The Kinetic Properties of Spinach Leaf Glyoxylate Acid Reductase

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SUMMARY

The kinetic properties of spinach leaf glyoxylate acid reductase have been evaluated. In the presence of pyridine nucleotides, the enzyme catalyzes the reversible reduction of glyoxylate to glycolate and hydroxypyruvate to d(-)-glycerate. The pH optima of the reductive reactions are between 6.0 and 6.5; the pH optima of the oxidative reactions are at 8.0. The enzyme is competitively inhibited by pyruvate, 3-mercaptoacrylate, and 3-fluoropyruvate and noncompetitively inhibited by dihydroxyfumarate. A spectral shift in protein absorbance, from 280 nm to 270 nm, is associated with the dihydroxyfumarate inhibition.

Ternary rate equations of the enzyme and a degraded form of the enzyme have been calculated and compared. Specific changes in the Michaelis constant for pyridine nucleotides suggest that the degraded form has undergone structural modifications in the pyridine nucleotide binding site.

All forms of the enzyme are anion-regulated in the direction of d(-)-glycerate or glycolate formation. Phosphate stimulates hydroxypyruvate reduction at all anion concentrations; chloride, bromide, sulfate, and nitrate stimulate at low concentrations but inhibit at high. The anion effects are pH-dependent and are competitive with hydroxypyruvate or glyoxylate. They are noncompetitive with reduced pyridine nucleotides. Evidence is presented which suggests that a ligand-induced conformational change is important in the functioning of anion effectors.

A homogeneous form of spinach leaf glyoxylate acid reductase has been isolated from commercial crystalline preparations (1). The enzyme catalyzes the reduction of hydroxypyruvate as well as glyoxylate and has a molecular weight of 97,500 ± 5,000. It is composed of two equal-sized subunits which are dissociated by exposure to 6.0 M guanidine hydrochloride-0.1 M mercaptoethanol, 8.0 M urea-0.1 M mercaptoethanol, or iodoacetate. Analytical disc gel analyses, amino-terminal end group analyses, and peptide mapping indicate that the polypeptide chains of each subunit are identical or very similar in their primary structure. The subunits can reassociate to yield the native enzyme dimer and a nearly inactive enzyme tetramer.

The present report characterizes the kinetic properties of this enzyme. It shows that the products of hydroxypyruvate and glyoxylate reduction are d(-)-glycerate and glycolate, respectively. Anion modification of the reductive reaction is shown and a mechanism for these effects is suggested. Noncompetitive inhibition by solutions of dihydroxyfumarate is indicated.

The data are compared with results obtained from similar evaluations of several bacterial "hydroxypyruvate reductases" (2-4) and a tobacco leaf glyoxylate reductase purified by Zelitch (5). The kinetic properties of the enzyme are also compared to the kinetic properties of three additional forms of the protein which have been isolated and characterized (1). Two of these forms are considered isozymes in nature since they have the same molecular weight and amino acid composition as the enzyme above. The third is concluded to be a degraded form of the enzyme since it has a lower molecular weight and a different amino acid content (1). These forms have been shown to be readily distinguished by their specific activities, isoelectric points, and stabilities.

MATERIALS AND METHODS

All chemicals were obtained from commercial sources or were prepared as described (1). Procedures are noted in context.

Standard Enzyme Assays—Enzyme activity was determined spectrophotometrically by measuring at 340 nm the quantity of DPNH utilized or the quantity formed. For assays of enzyme activity toward hydroxypyruvate, the incubation mixture contained: potassium phosphate at pH 6.4, 60 μmoles; lithium hydroxypyruvate, 5 μmoles; an appropriate amount of enzyme; DPNH, 0.2 μmole. For measurements of enzyme activity toward glyoxylate, the incubation mixture contained: potassium phosphate at pH 6.4, 10 μmoles; sodium glyoxylate, 50 μmoles; an appropriate amount of enzyme; DPNH, 0.2 μmole. For assays of enzyme activity toward d(-)-glycerate or glycolate, the incubation mixtures contained Tris-chloride at pH 8.5, 200 μmoles; d(-)-glycerate, 5 μmoles, or glycolate, 25 μmoles; an appropriate amount of enzyme; DPN, 2 μmoles. The final volume of all incubation mixtures was 1.0 ml; the temperature was 25°C.

One unit of enzyme activity is defined as that quantity of DPNH utilized or formed per minute at 25°C.
enzyme catalyzing the formation of 1.0 μmole of reduced or oxidized pyridine nucleotide per min in the standard assay mixtures. Specific activity is defined as units per mg of protein; protein was determined colorimetrically (6) with crystalline bovine serum albumin as the standard.

