Rat Liver ω-Amidase. Kinetic Evidence for an Acyl-Enzyme Intermediate†

Louis B. Hersh

ABSTRACT: Rat liver ω-amidase catalyzes hydrolysis and acyl-transfer reactions with the amide and monoalkyl esters of α-ketoglutarate, glutarate, and succinate. The specificity of the enzyme has been extended by demonstrating the reactivity of a number of para-substituted phenyl glutarates. *p*-Methoxy, *p*-chloro, *p*-methyl, and the unsubstituted phenyl ester of glutarate are hydrolyzed at about one-half the rate of the methyl ester, and show little dependence on the p*K* of the leaving group. Hydroxaminolysis of glutaramate and methyl-glutarate leads to a twofold increase in the total rate of substrate utilization (hydrolysis plus hydroxaminolysis), with a nonstoichiometric decrease in hydrolysis at lower hydroxylamine concentrations. On the other hand, hydroxylamine has no effect on the rate of *p*-cresol release from *p*-methylphenyl glutarate, and the rate of hydrolysis is decreased proportionally to the increase in hydroxaminolysis. The partitioning of the acyl group derived from glutaramate, methyl glutarate, and *p*-methylphenyl glutarate between water and hydroxylamine is identical for the three substrates. Nucleophiles such as hydroxylamine and methanol show uncompetitive activation for the total rate of glutaramate disappearance, and for a particular acyl donor V_{max} for acyl transfer is independent of the added nucleophile. These results are interpreted in terms of a two-step mechanism involving an acyl-enzyme intermediate.

Rat liver ω-amidase was first described by Meister and coworkers (Meister and Tice, 1950; Meister *et al.*, 1953, 1955; Meister, 1953, 1954). This enzyme, in conjunction with glutamine transaminase (Braunstein and Ting-Sen, 1960a,b; Yoshida, 1967; Cooper and Meister, 1972), comprises the enzyme system previously designated glutaminase II (Errera, 1949; Errera and Greenstein, 1949; Greenstein and Price, 1949), which appears to be involved in the metabolism of glutamine primarily in the liver and kidney.

The ω-amidase has recently been purified and characterized in this laboratory (Hersh, 1971a). In addition to the previously described amidase activity (Meister *et al.*, 1955), the enzyme also catalyzes hydrolysis and acyl-transfer reactions with a number of alkyl esters of α-ketoglutarate, glutarate, and succinate. The existence of acyl-transfer reactions, although readily interpretable in terms of a double-displacement reaction involving an acyl-enzyme intermediate, could proceed by direct nucleophilic attack. In order to distinguish between these two possibilities the kinetics of acyl transfer for a number of glutarate derivatives was investigated. The results of this study, which provide strong evidence for a mechanism involving an acyl-enzyme intermediate, are the subject of this paper.

Materials and Methods

Reagents. Para-substituted phenyl glutarates were prepared by the method of Gaetjens and Morawitz (1960). Ethyl α-ketoglutarate and glutaramate were prepared as previously described (Hersh, 1971a). Hydroxylamine hydrochloride and methylamine hydrochloride were recrystallized from ethanol–ether prior to use. All other reagents were obtained commercially and were generally recrystallized or redistilled before use.

Preparation of Rat Liver ω-Amidase. Rat liver ω-amidase was prepared as previously described (Hersh, 1971a), except that DEAE-Sephadex A-50 was used in place of DEAE-cellulose, and the hydroxylapatite step was omitted.

Enzyme Assays. HYDROLYSIS OF GLUTARATE DERIVATIVES. Ethyl α-ketoglutarate hydrolysis was assayed by following α-ketoglutarate liberation with glutamate dehydrogenase (Hersh, 1970). The hydrolysis of para-substituted phenyl glutarates was followed by measuring phenol release. The extinction coefficient of the phenols was determined with redistilled compounds: (compound, λ (nm), E(M)) phenol, 270, 1380; *p*-chlorophenol, 280, 1360; *p*-methylphenol, 277, 1540; *p*-methoxyphenol, 285, 2200).

Methyl glutarate hydrolysis was measured by following the disappearance of ester as determined by the alkaline hydroxylamine method of Hestrin (1949). Glutaramate hydrolysis was measured by following ammonia release with glutamate dehydrogenase (Su *et al.*, 1969).

