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PURIFICATION AND SOME PROPERTIES OF AN ALTERNATE FORM OF GLYCOLATE OXIDASE

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SUMMARY

An alternate form of glycolate oxidase (glycolate: O_2 oxidoreductase, EC 1.1.3.1) has been isolated and extensively purified from etiolated wheat by means of alumina C-γ gel adsorption, Sephadex G-200, and TEAE-cellulose chromatography. At any stage of purity the enzyme immediately lost all activity in the absence of substrate. The alternate form of glycolate oxidase was also inactivated by $(NH_4)_2SO_4$ and ethanol fractionation procedures which had been used to isolate the original form of the oxidase. Addition of FMN does not reactivate the protein.

The purified alternate form of glycolate oxidase did not have an absorption spectrum characteristic of FMN. Rather, maximum peaks were at 420, 320, and 278 mμ resembling spinach ferredoxin. The activity of the enzyme was enhanced by the addition of either Clostridium or plant ferredoxin but not FMN. By analysis the enzyme contained some FMN. The pH optimum was at 8.3 and the initial products of the reactions were glyoxylate and H_2O_2.

The 2 forms of glycolate oxidase can be differentiated by their spectral properties, by precipitation with $(NH_4)_2SO_4$, by their response to added FMN, and by inhibitors. The alternate form of the enzyme is inhibited by *p*-chloromercuribenzoate (PCMB), while the original form is relatively insensitive to this inhibitor. A preliminary survey indicated that the alternate form of the oxidase is most prevalent in young plant tissue.

INTRODUCTION

The glycolate pathway is an active and ubiquitous system in green plants[1]. The enzymic oxidation of glycolate to glyoxylate and hydrogen peroxide by glycolate oxidase (glycolate: O_2 oxidoreductase, EC 1.1.3.1) has been investigated in several laboratories. The demonstration of the oxidase in tobacco leaves[2,3], was followed by its further purification[4,5] and subsequent crystallization[6] from spinach leaves. Flavin mononucleotide (FMN) is required for maximum activity of the enzyme and has been the only known cofactor[4-6].

Later a substrate-protected form of glycolate oxidase was demonstrated in

Abbreviation: PCMB, *p*-chloromercuribenzoate.

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extracts from etiolated barley and wheat leaves[7,8]. The activity of this latter form of the oxidase, when isolated in the presence of substrate, was relatively unaffected by additional FMN. The present report describes the partial purification and some properties of this substrate-protected or alternate form of glycolate oxidase, as well as how this form of the enzyme can be differentiated from the enzyme originally described. Evidence is presented which implies the coupling of the alternate form of glycolate oxidase to a ferredoxin-like carrier.

EXPERIMENTAL METHODS

Sodium glycolate was purchased from Matheson, Coleman and Bell; aged alumina C-γ from Sigma Chemical Company; Sephadex G-200 from Pharmacia and TEAE-cellulose from Serva Company. Twice crystallized catalase was obtained from Worthington Biochemical Corporation. Spinach ferredoxin was a gift from Dr. N. GOOD of Michigan State University; Clostridium ferredoxin samples were gifts from Dr. R. H. BURRIS of the University of Wisconsin and from Worthington Biochemical Corporation.

Glyoxylate was determined by the method of DIXON AND KORNBERG[9]. Protein was estimated according to the method of WARBURG AND CHRISTIAN[10].

Glycolate oxidase or glycolate:O_2 oxidoreductase (EC 1.1.3.1) was measured manometrically[2]. In the main part of the Warburg vessel were placed 0.05–0.3 unit of enzyme and 2 μmoles of sodium glycolate in 2.5 ml of 0.08 M potassium phosphate buffer (pH 8.3). The maintenance of substrate with the enzyme at all times necessitated this addition of excess glycolate when preincubating the enzyme in the vessel. After 5 min equilibration at 30°, zero time was designated when the manometers were closed and an additional 0.5 ml of 0.02 M sodium glycolate was added from the side arm into the reaction mixture. The center well contained KOH. One unit of activity was defined as the amount of enzyme required for the uptake of 1 μmole of O_2 per min during a 25-min period of incubation under the above conditions.

