Orr, G. A., Simon, J., Jones, S. R., Chin, G. J., & Knowles, J. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2230–2233.
Rex Sheu, K. F., & Frey, P. A. (1977) *J. Biol. Chem. 252*, 4445–4448.
Rex Sheu, K. F., & Frey, P. A. (1978) *J. Biol. Chem. 253*, 3378–3380.
Richard, J. P., Ho, H., & Frey, P. A. (1978) *J. Am. Chem. Soc. 100*, 7756–7757.
Saenger, W., Suck, D., & Eckstein, F. (1974) *Eur. J. Biochem. 46*, 559–567.
Stahl, K. W., Schlimme, E., & Eckstein, F. (1974) *FEBS Lett. 40*, 241–246.
Usher, D. A., Erenrich, E. S., & Eckstein, F. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 115–118.
Wong, L. J., Rex Sheu, K. F., Lee, S. L., & Frey, P. J. (1977) *Biochemistry 16*, 1010–1016.

Purification and Characterization of Human Liver Glycolate Oxidase. Molecular Weight, Subunit, and Kinetic Properties†

Harvey Schwam,* Stuart Michelson, William C. Randall, John M. Sondey, and Ralph Hirschmann

ABSTRACT: Glycolic acid oxidase (EC 1.1.3.1, glycolate:oxygen oxidoreductase) has been purified 1000-fold from human liver to apparent homogeneity by gel electrophoresis. The molecular weight of the enzyme, determined by sedimentation equilibrium, by Sephadex gel filtration, and by dodecyl sulfate gel electrophoresis of the enzyme cross-linked with dimethyl suberimidate, was found to range between 160 000 and 179 000. A subunit molecular weight ranging from 40 000 to 43 000 was found by sodium dodecyl sulfate gel electrophoresis and sedimentation equilibrium in 5 M guanidine hydrochloride. A tetrameric structure for the active enzyme has been established by the molecular weight studies and enzyme cross-linking experiments with dimethyl suberimidate. The enzyme, shown to contain the FMN coenzyme by spectroscopic analysis, was found to be rapidly and irreversibly inactivated by the acetylenic substrate DL-2-hydroxy-3-butynoate. Enzyme turnover studies with the acetylenic substrate have shown that inactivation occurs after 130 catalytic events. In contrast, two 4-substituted 2-hydroxy-3-butynoates, DL-2-hydroxy-4-phenyl-3-butynoate, and DL-2-hydroxy-6-phenyl-3-hexynoate were shown not to be inhibitors of the oxidase. *N*-Octyloxamate was found to be a potent, novel type of inhibitor of the enzyme, but in contrast to hydroxybutynoate inhibition, this inhibitor is of the reversible, noncompetitive type with a K_I of 3.2×10^{-6} M. Both *N*-octyloxamate and DL-β-phenyllactate, also shown to be a reversible noncompetitive inhibitor of the enzyme, were shown to reduce the rate of irreversible inhibition of the enzyme by hydroxybutynoate.

Seventy years ago, Dakin (1907) demonstrated that dogs possess an activity capable of converting glycolate or glyoxylate to oxalate. The site(s) of the metabolic conversion was not known until Dohan (1940) noted that rabbit and rat liver exhibited an oxidase activity, independent of added cofactors, that metabolized glycolate. Kun (1952), twelve years later, showed that a supernatant from homogenized rat livers contained the glycolate oxidizing activity and molecular oxygen participated in the process. Zelitch & Ochoa (1953) and Kun et al. (1954), extending earlier work, achieved partial purifications of the rat liver glycolate oxidizing activity and presented evidence suggesting the oxidase activity to be a flavoprotein. Robinson & Sizer (1959), Keay et al. (1960), and Robinson et al. (1962) in a series of papers described the isolation and properties of a pig kidney cortex enzyme with FMN as the prosthetic group that catalyzed the oxidation of glycolate, other short chain L-α-hydroxy acids, and glyoxylate. Schuman & Massey (1971a,b) purified and characterized glycolic acid oxidase from pig liver. Their preparation was shown to contain 1 mol of FMN per 51 000 daltons. Holmes & Duley (1975) probed the physical properties of the purified rat liver enzyme and reported the holoenzyme to be composed of a set of four 43 000 molecular weight subunits. In this paper we report the isolation and purification of human liver glycolate oxidase and evaluate its structural, kinetic, and subunit properties.

Experimental Procedure

All experimental details are given in the supplementary material (see paragraph concerning supplementary material at the end of this paper).

Results

Table I shows an overall purification of about 400-fold when the 65% ammonium sulfate precipitate is taken as the starting point. Starting from the homogenization step, the purification achieved is greater, but since the dye reduction assay at this initial stage of the isolation scheme undergoes a spurious nonenzymatic reduction, the 65% ammonium sulfate precipitate step was chosen as the starting point for yield and purity calculations.

Lactate dehydrogenase, which can also oxidize glyoxylate to oxalate, was assayed throughout the purification scheme by the procedure of Gibbs & Watts (1973).

The enzyme was found to be homogeneous on 5, 6, 7, and 8% analytical disc gels stained for protein or enzyme activity, and on $NaDodSO_4$ gels only a single subunit band was detected.

