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# PURIFICATION AND CHARACTERIZATION OF GLYCOLIC ACID OXIDASE FROM PIG LIVER*

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## SUMMARY

1. A procedure for the isolation of glycolic acid oxidase (glycolate:$O_2$ oxidoreductase, EC 1.1.3.1) from pig liver is described. The enzyme has been crystallized and the amino acid composition has been determined.

2. Glycolic acid oxidase binds a variety of anions. Sulfate, sulfite, chloride, heptanoate, oxalate and phosphate each cause characteristic changes in the visible absorption spectrum of the enzyme. Studies on these effects have suggested that the FMN prosthetic group may be near one or more positively charged groups and also a hydrophobic region of the protein.

3. The p$K$ for the ionization of the 3-imino nitrogen in free FMN (p$K$ = 10.3) is shifted to about pH 8 in FMN bound to glycolic acid oxidase. The observed p$K$ is increased (to pH 9.3) when the enzyme titration is performed in the presence of oxalate.

4. Glycolic acid oxidase contains a second chromophore which has not yet been identified. FMN can be selectively removed from the protein to give a flavin-free protein which is green in color. This green material has absorption maxima at 328,425, and 600 m$\mu$ and a fluorescence emission maximum at about 450 m$\mu$.

5. The visible absorption of glycolic acid oxidase is bleached in an autogenous reaction. This reaction, which is spectrally similar to the bleaching that occurs when sulfite is added to the enzyme, has been partly characterized.

6. The unusual absorption spectrum of pig liver glycolic acid oxidase and possibly other glycolic acid oxidases is due, at least in part, to anion binding, the presence of the unidentified green chromophore, and autogenous bleaching.

## INTRODUCTION

Enzymes, which catalyze the oxidation of $\alpha$-hydroxy acids by $O_2$, have been

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

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isolated from a variety of plant[1-7] and animal sources[8-13]. The general reaction catalyzed by these enzymes is:

$$R\text{-}CHOH\text{-}COOH + O_2 \rightarrow R\text{-}CO\text{-}COOH + H_2O_2$$

In general, it has been found that the enzymes are flavoproteins (with FMN as the only known prosthetic group) and that they are stereospecific for the L-$\alpha$-hydroxy acid isomers. The enzymes are not specific for a single hydroxy acid but there are differences in chain length specificity. Enzymes which preferentially oxidize the short-chain aliphatic hydroxy acids and which show maximal activity with glycolic acid (R=H) are designated as glycolic acid oxidases (glycolate:$O_2$ oxidoreductase, EC 1.1.3.1).

The glycolic acid oxidase described in this communication has been isolated from pig liver by a modification of the method of DICKINSON[13]. The enzyme contains 2 moles of FMN per molecular weight of 100 000 (ref. 13). At an early stage in this work it became clear that the absorption spectrum of the purified enzyme varied considerably with each preparation, and that in all preparations the spectrum was quite different from the absorption spectra of other simple flavoproteins[14]. Spectral characterization of the pig liver enzyme was of particular interest since unusual spectral properties have been reported for glycolic acid oxidases from other animal[10] and plant[5] sources. We have found that the spectral complexity and variability of the pig liver enzyme can be largely explained in terms of the following three parameters: (1) anion and pH effects, (2) the presence of a chromophore in addition to FMN, and (3) an autogenous bleaching of flavin absorption. Studies on the interaction of the enzyme with anions have provided preliminary information about the nature of the forces which may be involved in substrate binding at the active site[15].

## METHODS AND MATERIALS

### *Assay of enzyme activity*

*$K_3Fe(CN)_6$ assay.* All assays were performed at 25° in a cuvette with a 1-cm lightpath using a Gilford recording spectrophotometer. Each assay contained: 200 $\mu$moles of potassium phosphate (pH 7.0), 1 mg of bovine serum albumin, 3 $\mu$moles of EDTA, 4 $\mu$moles of $K_3Fe(CN)_6$, 2 $\mu$moles of sodium glycolate, enzyme and water to a volume of 3.0 ml. One unit of activity is defined as an absorbance change at 420 m$\mu$ of 1.0 per min.

*2,6-Dichlorophenolindophenol (DCIP) assay.* A modification of the dye reduction method of ZELITCH AND OCHOA[4] was used. Assay conditions were identical to the $K_3Fe(CN)_6$ assay except that $K_3Fe(CN)_6$ was replaced by 0.1 $\mu$mole of DCIP. A decrease in absorbance at 600 m$\mu$ of 1.0 per min is defined as one unit of activity.

The $K_3Fe(CN)_6$ assay was used to monitor the yield and purification during each stage of enzyme preparation and for comparing the specific activity of samples isolated in successive preparations. The DCIP assay is approx. 6 times more sensitive than the $K_3Fe(CN)_6$ assay and it was used to assay enzyme in column fractions. Crude enzyme fractions exhibited a large, non-linear blank rate of dye reduction. This blank rate decreased and became nearly linear after a few minutes of reaction and substrate was then added. Enzyme activity was calculated by subtracting the

blank rate observed immediately before substrate addition. Specific activities were expressed as units of activity per unit of $A_{280\ m\mu}$.

*Enzyme purification*

The enzyme purification described below is a modification of a procedure developed by DICKINSON[13]. All steps of the purification were carried out at 2–4° in the presence of $3 \cdot 10^{-4}$ M EDTA.

*Homogenization, pH and $(NH_4)_2SO_4$ precipitations.* Pig liver was packed in cracked ice at the slaughterhouse and immediately transported to the laboratory. The liver was cut into small cubes and divided into ten 1-kg batches. Each batch was washed for 5 min in 2 l of 0.15 M KCl and after discarding the washings, it was homogenized in a further 2 l of 0.15 M KCl for 75 sec at medium speed in a 1-gallon-capacity Waring blendor. The foam was removed and the pH was adjusted to 4.8, with stirring, using 2 M acetic acid. The homogenate was centrifuged for 30 min at 10 000 rev./min in the 3RA-V head of a Lourdes Vacu-fuge. The precipitate was discarded and the pH of the supernatant was quickly adjusted to 8.0, with stirring, using 1 M NaOH. The precipitate which formed during neutralization was allowed to settle out overnight and the bulk of the extract could be siphoned off. The residual material was centrifuged for 20 min at 10 000 rev./min in the GSA head of a Sorvall refrigerated centrifuge and the supernatant was pooled with the bulk of the extract.

The $(NH_4)_2SO_4$ fractionation was conveniently carried out in 2-l aliquots. Solid $(NH_4)_2SO_4$ was added to 44% saturation and the mixture stirred for at least 15 min after the salt had dissolved. It was then centrifuged for 40 min at 2000 rev./min in the 4-l head of a refrigerated International centrifuge. The precipitate was discarded. $(NH_4)_2SO_4$ was added to the supernatant to 58% saturation and the mixture centrifuged as before, except the time was increased to 60 min. The supernatant was discarded. The precipitate was dissolved in a minimal volume of 0.1 M sodium phosphate (pH 6.3) and dialyzed for 24 h against four changes of a 10-fold excess of this buffer. This dialysis, and all subsequent stages of the preparation and handling, were done in darkness. A small precipitate was usually present after dialysis and this was removed by centrifugation.

*Calcium phosphate gel–cellulose chromatography.* The method used to prepare the columns was similar to the method of MASSEY[16]. A suspension containing 216 g of cellulose powder, 18 g of calcium phosphate gel and 2.4 l of water was used to obtain a packed column measuring 5.5 cm × 50 cm. Continuous stirring during column packing ensures a uniform column and reasonable flow rates (100–200 ml/h).

The dialyzed enzyme was applied to a calcium phosphate gel–cellulose column, previously equilibrated with 0.1 M sodium phosphate (pH 6.3). The column was washed with 0.1 M sodium phosphate (pH 6.3) containing 2% $(NH_4)_2SO_4$ until the $A_{280\ m\mu}$ of the eluate was less than 0.05. The enzyme was eluted in 0.1 M sodium phosphate (pH 7.0) containing 5% $(NH_4)_2SO_4$. Column fractions which had specific activities greater than one-fourth that of the best fractions were pooled. Glycolic acid oxidase was precipitated from the pooled fractions by addition of $(NH_4)_2SO_4$ to 80% saturation. The precipitate was collected by centrifugation (60 min at 10 000 rev./min in the 3RA-V head of a Lourdes Vacu-fuge) and resuspended in a minimal volume of 0.1 M sodium phosphate (pH 7.0).

