A Homolog of Old Yellow Enzyme in Tomato
SPECTRAL PROPERTIES AND SUBSTRATE SPECIFICITY OF THE RECOMBINANT PROTEIN*

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A cDNA was isolated and characterized from a tomato shoot cDNA library, the deduced amino acid sequence of which exhibited similarity with yeast Old Yellow Enzymes (OYEs) and related enzymes of bacterial and plant origin. Sequence identity was particularly high with 12-oxophytodienoate 10,11-reductase (OPR) from Arabidopsis thaliana. The cDNA-encoded protein was expressed as a glutathione S-transferase fusion protein in Esherichia coli and was purified from bacterial extracts. The protein was found to be a flavoprotein catalyzing the NADPH-dependent reduction of the olefinic bond of α,β-unsaturated carbonyl compounds, including 12-oxophytodienoic acid. Thus, the tomato enzyme was termed LeOPR. The catalytic efficiency of LeOPR was highest with N-ethylmaleimide followed by 12-oxophytodienoic acid and maleic acid as substrates. Photodegradation of the LeOPR-bound FMN resulted in the formation of a red, anionic semiquinone prior to the formation of the fully reduced flavin dihydroquinone. Spectroscopic characterization of LeOPR revealed the formation of charge transfer complexes upon titration with para-substituted phenolic compounds, a distinctive feature of the enzymes of the OYE family. The ligand binding properties were compared between LeOPR and OYE, and the findings are discussed with respect to structural differences between the active sites of OYE and LeOPR.

Old Yellow Enzyme (OYE)† (EC 1.6.99.1) was initially isolated from brewer’s bottom yeast and was the first enzyme shown to possess a flavin cofactor (1, 2). Despite extensive biochemical and spectroscopic characterization, the physiological role of the enzyme has remained obscure. OYE has been described as a diaphorase catalyzing the oxidation of NADPH in presence of molecular oxygen. However, molecular oxygen is commonly viewed as an opportunist electron acceptor, and the physiological oxidant has not been identified (3). A number of electron acceptors for reduced OYE are known including the olefinic bond of α,β-unsaturated ketones and aldehydes, suggesting that the physiological substrate may comprise such a structural feature (4, 5). A growing family of OYE homologs has been identified in organisms with both prokaryotic and eukaryotic origins (4, 6–9). Recently, the cloning and sequencing of a higher plant OYE homolog from Arabidopsis thaliana has been described (10). This enzyme catalyzes the NADPH-dependent reduction of 12-oxophytodienoate (OPDA) to yield 3-oxo-2(Z)-pentenyl-cyclopentane-1-octanoic acid and was therefore termed 12-oxophytodienoate 10,11-reductase (OPR). The OPR-catalyzed reaction is part of the biosynthetic pathway to jasmonic acid, a compound with multiple hormonal activities in higher plants (11). Two isoforms of this enzyme (OPRI and OPRII) exist in Arabidopsis and in Corydalis sempervirens (Rock Harlequin), only one of which (OPRII) reduces the 9S,13S enantiomer of OPDA, the precursor of biologically active 7-epi jasmonic acid (12–14). In contrast, OPRI does not accept the isomers of OPDA with the 13S-configuration as substrates, and the physiological role of this isoform remains obscure (13). We describe here the molecular cloning and overexpression in E. coli of an OPR from tomato plants, the purification of the recombinant enzyme, and the characterization of its substrate specificity.

One of the most salient features of yeast OYE is its ability to form charge transfer (CT) complexes with a variety of aromatic and heteroaromatic compounds carrying an ionizable hydroxyl group. Formation of the CT complex results in a long-wavelength optical transition in the absorbance spectrum. A positive correlation was observed between the absorbance maximum of the newly formed long wavelength transition and the Hammett para-constant of the p-substituted phenolic compounds, which can be considered as a measure of the phenolate ionization potential (15). This finding and the pH dependence of binding suggest that in the CT complex, the bound phenolate and the oxidized flavin act as the electron donor and acceptor, respectively. We report here on the spectral properties of the charge transfer complexes formed between recombinant LeOPR and phenolic ligands. This is the first detailed description of the properties of CT complexes formed by an OYE homolog isolated from a higher plant.