Kinetic studies used a Beckman model 1DU spectrophotometer equipped with a Gilford cuvette changer or a Cary model 14 recording spectrophotometer. Cells had a 1 cm light path. Control cuvettes contained no enzyme or boiled enzyme, and all studies utilized stock enzyme solutions which were diluted 1 hour before use into potassium phosphate, pH 7.0, containing 0.1% bovine serum albumin (w/v). Thus diluted, the enzymes were stable for 24 hours (1). Albumin did not affect the kinetic data.

Enzyme Preparation—The homogenous enzymes utilized in these studies were obtained from crystalline preparations of spinach leaf glyoxylic acid reductase (Boehringer Mannheim). They were isolated by gel filtration chromatography, preparative gel electrophoresis, or isoelectric focusing as described (1). On analytical disc gels, the RF of the major enzyme form was 0.22; it catalyzed the reduction of 274 μmole of hydroxypyruvate and 91 μmole of glyoxylate per min per mg. The RF values of two isozymes having the same molecular weight as this major form were 0.19 and 0.17; they catalyzed the reduction of 240 and 195 μmole of hydroxyypyruvate or of 84 and 40 μmole of glyoxylate per min per mg, respectively. The degraded enzyme form with a lower molecular weight had an RF value of 0.27 and catalyzed the reduction of 180 μmole of hydroxyypyruvate or 71 μmole of glyoxylate per min per mg.

Enzymes were stored at -20 °C in 0.04 M Tris-chloride, pH 7.4, containing 20% glycerol (v/v) and 1 mM mercaptoethanol. Protein concentration during storage was 3 to 5 mg per ml.

RESULTS

Kinetic Properties of Major Enzyme Form, V, 0.22

Specificity and Kinetics of Forward Reaction—In the presence of DPNH the enzyme catalyzed the reduction of hydroxy- pyruvate and glyoxylate. In the presence of 0.15 mM reduced pyridine nucleotide, the following compounds did not serve as substrates when each was tested at a concentration range between 0.1 and 100 mM: pyruvate, β-mercaptopyruvate, fluoro- pyruvate, bromopyruvate, α-ketobutyrate, β-keto pyruvate, oxalacetate; dihydroxyfumarate, β-ketoglutarate, α-ketoglutarate, α-ketoisocaproate, phoshoenolpyruvate, glyoxal, acetaldehyde, glycolaldehyde, malonic semialdehyde, α-ketovalerolactone, dihydroxyacetone, d-serine, or l-serine. A preparation of tartronic semialdehyde was one-third as active as an equimolar concentration of hydroxy- pyruvate; at best, it was concluded to be a poor substrate since the tannic semialdehyde preparations contained enough hydroxy- pyruvate (30%) to account for the activity found (2, 3).

At pH 6.4 and in the presence of 3 mM hydroxy- pyruvate, the operational K, value2 for DPNH was 3.5 × 10^-4 M; at pH 6.4

2 These values are considered operational since they were not obtained at "infinite" concentrations of the opposite substrate and since simple Michaelis assumptions do not apply in bimolecular reactions. Limiting Michaelis constants for the two substrates, A and B, can be obtained from the subsequent rate equations and are the K, and K, terms, respectively (7). Their significance will depend upon the reaction mechanism (7, 8).

and, in the presence of 50 mM glyoxylate, the K, value for DPNH was 7.7 × 10^-4 M. With 0.15 mM TPNH, the K, value for hydroxy- pyruvate was 5.0 × 10^-4 M and the K, value for glyoxylate was 5.0 × 10^-4 M.

TPNH replaced DPNH as the reduced pyridine nucleotide, but not readily. At pH 6.4 and in the presence of 0.15 mM TPNH, the K, value for hydroxy- pyruvate was 1.4 × 10^-4 M but the K, value for glyoxylate could not be calculated. In the presence of 3 mM hydroxy- pyruvate or 50 mM glyoxylate, the K, values for TPNH were 4 × 10^-4 M and 1 × 10^-4 M, respectively. Maximal velocity values were at least 10-fold lower than those obtained in the presence of DPNH.

Kinetic analysis (7) of the reduction of glyoxylate or hydroxy- pyruvate by DPNH allowed the determination of the constants, K, K, and K, for the general rate equation (Equation 1).

In this equation, V_\text{max} designates the maximum velocity at a given enzyme concentration and v represents the observed velocity. K, and K, may be considered "limiting Michaelis constants" for the Substrate A and B (7). K, is a reaction constant describing the kinetic effects of having both substrates on the enzyme at one time (7). Data for all studies were obtained for initial rates of reaction, i.e. before 10% of the substrates had been utilized.