Molecular Weight. The molecular weight of human glycolate oxidase was investigated by the gel filtration method utilizing Sephadex G-200 as the gel and xanthine oxidase, γ-globulin, lactic acid dehydrogenase, and bovine serum albumin as the protein standards. Human liver glycolate oxidase eluted from the column as a single, symmetrical peak corresponding to a molecular weight of 160 000.

When native human liver glycolate oxidase was treated with sodium dodecyl sulfate–mercaptoethanol and subjected to

† From the Department of Medicinal Chemistry, Merck Sharp & Dohme Research Laboratories, West Point, Pennsylvania 19486. *Received May 9, 1978; revised manuscript received February 13, 1979.*

0006-2960/79/0418-2828$01.00/0

Table I: Summary of the Purification of Glycolate Oxidase from Human Liver

stages		volume (mL)	protein		enzyme act.		sp act.	recovery (%)
			OD_{280}	total	units/mL	total		
(1)	65% $(NH_4)_2SO_4$ ppt	700	46	32 200	0.69	483	0.015	100
(2)	DEAE-Sephadex	220	4.3	946	2.0	440	0.47	91
(3)	calcium phosphate gel cellulose	135	0.27	36	1.67	225	6.2	46

electrophoresis, a single, faster moving polypeptide species was observed, suggesting that the native enzyme is composed of a number of identical or very similar molecular weight polypeptide subunits. Furthermore, the subunit molecular weight of human liver glycolate oxidase, when determined by the method of Weber & Osborn (1969), was found to correspond closely to that of the aldolase monomer (40 000).

These data, therefore, are consistent with a holoenzyme composed of four identical or very similar promoters. This model is entirely consistent with the results obtained from ultracentrifugation studies that gave values of 179 000 for the holoenzyme and 43 200 for the monomeric subunit as well as with the results described below from cross-linking experiments conducted with dimethyl suberimidate.

Subunit Structure. When human liver glycolate oxidase was allowed to react with dimethyl suberimidate at pH 8.5 under conditions similar to those reported by Davies & Stark (1970) and subjected to $NaDodSO_4$ disc gel electrophoresis, only two bands, corresponding to the monomer and dimer, were discernible. When the time of the reaction was increased up to 24 h and the concentration of dimethyl suberimidate was increased up to 8 mg/mL, similar results were obtained. In contrast, when aldolase was cross-linked under identical conditions, the four distinct bands ranging from the monomer to the tetramer, reported by Davies and Stark, were obtained.

Browne & Kent (1975) in their investigation of the reaction of ethyl acetimidate with primary alkylamines showed that as the pH was increased from 8 to 10, the half-life of the imidate increased and an apparent change in the reaction mechanism occurred, yielding *N*-alkylacetamidine at yields of up to 60%. Our study of the reaction of human liver glycolate oxidase and dimethyl suberimidate revealed that as the pH was raised in increments from 8.5 to 9.5, the reaction product contained greater amounts of the higher oligomers, trimer and tetramer. At pH 9.5 a band was observed with a mobility similar to that of the well-defined aldolase tetramer (M_r 160 000).

Spectroscopic Properties of the Enzyme. The spectrum of human liver glycolate oxidase in 0.1 M sodium phosphate, presented in Figure 1, shows the typical dominance of the flavin chromophore, with maxima at 450, 425, and 365 nm and marked shoulders at 480 and 335 nm. This resolved spectrum, however, is unusual in that the shoulder generally found at 430 nm is, instead, present as a peak at 425 nm. The reason for this is not entirely clear but may be due to the presence of a second chromophore. Schuman & Massey (1971a) showed that their preparation of pig liver enzyme contained a second chromophore with an absorption maximum at 425 nm, whose structure was reported by Mayhew and co-workers (Mayhew et al., 1974) to be 6-OH-FMN. The argument for the existence of a second chromophore in human liver glycolate oxidase is strengthened by the spectrum shown in Figure 1, curve 2, for the enzyme–sulfite complex. While typical flavoprotein and model flavin compounds show no absorption at wavelengths above 380 nm when treated with sulfite (Massey et al., 1969; Müller & Massey, 1969), the human liver glycolate oxidase–sulfite complex shows a maximum at 410 nm. The human liver glycolate oxidase

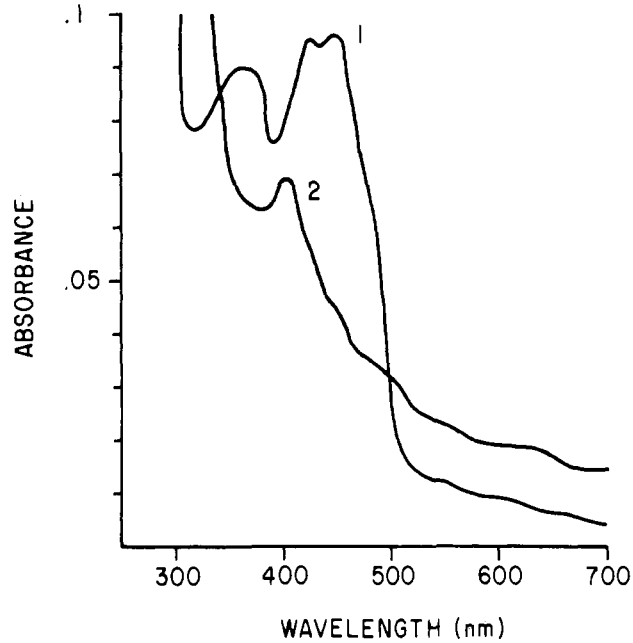

FIGURE 1: Absorption spectrum of glycolate oxidase. Curve 1: human liver glycolate oxidase in 0.1 M sodium phosphate, pH 7. Curve 2: human liver glycolate oxidase–sulfite in 0.1 M sodium phosphate, pH 7.

