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STUDIES ON GLYCOLLATE OXIDASE FROM PEA LEAVES

DETERMINATION OF STEREOSPECIFICITY AND MODE OF INHIBITION BY α-HYDROXYBUTYNOATE

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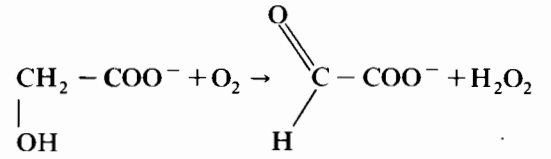
Glycollate oxidase (glycollate:oxygen oxidoreductase, EC 1.1.3.1) from *Pisum sativum* has an unusual absorption spectrum which suggests that the flavin N(3)-H function of the FMN coenzyme is ionized at pH 8.3. The enzyme is reduced rapidly by the substrate glycollate to yield a normal, reduced FMN coenzyme which is readily reoxidized by O_2. No evidence for the occurrence of covalent intermediates during reduction, as observed upon reduction of L-lactate oxidase from *Mycobacterium smegmatis* with the same substrate (Ghisla, S. and Massey, V. (1980) J. Biol. Chem. 255, 5688–5696), could be obtained. The enzyme was determined to be greater than 99.5% specific in the abstraction of the *Re*-hydrogen of glycollate. D-Lactate dehydrogenase from *Lactobacillus leichmanii* was shown to be only 97% selective for the *Si*-side in the reduction of glyoxylate. Glycollate oxidase was shown to be inhibited by α-hydroxybutynoate via covalent modification of the FMN coenzyme, in a fashion similar to that encountered with L-lactate oxidase (Schonbrunn, A., Abeles, R.H., Walsh, Ch.T., Ghisla, S., Ogata, H. and Massey, V. (1976) Biochemistry 15, 1798–1807). This inhibitor serves both as substrate and inactivator of the enzyme.

Introduction

Glycollate oxidase (glycollate:oxygen oxidoreductase, EC 1.1.3.1) is a flavin enzyme which occurs in mammalian peroxisomes of kidney and liver [1] and also in the green leaves of plants [2]. In general it is characterized by a specificity for the simplest α-hydroxycarboxylic acid, glycollate, the higher homologues being much poorer substrates [3,4]. The corresponding α-keto acid and hydrogen peroxide are the products of the catalytic oxidation according to the equation:



In spite of its wide occurrence, glycollate oxidase from plants has received little attention with respect to its mechanism of action. This might be due to its instability, which results from the relatively weak binding of FMN and to the tendency to aggregate more or less irreversibly [4,5]. Recently the X-ray crystallographic investigation of

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the spinach enzyme has been started [6]. In contrast to this, in recent years the related bacterial enzymes which metabolize L-lactate, and which also have FMN as coenzyme, have received considerable attention (see references in Ref. 7). In particular L-lactate oxidase from *Mycobacterium smegmatis* was found to recognize glycollate as a substrate, and to oxidize catalytically its *Re*-hydrogen, while a metastable adduct was formed arising from abstraction of the *Si*-hydrogen [8,9]. These results are of considerable relevance, as they provide evidence for a carbanion mechanism, and for covalent catalysis with this type of flavin enzyme. It was thus of interest to investigate whether glycollate oxidase might also oxidize glycollate via formation of covalent intermediates, and to correlate the reaction mechanisms of both enzymes. In addition it was our aim to determine the stereospecificity of the *Pisum sativum* enzyme by using chirally labeled glycollates and to compare it to the stereospecificity of other plant glycollate oxidases, which have been reported to be *Re*-specific, or at least *Re*-selective [10,11]. Glycollate oxidases from *P. sativum* [12] and human liver [13] have been reported to be inhibited by the suicide substrate α-hydroxybutynoic acid. With the human liver enzyme α-hydroxybutynoate was found to be a substrate and an irreversible inhibitor [13]. For the *P. sativum* enzyme no details were given on the mechanism of inactivation or the nature of the modification [12].

α-Hydroxybutynoic acid has been shown to inactivate L-lactate oxidase and D-lactate dehydrogenase, among other α-hydroxy acid oxidases [14–17], via formation of covalent flavin adducts. The structures of the inactivation products were shown to reflect the L- or D-specificity of the enzyme. Thus, the inactivation reaction in turn might be employed as a criterion for the determination of the stereospecificity of enzymes by use of resolved D- or L-hydroxybutynoic acid. In this context, it was our aim to attempt the resolution of the D,L-α-hydroxybutynoic acid, and to investigate its inactivation of *P. sativum* glycollate oxidase.

