

Role of tyrosine 129 in the active site of spinach glycolate oxidase

Peter MACHEROUX^{1,2}, Volker KIEWEG², Vincent MASSEY¹, Eskil SÖDERLIND³, Kaj STENBERG³ and Ylva LINDQVIST³

¹ Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI, USA

² Fakultät für Biologie, Universität Konstanz, Germany

³ Department of Molecular Biology, Swedish University of Agricultural Sciences, Uppsala Biomedical Center, Sweden

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The enzymatic properties and the three-dimensional structure of spinach glycolate oxidase which has the active-site Tyr129 replaced by Phe (Y129F glycolate oxidase) has been studied. The structure of the mutant is unperturbed which facilitates interpretation of the biochemical data. Y129F glycolate oxidase has an absorbance spectrum with maxima at 364 and 450 nm ($\epsilon_{\max} = 11400 \text{ M}^{-1} \text{ cm}^{-1}$). The spectrum indicates that the flavin is in its normal protonated form, i.e. the Y129F mutant does not lower the $\text{p}K_a$ of the N(3) of oxidized flavin as does the wild-type enzyme [Macheroux, P., Massey, V., Thiele, D. J., and Volokita, M. (1991) *Biochemistry* 30, 4612–4619]. This was confirmed by a pH titration of Y129F glycolate oxidase which showed that the $\text{p}K_a$ is above pH 9. In contrast to wild-type glycolate oxidase, oxalate does not perturb the absorbance spectrum of Y129F glycolate oxidase. Moreover oxalate does not inhibit the enzymatic activity of the mutant enzyme. Typical features of wild-type glycolate oxidase that are related to a positively charged lysine side chain near the flavin N(1)-C(2=O), such as stabilization of the anionic flavin semiquinone and formation of tight N(5)-sulfite adducts, are all conserved in the Y129F mutant protein. Y129F glycolate oxidase exhibited about 3.5% of the wild-type activity. The lower turnover number for the mutant of 0.74 s^{-1} versus 20 s^{-1} for the wild-type enzyme amounts to an increase of the energy of the transition state of about 7.8 kJ/mol. Steady-state analysis gave K_m values of 1.5 mM and 7 μM for glycolate and oxygen, respectively. The K_m for glycolate is slightly higher than that found for wild-type glycolate oxidase (1 mM) whereas the K_m for oxygen is much lower. As was the case for wild-type glycolate oxidase, reduction was found to be the rate-limiting step in catalysis, with a rate of 0.63 s^{-1} . The kinetic properties of Y129F glycolate oxidase provide evidence that the main function of the hydroxyl group of Tyr129 is the stabilization of the transition state.

The three-dimensional structure of glycolate oxidase has been determined by X-ray crystallography (Lindqvist and Brändén, 1989) and has now been refined to 0.2-nm resolution (Lindqvist, 1989). Comparison of properties with the extensively studied L-lactate oxidase and the topography of the active site and the amino acids surrounding the prosthetic group FMN led to the proposal (Lindqvist and Brändén, 1989; Ghisla and Massey, 1991) of a reaction mechanism that involves the amino acids Tyr24, Asp157, Tyr129, Lys230, His254 and Arg257 (see Fig. 1 and Scheme II). In this proposal the side chain of Lys230 provides a positive charge near the N(1)-C(2=O) locus which is thought to enhance the electrophilicity of the N(5) atom. At the same time, this positively charged side chain is held responsible for some of the most typical flavoprotein oxidase characteristics, such as stabilization of the anionic flavin semiquinone and

of the benzoquinoid form of 8-mercapto-flavin, as well as formation of tight sulfite-N(5) adducts. In a recent study of wild-type glycolate oxidase, it was confirmed that this enzyme exhibits all these features and can be regarded as a typical member of this class of flavoproteins (Macheroux et al., 1991). Moreover it was found that all its properties in solution are compatible with the X-ray crystal structure. Kinetic studies on the wild-type enzyme also revealed that catalysis proceeds via a ping-pong mechanism and that the reduction of FMN is rate-limiting.

The next step towards a more detailed understanding of the catalytic events and the involvement of active-site amino acids in catalysis is to replace them individually by appropriate amino acids, i.e. amino acids that cannot substitute for the original amino acid in terms of catalysis and/or binding of substrate(s). In this paper we report on the properties of a Y129F mutant glycolate oxidase. The hydroxyl group of Tyr129 was proposed to form a hydrogen bond to the α -hydroxy group of glycolate and to facilitate the breakdown of a putative N(5) adduct to yield reduced FMN and the product (glyoxylate). In order to assess the role of Tyr129, we replaced it with Phe using site-directed mutagenesis techniques. Our results confirm that Tyr129 participates in the reductive step of catalysis but the presence of the tyrosine hydroxyl group is not essential for substrate binding. As ex-

Correspondence to P. Macheroux, Fakultät für Biologie, Universität Konstanz, P. O. Box 5560, W-7750 Konstanz, Germany
Fax: +49 7531 882966.

Abbreviation. Y129F glycolate oxidase, glycolate oxidase with Tyr129 replaced by Phe.

Enzymes. Flavocytochrome b_2 , L-lactate cytochrome c oxidoreductase (EC 1.1.2.3); lactate oxidase, L-lactate 2-monooxygenase (EC 1.13.12.4); peroxidase from horseradish (EC 1.11.1.7); spinach glycolate oxidase short-chain L- α -hydroxy acid oxidase isozyme A (EC 1.1.1.15).

pected, the Y129F mutation did not abolish the flavoprotein oxidase characteristics mentioned above.