When necessary, enzyme can be stored at this stage as an $(NH_4)_2SO_4$ suspension

in 0.1 M sodium phosphate (pH 7.0) at −20°, without deterioration. Two preparations of enzyme, each made from 10 kg of liver, were pooled for the following purification steps.

*CM-cellulose chromatography.* The concentrated enzyme was dialyzed for 48 h *versus* eight changes of a 50-fold excess of 0.03 M sodium phosphate (pH 6.0). The precipitate which formed during dialysis was removed by centrifugation and was discarded. The supernatant was passed through a CM-cellulose column (3.5 cm × 30 cm), previously equilibrated with 0.03 M sodium phosphate (pH 6.0). Under these conditions glycolic acid oxidase passes through the column as a yellow band whereas a contaminating hemoprotein is adsorbed as a brown band at the top of the column. The yellow eluate from this column was dialyzed for 48 h *versus* eight changes of a 25-fold excess of 0.005 M sodium phosphate (pH 7.6).

*DEAE-cellulose chromatography.* The dialyzed enzyme was applied to a DEAE-cellulose column (3.5 cm × 33 cm), previously equilibrated with 0.005 M sodium phosphate (pH 7.6). The column was washed with this buffer until the $A_{280\ m\mu}$ of the eluate was less than 0.005. The enzyme was eluted from the column with a linear gradient, formed from 1 l of 0.01 M sodium phosphate and 1 l of 0.05 M sodium phosphate, both at pH 7.6. Most of the enzyme fractions eluted from the column had the same specific activity and these fractions were pooled. If the $A_{280\ m\mu}$ of the pooled eluate was greater than 1.0, $(NH_4)_2SO_4$ was added to 80% saturation and the precipitate was collected by centrifugation (60 min at 10 000 rev./min in the 3RA-V head of a Lourdes Vacu-fuge). However, if the $A_{280\ m\mu}$ of the pooled eluate was less than 1.0, better recovery of enzyme activity was achieved if $(NH_4)_2SO_4$ was added to 100% saturation. The precipitate was collected by passing the suspension through a column of Hyflo Super-Cel (1.0 cm × 3.5 cm), previously equilibrated with 0.1 M sodium phosphate (pH 7.0) saturated with $(NH_4)_2SO_4$. The precipitate was retained at the top of the column, and was eluted in a small volume of 0.1 M sodium phosphate (pH 7.0). Side fractions (generally used for preliminary experiments) were similarly pooled and concentrated.

Table I summarizes the steps in the purification procedure.

*Amino acid analyses*

Amino acid analyses were performed using analytical methods similar to those described previously[17]. The cystine and cysteine content was determined in a separate analysis as cysteic acid by the method of SPENCER AND WOLD[18]. Tryptophan was determined separately by a modification[19] of the method of SPIES AND CHAMBERS[20].

*Spectrophotometric measurements*

Spectral perturbations produced by $(NH_4)_2SO_4$, $K_2SO_4$, KCl, sodium oxalate, sodium heptanoate and $Na_2SO_3$ were obtained by adding equal volumes of the compound to sample and reference cuvettes. The compounds were made up in the same buffer as the enzyme. The effects of phosphate and pH on the spectrum were determined by diluting a concentrated solution of enzyme into buffers at the desired pH and phosphate concentration. Commercial FMN was purified by the method of MASSEY AND SWOBODA[21]. Unless otherwise noted, all absorption spectra were recorded at 10° using a Cary Model 14 recording spectrophotometer. Except in the case of the FMN titration, all buffers contained $3 \cdot 10^{-4}$ M EDTA. Spectra presented are corrected

TABLE I

PURIFICATION OF GLYCOLIC ACID OXIDASE

A and B refer to two separate 10-kg preparations which were purified until Stage 5 when they were pooled for final purification. Units of enzyme activity are based on the $K_3Fe(CN)_6$ assay. Enzyme activity prior to calcium phosphate gel chromatography can only be roughly estimated due to the presence of a large blank reaction. Estimates of percent activity recovered are based on the highest total activity measured prior to calcium phosphate gel chromatography.

| *Stage* | | *Vol. (ml)* | *Total activity (units)* | *Total* $A_{280\ m\mu}$ | *Specific activity (units/* $A_{280}m\mu$*)* | *% Recovery of activity* |
|---|---|---|---|---|---|---|
| 1. Supernatant after pH 4.8 precipitation and neutralization to pH 8.0 | A | 22 650 | 420 | $936 \cdot 10^3$ | $4.5 \cdot 10^{-4}$ | --- |
| | B | 22 750 | 490 | $940 \cdot 10^3$ | $5.2 \cdot 10^{-4}$ | --- |
| 2. 44% $(NH_4)_2SO_4$ supernatant | A | 23 770 | 523 | $401 \cdot 10^3$ | $1.3 \cdot 10^{-3}$ | --- |
| | B | 23 930 | 350 | $450 \cdot 10^3$ | $7.7 \cdot 10^{-4}$ | --- |
| 3. 44–58% $(NH_4)_2SO_4$ precipitate after dialysis | A | 1 750 | 525 | $124 \cdot 10^3$ | $4.2 \cdot 10^{-3}$ | 100 |
| | B | 1 530 | 475 | $120 \cdot 10^3$ | $4.0 \cdot 10^{-3}$ | 97 |
| 4. Calcium phosphate gel column eluate after concentration and dialysis | A | 64.5 | 328 | 800 | 0.41 | 63 |
| | B | 62.0 | 304 | 1060 | 0.28 | 62 |
| 5. Pool preparations A and B | | 126.5 | 632 | 1860 | 0.34 | 63 |
| 6. CM-cellulose eluate after dialysis | | 338 | 497 | 1110 | 0.45 | 49 |
| 7. DEAE-cellulose eluate after concentration | | | | | | |
| Best fraction | | 5.0 | 206 | 145 | 1.42 | 20 |
| Side fraction | | 8.3 | 94.5 | 90.5 | 1.04 | 9.3 |

for volume changes. Fluorescence activation and emission spectra were obtained using an Aminco–Bowman spectrofluorimeter and are uncorrected for photomultiplier response.

*Materials*

Glycolic, glyoxalic, and L-lactic acids and DCIP were purchased from Calbiochem. Reagent-grade $K_3Fe(CN)_6$ was obtained from Baker and Adamson. Cellulose powder (CF-2), DEAE-cellulose (DE-22) and CM-cellulose were from Whatman. $(NH_4)_2SO_4$ (enzyme grade) was obtained from Mann Research Laboratories. Hyflo Super-Cel was from Johns-Manville. Sodium and potassium phosphate were obtained from Baker Chemical Company. $Na_2SO_3$, KCl and KBr were purchased from Mallinckrodt Chemical Works. Oxalic acid was from Allied Chemical Company, heptanoic acid was from Eastman Organic Chemicals and $K_2SO_4$ was from the General Chemical Company. FMN was obtained from Sigma. Silica gel S-HR plates (starch as binder) were obtained from Brinkman Instruments. Glass-distilled water was used throughout.