Materials and Methods

Glycollate oxidase from *P. sativum* (common pea) was isolated from 2-week-old seedlings and assayed according to the method of Kerr and Groves [4]. FMN (10^{-4}–10^{-5} M) was added to all buffers in order to prevent dissociation of the coenzyme. The enzyme showed a single major band on SDS-polyacrylamide gel electrophoresis according to the method of Weber and Osborne [18], several non-identified minor bands accounted for the residual 5%, no band, however, exceeding 1%. This enzyme preparation showed with L-lactate an activity similar to that reported by Kerr and Groves [4]. Most experiments were carried out as soon as possible in order to avoid complications arising from the activity-loss characteristic of this enzyme. In fact, upon storage at 0°C in 50 mM Tris-HCl, pH 8.3, at concentrations of 12 mg/ml, and in the presence of 10^{-3} M FMN, the loss of activity obeyed a first-order decay law, and had a $t_{1/2}$ of 12 days.

Glycollate was from Fluka (Buchs, Switzerland) and was recrystallized from water/isopropanol before use. (2*R*)- and (2*S*)-[^{3}H]glycollates were prepared, and purified as described earlier [8,9].

Synthesis of 2-hydroxybutynoic acid and resolution of the enantiomers. Glyoxylate methyl ester was prepared by periodic acid oxidation of tartaric acid dimethyl ester (from Fluka, Buchs, Switzerland) [19]. Condensation with ethynyl-magnesium bromide [20] was carried out by the method of Verny and Vessiere [21], the crude product was distilled at 15 T and 70°C to yield the hydroxybutynoate methyl ester as a colourless liquid, which solidified upon cooling. Yield 5%, m.p. 55–58°C. (Found: C 52.20, H 5.41%. $C_5H_6O_3$ (M_r 114.10) requires C 52.63, H 5.30%.) NMR (C^2HCl_3); = 4.86 ppm (1 proton, quartet, αH, $J_{\alpha,\gamma} = 2.49$ Hz), 3.89 ppm (3H, singlet, $-COOCH_3$), 3.14 ppm (1H, doublet, $-OH$) and 2.53 ppm (1H, doublet, γH). Hydrolysis of the methyl ester was carried out in 16% HCl at ambient temperature and the course of the reaction was followed by thin-layer chromatography (Merck, silica plates, chloroform/methanol; 20:1); it was complete after 8 h. The hydrochloric acid was flash-evaporated and the residue was dried by azeotropic distillation with small amounts of absolute ethanol and benzene to yield the theoretical amount of the acid as a slightly yellow oil. The acid could be converted to the Li-salt by neutralization with LiOH, and precipitation at 0°C with ethanol. The Li-salt was

identical by its NMR and infrared spectra with the material obtained by the original procedure of Verny and Vessiere [21]. For the separation of the racemic mixture, 1.9 g (19 mmol) of the crude, oily hydroxybutynoic acid were dissolved in 20 ml water and then warmed to 30–35°C. 4.1 g (10 mmol) brucine hydrate (Fluka) was added in portions at the same temperature. The whole mixture was stirred at 30–35°C for an additional 50 min and then filtered. After filtration, methanol (3–4 ml) was added, and crystallization was induced by cooling to −20°C. The mixture was allowed to stand 1 h at 0°C. The crystals were filtered off, redissolved in 20–25 ml absolute methanol and allowed to crystallize for 20 h at 4°C. After filtration they were recrystallized again from 5 ml absolute methanol. Yield: 1.15 g of the brucine salt of L-α-OH-butynoic acid, m.p. 170°C. The free acid was obtained as a colourless oil by passing the brucine salt over a 2 × 4.5 cm column of Dowex 50-X-8, H^+-form. The acidic fractions were neutralized with LiOH, evaporated to dryness and the residue recrystallized from absolute ethanol to yield 90 mg (9.5%) of the Li salt of L-(+)-α-hydroxybutynoic acid (m.p. > 225°C decomp.). (Found: C 45.43, H 3.22%; $C_4H_3O_3Li$ (M_r 106.01); requires C 45.32, H 2.85%). NMR (^2H_2O): 4.71 ppm (1H, doublet, αH, $J_{\alpha,\gamma} = 2.5$ Hz), and 2.83 ppm (1H doublet, γH, $J_{\gamma,\alpha} = 2.5$ Hz). $[\alpha]_D^{25°C}$ (methanol, 4 mg/ml) = +29°. The D-enantiomer could be isolated in small amounts by repeating the above procedure, however, starting with equimolar concentrations of brucine and repeating the fractional crystallization until greater than 50% of the total α-hydroxybutynoate had been separated as the brucine salt. Careful repeated recrystallization of the remaining semi-solid residue from absolute methanol, and subsequent Dowex 50 W-X-8 chromatography yielded approx. 5% of the D-enantiomer. The optical purity of both enantiomers was estimated by a double test using D-lactate dehydrogenase from *Lactobacillus leichmanii* and L-lactate dehydrogenase from hog or rabbit muscle (both from Boehringer) according to methods in Refs. 23 and 35, and was found to be greater than 96% for the L- and approx. 90% for the D-enantiomer. The stereochemistry was confirmed by stereospecific reaction of the pure enantiomers with L-lactate oxidase [14,15] and D-lactate dehydrogenase [16,17].