ACYL-TRANSFER REACTIONS. The reaction of hydroxylamine with glutarate derivatives was assayed by measuring hydroxamate formation by the method of Lipmann and Tuttle (1945). Acyl transfer to methylamine was followed by measuring the formation of [^{14}C]-*N*-methyl glutaramate as previously described (Hersh, 1970). Methanolysis of glutaramate was determined by measuring the appearance of ester with alkaline hydroxylamine (Hestrin, 1949). Stock solutions of *p*-methoxy- and *p*-chlorophenyl glutarate were prepared in 40% dioxane, while *p*-methylphenyl and phenyl glutarate were prepared in 100% dioxane. The concentration of ester was adjusted such that the concentration of dioxane in the reaction mixture was 2% or less. Control experiments showed dioxane, up to a concentration of 5%, has no effect on the hydrolysis of ethyl α-ketoglutarate or glutaramate, and at 10% dioxane, only 5–10% inhibition was observed.

Results

Rat liver ω-amidase catalyzes the hydrolysis of the amide

† From the Department of Biochemistry, The University of Texas, Southwestern Medical School at Dallas, Dallas, Texas 75235. *Received February 1, 1972.* This work was supported in part by grants from the National Institutes of Health (AM 13443) and the Robert A. Welch Foundation (I 391), Houston, Texas.

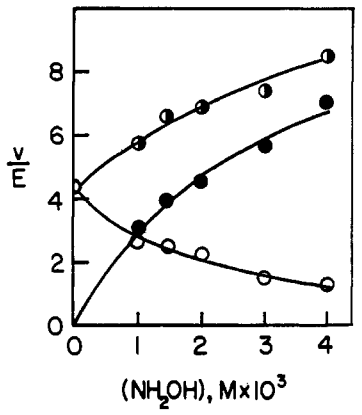


FIGURE 1: The effect of hydroxylamine on the reaction of methyl glutarate. Reaction mixtures contained 50 mM potassium phosphate buffer (pH 7.0), 20 mM methyl glutarate, hydroxylamine as indicated, and 56 μg of enzyme in a final volume of 2.0 ml. Hydrolysis (○) was measured by the disappearance of ester with alkaline hydroxylamine, and hydroxaminolysis (●) by hydroxamate formation. The total reaction rate (◑) was calculated from the equation V(total) = V(hydrolysis) + V(hydroxaminolysis). V/E expressed as μmoles/min per mg protein.

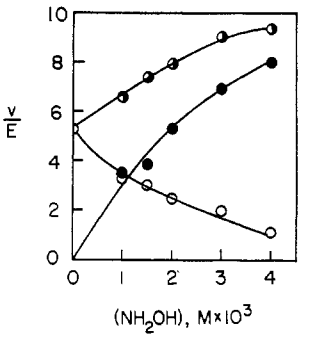


FIGURE 2: The effect of hydroxylamine on the reaction of glutaramate. Reaction mixtures were identical with those described in Figure 1 except 20 mM glutaramate replaced 20 mM methyl glutarate. Hydroxaminolysis (●) was measured by hydroxamate formation, and the total reaction rate (◑) by ammonia liberation. The rate of hydrolysis (○) was calculate from the equation: V(hydrolysis) = V(total) − V(hydroxaminolysis). V/E expressed as μmoles/min per mg of protein.

and monoalkyl esters of α-ketoglutarate, oxaloacetate (only the amide has been examined), succinate, and glutarate (Meister *et al.*, 1955; Hersh, 1971a). In order to learn more about the specificity of the enzyme and to provide a broader class of substrates to be examined, the hydrolysis of a number of para-substituted phenyl glutarates was studied. Table I lists the kinetic parameters, K_m and V_{max}, for the hydrolysis of *p*-chlorophenyl glutarate, phenyl glutarate, *p*-methylphenyl glutarate, and *p*-methoxyphenyl glutarate. The maximal velocities for the hydrolysis of these phenyl esters are approximately two- to threefold less than the V_{max} for the corresponding amide or methyl ester, and show only a slight dependence on the pK of the phenol.