PURIFICATION OF ENZYME

In the present work a form of the enzyme has been partially purified which has different properties and cofactor requirements than the FMN-requiring glycolate oxidase which has previously been isolated from many sources. In this manuscript the new form of the enzyme will be referred to as the "alternate form of glycolate oxidase". References to glycolate oxidase as described in previous literature will be referred to as the "original form of glycolate oxidase". The alternate form of glycolate oxidase also has been previously referred to as a proenzyme[8,11], but since an active oxidase can be obtained by preparation with substrate without addition of FMN, the proenzyme term is incorrect.

Crude extract

Etiolated wheat, *Triticum vulgare* L, variety Thatcher, was grown in moist vermiculite at 20° in total darkness for 7–8 days. The leaves were harvested, washed in cold tap water, and frozen in plastic bags at —20°. The frozen leaves may be stored for weeks before use. Prior to extraction, 4 kg of frozen tissue were thawed

at 2° and cut into inch segments. All subsequent steps were conducted in a 2° cold room. Four 1-kg weights of tissue, without added solvent, were each homogenized with 1 g of sodium glycolate in a large Waring blender. The homogenates were squeezed through several layers of cheese cloth, combined, and centrifuged at 15000 × *g* for 30 min to remove debris. The enzyme must be maintained in the presence of excess substrate (generally 0.01 M) through all extraction and purification procedures. Otherwise activity was lost even at 2°.

Acid treatment

To the crude wheat extract cold acetic acid was added until the pH was 5.3. After centrifuging at 15000 × *g* for 15 min to clarify, the supernatant fluid was adjusted to pH 6.5 with KOH. Due to instability of the enzyme at pH 5.3, this procedure was conducted as rapidly as possible. However, the acid treatment removed a large bulk of protein, and if omitted, subsequent purification steps were less efficient. After adjusting to pH 6.5, the enzyme was stable at this stage of purification if substrate were present and could be stored at −20° for several weeks with little loss in activity.

Fractionation with alumina C-γ gel

To the enzyme solution at pH 6.5 was added sufficient alumina C-γ gel (approx. 15 g dry weight) to effect total adsorption of the activity. The gel was removed by centrifugation, and in order to remove extraneous protein, the gel was washed 1 h with 4 l of 0.1 M Tris–acetate buffer (pH 7.0) containing 0.01 M sodium glycolate. Following centrifugation to discard the Tris–acetate wash, the enzyme was eluted from the gel by washing it for 90 min with 250 ml of 0.1 M potassium phosphate buffer (pH 8.3) containing 0.01 M sodium glycolate. The eluent was lyophilized with relatively small loss of activity.

Sephadex G-200 chromatography

The lyophilized alumina gel preparation was dissolved in 0.02 M sodium glycolate to a vol. of 30 ml and chromatographed on a Sephadex G-200 column (4 cm × 70 cm). The Sephadex was prepared by swelling for at least 3 weeks in advance in 0.1 M potassium phosphate buffer (pH 8.3) containing 0.01 M sodium glycolate. After packing the column, equilibration was achieved with the buffer containing substrate before sample introduction. If the Sephadex and subsequent TEAE-cellulose

TABLE I

PURIFICATION OF ENZYME

Fraction	*Total protein (mg)*	*Total units activity*	*Units per mg protein*	*Recovery (%)*
Leaf extract	114 300	768	0.0067	100
Acid treated	69 200	652	0.0094	85
Alumina C-γ gel	4 200	261	0.0622	34
Sephadex G-200	865	154	0.178	20
TEAE-cellulose	74	115	1.56	15
Sephadex G-200	12	38	3.16	5

columns were not completely equilibrated with substrate as well as buffer, activity was lost. Elution from the Sephadex was accomplished with the same buffer at a flow rate of 60 ml/h. Fractions (12 ml) with specific activities in excess of 0.13 μmole O_2 per min per mg protein were pooled for further purification.

