Biochimica et Biophysica Acta, 657 (1981) 438—447

BBA 69203

IDENTIFICATION OF THE FACTOR RESPONSIBLE FOR AUTOGENOUS BLEACHING OF GLYCOLLATE OXIDASE

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(Received August 26th, 1980)

Key words: Glycollate oxidase; Flavoprotein; Flavin bleaching; Flavin-sulfite complex

Summary

Autogenous bleaching of glycollate oxidase (glycollate:oxygen oxidoreductase, EC 1.1.3.1) is characterized by a loss of absorption due to enzyme-bound FMN and the presence of a lag in the catalytic assay. The extent of bleaching varies with different preparations. The following evidence indicates that bleaching is due to the presence of sulfite in the enzyme preparation which forms a reversible complex (EFMN + Y $\rightleftharpoons$ EFMN · Y, Y = sulfite) with enzyme-bound FMN. Both EFMN · Y and authentic enzyme-sulfite complex exhibit similar spectral and catalytic properties. At pH 7.0 both complexes are decomposed by agents (2,6-dichlorophenolindophenol, H_2O_2) which cause sulfite oxidation. In the absence of such agents the complexes are extremely stable at pH 7.0 even during extensive dialysis. A pronounced decrease in complex stability is observed at pH 9.0 and 25°C. Both sulfite and Y are readily removed by dialysis under these conditions. At lower temperatures both complexes exhibit an increase in stability at pH 9.0. Reversible association-dissociation is observed with EFMN · Y and EFMN · SO_3 at pH 9.0, when the temperature is varied between 25 and 0°C. The supernatant obtained after heat denaturation of EFMN · Y causes bleaching when mixed with EFMN, indicating that Y is present in the heat extract. A stable Bunte salt is formed by reacting sulfite with 4,4′-dithiodipyridine. Reaction of the latter with the heat extract yields a product with spectral and chromatographic properties identical to the authentic Bunte salt, indicating that the heat extract contains sulfite.

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Introduction

Pig liver glycollate oxidase (glycollate:oxygen oxidoreductase, EC 1.1.3.1) catalyzes the oxidation of various L-α-hydroxy acids coupled with the reduction of oxygen or other electron acceptors such as 2,6-dichlorophenolindophenol (DCIP). The enzyme contains FMN as its major prosthetic group with a variable minor component identified as 6-hydroxy-FMN [1,2]. The visible absorption spectrum observed for different preparations of glycollate oxidase varies depending on the 6-hydroxy-FMN content and on the extent of autogenous bleaching. The latter is associated with an apparent loss of absorption due to enzyme-bound FMN and the presence of a lag in the catalytic assay. The spectral changes and the effect on the catalytic assay are similar to that observed upon formation of a covalent complex between enzyme-bound FMN and sulfite. Characteristically observed with flavoprotein oxidases [3], the sulfite complex is formed via nucleophilic attack of sulfite at position N(5) of the flavin [4]. Schuman and Massey [1] found that both types of spectral bleaching could be reversed by adding anions (e.g., oxalate) which bind to positively charged groups near the flavin. This treatment would also abolish the lag period in the catalytic assay. The results suggested that enzyme preparations which exhibit autogenous bleaching might contain a factor capable of forming a reversible sulfite-like complex with the FMN moiety.

We recently isolated a preparation of glycollate oxidase which exhibited a substantial amount of autogenous bleaching. This prompted the studies described in this paper which provide evidence indicating that sulfite itself is the factor responsible for autogenous bleaching.

Experimental procedure

Materials

Sodium glycolate was purchased from Baker Chemical Company. DCIP was obtained from Sigma Chemical Co. Sodium sulfite and hydrogen peroxide were obtained from Mallinckrodt. 4,4′-Dithiodipyridine was purchased from Aldrich. Analytical TLC plates containing an ultraviolet indicator (silica gel 60 F-254) were purchased from Brinkman Instruments. All other materials were the best commercially available grade and used as received.

Methods

Glycollate oxidase was purified from pig liver and assayed by the procedures of Schuman and Massey [1]. Protein was determined by the method of Lowry et al. [5]. The enzyme preparations exhibited specific activity values similar to that previously reported for pure enzyme [1]. The activity of the preparation which exhibited autogenous bleaching was determined by measuring the rate observed after the lag period in the catalytic assay. Alternatively, the lag could be eliminated by prior incubation of the enzyme with oxalate (0.08 M) at pH 7.0. In agreement with previous studies [1], the initial rate observed with the oxalate-treated sample was the same as the activity observed after the lag period with untreated enzyme. Assays were performed using a Gilford spectrophotometer.