In Equations 2 and 3, respectively, values of the constants for the reduction of glyoxylate and hydroxy- pyruvate are presented. In these and subsequent equations, the values are the average of at least three separate experiments on different lots of enzyme and different batches of substrates. Experimental values were in satisfactory agreement with the curves calculated from these equations (Fig. 1). The results implicate the existence of a ternary complex involving enzyme, carbonyl substrate, and pyridine nucleotide but do not exclude a Theorell-Chance mechanism (7, 8).

Enzyme concentrations used to obtain data for Equation 2 were 10-fold higher than concentrations of enzyme needed to evaluate Equation 3. At the same enzyme concentration, the maximal velocity (V_\text{max}) of hydroxy- pyruvate reduction was 3.7-fold greater than that for glyoxylate catalysis.

Product—D(−)-Glyceric acid was identified as the product when the enzyme catalyzed the reduction of hydroxy- pyruvate; glycolic acid was produced from glyoxylate.

For the isolation of products, 3-ml cuvettes having a 1-cm light path were maintained at 25° and were monitored continuously by measurement of absorbance at 340 μM. DPNH
was the reduced pyridine nucleotide in all experiments; control cuvettes were identical with experimental ones, except for the substitution of boiled enzyme for active enzyme.

Four cuvettes were used to isolate the product of hydroxypyruvate reduction. In a total volume of 2 ml, each cuvette contained potassium phosphate at pH 6.4, 200 μmoles; lithium hydroxypyruvate, 150 μmoles; and enzyme, 0.05 mg. Over the course of 1 hour, 75 μmoles of reduced pyridine nucleotide were added to each cuvette in 5- to 10-μmole increments. Reactions were terminated by acidification to pH 2.0 with sulfuric acid. To monitor the isolation, tracer amounts of α-glyceric acid-1-14C (14 mCi per mmole) were added to one cuvette and the reaction mixtures were pooled, heated, charcoal-treated, absorbed to columns of Dowex 1-acetate, and eluted as previously described (2-4). Radioactivity was detected in the eluate with (9) or without (10) prior periodate treatment. A yield of 232 pmoles of glyceric acid was obtained, no glyceric acid being calculated that 283 pmoles of glycerate were produced from 300 μmoles of glyceric acid by means of the chromotropic acid reaction.

The product of hydroxypyruvate reduction had the same RF values as authentic DL-glyceric acid and DL-glyceric acid-3-14C when cochromatographed in the following solvent systems: (a) ethyl-formic-acid-water (5:2:1), RF 0.31; (b) 1-butanol saturated with 0.3 M formic acid, RF 0.26; (c) ethyl-acetic-acid-water (12:3:1), RF 0.13; (d) ethanol-ammonia-water (8:1:1), RF 0.46 and 0.17. The optical rotation [α]D, was +142.2 ± 1.0 for multiple samples of the sodium salt made from the product. In the presence of equimolar ammonium molybdate and product (18 to 25 mM), the [α]D for several samples was +111.2 and 120.8 when calculated for the sodium salt (2-4). At pH 8.5, in the presence of 2 mM DPN, 0.1 mM hydroxypyruvate, and the isolated product, lactic dehydrogenase did not cause an increase in 340 μm absorption; hydroxypyruvate reductase (2) did.

For the determination of the product of glyoxylate reduction experiments were identical with those above with the following modifications: the incubation mixtures contained 40 μmoles of potassium phosphate, pH 6.4, and 500 μmoles of glyoxylate; the heating step was eliminated; and glycolic acid-14C was used as the marker. Glycolic acid was eluted from the ion exchange columns between 150 and 180 ml (24) and was assayed by the spectrophotometrically pure, it was calculated that 283 μmoles of glycerate were produced from 300 μmoles of DPNH which had been observed, spectrophotometrically, to be oxidized.

The reaction was reversible with (−)-glyceric acid in the presence of DPN. It was linear with respect to time and proportional to protein concentration. At 25°C, an equilibrium constant of 1.61 × 1012 was calculated from the data in Table I and from Equation 4. This represents a ΔF of −16.7 kcal per mole at 25°C and of −7.1 kcal per mole at pH 7.0 at the same temperature.

\[
K_{\text{eq}} = \frac{[\text{glyceric acid}][\text{DPN}]}{[\text{hydroxypyruvic acid}][\text{DPNH}][\text{H}^+]} \quad (4)
\]

In the presence of DPN, the reaction was also reversible with glycolic acid, and at 25°C an equilibrium constant of 3.83 × 1014 was calculated from the data in Table II and from Equation 5. This represents a ΔF of −19.9 kcal per mole at 25°C and of −10.4 kcal per mole at pH 7.0 at the same temperature.