TEAE-cellulose chromatography

The combined active fractions from the Sephadex column were dialyzed 4 h against 20 vol. of solution containing 0.01 M sodium glycolate and 0.001 M cysteine. The enzyme was then passed through a TEAE-cellulose column (2.2 cm $\times$ 40 cm) which had been pre-equilibrated with 0.01 M potassium phosphate buffer (pH 8.3) containing 0.01 M sodium glycolate. The activity was not absorbed, and emerged with the front. The average results of several such preparations are presented in Table I. Although variations existed with initial specific activities and with the gel adsorption step, an over-all yield of 15 % with an increase in specific activity in excess of 250-fold over the crude extract was generally obtained.

Further purification may be obtained by lyophilizing the eluent from the TEAE-cellulose column and then passing the concentrated enzyme through a second Sephadex G-200 column as previously described. The increase in specific activity over the TEAE-cellulose-purified enzyme was 2-fold. However, there was generally a relatively great loss of active protein which accompanied this step. Enzyme of this purification (500-fold) was used to establish the spectral character of the alternate form of glycolate oxidase.

Difference in purification procedures

The alternate form of glycolate oxidase has been partially purified by a procedure which did not involve precipitation of the enzyme. Unlike the original glycolate oxidase, the enzyme reported here is unstable to fractionation by $(NH_4)_2SO_4$ (refs. 2, 4, 6) and ethanol precipitation[4,6], and for this reason has gone undetected in preparations of glycolate oxidase. Precipitation by ethanol of the alternate form of the enzyme resulted in total inactivation, irreversible with added FMN. If the alternate form of the enzyme in the crude extract were dialyzed or treated with $(NH_4)_2SO_4$, it was inactivated. If an excess of FMN were added to a redissolved fraction, which had been precipitated by 35–50 % satd. $(NH_4)_2SO_4$ from the crude extract, full recovery of enzyme activity was possible. However, the protein no longer had the properties of the alternate form of the enzyme. By contrast the original form of the enzyme would have been precipitated by 24–35 % satd. $(NH_4)_2SO_4$. These differences to $(NH_4)_2SO_4$ fractionation clearly differentiate between the 2 forms of the oxidase. The alternate form of the enzyme when partially purified as described in Table I could not be precipitated by $(NH_4)_2SO_4$ or dialyzed with retention of activity. Further the partially purified protein fractions after precipitation or dialysis could not be reactivated with excess FMN.

RESULTS AND DISCUSSION

Reactions catalyzed

The original form glycolate oxidase catalyzes the oxidation of glycolate to glyoxylate[3–5] and H_2O_2 (refs. 4, 5) and may further catalyze the oxidation of glyoxy-

late to oxalate[12]. The alternate form of the oxidase also catalyzes the initial formation of glyoxylate and H_2O_2. Glyoxylate formation was measured spectrophotometrically by trapping the glyoxylate with phenylhydrazine. H_2O_2 production was assumed upon the basis of catalase inhibition of O_2 uptake and CO_2 evolution (Fig. 1). The inhibition of O_2 uptake is due to H_2O_2 decomposition to O_2 so that the observed net O_2 uptake is less. The inhibition of CO_2 evolution is due to removal of H_2O_2 so that the non-enzymic oxidation of glyoxylate by H_2O_2 is inhibited.

Effect of pH

The effect of pH on the activity of the alternate form of glycolate oxidase is shown in Fig. 2. The maximum activity of the enzyme is at pH 8.3, the optimum for the original form of the oxidase[3]. The use of Tris buffer resulted in a 27 % inhibition of activity at pH 8.3. In one report the original form of the oxidase was also inhibited by Tris buffer[12], while in another report the enzyme activity was maximum in Tris[4].

Effect of inhibitors

The alternate form of glycolate oxidase is irreversibly inhibited by low concentrations of *p*-chloromercuribenzoate (PCMB). A concentration of $1 \cdot 10^{-4}$ M PCMB, without preincubation with the enzyme, caused a 50–100 % loss of activity which could not be reversed with excess cysteine. This was true for the enzyme at all stages of purification including the original plant extract. The original form of the oxidase