Part of this work was presented at the 10th Symposium on 'Flavins and Flavoproteins' in Como, Italy (1990).

MATERIALS AND METHODS

Materials

Glycolic acid, iodoacetamide and methyl methane thiol-sulfonate were from Aldrich. Horseradish peroxidase, *o*-dianisidine, FMN, phenylmethanesulfonyl fluoride, D-(+)-glucose, and all amino acids were from Sigma. Yeast nitrogen base without amino acids was from Difco Laboratories (Detroit, Michigan). Isopropyl β -D-thiogalactopyranoside, peptone, yeast extract and glycerol were from Roth (Karlsruhe, Germany). 8-Mercapto-FMN was freshly prepared from 8-chloro-FMN as described in the literature (Massey et al., 1979).

Oligonucleotide-directed mutagenesis

The gene of glycolate oxidase was cloned into the *EcoRI* restriction site of vector M13mp18. The orientation was determined with an asymmetric *HindIII/StyI* digest. A 21-base complementary oligonucleotide, AAGGTCGAGAAGCAT-ATGTTT, was synthesized on a Biolabs DNA synthesizer. The mutant codon is underlined. The mutant codon, TTC, was chosen to match the most frequently used Phe codon in *Saccharomyces cerevisiae* (Guthrie and Abelson, 1982). The Y129F mutant was constructed according to the manufacturer's instruction using the 'Oligonucleotide directed *in vitro* mutagenesis system, version 2' kit (code RPN, 1523, Amersham International plc, Amersham, UK). The mutant was identified by dideoxynucleotide sequencing. After cloning the mutant gene into the *EcoRI* restriction site of expression vector pGAO (Macheroux et al., 1991), the orientation was determined with an asymmetric *EcoRV* digest and the mutation was verified by dideoxy sequencing. Introduction of the Y129F mutation into the *Escherichia coli* expression plasmid (pPM1, see Macheroux et al., 1992) was achieved by exchanging the *NsiI-SacI* fragment of the the wild-type glycolate oxidase gene with the fragment containing the Y129F mutation.

Enzyme assays

Enzymes were assayed as described by Macheroux et al. (1991) except that a 50- μ l sample volume was used instead of the 10 μ l of wild-type glycolate oxidase. This was necessary since Y129F glycolate oxidase exhibited a comparatively low activity.

Expression of the mutant Y129F glycolate oxidase

The Y129F mutant was prepared either from transformed yeast cells (Macheroux et al., 1991) or from the recently developed *E. coli* expression system (Macheroux et al., 1992). The mutant enzymes from both sources were identical in their spectral and kinetic properties. However, the yield of Y129F glycolate oxidase from the yeast system was diminished considerably to approximately 2–3 mg compared to 12–14 mg of wild-type enzyme (from 200 g wet cell paste) whereas the *E. coli* expression system yielded the same amount of target protein (yield from 150 g wet cell paste was

about 50–60 mg). The reasons for the sharply reduced level of Y129F mutant protein in the yeast expression system are not known.

Photoreduction

Y129F glycolate oxidase was made anaerobic by alternate evacuation and flushing with oxygen-free argon. The photochemical reduction, using EDTA and 5-deazaflavin as catalyst, was then performed as described by Massey and Hemmerich (1978). Light irradiation was carried out with a sun gun (Smith Victor Corp., Griffith) at an intensity of about $0.6 \text{ J s}^{-1} \text{ cm}^{-2}$.

Preparation of 8-mercapto-FMN Y129F glycolate oxidase

An ammonium sulfate suspension (5 ml) containing approximately 0.4 mg Y129F glycolate oxidase was centrifuged at 20000 g for 10 min and the pellet was redissolved in 1 ml 0.1 M potassium phosphate, pH 8.3, containing a fourfold excess of 8-mercapto-FMN (based on the concentration of enzyme-bound FMN). The displacement of the native FMN with 8-mercapto-FMN was monitored spectrophotometrically by the increase of absorbance at 650 nm. After completion of the reaction ($\approx 4 \text{ h}$ at 4°C), the sample was passed through a Sephadex G-25 column to separate free 8-mercapto-FMN and released native FMN from the protein. The yield of 8-mercapto-FMN Y129F glycolate oxidase achieved with this method was greater than 90%.

Other methods

The absorption coefficient for Y129F glycolate oxidase was determined as described for wild-type glycolate oxidase (Macheroux et al., 1991).

Y129F glycolate oxidase was crystallized as for wild-type enzyme (Lindqvist and Brändén, 1979).

Absorbance spectra were recorded either with a Varian spectrophotometer, Cary model 219 or a Hewlett Packard diode array spectrophotometer, model 8452A. Excitation and emission spectra were recorded with a ratio-recording spectrofluorimeter designed and built by Dr D. P. Ballou and Mr G. Ford (University of Michigan).

Enzyme-monitored turnover studies were carried out in a temperature-controlled stopped-flow apparatus interfaced with a Nova 2 (Data General) computer system (Beatty and Ballou, 1981). The data obtained were analyzed according to the method described by Gibson et al. (1964).

Rapid-reaction studies were carried out with a temperature-controlled stopped-flow apparatus constructed by Raichle (1981) and equipped with a diode array (Spectroscopy Instruments GmbH, Germany). Data acquisition was performed with a Macintosh II cx computer using the POSMA software. The data were analyzed using the kinetic fitting program (program A) of Dr D. P. Ballou (University of Michigan). The observation path for both stopped-flow instruments was 2 cm.