EXPERIMENTAL PROCEDURES
Cloning and Overexpression of LeOPR—All basic molecular techniques were adapted from Sambrook et al. (16) or Ausubel et al. (17). A cDNA library was constructed from tomato Lycopersicon esculentum cv. Castlemart II shoot tissue in the yeast E. coli shuttle vector pYES2 (Invitrogen, Groningen, The Netherlands). The LeOPR cDNA was isolated serendipitously by transformation of a yeast reporter strain† using a selection system for proteases that interact with the peptide system (18). The cDNA sequence of LeOPR was determined on both strands using fluorescent dyeoxy chain terminators in the cycle sequencing reaction (Perkin-Elmer) and the Applied Biosystems model 373A DNA sequencer. Oligonucleotide sequencing and polymerase chain reaction primers were obtained from Microsynth (Balgrach, Switzerland). The open reading frame of the LeOPR cDNA was amplified by polymerase chain reaction (Perkin-Elmer)’s 5’ primer, ATGGAAAAATAAAGTCTGGTA; 3’ primer, GGGGTACCTCATGTCATGGTTTCTAG-

* This work was supported by Grant 31.46818.96 of the Swiss National Science Foundation (to A.S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ242551.

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† The abbreviations used are: OYE, Old Yellow Enzyme; CT, charge transfer; GST, glutathione S-transferase; OPDA, 12-oxophytodienoic acid; OPR, 12-oxophytodienoate 10,11-reductase.

‡ J. Straßner and A. Schaller, unpublished data.
Figure 1. Nucleotide sequence and deduced amino acid sequence of the LeOPR cDNA. The nucleotide sequence of the LeOPR cDNA is shown. The asterisks denote stop codons in the 5'-untranslated leader sequence and at the 3'-untranslated region following the stop codon (TGA in position 1399). The DNA gel blots were hybridized with the radiolabeled cDNA and washed under anaerobic conditions in argon-saturated phosphate buffer. EDTA (5 mM) was used as the electron donor. For photoreduction, the cuvette was illuminated (750 μmol m⁻² s⁻¹) using a slide projector. RESULTS

Molecular Cloning of LeOPR—A 1254-base pair cDNA was isolated from a tomato shoot cDNA library, and both strands were sequenced (Fig. 1, GenBank™ accession number AJ242551). This cDNA encompasses an open reading frame of 1128 nucleotides (ATG in position 108) that is preceded by an in-frame stop codon (TAA, position 81), indicating that the cDNA comprises the entire protein coding region. However, the 5'-untranslated region following the stop codon (TGA in position 1236) is unexpectedly short. Possibly, the A₃ stretch at the 3'-end does not represent the poly(A) tail of the mRNA but rather an oligo(A) stretch within its 3'-untranslated region. The amino acid sequence deduced from the open reading frame for LeOPR was found to be most closely related (71.4% identity) to that of OPR from A. thaliana (10). Therefore, as long as its physiological substrate remains unidentified, we will refer to the cDNA-derived protein as LeOPR. Sequence identities of 67–72, 29–33, and 18–40% were observed with other OYE-like enzymes of plant, fungal, and bacterial origin, respectively. A sequence alignment of the amino acid sequence deduced from

Characterization of a Tomato OYE Homolog

AA and cloned into the StuI and KpnI 1 restriction sites of pGEX-G (19), a derivative of pGEX-3X (Amersham Pharmacia Biotech). This vector allows expression of LeOPR in fusion with glutathione S-transferase under control of the isopropyl-1-thio-β-D-galactopyranosidase-inducible promotor. The expression construct was transformed into E. coli BL21. A 200-ml culture was grown to an OD₆₀₀ of 0.6 and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM. Cells were allowed to grow for another 6 h at 30 °C, when they were harvested by centrifugation and subjected to 80 °C heat treatment until proteinase activity stopped. After heat treatment, the cells were stored at 4 °C, when they were harvested by centrifugation and stored at 6°C until purification of LeOPR.