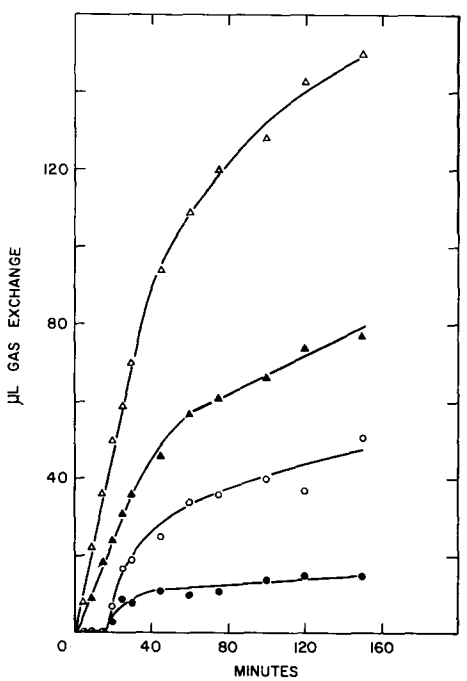

Fig. 1. Effect of catalase on O_2 uptake and CO_2 evolution by glycolate oxidase. The standard incubation mixture consisted of 150 μmoles of phosphate buffer (pH 8.3), 0.12 unit of TEAE-cellulose-purified glycolate oxidase and 10 μmoles of substrate in a vol. of 3 ml. Catalase was added at a final concn. of 1.2 mg/ml. In controls: △—△, O_2 uptake; ○—○, CO_2 evolution; in the presence of catalase: ▲—▲, O_2 uptake; ●—●, CO_2 evolution.

required a concentration of $5 \cdot 10^{-2}$ M PCMB and an hour preincubation before significant loss in activity could be demonstrated[6]. The use of PCMB at about $1 \cdot 10^{-3}$ M could provide an assay procedure to differentiate between the 2 forms of the enzyme in a mixture of both.

Other sulfhydryl reagents, including iodoacetate, 5,5'-dithiobis-(2-nitrobenzoate), and *N*-ethylmaleimide at $1 \cdot 10^{-3}$ M, had no inhibitory effect on the initial activity of the enzyme. Similarly the enzyme was uneffected by $1 \cdot 10^{-2}$ M sodium

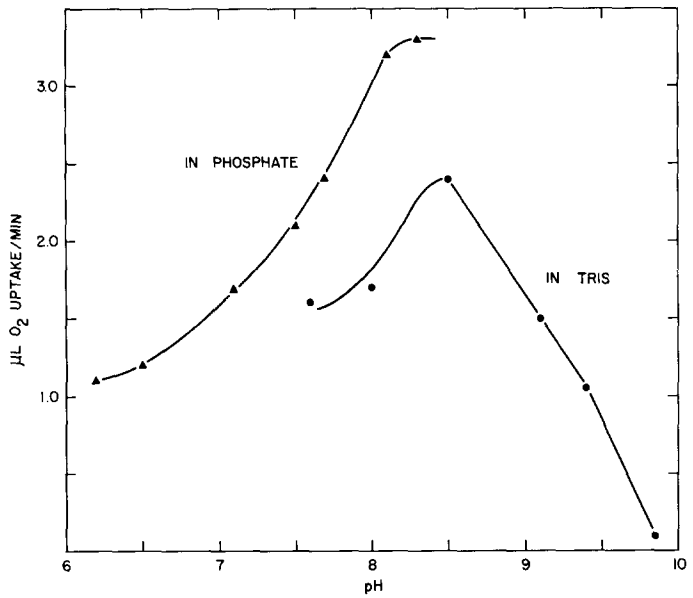

Fig. 2. Rate of oxidation of glycolate as a function of pH in phosphate and Tris–acetate buffers. The reaction vessels contained 200 μmoles of buffer, 0.15 unit of TEAE-cellulose purified glycolate oxidase and 10 μmoles of substrate in a vol. of 3 ml.