Reaction of sulfite with 4,4′-dithiodipyridine was conducted in 0.1 M sodium phosphate buffer, pH 7.0, at 25°C following a procedure similar to Humphrey et al. [6]. Excess 4,4′-dithiodipyridine ($8.0 \cdot 10^{-5}$—$2.8 \cdot 10^{-4}$ M) was added to the sulfite solution, the absorbance at 324 nm ($\epsilon = 2.2 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$) was recorded after 3 min and used to determine the amount of 4-mercaptopyridine formed. In the presence of FMN the amount of 4-mercaptopyridine was determined after correcting for the absorbance of the flavin at 324 nm. FMN did not otherwise affect the assay at concentrations similar to those present in the heat extract from glycollate oxidase. A suitable volume (0.35—2.0 ml) of the reaction mixture containing 12—50 nmol of the Bunte salt was applied to a TLC plate as a streak. The plate was developed with chloroform/methanol (7 : 3), dried, rotated by 90° and placed in a tank containing 100% methanol. The spot containing the Bunte salt was eluted with water (0.4 ml) and the silica gel was removed by centrifuging. The absorption spectrum of the supernatant (0.26—0.29 ml) was recorded vs. a reference cell prepared from the same area of a blank control plate. The yield of the Bunte salt was determined from its absorption at 265 nm ($\epsilon = 6000$ $M^{-1} \cdot cm^{-1}$ [6]). Absorption spectra were obtained using a Beckman 25 spectrophotometer.

The effects of pH and temperature on the dissociation constant of the enzyme-sulfite complex were determined by spectral titration [7], in 0.1 M sodium phosphate buffer, pH 7.0, or in 0.1 M sodium borate buffer, pH 9.0, using an enzyme preparation which did not exhibit autogenous bleaching. Except at pH 9 and 0°C, rapid equilibration was observed at each point in the titrations.

All buffer solutions used in these studies contained $3 \cdot 10^{-4}$ M EDTA.

Results and Discussion

Autogenous bleaching, as evidenced by a lag in the catalytic assay, has never been detected in the early stages of enzyme purification. Owing to the presence of a non-linear blank rate of DCIP reduction, a different procedure is required for crude enzyme samples which involves a short preincubation (2 min) of the enzyme with all assay components except glycollate (substrate-last assay) [1]. Assays with purified enzyme samples are normally initiated by adding enzyme (enzyme-last assay). We have found that enzyme which exhibits a lag in the enzyme-last assay does not show a lag in the substrate-last assay, indicating that the latter cannot be used to detect autogenous bleaching in crude enzyme samples. The lag is observed with the substrate-last assay provided DCIP is omitted during the preincubation period. The results suggest that apparent dissociation of the sulfite-like enzyme complex (EFMN · Y) occurs during preincubation with DCIP, possibly via oxidation of the factor (Y) responsible for autogenous bleaching. For comparison, experiments were conducted using authentic enzyme-sulfite complex prepared using another enzyme preparation which did not exhibit autogenous bleaching. The enzyme-sulfite complex exhibited a lag when assayed by the enzyme-last method, similar to previous studies [1]. However, no lag was detected when the complex was assayed by the substrate-last method. Other studies showed that sulfite would rapidly reduce DCIP, as evidenced by decreases in absorption at 600 nm when sulfite

(15 μM) was mixed with DCIP under the same conditions which eliminate the lag with EFMN · Y and EFMN · SO_3.

Hydrogen peroxide was tested to determine whether another oxidizing agent would also cause decomposition of EFMN · Y. In these studies a sample of autogenously-bleached enzyme was mixed with 5 mM H_2O_2 in 0.1 M sodium phosphate buffer, pH 7.0, at 25°C. A small aliquot, assayed after 5 min, showed that peroxide treatment eliminated the lag (Fig. 1). The lag did not reappear during subsequent incubation of the enzyme even if excess catalase was added to destroy any free H_2O_2. In similar studies it was found that H_2O_2 would also eliminate the lag observed with authentic enzyme-sulfite complex.