Determination of ^3H release from glycollates. The labeled glycollates were incubated at 22°C in the dark at the concentrations given in the legend of Fig. 2. At the intervals shown, 10-μl aliquots were withdrawn and applied directly to a Dowex AG 1-X2 column (OH^- form: bed volume = 0.5 ml) and washed with distilled water until no radioactivity was present in the eluate (4–5 ml). The bulk of $^3H^+$ radioactivity was eluted in the first ml, while the acidic fraction was eluted wtih 5 ml of 1 M HCl. By this method a complete separation was obtained and more than 95% of the total radioactivity applied to the column was recovered. The fractions were counted in 10 vol. liquid scintillator (ELS 294, Koch and Light) with a Packard Tri-Carb scintillation spectrometer model 30003. The vials containing appreciable amounts of ^3H were standardized internally using ^3H standards from New England Nuclear.

Absorption spectra were recorded with a Cary 118 recording spectrophotometer at the temperature given. Fluorescence spectra were recorded with a Perkin-Elmer MPF-3.

Results and Discussion

Some properties of the enzyme

Spectral properties of glycollate oxidase from *P. sativum* have not been previously reported. The enzyme showed a rather unusual spectrum in that the two main transitions in the visible and near ultraviolet were blue-shifted to an unusual extent (λ_{max} approx. 435, and 335 nm) (Fig. 1), compared to the glycollate oxidase from spinach [5], which was reported to have λ_{max} at 450 and 340 nm. A similar blue-shifted spectrum was observed with glycollate oxidase from pig liver at high pH [1] and was taken to indicate that the flavin position N(3)-H is ionized. No evidence for a second chromophore, (6-hydroxyflavin [1,24]), as was found with the pig and human liver enzyme [13], was observed. The FMN chromophore of the spinach enzyme was reported to be rather weakly bound to the enzyme; the resulting apoenzyme aggregated easily to an inactive form [5]. The lability of FMN binding of the *P. sativum* enzyme could be demonstrated directly by incubation of the enzyme with an excess of apoflavodoxin from *Megasphera elsdenii*, an apoenzyme which binds FMN rapidly

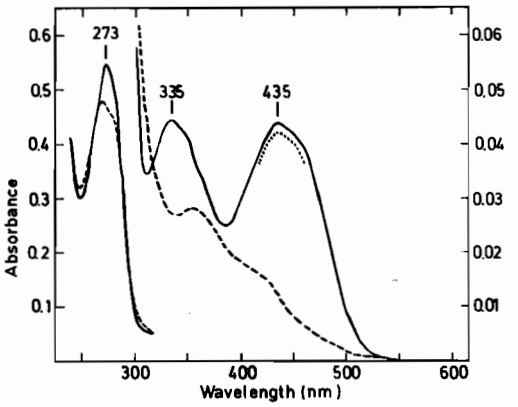


Fig. 1. Spectral properties of oxidized and substrate-reduced glycollate oxidase. The enzyme $8.8 \cdot 10^{-6}$ M, in 0.05 M Tris-HCl buffer, pH 8.3, was made anaerobic (————), and glycollate (10^{-3} M final concentration) was added from a side arm of the cuvette. The absorption at 460 nm was bleached rapidly to yield the spectrum of the reduced enzyme (- - - - - -). Admission of air immediately restored the absorption of the oxidized enzyme (· · · · · ·).