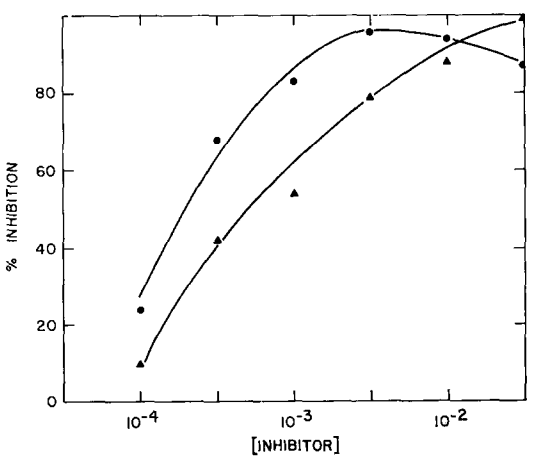

Fig. 3. Effect of sulfonate inhibitors on glycolate oxidase. Reaction mixtures consisted of 150 μmoles of phosphate buffer (pH 8.3), 0.12 unit of TEAE-cellulose-purified glycolate oxidase, 10 μmoles of substrate and inhibitor as indicated, in a total vol. of 3 ml. ●—●, 2-pyridylhydroxymethane sulfonate; ▲—▲, hydroxymethane sulfonate.

azide, sodium cyanide or EDTA. This insensitivity of the enzyme to these inhibitors is similar to the properties of the original oxidase.

ZELITCH[13] has used certain hydroxymethanesulfonic acid derivatives as competive inhibitors of the original glycolate oxidase. The effect of 2 of these inhibitors, α-hydroxy-2-pyridinemethanesulfonate and hydroxymethanesulfonate, on the alternate form of the enzyme is shown in Fig. 3. At concentrations of 1×10^{-3} M both compounds are potent inhibitors of enzymic activity.

Cofactor requirement and absorption spectra

One distinguishing characteristic between the alternate form of glycolate oxidase and the original oxidase is the different response of each to added FMN. Several 100-fold increases in activity and specific activity were obtained with the original form of the enzyme when FMN was added in concentrations of $2 \cdot 10^{-5}$–$2 \cdot 10^{-3}$ M. In fact crystallization of the enzyme was done in the presence of $2 \cdot 10^{-3}$ M FMN (ref. 6). In contrast to this was the relatively small response of the alternate form of the enzyme to FMN added at any stage throughout the purification sequence. The increase in activity of the enzyme due to added FMN was less than 20 % once purification was

TABLE II

EFFECT OF $1 \cdot 10^{-4}$ M FMN ON GLYCOLATE OXIDASE

Standard assay mixture contained in a final volume of 3 ml, 150 μmoles of phosphate buffer (pH 8.3), 10 μmoles of substrate and enzyme at the different stages of purification. To this assay FMN was added at a final concn. of $1 \cdot 10^{-4}$ M.

Stage of purification	*Increase in activity* (%)
Leaf extract	33
Alumina C-γ gel	16
Sephadex G-200	19
TEAE-cellulose	12

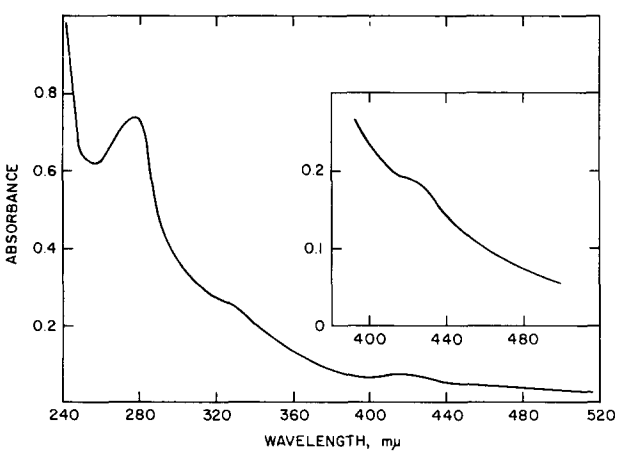

Fig. 4. Absorption spectrum of glycolate oxidase purified through the second Sephadex G-200 chromatography step; protein concn. was 0.6 mg/ml in 0.1 M phosphate buffer (pH 8.3) containing 0.01 M substrate. For data in the insert, the protein concn. was 1.8 mg/ml.

initiated (Table II). It has also been noted that FMN was unable to reverse loss of activity of the purified enzyme after dialysis against buffers not containing substrate.