Studies with oxidizing agents were conducted at pH 7 which is the same pH used for assay and storage of the stock of autogenously-bleached enzyme. At this pH the lag is not eliminated by dilution or by incubating the enzyme alone at 25 or 0°C. However, we find that the lag is rapidly eliminated by diluting the enzyme with 0.1 M sodium borate buffer, pH 9.0, at 25°C. On the other hand, the lag was not abolished if the enzyme was diluted into borate buffer at 0°C. However, if the temperature of the pH 9.0 solution at 25°C was lowered to 0°C, the lag reappeared within a few hours. No lag was detected when the temperature of the sample was again increased to 25°C and assayed after 5 min. The results suggest that the reaction responsible for autogenous bleaching is an equilibrium reaction (Eqn. 1), and that the apparent dissociation constant depends on pH and temperature, increasing at higher pH values and temperatures.

$$\text{EFMN} \cdot \text{Y} \rightleftharpoons \text{EFMN} + \text{Y} \qquad (1)$$

Changes in the visible absorption spectrum of the enzyme provide direct

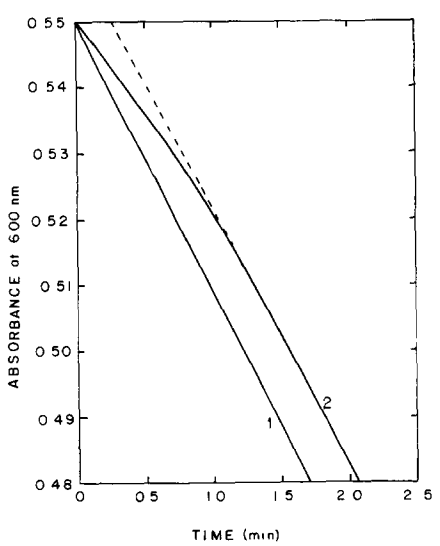
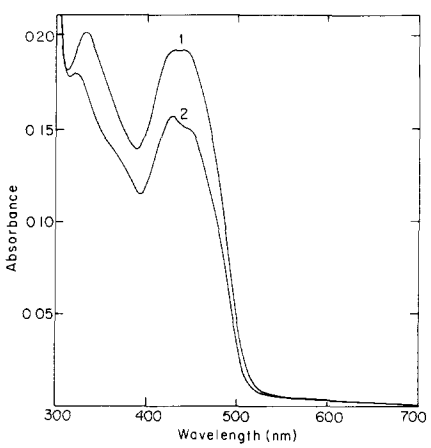


Fig. 1. Effect of hydrogen peroxide treatment on assays with autogenously-bleached enzyme. The enzyme was mixed with H_2O_2 as described in the text and assayed by the enzyme-last method (Line 1). Curve 2 was obtained with untreated enzyme.

evidence for the effect of temperature on the equilibrium concentration of EFMN · Y at pH 9.0 (Fig. 2). Bleaching of the enzyme is observed when the temperature of the solution is lowered from 25 to 0°C, as evidenced by a slow decrease in absorbance at 450 nm. The reaction is reversible as evidenced by the rapid return of absorbance at 450 nm when the temperature is increased back to 25°C. The results show that the elimination of the lag in the catalytic assay is accompanied by a decrease in the concentration of EFMN · Y, similar to results previously obtained for the effect of anions on the extent of autogenous bleaching at pH 7.0 [1].

A similar effect of temperature is observed with authentic enzyme-sulfite complex at pH 9.0 (Fig. 3). Only a small amount of complex is formed upon addition of sulfite (0.31 mol/mol FMN) at 25°C. The amount of complex increases when the temperature is lowered to 0°C, as evidenced by a slow decrease in absorption at 450 nm similar to the reaction observed with autogenously-bleached enzyme. When the temperature is increased to 25°C most of the complex rapidly dissociates, resulting in a spectrum identical to that originally observed at 25°C. That the observed changes reflect differences in complex stability is evidenced by the values obtained for dissociation constants, which, at 0°C, were found to be $9.9 \cdot 10^{-8}$ and $2.9 \cdot 10^{-6}$ M at pH 7.0