Purification of Recombinant LeOPR—E. coli cells (10 g) were resuspended in 20 ml of Buffer A (50 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM EDTA) containing 0.1 mg/ml DNaseI, 1 mg/ml lysozyme, and 1 mM phenylmethylsulfonyl fluoride. After 20 min at room temperature, cells were lysed by sonication. The cell debris was removed by centrifugation (25000 × g for 10 min at 4 °C), and the supernatant was subjected to affinity chromatography on a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech). After extensive washing with Buffer A and Buffer B (50 mM Tris-HCl, pH 8.0), the fusion protein (GST-OPR) was eluted with Buffer B containing 5 mM reduced glutathione. Factor Xa (Factor Xa cleavage and removal kit; Roche Diagnostics, Switzerland) was used at a ratio of 1 μg Xa per 10 μg GST-OPR during 16 h at 4°C in order to cleave the fusion protein at the predetermined site, releasing LeOPR with the authentic N-terminal methionine residue. The progress of cleavage was monitored by SDS-polyacrylamide gel electrophoresis. Processed LeOPR was purified by affinity chromatography on glutathione-Sepharose as before to remove the GST protein. The final purification step involved anion exchange chromatography on a MonoQ column (1-ml bed volume) (Amersham Pharmacia Biotech) (6-ml bed volume) equilibrated in Buffer A. The progress of protein purification was monitored by SDS-polyacrylamide gel electrophoresis performed on 10% polyacrylamide gels using the buffer system described by Laemmli (20). Gels were stained for proteins using Coomassie Brilliant Blue R250. Northern Blot Analysis—RNA was isolated from different tissues of tomato plants using a phenol-based extraction procedure (16). Total RNA (5 μg) was separated on formaldehyde-agarose gels and transferred to nitrocellulose membranes according to standard protocols. LeOPR transcripts were detected on the RNA gel blots using the radiolabeled cDNA (Prime-It DNA labeling system, Stratagene) as a probe. The blots were stained for proteins using Coomassie Brilliant Blue R250. Polyacrylamide gel electrophoresis performed on 10% polyacrylamide gels using the buffer system described by Laemmli (20). Gels were stained for proteins using Coomassie Brilliant Blue R250.
the tomato cDNA, of *Saccharomyces* OYE1–3, and of other plant-derived OYE-related sequences is shown in Fig. 2. Considerable sequence identity was observed over the entire lengths of the deduced protein sequences. Two of the three amino acid residues involved in substrate binding are conserved between the plant and yeast sequences (tyrosine 376 and histidine 192 (numbering refers to yeast OYE1 as shown in Fig. 2; the respective numbers are lower by 1 in Fig. 8 because the crystallized OYE lacked the N-terminal methionine residue)), whereas asparagine 195 is replaced by a second histidine and histidine 192 (numbering refers to yeast OYE1 as shown in Fig. 2; the respective numbers are lower by 1 in Fig. 8 because the crystallized OYE lacked the N-terminal methionine residue)).

The N-terminal amino acid sequences of the two polypeptides were determined (Fig. 1, underlined) and found to correspond to those predicted from the cDNA sequence, whereas weakly labeled bands point to the existence of at least one additional gene closely related to that of LeOPR per haploid genome in tomato.

Overexpression and Purification of LeOPR—LeOPR was cloned into the expression vector pGEX-G (19) and overexpressed in *E. coli* in N-terminal fusion with glutathione S-transferase. From 1 liter of *E. coli* culture, 64 mg of soluble GST-OPR were purified by affinity chromatography. LeOPR was cleaved from the GST moiety by proteolytic processing using factor Xa and was further purified by anion exchange chromatography. At this stage of purification, SDS-polyacrylamide gel electrophoresis revealed the presence of two polypeptides of 43 and 29 kDa, respectively (Fig. 4). The larger polypeptide constituted about 75% of the total protein, and its apparent molecular mass is in good agreement with the calculated Mr of 42,414 for LeOPR deduced from the cDNA sequence. The N-terminal amino acid sequences of the two polypeptides were determined (Fig. 1, underlined) and found to correspond to the N terminus and to an internal sequence of LeOPR, respectively. Therefore, the smaller polypeptide represents a truncated form of LeOPR and is most likely a degradation product. OYE is known to be a homodimeric protein in its native state (22). Therefore, co-purification of LeOPR and its degradation product during affinity and anion exchange chromatography is likely to be due to the formation of heterodimers between the two polypeptides.