Crystallographic data were collected on an *R*-axis IIC image plate on a Rigaku rotating anode and evaluated with the *R*-axis IIC processing software. Some more data were collected on an SDMS multwire area detector on a Rigaku rotating anode. These data were evaluated using the software supplied. All further crystal data was processed using the CCP4 program package (Science and Engineering Research

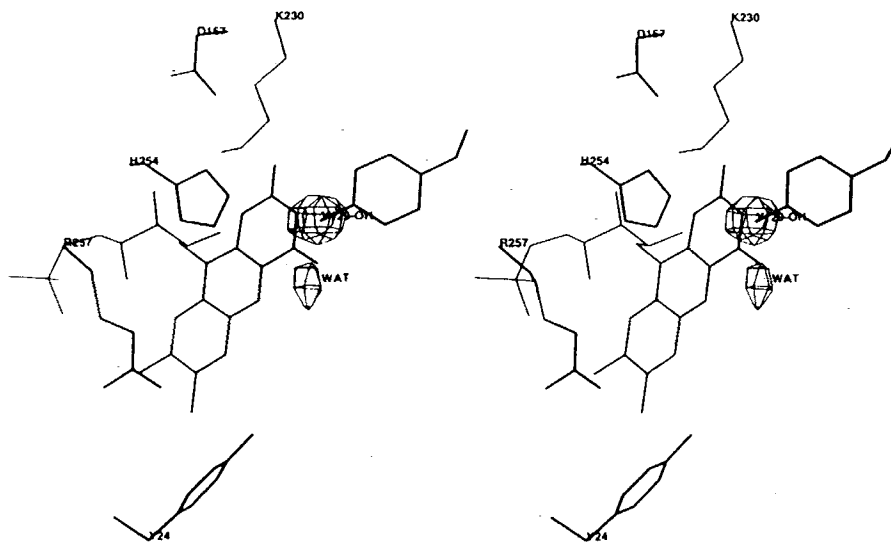


Fig. 1. Stereo view of active site of glycolate oxidase as determined by X-ray crystallography. The flavin ring system (center) and some of the surrounding amino acid residues are shown: Tyr24, Tyr129, Asp157, Lys230, His254 and Arg257. All amino acid residues depicted in this figure, except Lys230, are located on the *Si*-side of the isoalloxazine ring system. Included in the picture is the negative electron density contoured at 5σ of the difference Fourier with coefficients $|F_{\text{obs}} - F_{\text{calc}}| \alpha_{\text{calc}}$, where the structure factors were calculated from the wild-type model of glycolate oxidase. The only features are the peak for the missing Tyr129 OH and a peak showing lower occupancy in this mutant for a buried water on the *Re*-side.

Council 1979, Collaborative computing project no. 4 Daresbury Laboratory Warrington, England).

RESULTS

Crystal structure

The 44300 measurements were merged ($R_{\text{Intensity}} = 7\%$) to give 25200 independent reflections, comprising 85% of the data to 0.24-nm resolution (58% in the shell 0.25–0.24 nm). The *R*-factor after a few cycles of positional refinement with XPLOR (Brünger, 1989) is 18% including all data. A difference Fourier with coefficients $|F_{\text{obs}} - F_{\text{calc}}| \alpha_{\text{calc}}$, where the structure factors were obtained from the wild-type structure, was calculated. The main feature (Fig. 1) is a negative peak at the position of the Tyr129 OH group as expected. The only other significant feature is a negative peak, corresponding to a buried water, binding in a pocket in the wild-type enzyme which has been suggested (Lindqvist et al., 1991) to be where oxygen reacts with reduced enzyme on reoxidation of the coenzyme. No movement of any side-chains or of the cofactor is observed.

Spectral characteristics

The oxidized spectrum of Y129F glycolate oxidase differs considerably from wild-type glycolate oxidase in that the near-ultraviolet peak is shifted from 346 nm for wild-type glycolate oxidase to 364 nm for Y129F glycolate oxidase. The peak at 450 nm does not appear to be shifted, however. For Y129F glycolate oxidase this peak is slightly resolved, whereas wild-type glycolate oxidase exhibits a featureless absorbance band (see Fig. 2). The absorption coefficient for Y129F glycolate oxidase at 450 nm was determined to be $11400 \text{ M}^{-1} \text{ cm}^{-1}$, much higher than for wild-type glycolate oxidase ($9200 \text{ M}^{-1} \text{ cm}^{-1}$, Macheroux et al., 1991). The bathochromic shift of the near-ultraviolet peak and the higher absorption coefficient suggest that, unlike wild-type glycolate oxidase, the flavin N(3) position is not deprotonated

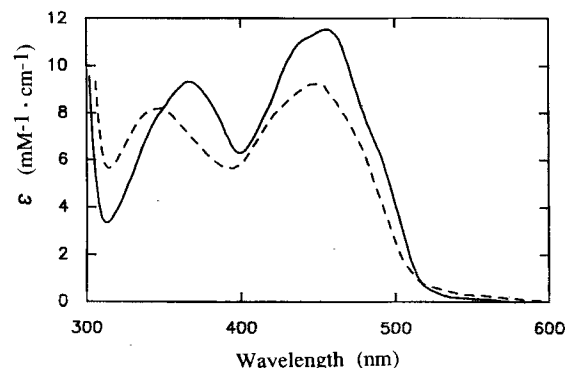


Fig. 2. Absorbance spectra of wild-type and Y129F glycolate oxidase. Both spectra were recorded in 0.1 M potassium phosphate pH 8.3 at 4°C. (---) Wild-type glycolate oxidase; (—) mutant enzyme.

(at pH 8.3). As was reported for wild-type glycolate oxidase, the oxidized cofactor, FMN, is not fluorescent when bound to the Y129F mutant protein.