with a $K_d = 4 \cdot 10^{-10}$ M [25]. When 10^{-6} M glycollate oxidase was mixed rapidly with apoflavoxodin ($8 \cdot 10^{-6}$ M) in the presence of $3 \cdot 10^{-6}$ M free, pure [26] FMN 75% of the fluorescence emission at 520 nm was quenched immediately. The residual fluorescence indicates that FMN bound to glycollate oxidase is also fluorescent and has a quantum yield similar to that of FMN itself. Subsequently this residual fluorescence decreased by further 50–60% with a $t_{1/2}$ of approx. 90 min at 10°C. This final value can be interpreted as representing an equilibrium condition between FMN bound to apoflavodoxin and to glycollate oxidase, and indicates that the binding constants to the two enzymes are comparable. In view of the instability of glycollate oxidase a precise determination by this method was impossible in our hands. As the fluorescence quenching due to binding to apoflavodoxin is much more rapid [25], the half-time of approx. 90 min should represent the rate-limiting release of FMN from glycollate oxidase.

Under anaerobic conditions the reaction of the enzyme with glycollate led rapidly to formation of the reduced species shown in Fig. 1. No intermediates similar to the covalent adducts observed with lactate oxidase [8,9] could be observed; if they were present, they could not be detected by normal spectral techniques. Admission of air to the reduced species led to immediate reformation of essentially the complete amount of oxidized enzyme (Fig. 1). The spectrum of the reduced species also confirms the absence of modified chromophores such as that observed with the pig liver enzyme [1] and the human liver enzyme [13].

Determination of stereospecificity of glycollate oxidase

The glycollate oxidases from spinach and from tobacco leaves [3,22] have been reported to oxidize the L-form of lactic acid and it was thus inferred that the (corresponding) (*R*)-hydrogen of glycollate would be removed in the catalytic reaction. Later, Rose [10] and others determined the stereospecificity of the tobacco enzyme to be $98 \pm 2\%$ for the *Re*-side using (*R*)-[^{3}H]glycollate. However, a double check using (*S*) labelled glycollate was not carried out, and possible errors due to oxidation of the product glyoxylate were neglected. The results in Fig. 2 show that the stereoselectivity of the enzyme from *P. sativum* for the (*R*)-hydrogen

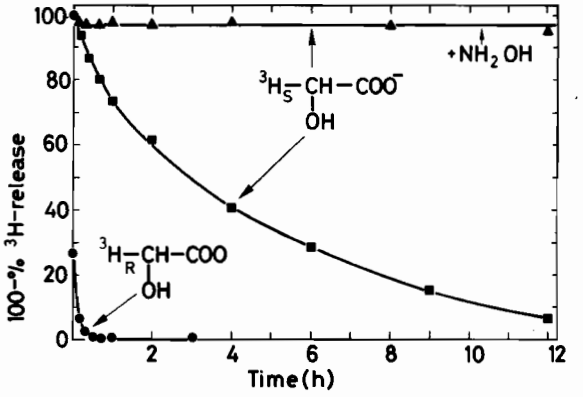
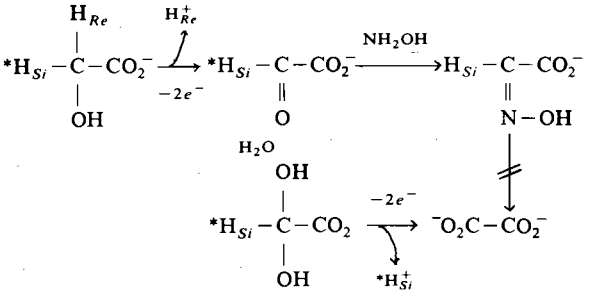