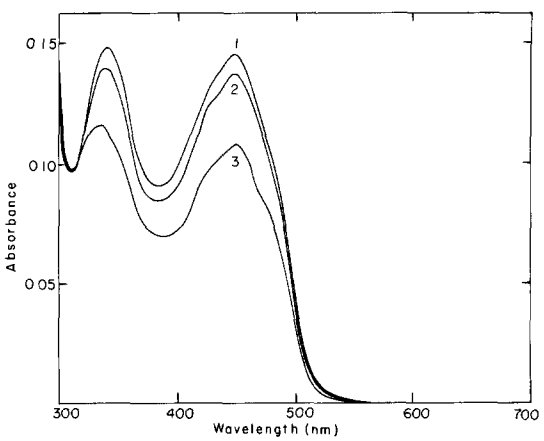


Fig. 2. Effect of temperature on the extent of autogenous bleaching at pH 9.0. An aliquot of autogenously-bleached enzyme was diluted into 0.1 M sodium borate buffer, pH 9.0, at 25° C and the spectrum shown in curve 1 was recorded. The temperature of the sample was then lowered to 0° C and curve 2 was recorded when the spectral changes were complete (2 h). Curve 1 was obtained when the temperature of the solution was increased back to 25° C. (The 375 nm absorption band observed for glycollate oxidase at pH 7.0 shifts to 335 nm at pH 9.0 due to ionization at the N(3) position of enzyme-bound FMN [1].)

Fig. 3. Effect of temperature on the apparent stability of the enzyme-sulfite complex at pH 9.0. Curve 1 is the spectrum of a sample of glycollate oxidase which did not exhibit autogenous bleaching in 0.1 M sodium borate buffer, pH 9.0, at 25° C. (The spectrum was not affected by lowering the temperature to 0° C.) Curve 2 was recorded immediately after addition of sulfite (0.31 mol/mol enzyme flavin). No further spectral changes were observed. The temperature of the sample was then decreased to 0° C and curve 3 was recorded when the spectral changes were complete (2 h). Curve 2 was obtained when the temperature was increased back to 25° C. (as compared with the preparation shown in Fig. 2, this preparation contains less 6-hydroxy-FMN and, therefore, exhibits somewhat different spectral properties particularly noticeable in the 430 nm region.)

and 9.0, respectively, and at 25°C were $3.6 \cdot 10^{-7}$ and $3.6 \cdot 10^{-5}$ M at the same respective pH values. These results show that complex stability decreases with increasing pH and temperature. The latter effect is especially pronounced at pH 9.0, where the dissociation constant observed at 25°C is more than 10-fold greater than the value obtained at 0°C. This effect is less marked at pH 7.0 where the complex is extremely stable even at 25°C, similar to results obtained in previous studies [1,7]. At pH 7.0 stoichiometric complex formation is observed except at ratios close to equimolar mixtures of sulfite and enzyme flavin. The stability of the enzyme-sulfite complex at pH 7.0 is dramatically illustrated by the fact that 80% of the complex remains intact even after dialysis for 48 h vs. 6 changes of a 50-fold excess of buffer at pH 7.0 and 5°C.

The final step in the preparation of glycollate oxidase involves dialysis at pH 7.0 and 5°C. The fact that some preparations contain EFMN · Y suggests that this complex is also very stable under these conditions. On the other hand, if Y is not a modified amino acid residue it might be removed by dialysis at pH 9.0 and 25°C, since the stability of EFMN · Y is considerably decreased under these conditions. To test this hypothesis a sample of autogenously-bleached enzyme was dialyzed at pH 9.0 and 25°C. The absorption spectrum of the dialyzed enzyme was similar to that observed for undialyzed enzyme at pH 9.0 and 25°C but, unlike the latter, did not alter when the temperature was lowered to 0°C. The dialyzed sample did not exhibit a lag even after incubation at 0°C for 24 h. An undialyzed control sample was maintained at pH 9.0 and 25°C for the same length of time as the dialyzed sample. A lag did reappear in this sample after the temperature was lowered from 25 to 0°C. The results indicate that the factor Y is not destroyed at pH 9.0 and 25°C, but is a small molecule which is removed by dialysis under these conditions. Studies with authentic enzyme-sulfite complex shows that sulfite can also be completely removed from the enzyme by dialysis at pH 9.0 and 25°C.