RESULTS AND DISCUSSION

*General properties*

Glycolic acid oxidase prepared by the method described in this paper has a specific activity of 1.2–1.4 (in the $K_3Fe(CN)_6$ assay). Such preparations give a single

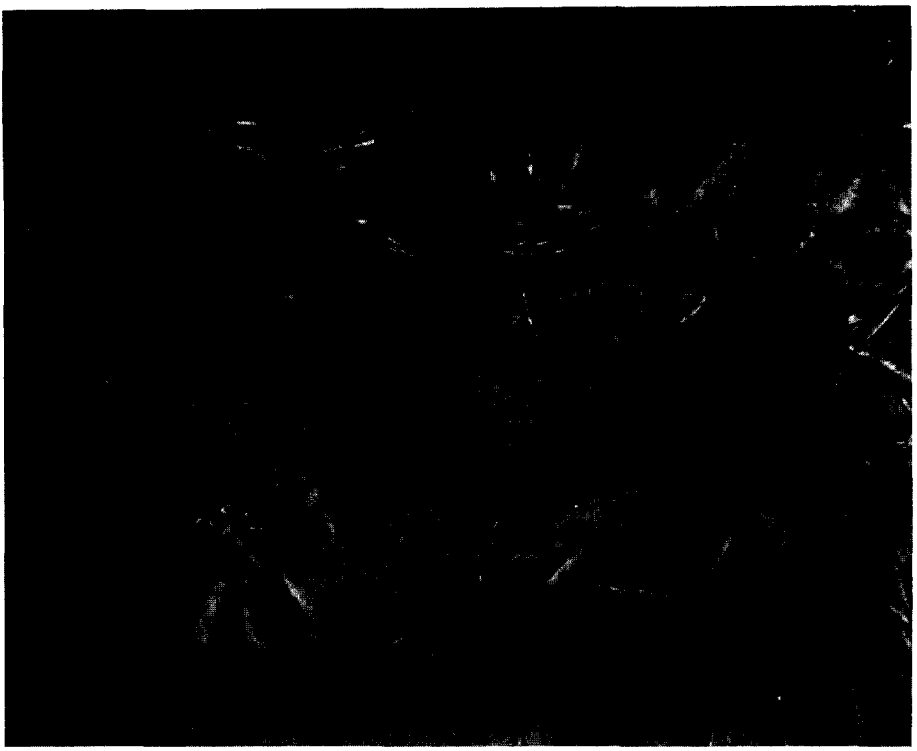

Fig. 1. Photomicrograph of glycolic acid oxidase crystals. The enzyme crystallized from a concentrated protein solution in 0.03 M sodium phosphate (pH 6.0). Magnification 500 ×.

protein band after electrophoresis in acrylamide gel at pH 9.3 and are judged to be pure.

The enzyme can be crystallized from concentrated solutions in sodium phosphate buffer (pH 6.0). When a solution having an $A_{280\ m\mu}$ of greater than 20 is left at 0° small crystals form during several days (Fig. 1). The crystalline material has the same specific activity as enzyme routinely isolated. This is the first mammalian glycolic acid oxidase to be obtained in crystalline form.

Glycolic acid oxidase can be stored frozen at −20° for several months without loss of activity if $(NH_4)_2SO_4$ is added to precipitate the enzyme or if the enzyme is stored in the presence of 2.5 M glycerol. In the absence of these protecting agents loss of enzyme activity, associated with the release of bound flavin, occurs upon freezing.

Flavin bound to pig liver glycolic acid oxidase appears to be sufficiently susceptible to photodecomposition to warrant precautionary measures. Enzyme placed in a stoppered quartz cuvette, 30 cm from a 15-W daylight bulb, loses over 50% of its absorption at 450 mμ in 5 days at 4°. When the sample had lost 70% of its 450-mμ absorption no activity could be detected whereas a dark control was fully active. Enzyme can be stored in darkness at 0° for several weeks without loss of activity.

The amino acid composition of the enzyme is shown in Table II. The molecular weight per mole of flavin calculated from the amino acid composition is 51 100. This

TABLE II

AMINO ACID COMPOSITION OF GLYCOLIC ACID OXIDASE

| *Amino acid* | *Residues per mole of flavin* |
|---|---|
| Aspartic acid | 43.1 |
| Threonine | 20.8 |
| Serine | 23.8 |
| Glutamic acid | 45.6 |
| Proline | 18.2 |
| Glycine | 33.2 |
| Alanine | 43.9 |
| Half-cystine* | 6.6 |
| Valine | 41.9 |
| Methionine | 11.8 |
| Isoleucine | 23.2 |
| Leucine | 46.9 |
| Tyrosine | 12.6 |
| Phenylalanine | 13.3 |
| Lysine** | 33.3 (32.4) |
| Histidine** | 5.1 (4.8) |
| Arginine** | 30.4 (28.6) |
| Tryptophan*** | 7.1 |

* Determined on a separate sample following oxidation to cysteic acid[18].

** Partial destruction of the basic amino acids during hydrolysis was compensated for by extrapolation of data from 24, 48, and 71 h of hydrolysis to zero time. Values in parentheses, obtained after 24 h of hydrolysis, are shown for comparison.

*** Determined colorimetrically by a modification[19] of the method of SPIES AND CHAMBERS[20].

is in good agreement with the minimum molecular weight of 50 000 obtained by DICKINSON[13] using the biuret reaction for protein estimation.

*Visible absorption spectra of oxidized and reduced enzyme*

The visible absorption spectrum of glycolic acid oxidase varies somewhat with different preparations but generally it has a spectrum similar to the one shown in Fig. 2. This spectrum shows absorption maxima at 375 and 452 mμ and marked

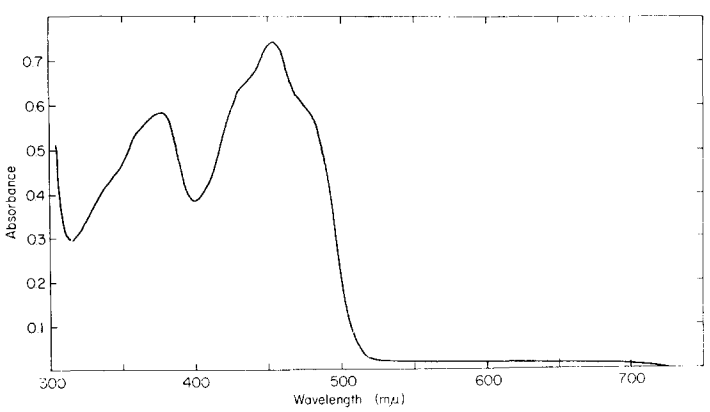


Fig. 2. Visible absorption spectrum of glycolic acid oxidase. The enzyme was dissolved in 0.1 M sodium phosphate (pH 7.0).

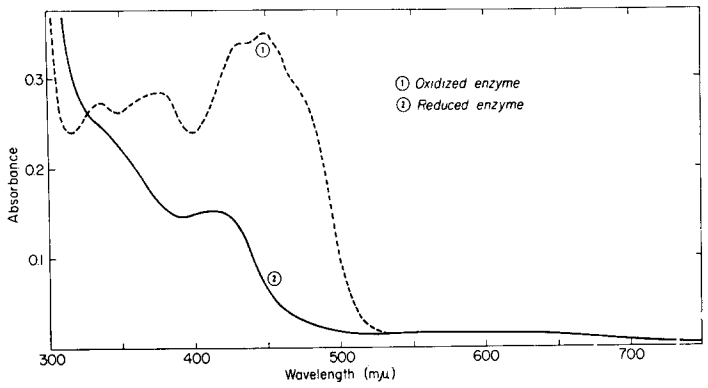


Fig. 3. Anaerobic reduction of glycolic acid oxidase by L-lactate. Curve 1, oxidized enzyme in 0.1 M sodium phosphate (pH 7.6); Curve 2, enzyme reduced by $3.5 \cdot 10^{-4}$ M L-lactate. The reduction was performed at 20°.

shoulders at 330, 430, and 480 mμ. It also has a broad and weak absorption band centered at 600 mμ. In some preparations the shoulders at 330 and/or 430 mμ appear as distinct peaks (Fig. 3). Such spectra are quite different from the two-banded absorption spectrum characteristic of most simple flavoproteins[14].

The spectrum of enzyme reduced anaerobically by a 10-fold excess of L-lactate is also shown in Fig. 3. Similar spectra are obtained upon reduction with other substrates such as glycolate or glyoxalate. In all cases the long-wavelength absorption band is unaffected by reducing substrates.

Initially, it was thought that the unusual spectrum of oxidized enzyme might be due to the presence of a metal ion since metalloflavoproteins exhibit complex absorption spectra[22]. However, metal analysis by emission spectroscopy indicated only trace amounts of metal in the enzyme preparation. It became clear that several other factors affect the visible absorption spectrum of this enzyme.