**Catalytic Activity of LeOPR—** LeOPR and the fusion protein GST-OPR were found to catalyze the oxidation of NADPH in presence of molecular oxygen. During the search for substrates of OYE, the C=C double bond in α,β-unsaturated carbonyl compounds was found to be an alternative electron acceptor (4,
Likewise, we observed an accelerated rate of NADPH oxidation by LeOPR in presence of \( \alpha, \beta \)-unsaturated carbonyl compounds. When monitoring the oxidation of NADPH spectrophotometrically, it is difficult to estimate the relative contribution of the unsaturated carbonyl substrates and the molecular oxygen to the observed reaction rates. Therefore, to exclude molecular oxygen as a potential electron acceptor, the determination of steady state rate constants with a consuming system (glucose/glucose oxidase, see “Experimental Procedures”).

Apparent \( K_m \) and \( k_{cat} \) values were derived from steady state kinetic analyses for a selection of \( \alpha, \beta \)-unsaturated carbonyl compounds and are summarized in Table I. GST-OPR was found to accept a wide range of structurally different \( \alpha, \beta \)-unsaturated carbonyl substrates was done in presence of an oxygen-consuming system (glucose/glucose oxidase, see “Experimental Procedures”).

The planar five-member ring and its substitution pattern resemble the cyclopentenone structure found in 12-oxophytodienoate, which is the substrate of OPR, the OYE homolog in Arabidopsis. Likewise, 12-oxophytodienoate (1-cyclopentene-1-octanoic acid) is the substrate of OPR, the OYE homolog in Arabidopsis.

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### Table I

| Substrate               | \( K_m \) (mM) | \( k_{cat} \) (s\(^{-1}\)) | \( k_{cat}/K_m \) (a.u.) |
|-------------------------|----------------|-------------------|------------------------|
| trans-Hex-2-enal\(^a\) | 2200           | 4.6               | 0.002                  |
| Cinnamaldehyde\(^b\)   | 570            | 6.9               | 0.012                  |
| trans-Dodec-2-enal\(^c\) | 250            | 11.3              | 0.045                  |
| Cyclohex-2-2- ones\(^d\) | 1310           | 0.7               | 0.001                  |
| \( N \)-Ethylmaleimide\(^e\) | 8              | 29.0              | 3.63                   |
| Maleic acid\(^f\)      | 47             | 13.8              | 0.29                   |
| 12-Oxophytodienoic acid\(^g\) | 15             | 15.5              | 0.62                   |
| Fumaric acid\(^h\)     | NR             | NR                | NR                     |
| Coumarin\(^i\)         | NR             | NR                | NR                     |

\(^a\) The assay contained GST-OPR at a concentration of 7.25 nm.

\(^b\) 0.725 nm GST-OPR.

\(^c\) 7.25 nm GST-OPR.

\(^d\) 30 nm GST-OPR.

\(^e\) NR, no reaction.

The formation of charge transfer transitions typically results in reciprocal changes in the absorbance of the interacting chromophores. The spectral characteristics of the CT complexes formed between \( \alpha, \beta \)-substituted phenols and (GST)-LeOPR, as well as their dissociation constants, are summarized in Table II. Although the dissociation constants of the CT complexes were generally lower for LeOPR in comparison to GST-OPR, both yielded similar long wavelength absorbance maxima. A linear relationship was observed between the absorbance maxima of the long wavelength transition and the Hammett \( \rho \)-constant (\( \sigma_p \)) (Fig. 6). Furthermore, the dissociation constant of the CT complex could be correlated to the phenolic \( \rho \)-constant (\( \sigma_p \)) (Table II), indicating that it is the phenolate that binds to the enzyme and that within the complex, the phenolate

![Figure 4. Purification of LeOPR after overexpression in E. coli. A Coomassie Brilliant Blue R250-stained SDS-polyacrylamide gel is shown. The crude cell extract (lane 2) was separated by centrifugation into the supernatant (lane 3) and the cell pellet (lane 4). Lanes 5–7 show the flow-through, the wash fraction, and the eluate of the glutathione-Sepharose column, respectively. Purified LeOPR after processing with factor Xa and MonoQ anion exchange chromatography is shown in lane 7. Protein standards are shown (lane M) and their masses are indicated in kDa.](image)

![Figure 5. Titration of LeOPR with \( \alpha, \beta \)-substituted phenols.](image)
and the flavin act as the charge donor and acceptor, respectively.