The absorption spectrum of autogenously-bleached enzyme at pH 7.0 and 25°C is compared in Fig. 4 with the spectrum of the supernatant obtained after heat denaturation. The latter spectrum is characteristic of protein-free uncomplexed FMN. Evidence for the presence of Y in the heat extract was sought by mixing the latter with a sample of enzyme from a preparation which did not exhibit autogenous bleaching. EFMN · Y was formed in this experiment as evidenced by the appearance of a lag in the catalytic assay. Since Y is present in the heat extract the results indicate that the stability of the FMN · Y complex is lost when the coenzyme is released from the protein. This is consistent with the observed similarities between EFMN · Y and EFMN · SO_3 and the fact that the affinity of free FMN for sulfite (K_d = 1.9 M) [4] is negligible as compared with FMN bound to glycollate oxidase. Previous studies indicate that EFMN · Y exhibits negligible absorbance at 450 nm, similar to EFMN · SO_3 [1]. The total concentration of enzyme-bound FMN (EFMN + EFMN · Y = $4.62 \cdot 10^{-5}$ M) was calculated based on the amount of free FMN in the heat extract. The concentration of EFMN ($3.46 \cdot 10^{-5}$ M) was estimated from the absorbance of the undenaturated sample at 450 nm ($\epsilon = 11.7 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$, determined for a preparation which did not exhibit autogenous bleaching [1]). This analysis yields a value of $1.16 \cdot 10^{-5}$ M for the concentration of EFMN · Y at pH 7.0, which corresponds to 25% of the total flavin in the preparation. The

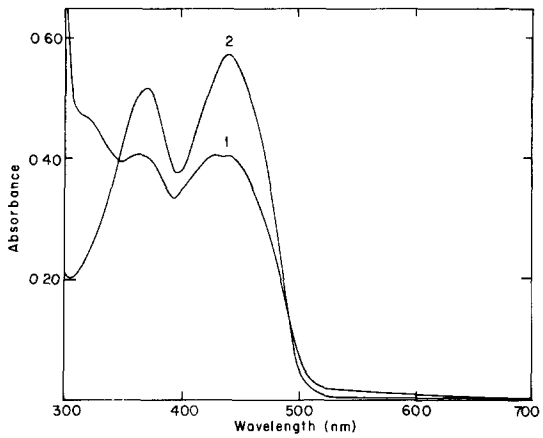


Fig. 4. Effect of heat denaturation on the spectrum of autogenously-bleached enzyme. Curve 1 is the spectrum of the enzyme in 0.1 M sodium phosphate buffer, pH 7.0, at 25° C. The sample was denatured by heating for 2 min at 100° C in a stoppered tube protected from light. The spectrum of the supernatant (Curve 2) was recorded after removing the protein precipitate.

same value was obtained for the percent of total flavin, present as EFMN · Y, in a separate analysis performed after diluting the enzyme 5-fold with buffer at pH 7.0. That the complex does not dissociate upon dilution provides further evidence for its stability at pH 7.0 and suggests that the concentration of Y in the heat extract is approximately stoichiometric with the amount of EFMN · Y in the undenaturated sample.

The observed similarities between EFMN · Y and EFMN · SO_3 prompted studies to determine whether the heat extract contained sulfite. Sulfite can be determined spectrophotometrically [6] based on the amount of 4-mercaptopyridine (λ_{max} = 324 nm) formed during reaction with 4,4′-dithiodipyridine (Eq. 2, R = C_5H_4N).

$$RSSR + SO_3^{2-} \rightarrow RS^- + RSSO_3^- \qquad (2)$$

However, a small amount of protein (approx. 5%) remains in solution after heat denaturation and residual protein sulfhydryl groups could interfere with this procedure. (The number of cysteine residues in glycollate oxidase is not known but the enzyme contains 7 half-cystine residues [1] vs. 0.25 mol Y/mol FMN estimated for the preparation of bleached enzyme). On the other hand, formation of the Bunte salt ($RSSO_3^-$) would provide unambiguous evidence for sulfite. The Bunte salt exhibits an absorption maximum at 265 nm (ϵ = 6000 $M^{-1} \cdot cm^{-1}$ [6]) and can be separated from other ultraviolet-absorbing reaction components by TLC (chloroform/methanol (7 : 3)). A spot attributed to the Bunte salt (R_F = 0.30 ± 0.02) was observed after reaction of excess 4,4′-dithiodipyridine with millimolar concentrations of sulfite but not after reaction with a similar amount of dithioerythritol. A faint spot, (R_F = 0.41) which migrates ahead of the Bunte salt, was observed in this experiment after reaction of 4,4′-dithiodipyridine with either sulfite or dithioerythritol and is probably due to 4-mercaptopyridine. Although this compound proved difficult