\[
K_{\text{eq}} = \frac{[\text{glycolic acid}][\text{DPN}]}{[\text{glyoxalic acid}][\text{DPNH}][\text{H}^+]} \quad (5)
\]
Specificity and Kinetics of Reverse Reaction—In Tris-chloride at pH 8.5, ternary kinetics was again observed and the following rate equations could be calculated for D(-)-glycerate or glycolic acid oxidation (Equations 6 and 7). In both cases, experimental data were in good agreement with the curves generated from these rate equations (Fig. 2).

\[ V_{\text{max}} = \frac{2.1 \times 10^4 \ \text{mM}}{\text{v}} + \frac{3.8 \times 10^4 \ \text{mM}}{\text{DPN}} + \frac{6.0 \times 10^{-4} \ \text{M}}{\text{[glycolate]}} \]  
\[ V_{\text{max}} = \frac{3.2 \times 10^4 \ \text{mM}}{\text{v}} + \frac{4.6 \times 10^4 \ \text{mM}}{\text{DPN}} + \frac{1.2 \times 10^{-5} \ \text{M}}{\text{[n-glycerate]}} \]  

The relative maximal velocity of the forward and reverse reactions was 30-fold greater in the direction of glycerate or glycolate formation under the stated conditions.

In the presence of 2 mM DPN and at pH 8.5 in Tris-chloride (0.2 mM) the following compounds did not serve as substrate at concentrations from 0.1 to 50.0 mM: ethanol, glycolaldehyde, isopropyl alcohol, glycerol, glyceraldehyde, 2-phosphoglyceric acid, 3-phosphoglyceric acid, phosphoglycerate, L(+)-glycerate, D- or L-lactate, malate, dihydroxyfumarate, L(+)-tartrate, n(-)-tartrate, or mesotartrate.

**pH Optima**—With standard assay conditions, the optimal pH for the reduction of glyoxylate was between 5.5 and 6.5 (Fig. 3A). For the reduction of hydroxypyruvate, the optimal activity was from pH 6.1 to 6.6 (Fig. 3B), and for the oxidation of glycerate or glycolate it was at pH 8.9 (Fig. 3C). Sensitivity of the pH curves to changes in the buffer concentration was noted and can be seen in Fig. 3B. Enzyme activity decreased much more sharply between pH 6.5 and 8.0 when the phosphate concentration was increased from 0.06 M to 0.1 M.

**Effect of Specific Anions and Anion Concentration on Enzyme Activity**—As with hydroxypyruvate reductase (2), tartronic semialdehyde reductase (3), and oxaloacetate reductive decarboxylase (4), the activity of the enzyme was markedly dependent upon the presence of various anions. With DPNH and hydroxypyruvate as substrates, the enzyme showed an optimum anion concentration beyond which inactivation occurred (Fig. 4). When glyoxylate was the carbonyl substrate, the activation phase was barely discernible with most anions (Fig. 4II) and the optimal enzymatic activity was obtained at lower anion concentrations. The most effective activator was phosphate and the most effective inhibitor was
Fig. 3. Activity as a function of pH with DPNH and glyoxylate as substrates (A), DPNH and hydroxypyruvate as substrates (B), and DPN and glyc erate or glycolate as substrates (C). TPNH could be substituted for DPNH and TPN for DPN without significantly changing the pH optima or the shapes of the curves. Buffers included sodium acetate (A), potassium phosphate (O), Tris-chloride (■), or sodium glycinate (△). Standard assay conditions were used. In B, the pH dependence under conditions in which the phosphate concentration was increased from 0.06 M to 0.1 M (— — —) is also presented.

Fig. 4. Effect of salts on the catalysis of hydroxypyruvate reduction (I) and glyoxylate reduction (II). Concentrations corresponding to zero salt represent basal buffer concentrations of either 25 mM potassium phosphate (I) or 10 mM potassium phosphate (II). Standard assay conditions were otherwise maintained. With the exception of ammonium bromide (△), for which only the noted cation was used, essentially identical curves were obtained with the ammonium, potassium, and sodium salts of the following anions; phosphate (•), sulfate (○), chloride (△), and nitrate (■). The pH was between 6.35 and 6.45 in each experiment.

Fig. 5. Enzyme activity as a function of increasing potassium phosphate concentration. Activity was measured under standard assay conditions except for the quantities and the pH of the potassium phosphate added. The data presented were obtained at pH 6.0 (■), pH 6.4 (○), pH 7.0 (△), and pH 8.0 (○); the data are plotted as a double reciprocal kinetic study to separate more clearly the effects at high and low anion concentrations.