Unlike the oxidized spectra of wild-type glycolate oxidase and Y129F glycolate oxidase, the reduced spectra are very similar, if not identical, with absorbance maxima at 365 nm for both the wild-type and the Y129F mutant (see also Fig. 3). Moreover, the reduced flavin is weakly fluorescent when bound to either enzyme with an emission maximum around 520 nm and an excitation spectrum identical to the absorbance spectrum (excitation $\lambda_{\text{max}} = 365 \text{ nm}$).

Lack of binding of oxalate

Oxalate binds and inhibits glycolate oxidase from various sources (Richardson and Tolbert, 1961; Nishimura et al., 1983). We have recently shown that this is also true for wild-type spinach glycolate oxidase (Macheroux et al., 1991). The Y129F mutant, however, did not exhibit any spectral perturbations upon addition of oxalate. Moreover the oxidation of

glycolate by Y129F glycolate oxidase was not inhibited by oxalate; even in the presence of 12 mM oxalate, no enzyme inhibition was observed. Thus it appears that the hydroxyl group of Tyr129 is essential for binding oxalate.

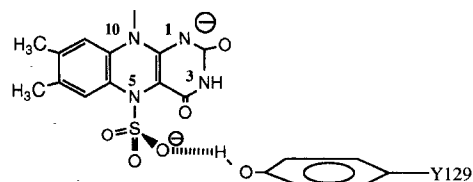
pH dependence of the absorbance spectrum of Y129F glycolate oxidase

The absorbance spectrum of oxidized Y129F glycolate oxidase at pH 8.3 (see above) indicated that the flavin ring system is not deprotonated at N(3) as is the case for the wild-type enzyme, which has an observable pK_a of 6.4 (Macheroux et al., 1991). When Y129F glycolate oxidase was subjected to a pH titration from pH 5 to pH 9.3 the absorbance spectrum did not change, demonstrating that the Y129F mutant enzyme does not have a pK_a in this range. If the pK_a is shifted in the Y129F mutant compared to free flavin ($pK_a = 10.3$, Schuman and Massey, 1971) it can only be a marginal shift. This finding provides strong evidence that the Tyr residue is involved in lowering the pK_a value found for wild-type glycolate oxidase, as was concluded recently (Macheroux et al., 1991).

The two latter differences between wild-type glycolate oxidase and the mutant raise the question of whether these two phenomena are related, i.e. whether oxalate binding itself perturbs the pK_a of the N(3) proton. This was reported earlier by Schuman and Massey (1971) who demonstrated that the pK_a of glycolate oxidase isolated from pig liver is shifted by 1.5 in the presence of oxalate. With the recombinant spinach glycolate oxidase (wild type) we found that the pK_a is also shifted by 1.5, from pH 6.4 to pH 7.9, in the presence of 20 mM oxalate. This fits nicely with the results reported above which demonstrate the significance of the Tyr129 residue in oxalate binding as well as in perturbing the pK_a of the N(3) proton. We assume that oxalate forms a hydrogen bond with the hydroxyl group of Tyr129, thereby attenuating its interaction with the C(4=O) oxygen, which in turn causes an increase of the pK_a of the N(3) proton.

Formation of a sulfite adduct

Titration of Y129F glycolate oxidase with sodium sulfite resulted in the formation of the flavin N(5)-sulfite adduct with spectral properties similar to those exhibited by the wild-type enzyme (Macheroux et al., 1991). The decrease in A_{450} as a function of sulfite concentration allowed the calculation of a K_d of 4 μM . This value is 15-times higher than the K_d for wild-type enzyme. It was interesting to study which contributions k_{on} and k_{off} provide to the higher K_d . Therefore we reacted the Y129F glycolate oxidase sulfite complex with methyl methanethiolsulfonate in order to determine the velocity of sulfite dissociation, in an experiment similar to that described for wild-type enzyme (Macheroux et al., 1991). This experiment revealed that k_{off} is about 10 times faster than with wild-type glycolate oxidase ($k_{off} = 7.7 \times 10^{-3} \text{ s}^{-1}$ for Y129F glycolate oxidase versus $0.825 \times 10^{-3} \text{ s}^{-1}$ for the wild-type enzyme), i.e. the largest contribution to the weaker binding of sulfite to Y129F glycolate oxidase stems from a faster dissociation rate of the complex. Consistent with that result we found a somewhat smaller k_{on} value of $1900 \text{ M}^{-1} \text{ s}^{-1}$ for the Y129F mutant protein, compared to the value of $3100 \text{ M}^{-1} \text{ s}^{-1}$ for wild-type enzyme (Macheroux et al., 1991). The 15-fold difference in K_d values for sulfite binding to wild-type and mutant enzyme corresponds to a binding energy of approximately 6.2 kJ/mol, typical of hydrogen-



Scheme I. Proposed structure of the flavin N(5)-sulfite complex of wild-type glycolate oxidase, with hydrogen-bond interaction between Tyr129 and an oxygen atom of sulfite contributing to the stabilization of the complex.

bonding interactions. This finding suggests that the sulfite complex is stabilized by a hydrogen bond between the hydroxyl group of the tyrosine residue and one of the oxygen atoms of the sulfite molecule as depicted in Scheme I.

Recent crystallographic studies on the sulfite complex of flavocytochrome b_2 (Tegoni and Mathews, 1988), which has a similar active-site structure (Xia and Mathews, 1990) showed that an equivalent Tyr in this enzyme (Y254) is close to one of the oxygen atoms (0.28 nm) of the sulfite group and forms a strong hydrogen bond. This finding supports our interpretation of the higher K_d value of the sulfite complex of Y129F glycolate oxidase.