Table II: Substrate Specificity and Kinetic Data for Human Liver Glycolate Oxidase

compd	rel rate (%)	rel[a] apparent K_m	rel[a] apparent V_{max}
glycolate	100	1.0	1.0
DL-2-hydroxy-4-phenyl-3-butynoate	77	0.87[b]	1.01[b]
L-α-hydroxyisocaproate	52	4.8[b]	0.91[b]
DL-2-hydroxy-6-phenyl-3-hexynoate	28	2.0[b]	0.54[b]
DL-2-hydroxy-4-phenyl-3-butenoate	13	2.1	0.10
DL-α-hydroxycaproate	8.8		
glyoxylate	6.9		
DL-α-hydroxyvalerate	6.8		
DL-α-hydroxybutynoate	6.6	9.3	0.07
DL-α-hydroxycaprylate	3.1		
DL-α-hydroxy-*n*-butyrate	1.9		
L-lactate[c]	1.7		

[a] Values for each substrate are divided by those determined with glycolate as the substrate under the same experimental conditions (25 °C; 5 × 10^{-5} M DCIP; 0.1 ionic strength).
[b] Excess substrate inhibition was observed for these compounds.
[c] The relative rates of DL-3,3-dichlorolactate, L-α-hydroxyisovalerate, DL-isocitrate, DL-maleate, L-alanine, and L-tartrate are less than 1%.

preparation exhibits an A_{280}/A_{450} ratio of 6.1 which compares favorably with the value of 7.3 reported for the pig liver enzyme (Jorns, 1975).

Substrate Specificity. The ability of human glycolate oxidase to oxidize hydroxy acids is reported in Table II as a percent of the rate observed with glycolate as the substrate. The human enzyme, like the rat liver enzyme (Kun et al., 1954; Ushijima, 1973) and pig liver enzyme (Dickinson, 1965), oxidized a number of L-α-hydroxy acids of medium chain

length, albeit at a lesser rate than glycolate. In fact, the relative oxidation rates for L-α-hydroxyisocaproate, DL-α-hydroxy-*n*-butyrate, L-lactate, and DL-α-hydroxyisovalerate are in the same decreasing order of substrate preference as that reported for a rat liver preparation by Ushijima (1973).

Table II also shows the results obtained when the k_{cat} inhibitor DL-α-hydroxybutynoate (Abeles & Maycock, 1976) and two analogues of this compound, DL-2-hydroxy-6-phenyl-3-hexynoate and DL-2-hydroxy-4-phenyl-3-butynoate, were tested as substrates. While hydroxybutynoate appeared to be about equal to glyoxylate as a substrate, DL-2-hydroxy-4-phenylbutynoate, an α-hydroxy acid which had not been previously described, was as good a substrate as glycolate and far better than the corresponding double-bonded compound DL-2-hydroxy-4-phenylbutenoate. In fact, a comparison of only the apparent Michaelis constant and V_{max} parameters with those obtained for glycolate indicates a glycolate oxidase preference for the 2-hydroxy-4-phenylbutynoate over glycolate. While the data on hand are insufficient to account for the high substrate activity of this compound, one may speculate that the flat phenylethynyl moiety attached to the hydroxy acid is in a favorable steric arrangement to help stabilize the enzyme–flavin–substrate ternary complex by π–π electron interaction between the phenylethynyl group and the flavin group of the cofactor.

The enzyme, as expected, showed little activity toward α-hydroxy di- or tricarboxylic acids, α-hydroxyalkenemono- or -dicarboxylic acids, and α-amino acids. It does, however, oxidize glyoxylate at about 7% the rate of glycolate and will, therefore, oxidize glycolate to oxalate via a rapid oxidation to glyoxylate and a slower oxidation step to oxalate.

The apparent Michaelis constants obtained with glycolate and five other α-hydroxy acids are also shown in Table II. For three of these compounds, DL-2-hydroxy-4-phenyl-3-butynoate, L-α-hydroxyisocaproate, and DL-2-hydroxy-6-phenyl-3-hexynoate, excess substrate inhibition was observed to a greater extent than that observed for glycolate. For these α-hydroxy acids, the Michaelis parameters were obtained by fitting the observed concentration–rate data to eq 1, which describes the

$$v = \frac{V[S]}{[S] + K_1 + [S]^2/K_2} \quad (1)$$

simplest case involving an inactive ternary complex of enzyme and two substrate molecules (Dixon & Webb, 1964).