*Spectral perturbations by anions*

Studies by HARBURY *et al.*[23] have shown that the absorption at 450 mμ in free flavins is very sensitive to solvent polarity. Thus in hydrophobic solvents shoulders appear on this maximum at 430 and 480 mμ. A flavin spectrum which has these shoulders is often referred to as a resolved flavin spectrum; if these shoulders are not present, the spectrum is said to be unresolved. The spectra of glycolic acid oxidase presented in Figs. 2 and 3 may be classified as resolved flavin spectra. However, these spectra were recorded in 0.1 M sodium phosphate buffer. Exhaustive dialysis *versus* $3 \cdot 10^{-4}$ M sodium phosphate (pH 7.0) results in enzyme having a totally unresolved 450-mμ band. The addition of phosphate results in the reappearance of a resolved 450-mμ band (Fig. 4). The $K_d$ for the enzyme–phosphate complex ($1 \cdot 10^{-2}$ M) was obtained by plotting the reciprocal of the observed absorption changes *versus* the reciprocal of the salt concentration, as described by BENESI AND HILDEBRAND[24] (see inset of Fig. 4).

A number of other inorganic and organic salts perturb the visible absorption spectrum of this enzyme. Studies on these effects were done in 0.1 M sodium phosphate buffer. The effect of $(NH_4)_2SO_4$ on the spectrum is shown in Fig. 5. Large absorption

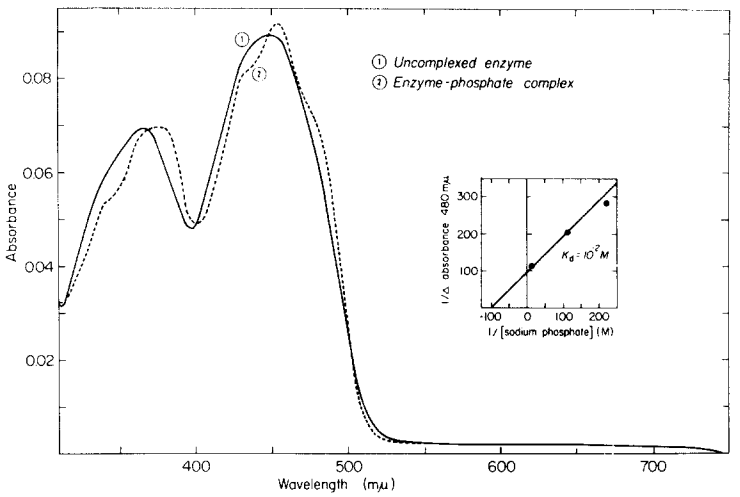


Fig. 4. Effect of phosphate on the spectrum of glycolic acid oxidase. Curve 1, $3 \cdot 10^{-4}$ M sodium phosphate (pH 7.0); Curve 2, $9.0 \cdot 10^{-2}$ M sodium phosphate (pH 7.0). The inset shows the Benesi–Hildebrand plot used to calculate $K_d$.

increases in the 450-m$\mu$ region are accompanied by decreases in absorption at wavelengths less than 380 m$\mu$. Very similar spectral changes are observed upon addition of $K_2SO_4$. KCl causes quite different changes in the spectrum, indicating that the qualitative aspects of the spectral change are due to the anion and not the cation. With KCl large absorption increases are seen in both the 450- and 380-m$\mu$ regions. Isosbestic points appear at 345 and 487 m$\mu$. The apparent dissociation constants

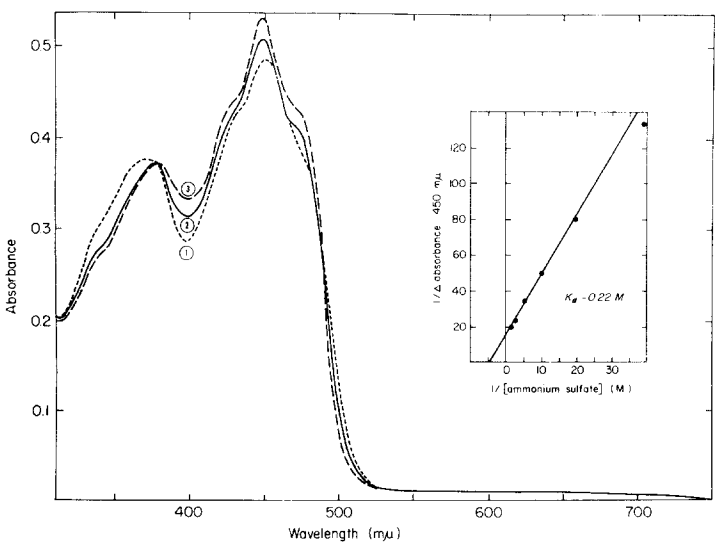


Fig. 5. Effect of $(NH_4)_2SO_4$ on the spectrum of glycolic acid oxidase. The enzyme was dissolved in 0.1 M sodium phosphate (pH 7.6). Curve 1, no additions; Curve 2, 0.10 M $(NH_4)_2SO_4$; Curve 3, 0.37 M $(NH_4)_2SO_4$. The inset shows the Benesi–Hildebrand plot used to calculate $K_d$.

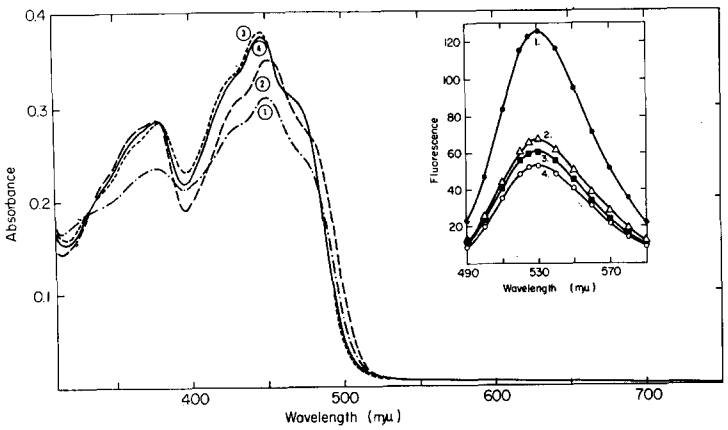


Fig. 6. Effect of heptanoate and oxalate on the spectrum of glycolic acid oxidase. The enzyme was dissolved in 0.1 M sodium phosphate (pH 7.6). Curve 1, no additions; Curve 2, $1.67 \cdot 10^{-2}$ M heptanoate; Curve 3, $9.91 \cdot 10^{-2}$ M oxalate; Curve 4, Curve 2 after addition of oxalate to a concentration of $9.91 \cdot 10^{-2}$ M. The inset shows the corresponding fluorescence emission spectra obtained by activating at 450 mμ.

for the complex of glycolic acid oxidase with $(NH_4)_2SO_4$, $K_2SO_4$ and KCl were determined from Benesi–Hildebrand plots. The values found were 0.22, 0.25 and 0.33 M for $(NH_4)_2SO_4$, $K_2SO_4$ and KCl, respectively.

Spectral perturbations are also observed in the presence of organic anions, such as aliphatic monocarboxylic and dicarboxylic acids. The spectral changes caused by excess sodium heptanoate and sodium oxalate are shown in Fig. 6. Oxalate and heptanoate also affect the weak flavin fluorescence of glycolic acid oxidase, which is decreased by approx. 50% in the presence of an excess of these reagents. In similarity with results obtained with inorganic anions, the spectral changes observed depend on the particular organic anion. For example, although heptanoate and oxalate increase the absorbance near 450 mμ, oxalate shifts the maximum to shorter wavelengths whereas heptanoate shifts the maximum to a slightly longer wavelength.

These results strongly suggest that one or more positively charged groups may be near the flavin chromophore. The binding of a given anion to such positively charged group(s) could cause a spectral perturbation by producing a specific change in the local environment of the flavin. This hypothesis suggests that all anions must share, at least in part, a common binding site. Under appropriate conditions, it should therefore be possible to displace one anion by another. This process can be conveniently monitored spectrally since each anion produces a characteristic spectral change. As seen in Fig. 6, the spectrum of an initial enzyme–heptanoate complex is converted to a spectrum characteristic of the enzyme–oxalate complex when excess oxalate is added. The conversion is nearly complete when the oxalate concentration is 7-fold higher than the heptanoate concentration. This is expected if heptanoate and oxalate cannot be bound at the same time since the dissociation constants for the binding of these two anions to glycolic acid oxidase are very similar (approx. $1 \cdot 10^{-4}$ M, see ref. 15).