While the originally purified oxidase is bright yellow in color, the alternate form of the oxidase when purified has been nearly colorless at equivalent concentrations. A comparison between the alternate form of the enzyme purified to a specific activity of 3.2 units/mg protein has been made with the published properties of the crystallized original oxidase with a specific activity of 9.4. A very pronounced FMN spectrum was obtained with 0.75 mg of the original oxidase[6]. The visible and ultraviolet absorption spectrum of the alternate form of glycolate oxidase (1.8 mg/ml) is presented in Fig. 4. There is no FMN spectrum, but absorption maxima at 420, 320 and 278 mμ are similar to those for spinach ferredoxin[14]. It should be noted that these spectral characteristics persist after 2 steps in the purification sequence which should have removed free plant ferredoxin from the enzyme; *i.e.*, TEAE-cellulose and Sephadex G-200 chromatography. The ferredoxin-like material was apparently necessary for both activity and stability of the enzyme and this made further purification difficult. Since the alternate form of the oxidase has not been crystallized nor proof of protein homogenity been obtained, a study of the function of the ferredoxin-like materials in the preparation was attempted by addition of ferredoxin. In the standard assay a 40 % increase in rate of activity of the enzyme was obtained with 0.41 mg/ml of Clostridium ferredoxin and 30 % increase with 0.08 mg of ferredoxin. Similar results were obtained with a spinach ferredoxin fraction. A ferredoxin-like material from our enzyme preparation stimulated activity of the alternate glycolate oxidase to a somewhat greater extent than the bacterial or spinach ferredoxin. This ferredoxin-like fraction was obtained best when the Sephadex G-200 chromatographic step of the purification procedure described in Table I was omitted. Then after removal of the alternate form of the oxidase from the TEAE-cellulose column, subsequent elution of the column with 0.1 M phosphate (pH 8.3) yielded a red-brown ferredoxin-like protein fraction.

Even though the alternate form of glycolate oxidase does not have an FMN spectrum and is not stimulated by FMN, a small amount of FMN can be obtained by mild acid hydrolysis from our most purified preparation of the alternate form of glycolate oxidase. The FMN was detected by its fluorescence after paper chromatography in propionic acid–butanol–water (1:2:1.4, by vol.). The R_F was 0.22 as compared with values of 0.20 for authentic FMN and 0.08 for FAD.

Enzymatic reduction of ferredoxin-like material has been achieved in the photosynthetic electron-transport system by ferredoxin:NADP reductase[14–16], in nitrogen fixation by a pyruvate:ferredoxin oxidoreductase[17,18], by acetaldehyde oxidation[19], and by the non-heme iron *plus* 'labile sulfide' characteristic of the mammalian NADH and succinic dehydrogenase systems[20]. All but one of these enzymatic systems have been demonstrated to be flavoproteins, and the possible association of ferredoxin with glycolate oxidase may be yet another case of a flavoprotein complex with ferredoxin. The purified alternate form of glycolate oxidase by itself could not reduce NAD or NADP in the presence or absence of added ferredoxin or in an aerobic or anaerobic atmosphere.

Distribution

Evidence for the alternate form of the enzyme in green as well as in etiolated

leaves has been obtained when a 50% increase in enzyme activity was obtained by grinding green wheat leaves[8] or Samsun tobacco leaves[11] with glycolate. Further, the differences in response to the 2 forms of the enzyme to $(NH_4)_2SO_4$ were used in a preliminary examination to determine the forms of the enzyme in Maryland Mammoth tobacco leaves. In extract from old leaves the glycolate oxidase activity was entirely precipitated by 21–33% satd. $(NH_4)_2SO_4$. In young tobacco leaves 75% of the enzyme was precipitated by 21–33% satd. $(NH_4)_2SO_4$, but 25% of the activity was in a second fraction precipitated by 35–50% satd. $(NH_4)_2SO_4$. These data suggest that more of the alternate form of the enzyme exists in younger green tissues. No information has been obtained to suggest a conversion of the alternate form of the enzyme *in vivo*, during aging, to the original form of the enzyme. However, as discussed with the purification of the enzyme, treatment of crude plant extracts with $(NH_4)_2SO_4$ apparently converts the alternate form of the oxidase to a form requiring excess FMN.

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