Evidence that phenyl glutarates are hydrolyzed by the amidase, rather than by a contaminating enzymatic activity, was obtained by showing that the ratio of specific activity for ethyl α-ketoglutarate hydrolysis compared to the specific activity for *p*-methylphenyl glutarate hydrolysis remains constant over the 100-fold purification required to obtain essentially pure enzyme (Table II).

TABLE I: Kinetic Constants for the Hydrolysis of Glutarate Derivatives.[a]

Substrate	K_m (M)	V_{max} (μmoles/min per mg of Protein)
p-Methoxyphenyl glutarate	8.0×10^{-3}	2.68
p-Methylphenyl glutarate	2.0×10^{-3}	2.76
Phenyl glutarate	8.0×10^{-3}	2.76
p-Chlorophenyl glutarate	3.7×10^{-3}	3.96
Methyl glutarate	3.0×10^{-3}	7.50
Glutaramate	2.0×10^{-3}	6.42

[a] Kinetic constants were determined in 50 mM potassium phosphate buffer (pH 7.0) at 30°.

Hydroxaminolysis. The effect of increasing concentrations of hydroxylamine on the hydrolysis, the hydroxaminolysis, and total reaction rate with glutaramate, methyl glutarate, and *p*-methylphenyl glutarate as substrates is shown in Figures 1–3. With glutaramate and methyl glutarate, the total reaction rate increases with increasing hydroxylamine concentration. Although the hydrolytic rate is decreased in the presence of hydroxylamine, this decrease is not stoichiometric with hydroxamate formation. On the other hand, with *p*-methylphenyl glutarate as substrate, the total reaction rate remains constant with increasing concentrations of hydroxylamine, and the rate of hydrolysis decreases stoichiometrically with hydroxamate formation. In all three of the above cases,

TABLE II: Constancy of the Specific Activity for Ethyl α-Ketoglutarate Hydrolysis Compared to the Specific Activity of *p*-Methylphenyl Glutarate Hydrolysis During Purification of Amidase.[a]

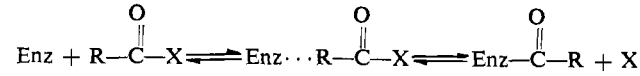
Step	Sp Act. for Ethyl α-Ketoglutarate Hydrolysis	Sp Act. for Ethyl α-Ketoglutarate Hydrolysis: Sp Act. for *p*-Methylphenyl Glutarate Hydrolysis
Extract	0.17	6.8
50:70% $(NH_4)_2SO_4$	0.85	6.9
DE-Sephadex	5.3	7.0
CM-Sephadex	17.4	6.9

[a] Assay for ethyl α-ketoglutarate hydrolysis: reaction mixture contained 50 mM potassium phosphate buffer (pH 7.4), 2 μM ethyl α-ketoglutarate, 0.125 μM DPNH, 10 mM ammonium chloride, 100 μg of glutamate dehydrogenase, enzyme, and water in a final volume of 1.0 ml. The reaction was measured by the oxidation of DPNH at 340 nm at 30°. Assay for *p*-methylphenyl glutarate hydrolysis: reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 5 mM *p*-methylphenyl glutarate, and enzyme in a final volume of 1.0 ml. The reaction was followed by measuring *p*-cresol liberation at 227 nm at 30°.

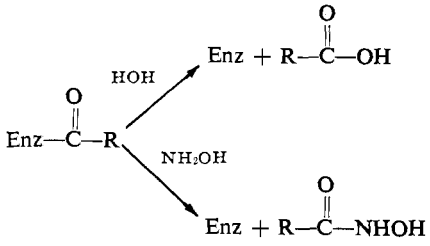
the maximal velocity for substrate utilization at infinite hydroxylamine is equal to the maximal velocity for the hydroxaminolysis reaction.