This displacement of heptanoate by oxalate complicates somewhat inter-

pretation of the spectral changes caused by sulfate, chloride, heptanoate and oxalate. These studies were done in phosphate which forms a weak complex with the enzyme. The spectral changes observed must therefore be due partly to the displacement of phosphate from the enzyme and partly to the new enzyme–anion complex. However, it is clear that the final spectrum of each new enzyme–anion complex exhibits a resolved 450-m$\mu$ peak, characteristic of flavins in hydrophobic solvents. Much kinetic evidence suggests the presence of a hydrophobic region near the flavin moiety of the enzyme[15]. At the present time it is not possible to specify whether the "hydrophilic-type" spectrum observed in the absence of anions is due to interaction of the flavin with the protein or the solvent. In the absence of anions the flavin may be accessible to the solvent (*i.e.* water). Binding of anions may induce a conformational change such that the flavin is no longer accessible to the solvent. Alternatively, the binding of anions may disrupt a flavin–protein interaction, possibly involving one of the positively charged groups near the flavin.

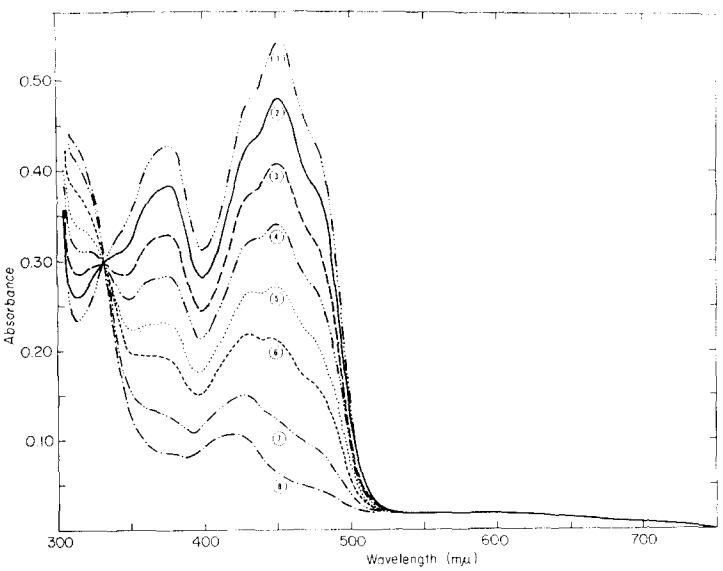


Fig. 7. Titration of glycolic acid oxidase with sulfite. Curve 1, untreated enzyme in 0.1 M sodium phosphate (pH 7.0); Curves 2–8 after the addition of sodium sulfite at concentrations yielding the following ratios of moles of sulfite per mole of enzyme-bound flavin: 0.139, 0.274, 0.417, 0.555, 0.694, 0.973, and 74.5, respectively.

The reaction of glycolic acid oxidase with sulfite is similar to the reaction observed with other flavoprotein oxidases[25,26] and with model flavin compounds[27]. The binding of sulfite is accompanied by a bleaching of flavin absorption and the formation of a new absorption band at about 320 m$\mu$ (Fig. 7). However, in other flavoproteins and model flavin compounds the fully formed complex has no absorption at wavelengths greater than 380 m$\mu$. In contrast, residual absorption above 380 m$\mu$ is observed with glycolic acid oxidase, even in the presence of a large excess of sulfite. The long-wavelength band remains unchanged in the presence of excess sulfite. In addition, a shift of the 450-m$\mu$ band to 430 m$\mu$ occurs during the titration and an

absorption band at 425 mμ is evident at the end of the titration. This aspect of the sulfite reaction will be considered later.

Evidence obtained with model compounds suggests that the sulfite reaction involves addition of sulfite to the flavin nucleus with the formation of a covalent bond at the N-5 position of the isoalloxazine ring[27]. In this respect the sulfite complex differs from other complexes formed by glycolic acid oxidase with anions. However, in similarity to D-amino acid oxidase[25], the positively charged groups near the flavin are also important in the binding of sulfite to glycolic acid oxidase. A gradual displacement of sulfite is observed when excess oxalate or $(NH_4)_2SO_4$ is added to enzyme partially bleached by sulfite. Oxalate causes a large increase in the apparent $K_d$ for the enzyme–sulfite complex. A value of $4.4 \cdot 10^{-5}$ M is obtained in the presence of 0.1 M oxalate whereas a value of about $8 \cdot 10^{-7}$ M (see ref. 25) is obtained when the titration is performed in the absence of oxalate. In addition to oxalate and $(NH_4)_2SO_4$, substrate can also displace sulfite. The spectrum characteristic of the enzyme–sulfite complex is converted to a spectrum characteristic of reduced enzyme when glycolate is added to enzyme bleached by sulfite. This displacement is also evidenced catalytically by an initial lag in the assay when an attempt is made to measure the activity of the enzyme–sulfite complex.

*Effect of pH on the absorption spectrum*

In addition to the effects of anions described above, the absorption spectrum of glycolic acid oxidase is also affected by pH (Fig. 8). As the pH is increased from pH 6 to pH 9, the absorption at 375 mμ decreases and a new maximum develops at 335 mμ. These changes are accompanied by a decrease in absorption at 450 mμ. They are completely reversible, and they have been observed with phosphate at different concentrations (4.5 and 90 mM), Tris–HCl, sodium pyrophosphate–HCl, or sodium borate–NaOH as buffering systems. These changes are probably due to an ionization of FMN since similar changes are observed when the pH of free FMN is

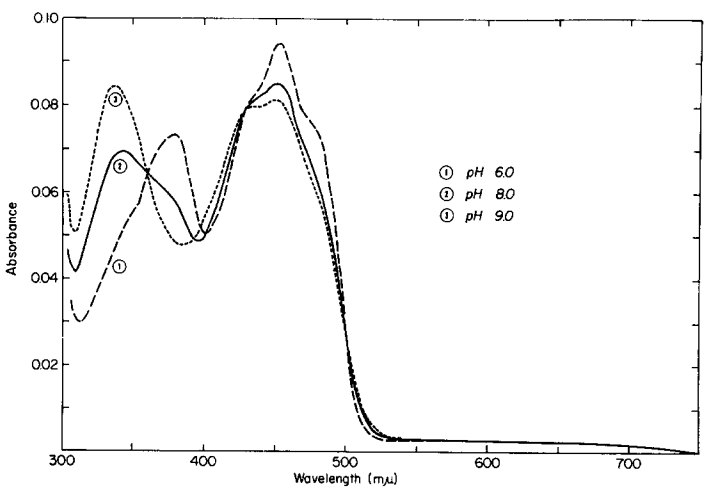


Fig. 8. pH titration of glycolic acid oxidase. The titration was performed in 0.09 M sodium phosphate.

increased from pH 9 to pH 12. In free FMN, this ionization has been ascribed to the 3-imino position in the isoalloxazine ring[28]. The p$K$ for this ionization in glycolic acid oxidase is different from the p$K$ in free FMN. A value for this p$K$ for free FMN was determined by spectrophotometric titration. The value found, p$K$ = 10.3, agrees well with values obtained by other methods which vary from p$K$ = 10.1 to p$K$ = 10.4 (see refs. 29–31). A p$K$ = 8.0 was observed for the ionization of glycolic acid oxidase in both 4.5 and 90 mM sodium phosphate buffers (Fig. 9).