Fig. 6. Effect of salts on the catalysis of 3-glycerate oxidation. Zero salt represents a 20 mM Tris-chloride concentration in otherwise standard assay conditions. No difference was detected between the sodium or potassium salts of nitrate (■), chloride (△), or phosphate (○).

Anion inhibition by nitrate was competitive with hydroxypyruvate or glyoxylate (K_i, 1.5 × 10^{-4} M) and was noncompetitive with the reduced pyridine nucleotide (K_i, 4 × 10^{-4} M). Inhibition by all other anions was also competitive with the carbonyl substrates, but not pyridine nucleotides.

Similar anion effects could not be shown in the direction of 3-glycerate oxidation when the reactions were examined at pH 8.0 and 8.8. Like tartronic semialdehyde reductase (3), however, significant increases in enzyme activity occurred with an increasing concentration of Tris-chloride buffer (Fig. 6). Since potassium and sodium chloride had no such effect, this activation is probably attributable to the tris(hydroxymethyl)-aminomethane moiety.

Effect of Anion Concentration on Enzyme Structure—No significant changes in sedimentation or diffusion coefficients were found under the different conditions of anion concentration.
GEL FILTRATION

FIG. 7. Gel filtration chromatography of tritiated glyoxylic acid reductase in low salt (A and B) and high salt (C and D) at pH 7.3. Sephadex G-25 columns, 2 X 15 cm, were used; the protein load was 2.5 mg. Protein was tritiated in low salt before filtration on Columns A and C and in high salt for Columns B and D. The temperature was 25°, and the flow rate was 1 ml per min. Fractions of 2 ml were collected. Low salt was 0.01 M Tris-chloride; high salt was 0.2 M Tris-chloride.

FIG. 8. Gel filtration chromatography of tritiated glyoxylic acid reductase in increasing concentrations of potassium phosphate, pH 6.4. Sephadex G-25 columns, 2 X 15 cm, were used; protein load was 2.5 mg. Protein was tritiated and equilibrated in 7.5 mM potassium phosphate, pH 6.4, before gel filtration. The temperature was 25°, and the flow rate was 1 ml per min. Fractions of 2 ml were collected. Columns run at 50, 100, and 150 mM potassium phosphate were the same as the column run at 40 mM potassium phosphate.

(0.01 to 0.2 M potassium phosphate at pH 6.4 or pH 6.0); however, by using tritium labeling and gel filtration chromatography (13), effects of ionic strength on secondary structure (14, 15) could be shown. Glyoxylic acid reductase was equilibrated with 0.01 M Tris-chloride, pH 7.3 (low salt concentration), or with 0.2 M Tris-chloride, pH 7.3 (high salt concentration), or both, by dialysis for 24 hours in a 6000-fold excess of buffer. Ten microliters of tritiated water (100 mCi per g) were added to 5-mg aliquots of the enzyme and the solutions were incubated for several days at 0-2° in order to establish the hydrogen-tritium exchange equilibrium. Aliquots of the labeled proteins were applied to columns (2 X 15 cm) of Sephadex G-25 and eluted at a flow rate of 1 ml per min; one column was equilibrated with 0.01 M Tris-chloride at pH 7.3 and the other with 0.2 M Tris-chloride at pH 7.3. Effluent samples were evaluated for enzymatic activity, 280 μm absorbance, and radioactivity; the counting techniques and corrections were the same as those described (13).

As can be seen in Fig. 7, A and B, exposure of the tritiated proteins to low salt conditions resulted in a high exchange of label with the medium regardless of the initial conditions of incubation. Exposure to conditions of high ionic strength resulted in a very much lower exchange of label (Fig. 7, C and D). Similar data were obtained with these buffers in the presence of 20% glycerol (v/v) and in the presence of 0.01 and 0.2 M potassium phosphate at pH 6.4. The transition between the high and low rate of exchange seemed to lie between 20 and 35 mM potassium phosphate (Fig. 8).

Enzyme Inhibition—With hydroxypyruvate as substrate, the reductive reaction was inhibited in a competitive manner by glyoxylate. Pyruvate, 3-mercaptopyruvate, and 3-fluoropyruvate were competitive inhibitors of the enzyme when either hydroxypyruvate or glyoxylate was substrate; dihydroxyfumarate inhibited noncompetitively. The $K_i$ values for these compounds are presented in Table III.