Photoreduction of LeOPR—Reduction of LeOPR-bound FMN was achieved under anaerobic conditions by irradiation with white incandescent light in the presence of EDTA as a source of electrons (Fig. 7). Initially, the reduction of the flavin cofactor resulted in a decrease in the absorbance at the 465 nm absorbance maximum concomitant with an increase at 380 nm, indicative of the formation of a red, anionic flavin semiquinone.

Assuming that the flavin semiquinone is the only flavin species absorbing at 385 nm, we derived an extinction coefficient of 12900 M$^{-1}$ cm$^{-1}$ for the semiquinone at this wavelength. The extinction coefficient is thus similar to that observed for the OYE-bound flavin semiquinone (29). The generation of the flavin semiquinone was followed by a further reduction to the fully reduced flavin. From a plot of the absorbance at 385 nm versus the time of irradiation, about 80% flavin semiquinone was estimated to be formed during photoreduction (Fig. 7). In the presence of the electron mediator methyl viologen (0.5 mM), no flavin semiquinone was generated during photoreduction, indicating that the radical species is stabilized kinetically rather than thermodynamically.

### Table II

| Ligand           | $pK_a$ | $\sigma_p$ | $K_D$ (M) | $\lambda$ (nm) | $\epsilon$ (M$^{-1}$ cm$^{-1}$) | Enzyme |
|------------------|--------|------------|-----------|----------------|---------------------------------|--------|
| p-Cresol         | 10.3   | -0.170     | 1060      | 620            | 1300                            | GST-OPR|
| p-Methoxyphenol  | 10.2   | -0.268     | 970       | 640            | 2100                            | GST-OPR|
| p-Fluorophenol   | 9.43   | 0.062      | 3780      | 600            | 950                             | GST-OPR|
| p-Chlorophenol   | 9.34   | 0.227      | 2040      | 600            | 550                             | GST-OPR|
| p-Hydroxyacetophenol | 8.05  | 0.516      | 90        | 535            | 3500                            | GST-OPR|
| p-Hydroxybenzaldehyde | 7.62  | 0.216      | 21        | 532            | 4100                            | GST-OPR|
| p-Nitrophenol    | 7.15   | 0.778      | 88        | 5300           | 3800                            | GST-OPR|

$^a$ Phenolic $pK_a$, as reported by Abramovitz and Massey (23).

$^b$ Hammett para-constant $\sigma_p$, as reported by Jaffe (32) and Hausch et al. (33).

**FIG. 6.** Correlation of the Hammett para-constant to the absorbance maximum of the charge transfer band. The wave numbers of the absorbance maxima of the long wavelength transitions were plotted against the Hammett para-constants ($\sigma_p$, as reported by Jaffe (32) and Hausch et al. (33)) of the respective ligand. The ligands used (and their respective $\sigma_p$ constants) were p-methoxyphenol (−0.268), p-methylphenol (−0.170), p-fluorophenol (0.062), p-hydroxybenzaldehyde (0.216), p-chlorophenol (0.227), p-hydroxyacetophenone (0.516), and p-nitrophenol (0.778). Absorbance maxima for the LeOPR (this paper) and OYE CT complexes (23) are represented by filled triangles and open squares, respectively. Linear regression was performed with the software SigmaPlot (version 4.1, Jandel Scientific), and the fits are shown by a solid line (OYE CT complexes) and a dashed line (LeOPR CT complexes), respectively.

**FIG. 7.** Photoreduction of LeOPR. LeOPR was illuminated under anaerobic conditions with 750 μmol m$^{-2}$ s$^{-1}$ of white light. Absorbance spectra were recorded from 300 to 750 nm before illumination (a) and after 20 (b), 45 (c), 85 (d), and 180 min (e) of illumination. The inset shows a plot of the absorbance at 380 nm as a function of the duration of light exposure. A comparison between the maximum absorbance observed at 380 nm with the extrapolation allows an estimation of the degree (81%) to which the anionic flavin semiquinone is stabilized by LeOPR.
DISCUSSION