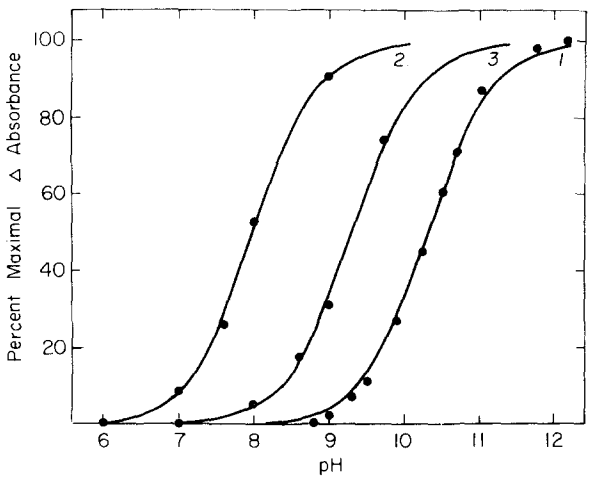


Fig. 9. Ionization of the 3-imino group of the isoalloxazine moiety in FMN (1) and glycolic acid oxidase in the absence (2) and the presence (3) of oxalate. FMN was titrated by addition of concentrated NaOH. This titration was performed at 24° in a solution containing $1.87 \cdot 10^{-2}$ M sodium borate–NaOH and $2.5 \cdot 10^{-2}$ M sodium phosphate. The enzyme titrations shown were performed in 0.09 M sodium phosphate. The oxalate concentration in Curve 3 was $4.5 \cdot 10^{-2}$ M. Changes in absorbance were measured at 350 and 335 m$\mu$ for FMN and enzyme, respectively. The curves drawn are theoretical titration curves. The maximal absorbance changes in the enzyme titrations were estimated from plots of change in $A_{335\ m\mu}$ *versus* pH.

The observed p$K$ for glycolic acid oxidase is shifted considerably by oxalate, an anion which is bound rather tightly (Fig. 9). This suggests that a specific interaction of the flavin with one of the positively charged groups may facilitate ionization of the 3-imino flavin nitrogen. For example, flavin ionization may be facilitated by hydrogen bonding of a positively charged group at the 2- or 4-carbonyl position of the isoalloxazine ring. If it is assumed that the enzyme–oxalate complex does not undergo ionization, Eqn. 1 can be used to predict the magnitude of this shift.

$$pK' = pK - \log K_d + \log[\text{oxalate}] \qquad (1)$$

From the known $K_d$ for the enzyme–oxalate complex ($4.4 \cdot 10^{-4}$ M, ref. 15) and the observed p$K$ of 8.0 for the flavin ionization of the enzyme in the absence of oxalate, a p$K'$ of 10 can be calculated for the ionization in the presence of $4.5 \cdot 10^{-2}$ M oxalate. The agreement with the observed value of 9.3 is reasonable; measurements with the enzyme are complicated by the fact that at high pH values glycolic acid oxidase is denatured and FMN is liberated.

At present we have no explanation for the fact that the p$K$ observed for flavin

ionization is unaffected by the phosphate concentration. However, catalytically, it is found that the interaction of the enzyme with phosphate differs significantly from that observed with other anions[15].

*Evidence for a second chromophore*

Several observations suggested that pig liver glycolic acid oxidase might contain a chromophore in addition to FMN. Firstly, not all features of the absorption spectrum can be explained on the basis of the specific ion and pH effects previously described. For example, although the spectrum of the freshly made enzyme is usually similar to the spectrum in Fig. 2, some preparations have distinct peaks at 330 and/or 430 mμ, rather than the shoulders shown in Fig. 2. In addition, all preparations have a broad absorption band centered at 600 mμ. This band is not affected by the anions, pH changes or reducing substrates which affect other regions of the spectrum. The spectrum of the enzyme–sulfite complex especially suggests a second chromophore. As mentioned earlier, in other flavoproteins and model flavin compounds, which complex with sulfite, the fully formed complex has no absorption at wavelengths greater than 380 mμ (refs. 25, 27). In contrast the complex of glycolic acid oxidase and sulfite does absorb above 380 mμ, the spectrum showing a maximum at 425 mμ. It seemed very likely that this residual absorption might be due to another component of the protein.

Conclusive evidence for a second chromophore was obtained by selective removal of FMN from the protein. This was achieved by dialyzing the protein against KBr as described by MASSEY AND CURTI[32]. During the dialysis the yellow color of native glycolic acid oxidase was lost, leaving a protein preparation which was distinctly green in color.

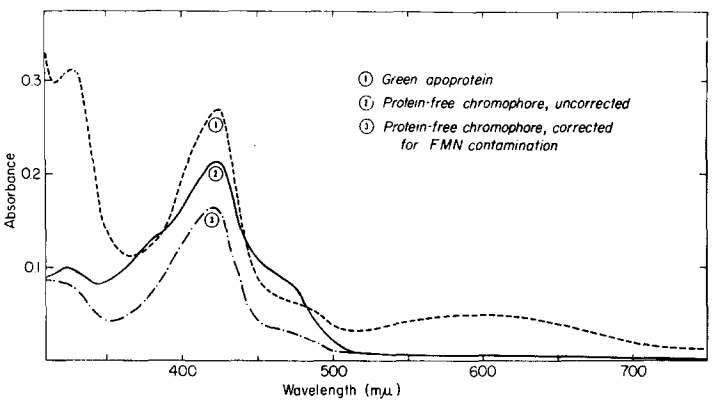


Fig. 10. Absorption spectra of green apoprotein before and after heating. FMN was removed by by dialyzing holoenzyme for 1 week at 4° *versus* five changes of a 100-fold excess of 0.1 M sodium phosphate (pH 7.0) containing 1.0 M KBr. The KBr was then removed by dialyzing the apoprotein preparation *versus* four changes of a 1000-fold excess of 0.1 M sodium phosphate (pH 7.0). The protein-free chromophore was obtained by boiling the apoprotein preparation for 5 min in a sealed tube, protected from light. A white protein precipitate was removed by centrifugation and the spectrum of the supernatant was recorded. Curve 1, green apoprotein; Curve 2, protein-free chromophore; Curve 3, spectrum of protein-free chromophore after subtracting the contribution from contaminating FMN. The FMN concentration ($5.3 \cdot 10^{-6}$ M) was estimated by comparing fluorescence emission at 520 mμ of the chromophore solution with a standard FMN curve (activating wavelength, 450 mμ).

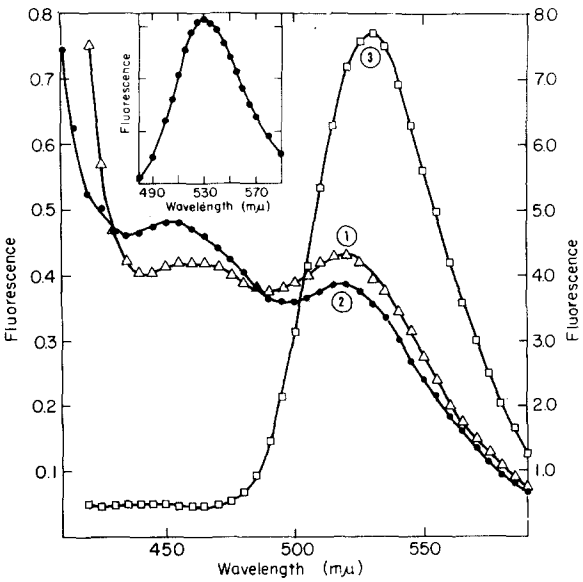


Fig. 11. Fluorescence emission spectra of green apoprotein before and after heating. Emission spectra obtained before heating by activating at 370 and 350 mμ are plotted in Curves 1 and 2, respectively. Curve 3 was obtained after heating green apoprotein. The activating wavelength is 350 mμ. Emission intensity for Curves 1 and 2 is represented by the left-hand scale whereas Curve 3 is plotted according to the scale on the right. Spectra were recorded in 0.1 M sodium phosphate (pH 7.0) at 0–1°. The inset shows the emission spectrum of an authentic sample of FMN.

This green apoprotein* has absorption maxima at 328 and 425 mμ, and a broad absorption band centered at 600 mμ (Fig. 10). The preparation is weakly fluorescent, with fluorescence emission maxima at 450 and 520 mμ. The shape of the fluorescence emission spectrum depends on the wavelength of the exciting light. Thus, excitation at 370 mμ enhances the emission at 520 mμ, while exciting light at 350 mμ enhances the emission at 450 mμ (Fig. 11). This suggests the presence of more than one fluorescent chromophore. The fluorescence emission at 520 mμ is probably due to residual FMN in the preparation, while the emission at 450 mμ is probably due to the unknown chromophore. The fluorescence excitation spectra provide further evidence for contamination by FMN (Fig. 12). The excitation spectrum for the fluorescence at 520 mμ shows maxima at 370 and 460 mμ, features which are characteristic of FMN (see inset to Fig. 12). This spectrum also shows a weak band at 290 mμ. When the emitted light is at 450 mμ, the excitation spectrum shows a much more intense band at 290 mμ, and a second band at 340 mμ.