Associated with dihydroxyfumarate inhibition there was a change in the absorption spectrum of the enzyme, the absorption maximum shifting from 280 to 270 μm (Fig. 9). After passage of the dihydroxyfumarate-enzyme solutions through Sephadex G-25 columns, the spectrum reverted to that of the native enzyme, as did the specific activities and kinetics. The spectral change occurring in the presence of dihydroxyfumarate

### Table III

| Inhibitor            | Substrate            | $K_i$ (comp) | $K_i$ (noncomp) |
|----------------------|----------------------|-------------|-----------------|
| Pyruvate             | Hydroxypyruvate      | 1.8 X 10^-4 | 2.1 X 10^-4     |
| Pyruvate             | Glyoxylate           | 3.9 X 10^-4 | 3.3 X 10^-4     |
| 3-Mercapto pyruvate  | Hydroxypyruvate      | 8.3 X 10^-4 | 6.8 X 10^-4     |
| 3-Mercapto pyruvate  | Glyoxylate           | 1.4 X 10^-4 | 2.5 X 10^-4     |
| 3-Fluoropyruvate     | Hydroxypyruvate      | 1.7 X 10^-3 | 1.8 X 10^-3     |
| 3-Fluoropyruvate     | Glyoxylate           | 1.8 X 10^-3 | 2.3 X 10^-3     |
| Dihydroxyfumarate    | Hydroxypyruvate      | 1.3 X 10^-4 | 1.5 X 10^-4     |
| Dihydroxyfumarate    | Glyoxylate           | 3.2 X 10^-4 | 3.1 X 10^-4     |

**Fig. 9.** Absorption spectrum of glyoxylic acid reductase in the presence (---) and absence (-----) of dihydroxyfumarate, 1 X 10^-4 M. The buffer is 0.1 M potassium phosphate at pH 7.0. Control cuvettes contained buffer or buffer plus dihydroxyfumarate, 1 X 10^-4 M, as appropriate. The effect on native enzyme, $R_p$ 0.22, was the same as on the degraded isozyme, $R_p$ 0.27.
was the same as had been observed with hydroxypruvate reductase (2) and tartaric semialdehyde reductase (3), enzymes which yield n(-)-glycerate from the catalytic reduction of hydroxypruvate. In contrast, enzymes such as lactic and alcohol dehydrogenase which catalyze the reduction of hydroxypruvate to L(+)-glycerate, were not inhibited by dihydroxyfumarate and do not show a spectral shift in its presence. Similarly, 3-phosphoglycerate dehydrogenase and malic dehydrogenase are unaffected by dihydroxyfumarate.

Compounds which failed to inhibit the reduction of either hydroxypruvate or glyoxylate (at concentrations between 0.1 and 20 mM) were 3-bromopyruvate, phosphoenolpyruvate, n-serine, glyceraldehyde, a-ketobutyrate, a-ketovalerate, and a-ketosuccinate. No significant inhibition of the enzyme was found at 1 mM concentrations of sodium EDTA or potassium arsenite.

The reverse reaction, the oxidation of n(-)-glycerate or glycolate, was competitively inhibited by lactate, phosphoglycerate, and propionic acid; however, inhibition was less than 20% at 10 mM concentrations of each compound.

Kinetic Properties of Other Forms of Glyoxylic Acid Reductase

Degraded Form—This form of the enzyme, Rp 0.27, on analytical gels, had a molecular weight of 83,000 ± 5,000 and was composed of two subunits, molecular weight 40,000 ± 3,000 (1). Peptide mapping studies suggested that this species was a degraded product of the major enzyme, Rp 0.22, described above.

The protein catalyzed the reduction of hydroxypruvate and glyoxylate in the presence of DPNH. In the presence of 0.15 mM reduced pyridine nucleotide, compounds which were not substrates with this isozyme; the concentrations of the compounds tested were the same as those previously described. At pH 6.4 and in the presence of 3 mM hydroxypruvate, the K_m value for DPNH was 5.2 × 10^{-6}; in the presence of 50 mM hydroxypruvate, the K_m value for DPNH was 2.5 × 10^{-6} M. With 0.15 mM DPNH, the K_m values for hydroxypruvate and glyoxylate were 5 × 10^{-6} and 5 × 10^{-2} M, respectively.

Kinetic analyses (7) of the reduction of hydroxypruvate or glyoxylate in the presence of DPNH allowed the calculation of the rate equations below (Equations 8 and 9). The experimental values were in satisfactory agreement with the curves calculated from these equations and again suggested that a ternary complex (7, 8) had been formed.

\[
\frac{V_{\text{max}}}{v} = 1 + \frac{1.5 \times 10^{-4}}{[\text{glyoxylate}]} + \frac{1.3 \times 10^{-4}}{[\text{DPNH}]} + \frac{4.1 \times 10^{-4}}{[\text{glyoxylate}[\text{DPNH}] (8)
\]

\[
\frac{V_{\text{max}}}{v} = 1 + \frac{2.0 \times 10^{-4}}{[\text{hydroxypruvate}]} + \frac{1.9 \times 10^{-4}}{[\text{DPNH}]} + \frac{6.5 \times 10^{-4}}{[\text{hydroxypruvate}[\text{DPNH}]} (9)
\]