Recently, plant proteins sharing high similarity with yeast OYE were characterized from C. sempervirens and cloned from A. thaliana, and they were found to catalyze preferentially the reduction of cis-(-)-OPDA (9R,13R)-OPDA (10, 12). These enzymes, termed OPRI, showed very little activity with the cis- (+)-stereoisomer (9S,13S)-OPDA), which is an intermediate of the octadecanoid pathway in the biosynthesis of jasmonic acid. More recently, the same laboratory reported the occurrence of an isozyme, called OPRII, catalyzing the reduction of the latter OPDA isomer (13). Therefore, OPRII was proposed to be the isozyme involved in jasmonic acid biosynthesis, whereas the role of OPRI in the octadecanoid pathway is unclear. The undesirable cis-(−)-isomer may be generated fortuitously by uncoupling of the allene oxide synthase/allene oxide cyclase reactions, and thus, a role for OPRI has been suggested in maintaining a pool of stereochemically pure OPDA for the biosynthesis of jasmonic acid (13). LeOPR, cloned and characterized here, was found to reduce commercially available OPDA, which is the racemic mixture of the two cis stereoisomers of OPDA. Preliminary data3 indicate that the stereoselectivity of LeOPR resembles that of OPRI in C. sempervirens and A. thaliana, i.e. it reduces the cis-(−)-stereoisomer preferentially over the cis-(+)-stereoisomer. However, Southern blot analysis (Fig. 3A) revealed the presence of at least two closely related genes within the haploid tomato genome, indicating the occurrence of LeOPR isozymes. The identification and characterization of the LeOPR isozyme corresponding to OPRII in C. sempervirens will be helpful in establishing the respective roles of these reductases in plant metabolism. Considering the broad substrate specificity of LeOPR (cf. Table I), it is tempting to speculate that other physiological relevant compounds may be reduced by this enzyme. Plants contain a multitude of substances with the basic substrate recognition motif, i.e. an α,β-unsaturated carbonyl function, and hence, other physiological substrates of LeOPR (and possibly also OPRI) may exist in planta. In view of this uncertainty, it remains to be seen whether the search for physiological substrates of LeOPR and OPRI will provide clues with respect to the in vivo substrate of yeast OYE, which, after several decades of research, still eludes discovery.

A distinctive feature of yeast OYE is the formation of CT complexes with phenolic ligands (24–26). We show here that a series of phenolic ligands form CT complexes with LeOPR. This is the first demonstration of such complexes with a plant flavoprotein, providing further biochemical evidence for the close relationship between yeast OYES and their plant homologs that was suggested recently (10). The absorbance maxima and the extinction coefficients of the LeOPR CT complexes were consistently lower than those reported for OYE (cf. Table II). Furthermore, the dissociation constants were considerably higher for the CT complexes with LeOPR. Despite these differences, a plot of the charge transfer absorbance maxima against the Hammett ζp parameters of the substituents at the aromatic ring of the phenolic ligands yielded a linear dependence with a slope similar to the one obtained for OYE (Fig. 6). This result indicates that the flavin moiety acts as the acceptor and the phenolic ligand as the donor in the LeOPR CT complexes, as was concluded earlier for yeast OYE (26). The hypochromic shift of the CT complex absorbance maxima in case of LeOPR indicates that the energy difference between the lowest unoccupied molecular orbital of FMN and the highest occupied molecular orbital of the phenolic ligand is larger for LeOPR than for yeast OYE. In other words, the redox potential of the enzyme-bound FMN is more negative and/or the redox potential of the phenolic ligand is more positive in LeOPR as compared with yeast OYE CT complexes.

According to the three-dimensional structure of yeast OYE, p-hydroxybenzaldehyde is bound on the si-face of the flavin ring system (27) (Fig. 8). The aldehyde group forms a hydrogen bond to the hydroxyl group of the side chain of tyrosine 375, whereas the p-hydroxy group is engaged in two hydrogen bond interactions with the side chains of histidine 191 and asparagine 194, respectively. Tyrosine 375 is also present in LeOPR (numbering scheme of LeOPR): at position 188, Ser → Gly; position 190, Asn → His; and position 247, Phe → Tyr. Note that amino acid Phe-296 in the yeast OYE structure has no counterpart in the plant homolog (see gap in the alignment shown in Fig. 2). The program Molscript was used to generate the structural representations.