Determination of Michaelis Parameters. Standard double-reciprocal plots of $1/v$ vs. $1/[\text{glycolate}]$ for human liver glycolate oxidase at the specified fixed concentrations of 2,6-dichlorophenolindophenol (DCIP) yielded apparent parallel lines as shown in Figure 2. These findings are in contrast to the converging line kinetics obtained in our own laboratory (unpublished experiments) and those reported by Schuman & Massey (1971b) for the pig liver enzyme. A nonlinear regression technique, described in the supplementary material, was employed to fit the observed initial rate data to the equation expected for a two-substrate mechanism involving only binary complexes (Plowman, 1972):

$$v = \frac{V[\text{DCIP}][\text{glycolate}]}{[\text{glycolate}][\text{DCIP}] + K_{Gly}[\text{DCIP}] + K_{DCIP}[\text{Gly}]} \quad (2)$$

The resulting parameter values giving the best fit to the data are $V = (2.4 \pm 0.1) \times 10^3$ (1250) mol of DCIP per mol per monomer per min, $10^4 K_{Gly} = 2.0 \pm 0.2$ (4.2) M, and $10^4 K_{DCIP} = 0.73 \pm 0.09$ (2.8) M, where the parenthetical values refer to those previously obtained for the pig liver enzyme (Schuman & Massey, 1971b). If these data are evaluated in terms of

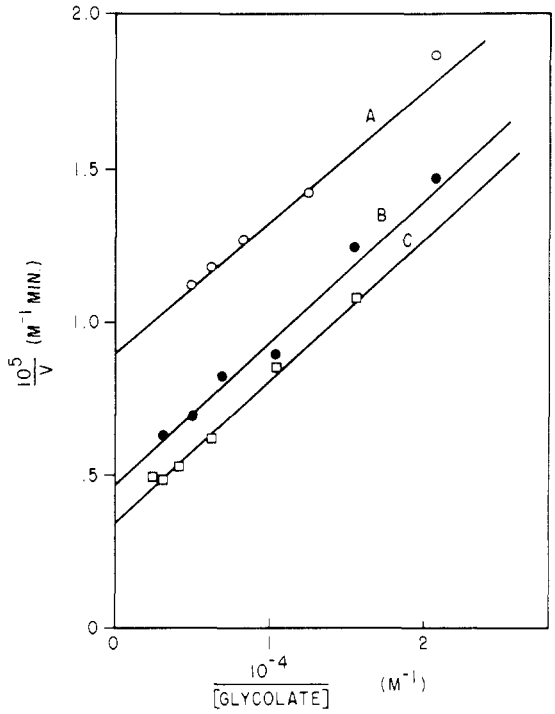

FIGURE 2: Double-reciprocal plot of the dependence of the rate of glycolate oxidase mediated oxidation of glycolate upon glycolate concentration at three concentrations of DCIP. (A) 0.375×10^{-4} M; (B) 0.75×10^{-4} M; (C) 1.50×10^{-4} M.

a two-substrate ordered sequential mechanism involving a ternary complex (Plowman, 1972), eq 3, the V, K_{Gly}, and K_{DCIP}

$$v = \frac{V[\text{DCIP}][\text{glycolate}]}{[\text{glycolate}][\text{DCIP}] + K_{Gly}[\text{DCIP}] + K_{DCIP}[\text{Gly}] + K_i} \quad (3)$$

parameters remain essentially unchanged and K_i ($= K_{i,Gly}K_{Gly}$ or $K_{i,DCIP}K_{DCIP}$, depending on the obligatory order of addition) is found to be $(0.08 \pm 0.15) \times 10^{-4}$ M^2, a value less than its computed standard error. Therefore, both the double-reciprocal plot of Figure 2 and the nonlinear regression analyses do not support the existence of an experimentally detectable term involving the product of reductant and oxidant.

The shift from convergent to parallel line kinetics does not, however, necessarily imply a fundamental change in the reaction mechanism, since it has been shown that a mechanism involving ternary complexes may, under some conditions, give rise to parallel line kinetics (Palmer & Massey, 1968).

Reversible Inhibition of Human Liver Glycolate Oxidase. At constant substrate concentrations of 5×10^{-5} M DCIP and 2×10^{-4} M sodium glycolate, both DL-β-phenyllactate (Liao & Richardson, 1973) and *N*-octyloxamate inhibit human liver glycolate oxidase according to a simple hyperbolic binding model with I_{50} values of $(3.2 \pm 0.2) \times 10^{-4}$ and $(3.3 \pm 0.3) \times 10^{-6}$ M, respectively. The dependence of the rate of reduction of DCIP on glycolate concentration is shown for specified concentrations of *N*-octyloxamate in Figure 3. Since a similar pattern was exhibited for fixed concentrations of DL-β-phenyllactate, both inhibitors are pure noncompetitive inhibitors of the enzyme, and therefore their I_{50} and K_I values are identical at a given DCIP concentration (Chou, 1974).