Equations 10 and 11 represent the rate equations for the oxidation of n(-)-glycerate and glycolate by the same isozyme. The relative maximal velocity was 30-fold greater in the direction of glyceraldehyde or glycolate formation under these conditions.

\[
\frac{V_{\text{max}}}{v} = 1 + \frac{2.0 \times 10^{-4}}{[\text{glycolate}]} + \frac{1.5 \times 10^{-4}}{[\text{DPNH}]} + \frac{5.4 \times 10^{-4}}{[\text{glycolate}[\text{DPNH}] (10)
\]

Comparisons of the kinetics of this isozyme with the kinetics of the Rp 0.22 enzyme described above revealed several consistent relationships. First, there was no significant difference in maximal velocity (V_{\text{max}}) between the two proteins when the same substrates were utilized and when similar enzyme concentrations were present; i.e. the V_{\text{max}} terms of Equations 2 and 8, of Equations 3 and 9, etc., were identical when compared. Second, the V_{\text{max}} in the presence of hydroxypruvate was 3- to 4-fold higher than the V_{\text{max}} obtained when glyoxylate was substrate, independent of the enzyme forms. Lastly, there was no significant difference in the K_m terms, the limiting Michaelis constants, between the two proteins but there was a consistent 2-fold difference in the K_m terms; i.e. the K_m value for DPNH was 3.4 × 10^{-4} as opposed to 1.3 × 10^{-4} in Equations 2 and 8, respectively, and was 5.2 × 10^{-4} as opposed to 1.9 × 10^{-6} in Equations 3 and 9, respectively, etc.

The specificity of the reverse reaction, the oxidation of n(-)-glycerate or glycolate, was the same for this enzyme form and for the Rp 0.22 enzyme. Inhibition properties were essentially identical (Table III) and dihydroxyfumarate solutions induced a shift in the absorption spectra analogous to that seen in the Rp 0.22 enzyme (Fig. 9). The pH dependence, the salt effects, and the tritium exchange data were also not significantly different from those described for the native enzyme.

Isozymes Rp 0.19 and 0.17—Although similar in molecular weight, amino acid composition, and subunit structure, these isozymes could be distinguished from each other by their specific activities and kinetic properties. The Rp 0.19 isozyme was identical with the Rp 0.22 enzyme in all respects, i.e. in its substrate specificity, pH optima, salt effects, ternary kinetics, and rate equations. The Rp 0.17 isozyme was also identical with the Rp 0.22 enzyme in its substrate specificity, pH optima, and salt

\[
\frac{V_{\text{max}}}{v} = 1 + \frac{1.0 \times 10^{-4}}{[\text{glycolate}]} + \frac{1.6 \times 10^{-4}}{[\text{DPNH}]} + \frac{1.0 \times 10^{-4}}{[\text{glycolate}[\text{DPNH}] (11)
\]

Fig. 10. Double reciprocal plot of n(-)-glycerate catalysis by the glyoxylic acid reductase isozymes. The DPNH concentration was 2 mM. Approximately equal amounts of enzyme protein were utilized for the Rp 0.19 and 0.22 isozymes; enzyme protein was 4- to 5-fold higher for the Rp 0.17 isozyme. Assays were performed at pH 8.8 in 0.2 M Tris-chloride.
Effects; however, distinct differences were evident in its rate equations. Maximal velocity values were 2- to 3-fold lower, and the $K_v$ values for hydroxypyruvate, glyoxylate, $p(-)-$glyceraldehyde, and glyceraldehyde were 2-fold higher. The $K_v$ values were similar in all rate equations. Double reciprocal plots readily show these differences (Fig. 10).

**DISCUSSION**

Spinach leaf glyoxylic acid reductase is similar to several bacterial enzymes capable of the catalytic reduction of hydroxypyruvate, i.e. hydroxypyruvate reductase (2), tartronic semialdehyde reductase (3), and oxalylglycolate-reductive decarboxylase (4). Like these enzymes, it catalyzes the formation of $p(-)-$glyceraldehyde from hydroxypyruvate, is anion-regulated, and is inhibited by dihydroxystearate. The dihydroxystearate inhibition is noncompetitive and is associated with the same spectral shift previously observed with bacterial preparations.