According to Scheme I, the replacement of Tyr129 with Phe does not interfere with the ability of the protein to stabilize a negative charge at the N(1)-C(2=O) locus, as is evidenced by the formation of a sulfite complex, but our experiments demonstrate that the removal of a hydrogen bond donor in the vicinity of the C(4=O)-N(5) region alters the stability of the complex.

Stabilization of the anionic flavin semiquinone

Photoreduction of Y129F glycolate oxidase according to the method of Massey and Hemmerich (1978) produced the red anionic flavin radical, as was observed with wild-type glycolate oxidase. In fact, the spectrum of Y129F glycolate oxidase semiquinone was very similar to the wild-type glycolate oxidase semiquinone (data not shown) despite the differences of the spectra in the oxidized state (see Fig. 2).

Stabilization of the benzoquinoid form of 8-mercapto-FMN bound to Y129F glycolate oxidase

Like the spectra of the flavin semiquinones, the spectra of the 8-mercapto-FMN wild-type and Y129F proteins are almost identical with absorbance peaks at 366 nm, 430 nm and 600 nm and a pronounced shoulder at 645 nm (data not shown) and are typical of flavoproteins oxidase, which uniformly stabilize the benzoquinoid form of the 8-mercapto-flavin (Massey et al., 1979).

The 8-mercapto-FMN Y129F glycolate oxidase binds sulfite very weakly with a K_d of 330 mM. This K_d is about 2.7-fold higher than the one found for wild-type glycolate oxidase and follows the tendency of weaker binding to the Y129F mutant observed with native FMN. Titration of 8-mercapto-FMN-containing Y129F glycolate oxidase with sulfite did not give rise to the formation of 8-sulfonyl-FMN, a result like that found with wild-type glycolate oxidase. In general, 8-mercapto-flavoprotein oxidases do not yield 8-sulfonyl-flavins when reacted with sulfite, in contrast to the results with flavoproteins of other reaction categories (Fitzpatrick and Massey, 1983).

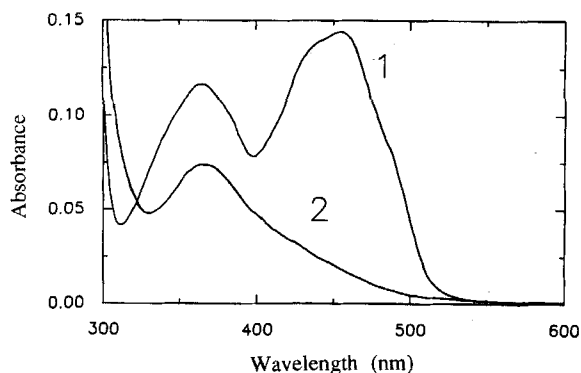


Fig. 3. Anaerobic reduction of Y129F glycolate oxidase with glycolate (single turnover experiment). 12.3 μM Y129F glycolate oxidase in 0.1 M potassium phosphate pH 8.3 (1 ml) at 4°C was made anaerobic by repeated cycles of evacuation and flushing with oxygen-free argon. After anaerobiosis was established, spectrum 1 (solid trace) was recorded; 10 μl 0.1 M sodium glycolate (adjusted to pH 8.3) was then mixed from a side arm. Spectrum 1, before and, spectrum 2, 10 min after mixing with glycolate.

In summary, although the catalytic velocity is lowered some 30-fold, the mutation of the Tyr129 residue to Phe in glycolate oxidase does not significantly affect the most typical flavoprotein oxidase properties, i.e. Y129F glycolate oxidase still behaves like a typical member of this group of proteins in terms of the criteria mentioned in the introduction.

Reduction of the Y129F glycolate oxidase with glycolate (reductive half reaction)

Under anaerobic conditions Y129F glycolate oxidase is readily reduced by glycolate as is shown in Fig. 3. Rapid reaction studies showed that reduction of the enzyme is monophasic and shows substrate saturation at higher glycolate concentrations. A double-reciprocal plot of $1/k_{\text{obs}}$ versus $1/[\text{glycolate}]$ yields a straight line with a y-intercept of 1.6 s ($= 1/k_3$; $k_3 = 0.63 \text{ s}^{-1}$). From the same plot, a K_d value of 1.0 mM for glycolate can be calculated from the slope divided by the intercept (see next section for definition of rate constants)

Reoxidation of reduced Y129F glycolate oxidase with molecular oxygen (oxidative half reaction)

The rate of reoxidation of Y129F glycolate oxidase reduced anaerobically with a twofold excess of glycolate was determined as described for wild-type enzyme (Macheroux et al., 1991). As with the wild-type enzyme, the reoxidation showed a linear dependency on the oxygen concentration. A plot of k_{obs} versus the oxygen concentration passed through zero and the slope yields a second-order rate constant of $k = 7.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$.