Irreversible Inhibition of Human Liver Glycolate Oxidase. Incubation of human liver glycolate oxidase with 4×10^{-5} M 2-hydroxybutynoate (Figure 4) resulted in a progressive loss of enzymatic activity with increasing incubation time. Also, since the enzymatic inactivation produced by 2-hydroxybutynoate during the incubation phase was not diminished

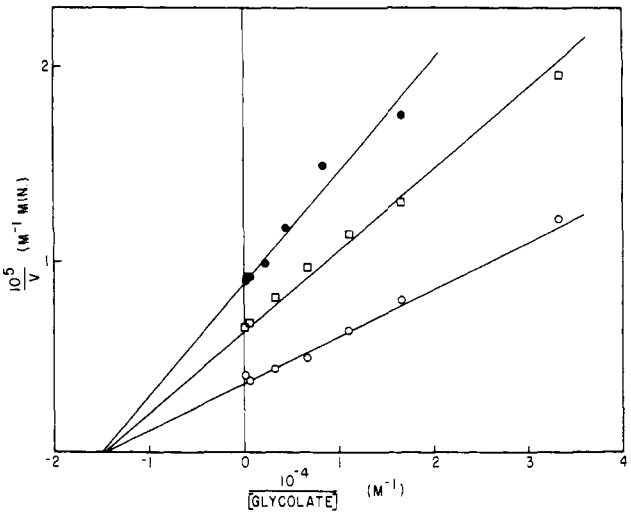

FIGURE 3: Double-reciprocal plot of the rate of reduction of an initial 5×10^{-5} M concentration of DCIP by human liver glycolate oxidase as a function of glycolate concentration. The *N*-octyloxamate concentration is 0.0 (⊙), 2.5×10^{-6} (□), and 5.0×10^{-6} M (●).

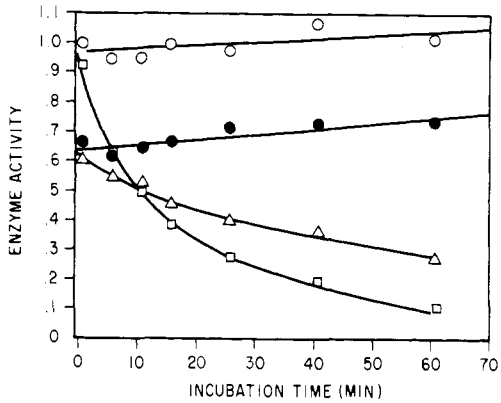

FIGURE 4: Irreversible inhibition of human liver glycolate oxidase by 2-hydroxybutynoic acid. Fractional enzyme activity after the indicated times of incubation for four incubation conditions: (⊙) without inhibitors; (●) in the presence of 1.76×10^{-5} M *N*-octyloxamate; (□) in the presence of 4.0×10^{-5} M 2-hydroxybutynoic acid; (△) in the presence of both 1.76×10^{-5} M *N*-octyloxamate and 4.0×10^{-5} M 2-hydroxybutynoic acid.

upon a rapid 10-fold dilution which occurs upon passage to the assay conditions, it is apparent that 2-hydroxybutynoate behaves as an irreversible inhibitor of human liver glycolate oxidase. In contrast, incubation of the enzyme with 1.76×10^{-5} M *N*-octyloxamate also resulted in a decreased enzymatic activity, Figure 4, but the decreased activity obtained was not progressive with increasing incubation time. In fact, the inhibition produced by *N*-octyloxamate or DL-β-phenyllactate (data not shown) is that which would be predicted to occur under the assay conditions and the I_{50} values found above. These compounds, therefore, unlike 2-hydroxybutynoate, show the characteristics of rapid, reversible inhibition. The irreversible inactivation by 2-hydroxybutynoate is shown in Figure 4 to be considerably slower when the incubation is conducted in the presence of the reversible inhibitor *N*-octyloxamate. A similar reduction of the inactivation rate was found when the incubation was conducted in the presence of DL-β-phenyllactate (not shown). The apparent first-order rate constants for enzyme inactivation may be converted to second-order rate constants for the aerobic destruction of human glycolate oxidase by 2-hydroxybutynoate to yield 1.0×10^3 M^{-1} min^{-1} in the absence of reversible inhibitor, 0.38×10^3 M^{-1} min^{-1} in the presence of 1.76×10^{-5} M *N*-octyloxamate, and 0.58 $\times 10^3$ M^{-1} min^{-1} in the presence of 6.47×10^{-4} M DL-β-phenyllactate.