Tobacco leaf glyoxylic acid reductase, an enzyme previously described (5), also catalyzes the reversible reduction of glyoxylate and hydroxypyruvate. Although dihydroxystearate inhibition has not been evaluated, the tobacco preparation yields $p(-)-$glyceraldehyde from hydroxypyruvate and is anion-modulated. It is different from the spinach protein in that arsenite inhibits its enzymatic activity and in that TPNH cannot replace DPNH as the reduced pyridine nucleotide. In addition, the anion modulation involves activation only, nitrate being the most effective activator and phosphate the least; i.e. the order of anion activation is exactly reversed. The kinetics of spinach leaf glyoxylic acid reductase are ternary; i.e. catalysis occurs only when both substrates are present on the enzyme at the same time (7, 8). Although all forms of the spinach enzyme exhibit these kinetic properties, differences which exist in the rate equations suggest specific structural modifications in two of the additional enzyme forms. When compared to the $R_{p} 0.22$ protein, the low molecular weight enzyme, $R_{p} 0.27$, has a 2-fold difference in the limiting Michaelis constant for pyridine nucleotides ($K_m$) in the absence of a detectable difference in the analogous constant for carbonyl substrates ($K_s$) and in the absence of a change in enzyme maximal velocity ($V_{max}$). Whether a random or an ordered mechanism (7, 8) is operative, the altered rate constants necessary to produce this difference should reflect a unique DPNH-enzyme interaction, i.e. a structural change in the binding site for pyridine nucleotides. In the same vein, the $R_{p} 0.17$ isozyme has an altered Michaelis constant for the carbonyl substrate but no change in the constant for DPNH. Although this isozyme has a maximal velocity different from that of $R_{p} 0.22$ enzyme, structural changes should have affected the carbonyl site more than the DPNH site. The data suggest that studies characterizing the structural differences between these forms will offer significant information concerning the active center of the spinach enzyme. Sulphydryl studies of these forms have already yielded interesting differences in this regard (16).

Anion effects on the spinach enzyme are extremely complex. At low salt concentrations, hydroxypyruvate reduction is activated in a similar quantitative fashion by all anions (Fig. 4). Double reciprocal plots emphasize this since the maximal enzyme velocity is independent of the anion present. As the salt concentration increases, each anion affects the activity differently, i.e. activating further or inhibiting (Figs. 4 and 5). The shift in anion modulation coincides with a change in enzyme maximal velocity and a change in enzyme conformation (Figs. 7 and 8). Similar enzyme changes in the presence of an effector have been termed negative cooperativity (12, 17, 18), and a ligand-induced conformational change is presumed responsible once the identity of the peptide chains has been shown and when isoenzyme contamination and pleomorphic forms have been ruled out (12, 17, 18). Coupling the data and this presumption (19), a model explaining the anion regulation is proposed. The enzyme is presumed to exist in two distinct conformational states which have different maximal velocities. A specific anion binding site is located near the carbonyl substrate site and a positively charged amino acid is present at that point or nearby on the enzyme molecule. The association of the anion and substrate sites is based on the competitive kinetics with carbonyl substrates; the existence of the positively charged residue is implied from electrostatic considerations and from the pH sensitivity of anion modulation. The suggestion that there is only one anion binding site is arbitrary, and the mechanism of the ligand-induced conformational change is unknown. Since the conformational change is independent of pH and since it apparently occurs with all anions and cations tested, changes in salt concentrations are presently presumed to be causal.

In the "low salt configuration," every anion binding to the enzyme will increase catalytic activity by increasing the affinity of the enzyme for its carbonyl substrates. In the "high salt configuration," every anion will compete with the carbonyl substrate for enzyme attachment since the anion and substrate sites are conformationally different. In both configurations, a decrease in hydrogen ion concentration eliminates the charge on the amino acid residue responsible for anion binding and eliminates the activation and inhibition. In both configurations, the anion with the highest affinity for the enzyme is the worst activator or best inhibitor.

The model is applicable to a consideration of the structure-function relationships of anion modulation in the bacterial and tobacco enzymes previously mentioned (2-5). Hydroxypyruvate reductase (2) and tobacco glyoxylic acid reductase (5) are anion-activated only; tartronic semialdehyde reductase (3) is either activated or inhibited by a particular anion, but not both. With no negative cooperativity, these enzymes should have no ligand-induced conformational shift, and trinitium exchange experiments should not be affected by increasing salt concentrations. Hydroxypyruvate reductase (2) should exhibit a low salt exchange pattern, whereas tartronic semialdehyde reductase (3) should exhibit a high salt pattern. Since anion modulation of oxalylglycolate reductive decarboxylase is similar to anion regulation of the spinach enzyme, trinitium exchange experiments should show a shift between 20 mM and 75 mM salt concentrations. In all cases, the anions should exhibit competitive kinetics when evaluated against carbonyl substrates.

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