Steady-state kinetics of Y129F glycolate oxidase

The anaerobic reduction of Y129F glycolate oxidase with glycolate described in a previous section demonstrated that the mutation led to only a partial loss of enzymatic activity. In order to determine the turnover number of Y129F glycolate oxidase and the K_m values for the substrates (glycolate and oxygen) we carried out an enzyme-monitored turnover experiment. When Y129F glycolate oxidase (7.5 μM) was

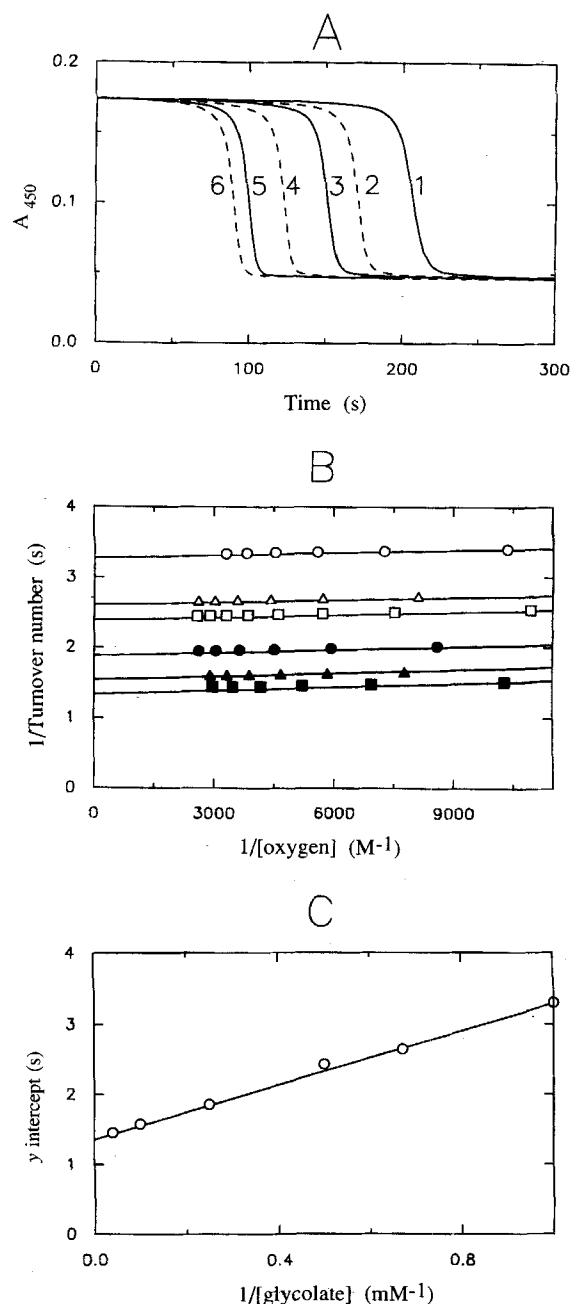


Fig. 4. Steady-state kinetics of Y129F glycolate oxidase. Y129F glycolate oxidase (7.5 μM final concentration) in air-saturated (21% oxygen = 0.41 mM) 0.1 M potassium phosphate pH 8.3 at 4°C was mixed with various glycolate concentrations in the same air-saturated buffer. (A) Enzyme monitored turnover traces from the reaction of Y129F glycolate oxidase with glycolate and oxygen. The concentrations of glycolate used (after mixing) were 1 mM (trace 1), 1.5 mM (trace 2), 2 mM (trace 3), 4 mM (trace 4), 10 mM (trace 5), and 25 mM (trace 6). (B) Double-reciprocal plot of the data obtained from the traces in A. The lines were obtained at the following glycolate concentrations: 1 mM (\circ), 1.5 mM (Δ), 2 mM (\square), 4 mM (\bullet), 10 mM (\blacktriangle), and 25 mM (\blacksquare). (C) Replot of the data obtained from B. The y-intercept at each glycolate concentration was plotted against $1/[\text{glycolate}]$. From this plot a turnover number of 0.74 s^{-1} and a K_m for glycolate of 1.5 mM was calculated.

reacted with various concentrations of glycolate in air-saturated ($\text{O}_2 = 0.41 \text{ mM}$) 0.1 M potassium phosphate, pH 8.3 at 4°C, the enzyme became reduced at various times as a function of substrate concentration, as is shown in Fig. 4A. Data

Table 1. Comparison of the catalytic properties of wild-type and the Y129F mutant glycolate oxidase.

Enzyme	Turnover number ($\approx k_3$)	k_{red} (k_3)	k_{ox} (k_5)	K_m for		
				oxygen (k_3/k_5)	glycolate [(k_2+k_3)/ k_1]	glycolate (k_2/k_1)
	s^{-1}		$\text{M}^{-1} \cdot \text{s}^{-1}$	μM	mM	
Wild-type	20	25	8.5×10^4	210	1.0	0.94
Y129F	0.74	0.63	7.3×10^4	7	1.5	1.0

analysis according to the method of Gibson et al. (1964) produced the family of parallel lines depicted in Fig. 4B. In this analysis, the steady-state level of oxidized enzyme existing through most of the time courses shown in Fig. 4A is a balance of the rate of reduction of the enzyme flavin by glycolate and reoxidation by molecular oxygen, and persists until the substrate present in limiting concentration (molecular oxygen) is exhausted, whereupon the oxidation state falls to that of the reduced enzyme, because the reducing substrate, glycolate, is present in excess. The area (in arbitrary units) swept out between the initial steady-state and final level is taken to equal that of the initial oxygen concentration (0.41 mM). As the enzyme concentration is known, each curve yields a set of turnover numbers at different concentrations of molecular oxygen, as the latter is consumed in the reaction. These are the data shown in Fig. 4B. A replot of the y -intercepts versus the glycolate concentrations is shown in Fig. 4C. A comparison with wild-type glycolate oxidase (Macheroux et al., 1991) shows that the kinetic pattern obtained with the Y129F mutant enzyme is qualitatively the same. Since Lineweaver-Burk plots and the corresponding replots from steady-state experiments bear diagnostic information as to the enzymatic mechanism, this finding implies that both wild-type and Y129F glycolate oxidase operate via the same mechanism. From Fig. 4, B and C, we can deduce the following information. The y -intercept of the line in Fig. 4C yields the turnover number of the enzyme (turnover number = 0.74 s^{-1}) and the slope divided by this intercept gives the K_m value for glycolate ($K_{m,\text{glycolate}} = 1.5 \text{ mM}$). The K_m value for oxygen can be derived from the slope of any of the lines in Fig. 4B divided by the intercept from the line in Fig. 4C (= turnover number). The K_m found for oxygen is $K_{m,\text{oxygen}} = 7 \mu\text{M}$. The kinetic parameters of wild-type and Y129F glycolate oxidase are compiled in Table 1 for comparison. The turnover number for the Y129F glycolate oxidase is 27 times lower than for the wild-type enzyme, indicating that Tyr129 is involved in catalysis. However, it is clear that Tyr129 is not essential for the oxidation of substrate since the residual activity of the Y129F mutant enzyme is relatively high. In terms of stabilization of the transition state, ΔG^\ddagger , the results indicate an increase of energy of about 7.8 kJ/mol or about 15% higher than for the wild-type enzyme.