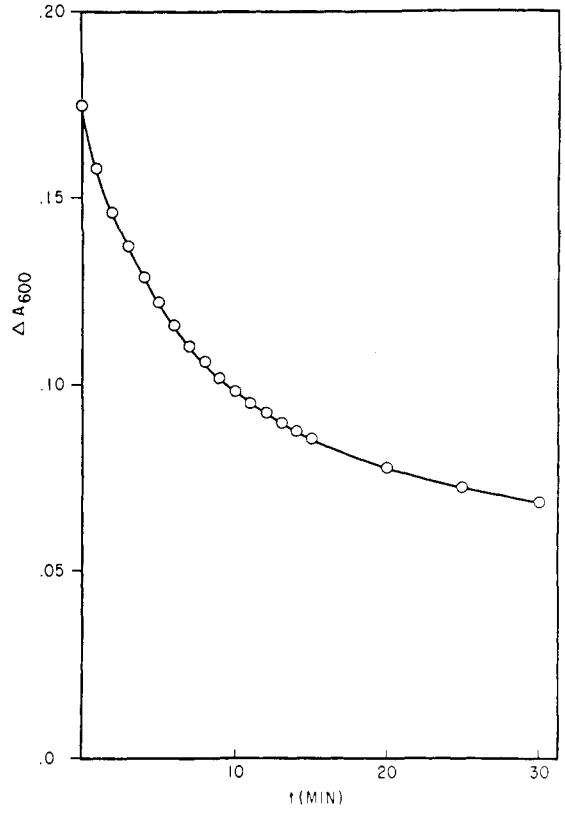

FIGURE 5: Absorbance changes at various times produced by the anaerobic oxidation of 2.50 mM 2-hydroxybutynoic acid in the presence of 5.0×10^{-5} M DCIP and 2 μg/mL human liver glycolate oxidase at pH 7.0, 0.10 ionic strength. These absorbance–time data have been corrected for the blank reaction occurring between 2-hydroxybutynoic acid and DCIP in the absence of the enzyme. The solid line represents the best fit obtained for these data to a two-exponential decay equation: $\Delta A_{600} = B_1 e^{-k_1 t} + B_2 e^{-k_2 t}$.

Determination of Total Turnover Number. The absorbance–time data for a typical human liver glycolate oxidase mediated anaerobic reduction of DCIP by 2-hydroxybutynoate are shown in Figure 5. The absorbance–time data, from which the blank reaction between DCIP and 2-hydroxybutynoate has been subtracted, can be fit to a two-exponential decay curve, eq 4, by nonlinear least-squares analysis. The average pa-

$$\Delta A_{600} = B_1 e^{-k_1 t} + B_2 e^{-k_2 t} \quad (4)$$

rameter values from quadruplicate determinations are B_1 = 0.10, k_1 = 0.19 min^{-1}, B_2 = 0.08, and k_2 = 0.016 min^{-1}.

If it is assumed that the rapid exponential decay is associated with the enzyme-mediated reduction of DCIP by 2-hydroxybutynoate and k_1 is the pseudo-first-order rate constant for inactivation of the enzyme, then B_1 represents the absorbance decrease associated with the total reduction of DCIP by this process. This corresponds to a molar decrease of 6.30×10^{-6} M in DCIP concentration based on an experimentally determined molar extinction coefficient of 1.62×10^4 M^{-1} cm^{-1} for DCIP under these conditions (data not shown). The glycolate oxidase concentration employed in these determinations, 2 μg/mL, represents a molar concentration of 4.8×10^{-8} based on a monomer molecular weight of 42 000. The turnover number is, therefore, 130 molecules of DCIP reduced (and therefore hydroxybutynoate molecules oxidized) before inactivation of one monomer of this enzyme.

The turnover number for human liver glycolate oxidase is similar to the 110 turnovers reported for the 2-hydroxy-

butynoate inactivation of *Mycobacterium smegmatis* L-lactate oxidase (Ghisla et al., 1976) but is substantially greater than the turnover number of 25 found by Cromartie & Walsh (1975) for the inactivation of the related flavin enzyme, rat kidney long-chain L-α-hydroxyacid oxidase. These authors also reported turnover numbers for a number of analogues of 2-hydroxybutynoate with alkyl groups attached to C-4. Their data showed that all alkyl groups substituted for the C-4 hydrogen resulted in at least a 200-fold increase in the turnover number. Since C-4 aryl substitutions for the C-4 hydrogen of hydroxybutynoate have not been previously reported, the reaction of DL-2-hydroxy-4-phenylbutynoate with human liver glycolate oxidase was investigated. Anaerobic incubation of 0.71 μg/mL of the enzyme with 1.5×10^{-4}M DL-2-hydroxy-4-phenylbutynoate resulted in complete reduction of the DCIP with no evidence of enzyme inactivation. Under the conditions of this experiment the turnover number, therefore, must be in excess of 8900, and no inactivation was discernible to that point.

Discussion

The purity of human liver glycolate oxidase was assessed by its behavior on gel filtration where it was shown that the symmetrical peak eluted from the column had a constant specific activity throughout the entire peak that was equal to the specific activity of the sample applied to the column. It was also demonstrated that the enzyme, upon electrophoresis in 6% gel, showed a single protein band when stained with Coomassie blue and that the protein band was enzymatically active toward glycolate by the nitro blue tetrazolium procedure. However, the appearance of a single band on disc gel electrophoresis cannot be interpreted as unequivocal evidence of protein homogeneity, since it is possible that charge differences and size compensate in such a way that two or more proteins may bear the same mobility under a single set of conditions. A more precise demonstration of protein homogeneity can be made by disc gel electrophoresis if a single band is observed at a number of different gel concentrations. When human liver glycolate oxidase was subjected to electrophoresis under conditions whereby all parameters were held constant, except that the gel concentrations were varied from 5 up to 8%, a single band was obtained in each case.