Fig. 2. Stereospecificity of glycollate oxidase. The enzyme, 0.4 units (1 nmol, $9 \cdot 10^{-6}$ M) in 150 µl 0.1 M Tris-HCl buffer, pH 8.3, was incubated in the presence of $5 \cdot 10^{-5}$ M FMN at 25°C in the dark with (2*S*)-[^{3}H]glycollate, $3 \cdot 10^{-5}$ M (3.5 nmol, $6.6 \cdot 10^4$ dpm/nmol) in the absence (■————■) and presence of $8 \cdot 10^{-3}$ M hydroxylamine (▲————▲). The results of a similar experiment carried out using $3 \cdot 10^{-5}$ M (2*R*)-[^{3}H]glycollate are shown by the curve ●————●. In this latter case hydroxylamine had no effect on the extent or rate of the reactions. The analysis of the data was carried out as described in the experimental section. Similarly, catalase and superoxide dismutase had no effect on the course of all reactions. The slow release of label from [$^3H_{si}$]glycollate is attributed to secondary oxidation of glyoxylate to oxalate. See text for further details.

is greater than 99.5%; the ^{3}H-label released into the solvent corresponds to this amount. The data of Fig. 2 show that the (*S*)-hydrogen is also removed by the enzyme, albeit at a much slower rate. This arises from oxidation of the labelled glyoxylate product to unlabelled oxalate:



This slow reaction, however, is completely inhibited by hydroxylamine, which traps the glyoxylate formed from the primary reaction. Accordingly, in the presence of NH_2OH 97.0 ± 0.5% of the label of (*S*)-[^{3}H]glycollate is retained and 3% is released. Exactly the same results were obtained using a homogeneous enzyme from a different source, L-lactate oxidase from *M. smegmatis*, for the oxidation of the same batch of stereospecifically labelled glycollates [9]. It is thus very improbable that the discrepancy arises from an impurity present in the glycollate oxidase preparation. Consequently this discrepancy in the abstraction of label using (*R*)- or (*S*)-[^{3}H]glycollates must originate in the (*S*)-[^{3}H]glycollate itself having the label 97% at the *S*- and 3% at the *R*-position. Therefore, we conclude that the D-lactate dehydrogenase from *L. leichmanii* used to prepare the L-labelled glycollate is only 97% selective for the *Si*-side in the reduction of glyoxylate, i.e., this enzyme is comparatively unspecific. On the other hand, the stereospecificity of muscle L-lactate dehydrogenase and of glycollate oxidase from *P. sativum* is greater than 99.5% *Re*.

Inactivation of the enzyme with α-hydroxybutynoic acid

Jewess et al. [12] have shown that pea glycollate oxidase is irreversibly inhibited in vitro and in vivo by α-hydroxybutynoate and report that this inhibitor was not oxidized catalytically at a measurable rate when the oxidation was followed by uptake of oxygen. In our opinion, the reported experiments [12] can be taken only as an indication that catalytic oxidation of α-hydroxybutynoic acid cannot be much faster than the rate of inactivation. That this is indeed the case could be demonstrated experimentally: when the oxidized enzyme was incubated anaerobically with a 7-fold excess of the inhibitor a rapid bleaching of the 450 nm absorption of the enzyme occurred (Fig. 3), which was accompanied by an increase in absorption in the 300 to 350 nm region. The resemblance of this spectrum to that of normal reduced enzyme indicated that normal enzyme reduction had occurred (cf. Fig. 1). Admission of oxygen immediately restored approx. 50% of the original absorption of the oxidized enzyme. This indicated that about 50% of the flavin had not been modified but had undergone normal (reversible) reduction. Upon further aerobic incubation the absorption at 400 nm was irreversibly bleached to approx. 15% the original

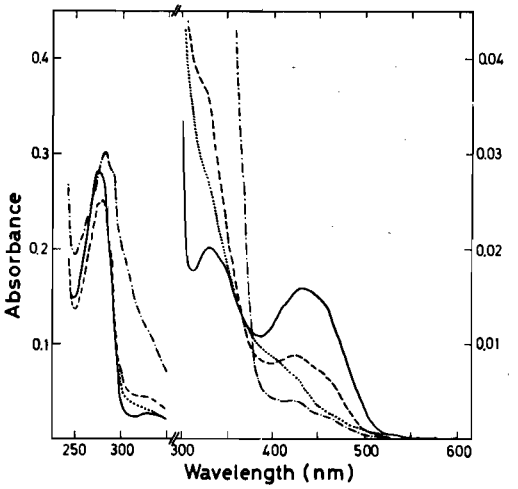


Fig. 3. Spectral course of inactivation of glycollate oxidase by α-hydroxybutynoic acid. The oxidized enzyme, $1.6 \cdot 10^{-6}$ M, 1.3 nmol in 0.8 ml $5 \cdot 10^{-3}$ M Tris-HCl buffer, pH 8.3, was made anaerobic (————) and then L-α-hydroxybutynoate (final concentration $1 \cdot 10^{-5}$ M, 9 nmol) was added from the side arm, at 2°C. Within 30 s the spectrum of curve (· · · · · ·) had formed. This species did not undergo further changes over a period of 30 min at 2°C. After this time oxygen was admitted and this restored approx. 50% of the original absorption of the oxidized enzyme (- - - - - -). Upon further standing under aerobic conditions the final spectrum of curve (· – · – ·) had developed after 2 h and did not change further.