The chromophore giving the green color can be obtained free in solution by heat denaturation of the apoprotein. In the free chromophore, the absorption band at 600 mμ disappears and the intensity of absorption at 425 and 328 mμ is decreased (Fig. 10). The loss of protein end-absorption may partially account for the large decrease in extinction of the 328-mμ peak. The fluorescence emission is almost

* The term apoprotein refers to the product derived from glycolic acid oxidase holoenzyme by the removal of its flavin. The removal of all non-protein components is not implied.

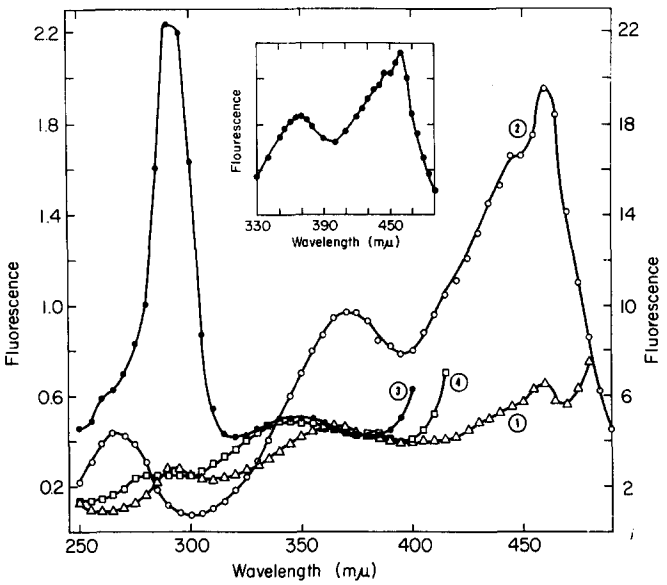


Fig. 12. Fluorescence activation spectra of green apoprotein before and after heating. Activation spectra obtained by measuring emission at 520 mμ before and after heating are plotted in Curves 1 and 2 ,respectively. Curves 3 and 4 were obtained by measuring emission at 450 mμ before and after heating, respectively. Emission intensity for Curves 1, 3 and 4 is represented by the left-hand scale whereas Curve 2 is plotted according to the scale on the right. Spectra were recorded in 0.1 M sodium phosphate (pH 7.0) at 0–1°. The inset shows the activation spectrum of an authentic sample of FMN.

unchanged at 450 mμ, but it is increased more than 20-fold at 520 mμ (Fig. 11). This change in fluorescence at 520 mμ is consistent with the idea that the green apoprotein contains residual FMN. Nearly all the fluorescence of free FMN is quenched when bound to glycolic acid oxidase. Thus, large increases in fluorescence at 520 mμ are to be expected when it is released from the protein. Furthermore, the excitation spectrum for the fluorescence at 520 mμ, which shows maxima at about 370, 460 and 265 mμ, is very similar to the fluorescence excitation spectrum of authentic FMN (Fig. 12). The excitation spectrum for the fluorescence at 450 mμ now shows only a single maximum at 340 mμ. The maximum at 290 mμ in the excitation spectrum of the green apoprotein is eliminated.

Thin-layer chromatography of the protein-free chromophore on silica plates, using a butanol–acetic acid–water solvent system (4:3:3, by vol.) confirmed the presence of two components. One component, visualized by its intense green fluorescence, had an $R_F$ value identical to an authentic sample of FMN ($R_F = 0.51$). The second component is probably due to the unknown chromophore. It is visualized by the development of a brown spot upon immersion in an iodine vapor chamber and had an $R_F$ value of 0.56. The $R_F$ values for the two compounds, although similar, are reproducibly distinct. Furthermore FMN does not give a positive spot test in the presence of iodine vapor.

The residual FMN in the protein-free chromophore preparation was estimated to be $5.3 \cdot 10^{-6}$ M by comparing fluorescence emission at 520 mμ with a standard

FMN curve. When the absorption spectrum of the unknown chromophore is corrected for this amount of FMN, the absorption maximum is shifted from 425 to 420 mμ, and the shoulders near 380 and 460 mμ become less pronounced (Fig. 10).

In addition to its absorption spectrum, green apoprotein has several other properties predicted from observations on holoenzyme preparations. Green apoprotein cannot be reduced by substrate, although it is readily reduced on addition of dithionite. The long-wavelength absorption band disappears on addition of dithionite and a featureless spectrum, similar to reduced flavin, is observed. The reduced protein is completely reoxidized by air. The spectrum of green apoprotein does not change in the pH range 6.2–9.6 or upon addition of oxalate.

The green apoprotein is also not susceptible to bleaching by sulfite. This observation shows that the residual absorption above 380 mμ when excess sulfite is mixed with glycolic acid oxidase is due to the green component. It is therefore possible to calculate a theoretical spectrum for enzyme–flavin by subtracting the residual absorption after addition of sulfite from the initial, sulfite-free spectrum. This is illustrated in Fig. 13. When the contribution from the green component is

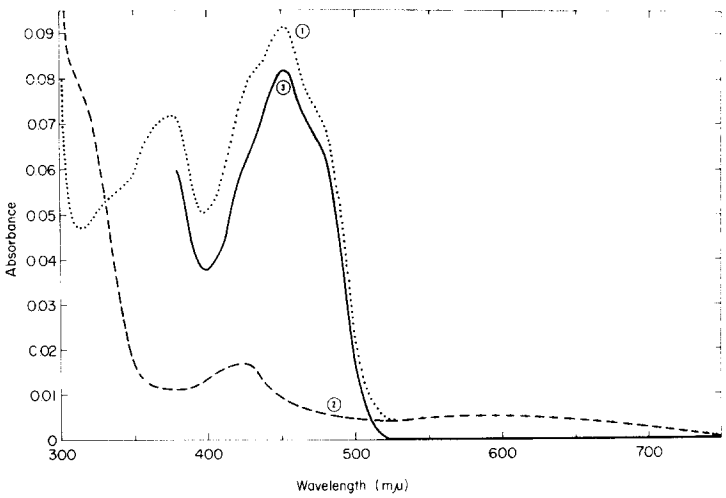


Fig. 13. Theoretical spectrum of flavin bound to glycolic acid oxidase. Curve 1, native enzyme in 0.1 M sodium phosphate (pH 7.0); Curve 2, after addition of 1 μmole of sulfite; Curve 3, difference spectrum obtained for enzyme-flavin by subtracting Curve 2 from Curve 1.

subtracted, the shoulder at 430 mμ and the long-wavelength absorption band are eliminated and a spectrum typical of simple flavoproteins is obtained. The resultant spectrum, calculated for pure yellow glycolic acid oxidase above 380 mμ, was the same for a large number of preparations.

The presence of a band at 290 mμ in the fluorescence activation spectrum of green apoprotein suggests that energy is first absorbed by an aromatic amino acid residue and then transferred to the unknown chromophore. Heat treatment releases the chromophore from the protein and eliminates this band. The long-wavelength absorption band of green apoprotein is also eliminated on heating. Since neither the

protein nor the chromophore alone absorb in this region, the long-wavelength band may be due to a charge-transfer complex between the protein and the unknown chromophore. This hypothesis is supported by observations that the long-wavelength band is lost when the green apoprotein is treated with dithionite and reappears when the protein is reoxidized. Transfer of electronic excitation energy is expected for the components of a charge-transfer complex. Heating destroys the charge-transfer complex between the chromophore and the protein and thereby eliminates the 290-m$\mu$ band in the activation spectrum.

Alternatively, it may be argued that heat treatment destroys the component of the unknown chromophore which absorbs at 290 m$\mu$. This argument must assume that this component also absorbs at 600 m$\mu$ or that an internal charge-transfer complex exists between the 290-m$\mu$ component and the rest of the chromophore. This hypothesis would be disproved if reconstitution of green apoprotein could be achieved using the heat-liberated chromophore. Unfortunately, it has not been possible to remove the chromophore without denaturing the protein and hence this experiment has not been done.