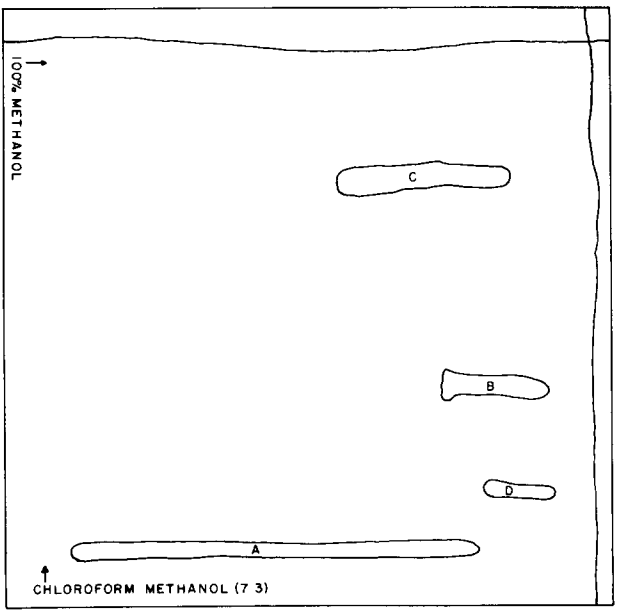
to detect, other studies showed that spots containing as little as 5 nmol of the Bunte salt could be easily detected.

A modified two-dimensional chromatography procedure was developed for analysis of dilute solutions of the Bunte salt. In this procedure the reaction mixture is applied as a streak, the plate is developed using the solvent system described above, and then the separated product streaks are concentrated into small spots with methanol (Fig. 5). Based on the observed R_F values, spots B and C in Fig. 5 are attributed to the Bunte salt and unreacted 4,4′-dithiodipyridine, respectively. Spot B was eluted from the plate and showed an absorption maximum at 265 nm as expected for the Bunte salt (Fig. 6). The yield of the Bunte salt varied between 38—100% in 10 separate experiments with an average of 58%. The time required to streak and develop the plates by this procedure may cause some decomposition as evidenced by spot D which was not identified. A spot due to 4-mercaptopyridine was not detected.

Addition of 4,4′-dithiodipyridine to the heat extract from autogenously-bleached enzyme results in the formation of 4-mercaptopyridine as evidenced by increases in absorption at 324 nm. The results shown in Fig. 7 were obtained when the reaction mixture was subjected to the modified two-dimensional chromatography procedure. Based on the observed R_F values, spots B′, C′ and D′ correspond to spots B, C and D observed for the reaction of sulfite with 4,4′-dithiodipyridine. The material eluted from spot B′ (R_F = 0.29) exhibits an absorption spectrum identical to the authentic Bunte salt (Fig. 6). The eluates from spots B and B′ were rechromatographed using a different solvent system (ethanol/dichloromethane (3 : 2)). The material from the heat extract of glycollate oxidase showed a single spot with the same R_F value (0.52) as the authentic Bunte salt.

The amount of the Bunte salt recovered from spot B′ (9.5 nmol) is about 1.5-fold greater than the amount of Y estimated for the sample used in these studies (6.0 nmol). A yield of 37% is calculated for the Bunte salt based on the amount of 4-mercaptopyridine formed during reaction of the extract with 4,4′-dithiodipyridine. The observed yield is less than the average (58%) but almost within the range (38—100%) observed in control studies. Based on these results it is difficult to determine whether some of the 4-mercaptopyridine was formed by reaction of 4,4′-dithiodipyridine with residual protein sulfhydryl groups. However, the amount of the Bunte salt recovered can be regarded as a minimal estimate of the amount of sulfite present in the heat extract. The fact that this is greater than the amount predicted, based on the concentration of bleached enzyme observed before denaturation, suggests that additional sulfite was released during the heat step. That the purified enzyme may contain a potential source of sulfite which is slowly released at 0°C is suggested by the fact that bleaching is observed for some preparations during several weeks of storage at 0°C [1]. It is significant that the enzyme is typically stored under conditions where it forms an extremely tight complex with sulfite and that EDTA is included in all buffers. Humphrey et al. [6] have shown that the instability observed for aqueous solutions of sulfite is due to oxidation catalyzed by metal ions and that sulfite solutions are stable in the presence of EDTA.

