., and Kubo, A. (2004) J. Appl. Microbiol. 96, 693–699
48. Bowman, J. P., McCammon, S. A., Nichols, D. S., Skerrat, J. H., Rea, S. M., Nichols, P. D., and McMeekin, T. A. (1997) Int. J. Syst. Bacteriol. 47, 1040–1047
49. Abboud, R., Popa, R., Souza-Egipsy, V., Giometti, C. S., Tollaksen, S., Mosher, J. J., Findlay, R. H., and Nealson, K. H. (2005) Appl. Environ. Microbiol. 71, 811–816
50. Meah, Y., Brown, B. J., Chakraborty, S., and Massey, V. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 8560–8565
51. Bletter, D. J., and Knoke, K. L. (1997) J. Bacteriol. 179, 6912–6920
52. Marshall, S. J., Krause, D., Blencowe, D. K., and White, G. F. (2004) J. Bacteriol. 186, 1802–1810