Several results suggest that human liver glycolate oxidase is composed of protomers of the same molecular size and suggest that quite likely the protomers are identical species. Disruption of the native enzyme with sodium dodecyl sulfate yielded, upon electrophoresis, a single compact band. If the enzyme contained protomers of more than one size, they would have been detected by electrophoresis as equally conspicuous bands. Furthermore, disruption of the intramolecular cross-linked human liver glycolate oxidase with sodium dodecyl sulfate gave an electrophoretic pattern of protein bands that were related via whole-number multiples. This would not be possible if human liver glycolate oxidase contained different-sized protomers.

When human liver glycolate oxidase was intramolecularly cross-linked with dimethyl suberimidate and subjected to electrophoresis, four distinct bands were discernible. The four bands indicate a tetrameric structure for the enzyme. When conditions for the cross-linking were optimized at pH 9.2, tetramer was clearly visible and there were no bands corresponding in size to anything greater than the tetramer. The molecular weight of the least mobile band in cross-linked and dissociated human liver glycolate oxidase agreed well with the molecular weight of 160 000 found by gel filtration and reasonably well with the 179 000 obtained from ultracentrifugation equilibrium sedimentation methods.

When these findings are coupled with the molecular weight data obtained for the subunit by a variety of techniques, including sodium dodecyl sulfate gel electrophoresis of cross-linked (43 000) and non-cross-linked (40 000) oxidases and equilibrium centrifugation in guanidine hydrochloride (43 200), it becomes quite clear that the molecular weight ratio of native human liver glycolate oxidase to its subunit is 4.

The specific activity of human liver glycolate oxidase purified in this study compares favorably with those obtained from other species. The maximum specific activity for the human preparation is about equal to that reported for the crystalline pig enzyme (Schuman & Massey, 1971a), and it is greater than those reported for a rat liver preparation (Duley & Holmes, 1976) and the enzyme obtained from spinach leaves (Frigerio & Harbury, 1958). It is interesting to note that, although the physical properties of these enzymes differ significantly, the catalytic efficiency of glycolate oxidase from the various sources cited is quite similar.

Reports on molecular weight studies conducted with glycolate oxidase obtained from various animal sources have yielded widely divergent results. Dickinson (1965) reported the molecular weight of highly purified porcine glycolate oxidase to range between 342 000 and 400 000 by ultracentrifugation procedures and the oxidase to behave as a 100 000-dalton protein by gel filtration techniques. The explanation proposed for this huge discrepancy was that the enzyme was capable of existing in a rapidly attained monomer–polymer equilibrium. Nakano and co-workers (Nakano et al., 1968) reported the molecular weight of a rat glycolate oxidase preparation determined by gel filtration to be 300 000. Ushijima & Nakano (1969) reinvestigated this enzyme and reported the minimum molecular weight based on FMN content to be 150 000. Recently, Phillips and co-workers (Phillips et al., 1976), utilizing a highly purified preparation of the rat liver enzyme, addressed themselves to the problems encountered by previous investigators of glycolate oxidase. Their results showed quite conclusively that the rat enzyme self-associates at all finite concentrations, and only upon extrapolation to infinite dilution could a reliable molecular weight for the native enzyme be obtained.

Furthermore, their data show that at enzyme concentrations up to 0.5 mg/mL small changes in concentration result in significant changes in the apparent molecular weight. Although specific experiments of the type described by Phillips (Phillips et al., 1976) were not carried out for the human enzyme, the molecular weight values obtained for the native enzyme by three different methods at concentrations ranging from 0.04 to 1.2 mg/mL of protein indicate that unlike the rat enzyme, human enzyme does not self-associate at these concentrations.

The molecular weight range for the human enzyme (160 000–179 000) and its subunit (40 000–43 000) correlates well with the data obtained in this laboratory (unpublished experiments) for the pig liver enzyme (160 000–166 000) and its subunit (40 000–42 000) and those reported by Phillips and co-workers (Phillips et al., 1976) for the rat enzyme (150 000–180 000) and its subunit (43 000).

A comparison of the K_I for *N*-octyloxamate to the K_I of the best previously reported inhibitors of glycolate oxidase shows *N*-octyloxamate to be a better inhibitor by about 2 orders of magnitude. The reason for this large enhancement of inhibitor activity is not entirely clear but appears to be due to at least two factors. In our study of a series of *N*-alkyl- and *N*-aryloxamates (unpublished experiments), the inhibition of pig

liver glycolate oxidase, expressed as $-\log I_{50}$, was found by regression analyses of the calculated Hansch π values (Leo et al., 1971) to be positively correlated with the hydrophobic nature of the substituent. An analogous dependence of K_I on the number of carbon atoms in the alkyl residues was shown by Schuman & Massey (1971b) for the inhibition of the pig liver enzyme by monocarboxylic acids. Therefore, one important factor that contributes significantly to the strong inhibtion shown by *N*-octyloxamate is the presence of the large hydrophobic octyl group. Additional information can be obtained from the second constant of the regression equation, which represents all the average contributions to the observed inhibition, except those due to hydrophobic effects. The fact that the second constant term of the regression equation obtained for the *N*-alkyl- and *N*-aryloxamate series was more positive than the values obtained for a similar series of both α-hydroxy and α-keto acids indicates that the oxamate moiety is an intrinsically better inhibitor than α-hydroxy and α-keto carboxylate groups.