Although the catalytic significance of the green component remains obscure, it is likely that a similar chromophore may be present in other glycolic acid oxidases. In fact, it was originally proposed by ROBINSON *et al.*[10] that the unusual spectral properties of the hog renal cortex enzyme (which exhibits a peak at 430 m$\mu$ and a pronounced shoulder at 325 m$\mu$) might be due to an additional undetected component of the prosthetic group. The glycolic acid oxidase from spinach exhibits absorption maxima at 340 and 445 m$\mu$ (ref. 5) and is the only enzyme in a survey of flavoproteins where the 373- or 375-m$\mu$ peak of free flavins has been shifted to wavelengths below 350 m$\mu$ (ref. 14). In addition, a highly labile glycolic acid oxidase has been isolated from etiolated plants which has absorption maxima at 320 and 420 m$\mu$ (ref. 33), similar to the green component of pig liver glycolic acid oxidase.

*Autogenous sulfite-like bleaching reaction*

It was initially thought that the spectral variability of glycolic acid oxidase might reflect variable ratios of green and yellow components. The amount of green component in a preparation can be estimated from the absorption at 420 m$\mu$ after addition of excess sulfite. The absorption at 280 m$\mu$ before adding sulfite relative to the absorption at 420 m$\mu$ after adding sulfite is a measure of the amount of the green component. This $A_{280\ m\mu}/A_{420\ m\mu}$ ratio varied between 46 and 56 for nine different preparations of enzyme, indicating that the amount of the green component does vary somewhat.

The contribution of the green component to the absorption at 450 m$\mu$ is small. Thus the ratio of absorption at 280 m$\mu$ to the absorption at 450 m$\mu$ should give an indication of the amount of FMN in the sample. This ratio, measured in 0.1 M sodium phosphate buffer (pH 7.0), varied from 7.3 to 9.2 for nine different preparations, suggesting that they contained different amounts of FMN. However, it has been found that the absorption at 450 m$\mu$ is not a good indication of the amount of flavin present, since the flavin absorption is sometimes bleached during storage, even when the enzyme is protected from light. The spectral changes which occurred during 5 h of storage in one preparation are shown in Fig. 14. The loss of absorption in the 450-m$\mu$ region and the concomitant increase in absorption in the 320-m$\mu$

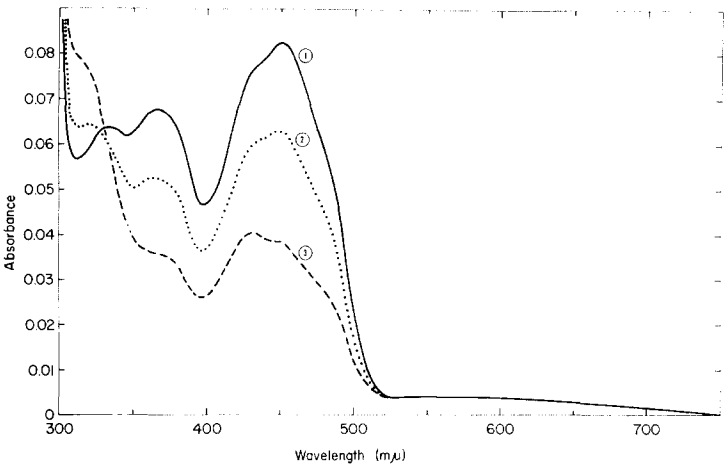


Fig. 14. Autogenous bleaching of glycolic acid oxidase. Curve 1, initial spectrum in $7 \cdot 10^{-3}$ M sodium phosphate (pH 7.0); Curves 2 and 3 were recorded 2 and 5 h later, respectively. The spectrum recorded after 12 h was identical to Curve 3.

region bear a striking resemblance to the bleaching which occurs when sulfite is added to the enzyme. The $A_{280\ m\mu}/A_{450\ m\mu}$ ratio increased from 8.6 to 18.3 during bleaching, but there was no change in the amount of the green component as measured by the $A_{280\ m\mu}/A_{420\ m\mu}$ ratio. When the spectra in Fig. 14 are corrected for absorption by the green component, they are qualitatively very similar and can in fact be superimposed if they are first normalized at 450 m$\mu$. Since the amount of the green component is not affected by the extent of bleaching, this is only possible if the product of the autogenous bleaching reaction, like the sulfite complex, has no absorption at wavelengths above 380 m$\mu$. This result explains why the theoretical spectrum calculated for yellow enzyme is constant over a large number of preparations, despite considerable variation in the extent of bleaching in different preparations.

Bleaching by sulfite and autogenous bleaching are similar in a number of other respects. Thus both can be reversed by anions which form complexes with the positively charged groups near the flavin. When $(NH_4)_2SO_4$ was added to enzyme partially bleached in the autogenous reaction the absorption increased during about 4.5 h to give a final spectrum similar to that observed before bleaching. Addition of $(NH_4)_2SO_4$ to freshly prepared enzyme also prevents autogenous bleaching. Like the enzyme–sulfite complex, enzyme bleached in the autogenous reaction shows a lag period in catalytic assay. During the assay, the rate increases to give a maximum rate identical with that observed before bleaching. The lag period is abolished if the enzyme is first treated with either $(NH_4)_2SO_4$ or substrate.

Enzyme preparations which exhibit extensive bleaching are atypical. Most preparations show only 10–15% loss of absorption at 450 m$\mu$ during several weeks of storage. The spectral changes in these preparations are similar to those observed in preparations which undergo extensive bleaching and they are also reversed by treatment with $(NH_4)_2SO_4$. However, 10–15% bleaching of enzyme-flavin can contribute significantly to the apparent spectral variability of the enzyme in the presence of a constant proportion of the green component. This amount of bleaching

of absorption at 450 mμ results in a more pronounced shoulder at 430 mμ and increased absorption around 320 mμ. In samples which exhibit extensive bleaching the shoulder at 430 mμ appears as a distinct peak. DICKINSON[13] reported that enzyme with a peak at 430 mμ was occasionally isolated. It is likely that these preparations are similar to the few atypical enzyme samples isolated in this laboratory in which extensive bleaching of enzyme-flavin has been observed. A similar argument might explain the peak at 430 mμ in the spectrum obtained for the glycolic acid oxidase from hog renal cortex[10].

In contrast to some preparations which undergo extensive bleaching at least one preparation showed no evidence of bleaching over a period of several months. These results suggest that the potential for autogenous bleaching is not a property of the native enzyme, but is acquired, in an as yet unidentified manner, during the enzyme preparation. Since the autogenous bleaching reaction bears a striking similarity to the sulfite addition reaction, it is possible that variable oxidation of a suitably located sulfhydryl group occurs during enzyme preparation. An autogenous sulfite-like bleaching reaction has not been reported for any other flavoprotein. The occurrence of this reaction in preparations of glycolic acid oxidase may simply be related to the fact that, of all the oxidases tested, this enzyme shows the greatest affinity for sulfite[25]. The reaction of glycolic acid oxidase with sulfite is stoichiometric up to nearly 70% complex formation at an enzyme-flavin concentration of $5.0 \cdot 10^{-5}$ M. The presence of small amounts of an oxidized thiol, which could potentially form a sulfite-like complex, may be sufficient to cause bleaching of enzyme-flavin.

The presence of variable amounts of bleached enzyme-flavin means that absorption at 450 mμ is not an accurate method of estimating the amount of bound flavin present. When a precise estimate of the amount of enzyme-flavin present is required the extinction coefficient at 450 mμ must be determined directly on the enzyme sample to be used. The best enzyme preparation isolated in this laboratory had an $A_{280\ m\mu}/A_{450\ m\mu}$ ratio of 7.3 and showed no evidence of bleaching during several months of storage. The extinction coefficient at 450 mμ in 0.1 M sodium phosphate (pH 7.0) was determined for this preparation by two different methods: (1) trichloroacetic acid precipitation and (2) boiling for 5 min. Both methods yielded a molar extinction coefficient of $11.7 \cdot 10^3$. Since the evidence suggests that this preparation contained little, if any, bleached flavin, this value must closely approximate the true extinction coefficient of FMN bound to glycolic acid oxidase.

ACKNOWLEDGMENTS

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