In the mutant we have lower occupancy for a buried water molecule on the *Re*-side. This is probably due to the possibility for free movement of this water now that the Tyr129 OH group is not present. This water has been suggested (Lindqvist et al., 1991) to occupy the position of oxygen which, in the oxidative half reaction, reoxidizes the reduced flavin of glycolate oxidase. The rate of reoxidation of reduced FMN by molecular oxygen, however, is only slightly slower in the mutant (see Table 1). This result suggests that

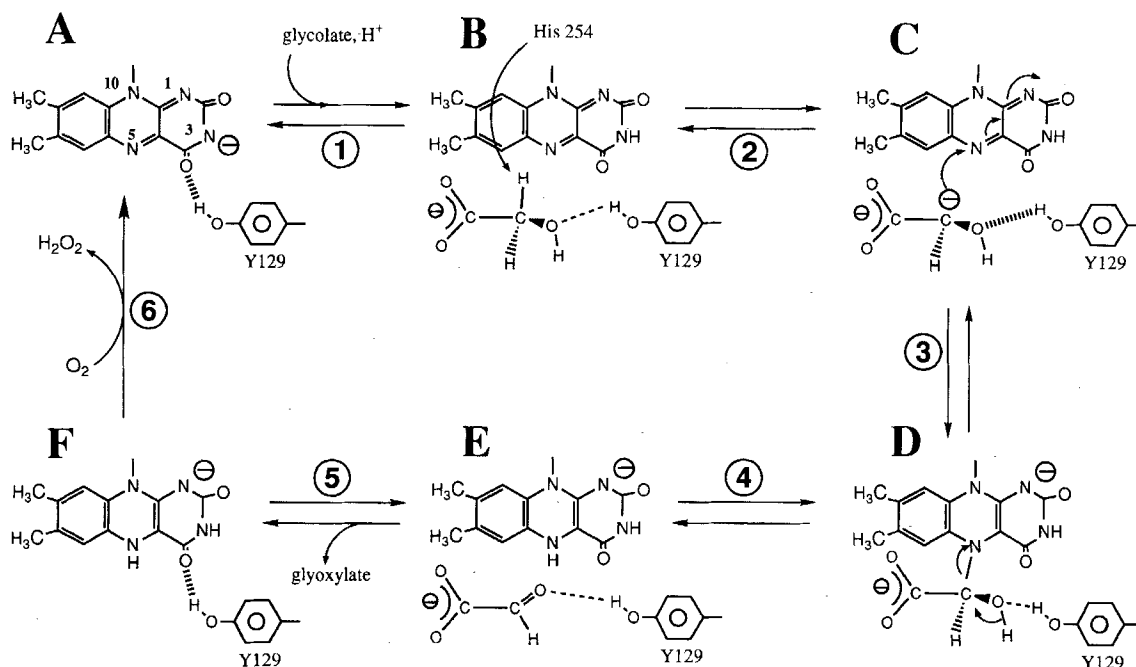
if displacement of this water by molecular oxygen is part of the catalytic cycle, it does not have much effect on the rate of reoxidation.

DISCUSSION

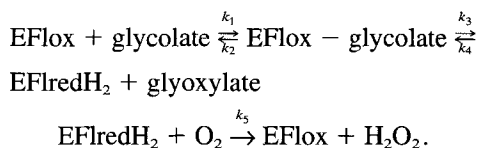
Replacing Tyr129 of glycolate oxidase with Phe has no effect on some properties whereas other features of the enzyme are changed dramatically. First, let us discuss the former group of properties. As mentioned in the introduction, the stabilization of the anionic semiquinone and of the benzoquinoid form of 8-mercapto-FMN, as well as the formation of a tight N(5)-sulfite adduct, can all be ascribed to a positively charged lysine residue (Lys230) in the vicinity of the N(1)-C(2=O) locus. Therefore one would expect that the Y129F mutation would not abolish these features. Clearly, the experiments presented in this paper prove that this is the case. The only minor difference was the finding that the K_d for the N(5)-sulfite complex was higher. However, this can be rationalized on the basis of the three-dimensional structure of the active site of glycolate oxidase: according to the structure it is likely that the hydroxyl group of Tyr129 forms a hydrogen bond to the sulfite group bound to N(5), resulting in the stabilization of the N(5)-sulfite adduct (Scheme I). This shows quite nicely that, although the positively charged Lys230 is probably the governing factor for the formation of the N(5)-sulfite adduct, Tyr129 modulates the strength of the complex by hydrogen bond formation. To further substantiate the role of Lys230 a thorough investigation of appropriate mutant proteins is under way.