At the present state of our knowledge it is not possible to delineate the specific factors that contribute to the greater intrinsic activity of the oxamate moiety, but one may speculate that when nitrogen is substituted for the β carbon of an α-keto acid, yielding an oxamate, the expected changes such as increased electron density about the carbonyl oxygen, increased capacity for hydrogen bonding, and the large geometric differences may all contribute to the greater activity of the oxamate group.

Acknowledgments

We are grateful to Dr. Christopher T. Walsh and Alfred W. Alberts for many helpful suggestions and the critical reading of the manuscript. We thank Donald W. Denny, Coordinator, Delaware Valley Transplant Program, for his aid in obtaining the human liver specimens and Dr. C. Stanley Rooney, Haydon W. Williams, and Carl Ziegler, of these laboratories, for the syntheses of DL-α-hydroxybutynoate, DL-2-hydroxy-4-phenyl-3-butynoate, and DL-2-hydroxy-6-phenyl-3-hexynoate. Also, we thank Jordan M. Hirshfield for the sedimentation equilibrium experiments and Albert Augenblick for the gas chromatography analyses.

Supplementary Material Available

All experimental details of this work including materials, enzyme activity assays and purification, gel electrophoresis, molecular weight determinations, ultracentrifugation, enzyme kinetics, and computations (10 pages). Ordering information is given on any current masthead page.

References

Abeles, R. H., & Maycock, A. L. (1976) *Acc. Chem. Res. 9*, 313.

Browne, D. T., & Kent, B. H. (1975) *Biochem. Biophys. Res. Commun. 67*, 126.

Chou, T.-C. (1974) *Mol. Pharmacol. 10*, 235.

Cromartie, T. H., & Walsh, C. T. (1975) *Biochemistry 14*, 3482.

Dakin, H. D. (1907) *J. Biol. Chem. 3*, 57.

Davies, G. E., & Stark, G. R. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 651.

Dickinson, F. M. (1965) Doctoral Dissertation, University of Sheffield, England.

Dixon, M., & Webb, E. C. (1964) *Enzymes, 2nd Ed.*, p 75, Academic Press, New York.

Dohan, J. S. (1940) *J. Biol. Chem. 135*, 795.

Duley, J. A., & Holmes, R. S. (1976) *Eur. J. Biochem. 63*, 163.

Frigerio, N. A., & Harbury, H. A. (1958) *J. Biol. Chem. 231*, 135.

Ghisla, S., Agata, H., Massey, V., Schonbrunn, A., Abeles, R. H., & Walsh, C. T. (1976) *Biochemistry 15*, 1791.

Gibbs, D. A., & Watts, R. W. E. (1973) *Clin. Sci. 44*, 227.

Holmes, R. S., & Duley, J. A. (1975) in *Isozymes* (Markert, C. L., Ed.) p 191, Academic Press, New York.

Jorns, M. S. (1975) *Methods Enzymol. 41B*, 337.

Keay, L., Robinson, J. C., Molinari, R., & Sizer, I. W. (1960) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 19*, 26.

Kun, E. (1952) *J. Biol. Chem. 194*, 603.

Kun, E., Dechary, J. M., & Pitot, H. C. (1954) *J. Biol. Chem. 210*, 269.

Leo, A., Hansch, C., & Elkins, D. (1971) *Chem. Rev. 71*, 525.

Liao, L. L., & Richardson, K. E. (1973) *Arch. Biochem. Biophys. 154*, 68.

Massey, V., Müller, F., Feldberg, R., Schuman, M., Sullivan, P. A., Howell, L. G., Mayhew, S. G., Matthews, R. G., & Foust, G. P. (1969) *J. Biol. Chem. 244*, 3999.

Mayhew, S. G., Whitfield, C. D., & Ghisla, S. (1974) *Eur. J. Biochem. 44*, 579.

Müller, F., & Massey, V. (1969) *J. Biol. Chem. 244*, 4007.

Nakano, M., Ushijima, Y., Saga, M., Tsutsumi, Y., & Asami, H. (1968) *Biochim. Biophys. Acta 167*, 9.

Palmer, G., & Massey, V. (1968) in *Biological Oxidations* (Singer, T. P., Ed.) p 263, Interscience, New York.

Phillips, D. R., Duley, J. A., Fennell, D. J., & Holmes, R. S. (1976) *Biochim. Biophys. Acta 427*, 679.

Plowman, K. M. (1972) *Enzyme Kinetics*, McGraw-Hill, New York.

Robinson, J. C., & Sizer, I. W. (1959) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 18*, 311.

Robinson, J. C., Keay, L., Molinari, R., & Sizer, I. W. (1962) *J. Biol. Chem. 237*, 2001.

Schuman, M., & Massey, V. (1971a) *Biochim. Biophys. Acta 227*, 500.

Schuman, M., & Massey, V. (1971b) *Biochim. Biophys. Acta 227*, 521.

Ushijima, Y. (1973) *Arch. Biochem. Biophys. 155*, 361.

Ushijima, Y., & Nakano, M. (1969) *Biochim. Biophys. Acta 178*, 429.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.

Zelitch, I., & Ochoa, S. (1953) *J. Biol. Chem. 201*, 707.