value, while the absorbance in the near ultraviolet increased strongly. We believe this is due to irreversible and covalent modification of the enzyme flavin chromophore and to catalytic formation of α-ketobutynoic acid, which can reasonably be expected to have a strong absorption in the 300 nm region [27]. At the end of this experiment the catalytic activity in the standard assay (cf. Materials and Methods) was 15–20% the original activity, while in the presence of excess unmodified FMN it was 40–50% of the original value. Apparently, the modified FMN can be released from the holoenzyme and replaced by normal FMN. When a 100-fold excess of inhibitor was used under anaerobic conditions, essentially complete inactivation was obtained after 2 h at 10°C. An estimate can be made of the moles of α-ketobutynoate formed during the inactivation by assuming an $\epsilon_{310} \geqslant 10000$ for the sum of its decay products. α-Ketobutynoate in fact is quite unstable and such an ϵ-value should be a reasonable one, and a low estimate as judged by comparison with similar ketoacid decay products [27]. In the experiment shown in Fig. 3, 9 nmol L-hydroxybutynoate were incubated with 1.3 nmol enzyme; taking the minimal ϵ_{310} value, 9 nmol of product, i.e., essentially all of the inhibitor, appear to have been oxidized and the enzyme was inhibited to 80–85%. Hence, the ratio of catalytic oxidation to inactivation [28] can be estimated to be $\geqslant 6$. A similar value was obtained by comparing the degree of inactivation and the molar excess of inhibitor used. Thus α-hydroxybutynoic acid serves both as substrate and as inhibitor of glycollate oxidase; and, consequently, the mechanism of inactivation can be categorized as belonging to the 'suicide' type [28].

Denaturation of the inactivated protein with trichloroacetic acid released a mixture of compounds which had no distinct absorption in the visible or near ultraviolet part of the spectrum. The mixture had a fluorescence emission with maxima at 525 (identical to that of unmodified FMN) and 420 nm. The latter emission has an excitation spectrum with $\lambda_{max} \approx 340$ nm, which presumably originated from the presence of several decay products of the modified FMN, as in the case of lactate oxidase [14,15]. Thus glycollate oxidase is apparently inactivated by a mechanism similar to that operative with L-lactate oxidase, where a cyclic 4a,5-adduct of the enzyme-bound flavin is formed initially [13,14]. In the present case, however, isolation and identification of the modification products were complicated by the instability of the enzyme and the modified flavin, and therefore were not attempted.

Comparison of glycollate oxidase from pea leaves and from other sources [1,5,13] with related enzymes which oxidize L-lactate, e.g., with L-lactate oxidase from *Mycobacteria* [29–31] or with flavocytochrome b_2 from yeast [32], reveals some interesting aspects: FMN is the coenzyme in most cases, and in particular when glycollate is the preferred substrate, suggesting a correlation of the two properties. With all glycollate oxidase and with *M. smegmatis* L-lactate oxidase, the (*R*) proton of glycollate is removed catalytically. With these enzymes, as well as with others which oxidize L-lactate, the same type of inactivation with α-hydroxybutynoate is encountered [33]. In contrast to this, D-lactate dehydrogenase from the anaerobe *M. elsdenii*, an FAD enzyme, yields a completely different type of adduct with the same inhibitor [17]. These similarities in stereospecificity and mode of inactivation of the FMN-containing α-hydroxy acid oxidases suggests a related evolution of the active centers.

A major difference between the enzymes oxidizing L-lactate as compared to glycollate, however, exists with respect to the binding of FMN to the apoenzyme and suggests major differences in the FMN-protein interactions. Thus, while with bacterial lactate oxidase [30,34] and with the yeast enzyme [32] the redox coenzyme is bound tightly, with the glycollate oxidases of plant origin coenzyme dissociation and comparatively weak binding appear to predominate.

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