On the other hand, we have demonstrated that the Y129F mutant lacks the binding of oxalate and that the pK_a of the N(3) of the flavin ring system is shifted to or near the pK_a of free flavin (= 10.3). We have recently put forward a model to explain the low pK_a of the N(3) of FMN bound to wild-type glycolate oxidase in which Tyr129 stabilizes the deprotonated form of the oxidized flavin by hydrogen bonding to the C(4=O), thus lowering the pK_a (Macheroux et al., 1991). The finding of a higher pK_a in the Y129F mutant is in good agreement with this model. However the magnitude of the pK_a shift, from pH 6.4 to ≈ 10 , corresponds to a free energy difference of 20–25 kJ/mol, suggesting that some other interaction in addition to the proposed hydrogen bonding must be involved. In fact, the crystal structure of the Y129F protein shows a lower density for a buried water molecule which in the wild-type glycolate oxidase forms a hydrogen bond with C(4=O).

The much lower K_m value for oxygen in the Y129F mutant seems at first unexpected. However, a recent kinetic study (Macheroux et al., 1991) showed that the kinetic mechanism of the wild-type enzyme can be described by two half reactions:



Scheme II. Proposed role of Tyr129 in the reductive half reaction of glycolate oxidase. Note that reduction of the flavin system is accompanied by protonation of the N(3) position (step 1 and 2) since the pK_a for reduced flavin is much higher than for the oxidized form (Macheroux, P., Sanner, C., Rüterjans, H., Ghisla, S., and Müller, F., unpublished results).



The K_m of oxygen is governed by k_3 and k_5 (Macheroux et al., 1991):

$$K_{m,\text{oxygen}} = k_3/k_5$$

with k_3 being the rate of reduction and k_5 the rate of reaction of reduced enzyme with molecular oxygen. Since reduction proceeds at a much smaller rate ($k_3 = 0.63 \text{ s}^{-1}$), as determined in the rapid reaction studies described above, and the rate of reoxidation ($k_5 = 7.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) was found to be similar to wild-type enzyme, the K_m for oxygen can be calculated as follows: $K_{m,\text{oxygen}} = 0.63 \text{ s}^{-1}/7.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1} = 8.6 \text{ } \mu\text{M}$. This calculated value is in excellent agreement with the K_m value obtained from the steady-state experiments.

The Y129F glycolate oxidase neither binds oxalate, as judged by the absence of any perturbation of its absorbance spectrum, nor does oxalate show any inhibitory effect on its catalytic activity. This observation seems at first inconsistent with the small effect of the Tyr \rightarrow Phe replacement on the K_m (and K_d) of glycolate, since both the substrate and the inhibitor were thought to be anchored between Tyr24 and Arg257 on one side and Tyr129 on the other side (Lindqvist and Brändén, 1989; Ghisla and Massey, 1991). Therefore one would expect that the removal of Tyr129 would not lead to a complete loss of oxalate binding. Based on extensive experimental data with lactate oxidase, Ghisla and Massey (1975) have suggested that oxalate acts as a transition state analogue. In their model oxalate mimicks the putative carbanion transition state and is therefore much more tightly bound to the active site than the substrate. Binding of oxalate to lactate oxidase was found to be a two-step process with a first, pH-independent, and a second, pH-dependent, step. The

second step is thought to reflect the interaction of oxalate with the active-site His. The first step, however, may result from the interaction of oxalate with other amino acid side chains in the active site. Our results, that the Tyr129 side chain stabilizes the oxalate-glycolate-oxidase complex, clearly support this view and are readily interpretable on the basis of oxalate being a transition state analogue. Since the main function of the tyrosine hydroxyl group appears to be stabilization of the transition state and not binding of the substrate (see also below), binding of oxalate is much more affected than the binding of the substrate, as was found in this investigation.

Scheme II shows a possible scenario for the catalytic oxidation of glycolate in the active site of glycolate oxidase. The hydroxyl group of tyrosine (on the right-hand side of each structure) hydrogen bonds with the C(4=O) in the oxidized state (structure A). In the initial Michaelis complex, this hydroxyl group forms a weak hydrogen bond with the substrate hydroxyl group as shown in structure B. Structure C depicts the negatively charged transition state which is stabilized through hydrogen-bond interaction by the Tyr side chain. In step 3 this carbanion species then transfers electrons either by formation of a covalent adduct (Ghisla and Massey, 1991), as shown in Scheme II, or by two succeeding electron-transfer steps to the flavin ring system (Dubois et al., 1990). The putative covalent adduct shown in Scheme II, structure D, collapses to yield the product molecule (glyoxylate) and reduced flavin (structure E). In the original proposal (Lindqvist and Brändén, 1989; Ghisla and Massey, 1991) the Tyr side chain was thought to facilitate this breakdown by acting as a general base, as shown in Scheme II. Our results indicate that the Tyr side chain is protonated and hence it can be concluded that the Tyr residue is probably not contributing much to this process.

Recently Dubois et al. (1990) reported similar findings for an equivalent Tyr \rightarrow Phe mutant of flavocytochrome b_2 , which has a similar active-site structure (Xia and Mathews,

1990). In their case k_{cat} was reduced by a factor of 40 and the K_m for the substrate (L-lactate) was slightly lower. Although glycolate oxidase and flavocytochrome b_2 belong to different classes of flavoproteins (oxidases and dehydrogenases, respectively), the similarity of the results strongly suggest that this Tyr residue plays a comparable role in these two enzymes, both in terms of catalysis and substrate binding.

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