The presence of variable amounts of sulfite in glycollate oxidase preparations can account for the observed variability in the extent of autogenous bleaching



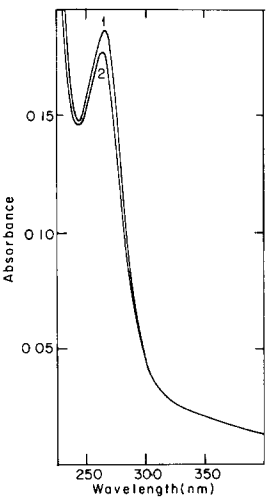


Fig. 5. Chromatography of dilute reaction products formed with 4,4′-dithiodipyridine and sulfite. The reaction mixture, prepared as described in Methods, was applied to the plate as a streak. The chromatogram was developed with chloroform/methanol (7 : 3) and the streaks were then concentrated with methanol in the directions indicated. The position of the original streak is indicated by FMN (A) which does not migrate in either solvent system. FMN was detected by its fluorescence. The other compounds were detected as dark spots under short wavelength ultraviolet light.

Fig. 6. Absorption spectra of the isolated Bunte salt. Curve 1 and 2 were obtained for the material eluted from spot B′ (Fig. 7) and spot B (Fig. 5), respectively.

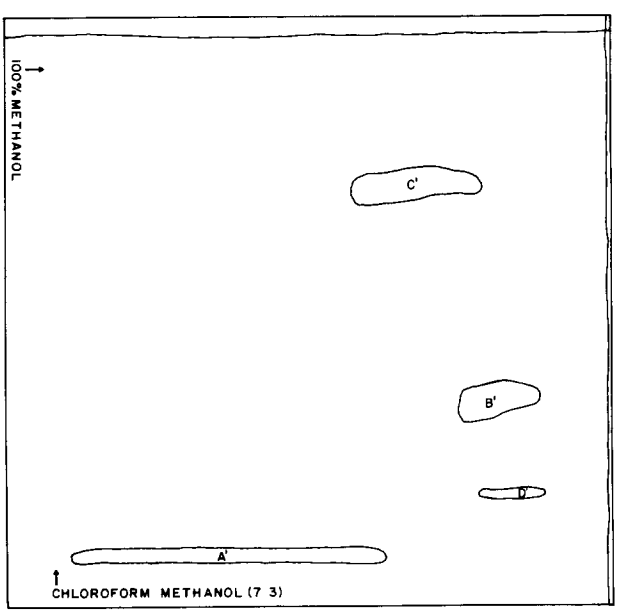


Fig. 7. Chromatography of reaction products formed with 4,4′dithiodipyridine and the heat extract from bleached enzyme. The heat extract (Fig. 4) was reacted with 4,4′-dithiodipyridine ($2.8 \cdot 10^{-4}$ M), the sample (0.52 ml) was applied to the plate as a streak and the chromatogram developed as described in Fig. 5.

including the fact that some preparations never exhibit evidence for this phenomenon. The results of these studies show that the effect of sulfite on the catalytic and spectral properties of the enzyme can be eliminated by dialysis under conditions which decrease the stability of the complex formed with enzyme-bound FMN. While the source of sulfite in glycollate oxidase preparations remains unclear, it may be pertinent that oxidation of cystine under certain conditions yields *S*-sulfocysteine ($CyS\text{-}SO_3^-$) as a major product [8] since sulfite could be generated from the latter via nucleophilic attack at the labile *S*-sulfo linkage.

Acknowledgements

This research was supported in part by a grant from the National Institutes of Health (GM 22662).

References

1 Schuman, M. and Massey, V. (1971) Biochim. Biophys. Acta, 227, 500—520
2 Mayhew, S.G., Whitfield, C.D., Ghisla, S. and Schuman-Jorns, M. (1974) Eur. J. Biochem. 44, 579—591
3 Massey, V., Müller, F., Feldberg, R., Schuman, M., Sullivan, P.A., Howell, L.G., Mayhew, S.G., Matthews, R.G. and Foust, G.P. (1969) J. Biol. Chem. 244, 3999—4006
4 Müller, F. and Massey, V. (1969) J. Biol. Chem. 244, 4007—4016
5 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
6 Humphrey, R.E., Ward, M.H. and Hinze, W. (1970) Anal. Chem. 42, 698—702
7 Jorns, M.S. and Hersh, L.B. (1976) J. Biol. Chem. 251, 4872—4881
8 Savige, W.E. and Maclaren, J.A. (1966) in The Chemistry of Organic Sulfur Compounds (Kharasch, N. and Meyers, C.Y., eds.), Vol. 2, pp. 367—402, Pergamon Press, Oxford