3 F. Schaller and E. Weiler, unpublished data.
8A), the two amino acid replacements (i.e. N194H and S192G) were modeled into the three-dimensional structure. It appears that the somewhat bulkier and less flexible histidine residue in LeOPR introduces a perturbation of the hydrogen bond network in the binding site of the phenolic ligand, which may be responsible for the higher dissociation constants and the altered properties of the charge transfer interactions between the flavin and the phenolic ligands. Interestingly enough, replacement of asparagine 194 with histidine in OYE1 was reported to result in a low yield of purifiable protein (28). It was suggested that the bulkier histidine residue interferes with flavin binding, resulting in the low yield of holoprotein (28). In LeOPR and other plant OYEs, the second amino acid replacement (S192G) may compensate for the unfavorable N194H exchange. The S192G replacement may result in a higher flexibility in this part of the protein, allowing for a second histidine in the binding site. Our findings demonstrate that the N194H replacement does not impede the formation of CT complexes. Therefore, the formation of CT complexes can be expected for all members of the OYE family featuring a histidine and/or an asparagine residue in positions 191 and 194 provided that the structural environment is properly set up to accommodate two histidine residues as discussed above. Another interesting difference between the modeled active-site structures of OYE (Fig. 8A) and LeOPR (Fig. 8B) is the absence in LeOPR of a residue corresponding to Phe-296 in OYE. This difference is also apparent from the sequence alignment shown in Fig. 2, where a gap (corresponding to OYE residues 294–299) had to be introduced in this region to allow for an optimal alignment with the yeast sequences. The active site of LeOPR thus appears to be more accessible to bulky substrates than that of OYE.

Reduction of LeOPR results in the formation of an anionic flavin semiquinone prior to the reduction to the flavin dihydrouquinone (Fig. 7). Formation of the red flavin semiquinone was also reported for yeast OYE (29). The occurrence of an anionic flavin semiquinone in the flavin binding pocket is attributed to the presence of the positively charged side chain of an arginine or lysine residue in the vicinity of the N(1)–C(2)=O locus of the flavin ring, which provides the necessary stabilization for the formation of a negative charge in this region (30, 31). In fact, the three-dimensional structure of yeast OYE reveals the presence of an arginine (position 243), and of a glutamine residue (position 114) in close proximity (2.8–3.2 Å) of the N(1)–C(2)=O locus of the flavin ring system. Both amino acids are strictly conserved in yeast OYEs and the plant homologs (Fig. 2) and thus, the observation of an anionic flavin semiquinone in these proteins can be rationalized by the presence of an appropriate flavin environment. The stabilization of the red anionic flavin semiquinone is, however, kinetic in nature as was shown in photoreduction experiments in the presence of an electron mediator. Under these conditions no anionic semiquinone could be detected. In case of OYE, 15–20% of the anionic flavin semiquinone was generated in the presence of an electron mediator, indicating that the nature of stabilization is predominantly kinetic (29). The amount of stabilization of the anionic flavin semiquinone is a consequence of the two one electron couples E1 (the midpoint potential of $\text{EFMN}_\text{red} + e^{-} \equiv \text{EFMN}^{*}$) and E2 (the midpoint potential of $\text{EFMN}^{*} + e^{-} \equiv \text{EFMN}_\text{red}$). In yeast OYE, these couples are separated by 30 mV ($E_1 = -245\ mV$; $E_2 = -215\ mV$ (29)) giving rise to the observed stabilization (15–20%). In case of LeOPR, the absence of a flavin semiquinone during photoreduction in presence of an electron mediator indicates that $E_2 \gg E_1$, i.e. the kinetic stabilization of the flavin semiquinone is even more pronounced in LeOPR as compared with OYE.

**Acknowledgments**—The excellent technical assistance of David Frasen is gratefully acknowledged. We thank Dr. Tim Clausen (Max-Planck Institute, Martinsried, Germany) for help in the preparation of Fig. 8 and Drs. Elmar W. Weiler and Florian Schaller (Ruhr-Universität Bochum, Germany) for a preliminary analysis of LeOPR stereospecificity and helpful discussions.

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J. Biol. Chem. 1999, 274:35067-35073.
doi: 10.1074/jbc.274.49.35067

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