These results are most readily interpreted in terms of a two-step mechanism involving an acyl-enzyme intermediate

Acylation:



Deacylation:



When glutaramate and methyl glutarate serve as acyl donors hydrolysis proceeds with both acylation and deacylation partially rate limiting. The addition of a second acyl acceptor to the system (*i.e.*, hydroxylamine) causes an increase in the total reaction rate; deacylation by reaction with water plus deacylation by reaction with hydroxylamine. Since acylation is only partially rate determining, hydroxaminolysis initially proceeds with only a slight decrease in the hydrolytic rate; however, as the hydroxylamine concentration is increased, acylation becomes more and more rate limiting, until eventually hydrolysis decreases at the expense of hydroxaminolysis, and V(total) equals V(hydroxaminolysis). The situation with *p*-methylphenyl glutarate as acyl donor is different in that acylation is always the rate-determining step. Under these conditions the acyl-enzyme reacts as soon as it is formed so that hydroxaminolysis can only occur at the expense of hydrolysis.[1]

Regardless of whether acylation or deacylation is rate limiting, if the reaction proceeds through a common acyl-enzyme intermediate, the partitioning of this intermediate between two different acyl acceptors (*i.e.*, water and hydroxylamine) should be independent of the leaving group. The data in Figure 4 show that over the range of 1–4 mM hydroxylamine the ratio of hydroxaminolysis to hydrolysis is identical for the amide, the methyl ester, and the *p*-methylphenyl ester of glutarate.

The hydrolysis of amides and esters *via* an acyl-enzyme mechanism can be kinetically classified as a special case of a "double displacement" or "Ping-Pong" mechanism. The inability to demonstrate Ping-Pong kinetics is, of course, due to the fact that the acyl acceptor, water, cannot be varied. If an acyl acceptor other than water can participate in the reaction, and the rate of acyl transfer to this alternate substrate is as fast or faster than the transfer to water, the reaction will obey eq 1 (Chung *et al.*, 1970), where A = the ester or amide substrate, B = the added nucleophile, and K_A, K_B, and K_{IB} are constants as defined by Chung *et al.* (1970). This equation describes hyperbolic uncompetitive activation by the added nucleophile B, so that double-reciprocal plots of $1/v$ *vs.* 1/[A] at fixed levels of B should result in a series of parallel lines. As shown in Figure 5, double-reciprocal plots of 1/rate of glutaramate disappearance *vs.* 1/[glutaramate] at varying levels of hydroxylamine do indeed approximate a series of parallel lines. The theoretical lines drawn through the experimental points in Figure 5, were obtained by fitting the data to eq 1 with the computer program of Knot and Reace (1971). The following kinetic constants were obtained: V_H = 5.55 ± 0.13 μmoles/min per mg of enzyme, K_{AH} = 2.0 ± 0.08 mM, and K_{IB} = 4.9 ± 0.8 mM. (It was not technically feasible to obtain an accurate determination of K_B.)

$$v = \frac{V_H[A]}{K_{AH} + [A]\left[\frac{1 + [B]/K_B}{1 + [B]/K_{IB}}\right]} \quad (1)$$

Alcoholysis and Aminolysis. The observation that the ω-amidase catalyzes hydrolysis of glutaramate and methyl-glutarate with nearly identical maximal velocities (Table I)

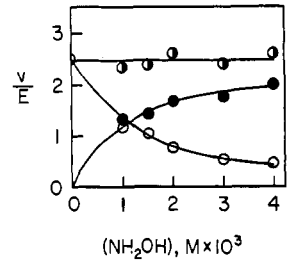


FIGURE 3: The effect of hydroxylamine on the reaction of *p*-methylphenyl glutarate. Reaction mixtures were identical to those described in Figure 1 except 5 mM *p*-methylphenyl glutarate replaced methyl glutarate. Two separate runs were made for each hydroxylamine concentration. In the first, V(total) (◑) was measured by *p*-methylphenol release, while in the second run the rate of hydroxaminolysis (●) was measured by hydroxamate formation. The rate of hydrolysis (○) was calculated as described in Figure 2. V/E expressed as μmoles/min per mg of protein.

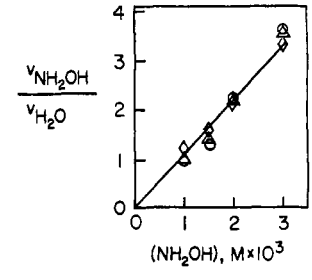


FIGURE 4: Partitioning of the glutaryl group derived from methyl glutarate, glutaramate, and *p*-methylphenyl glutarate between hydroxylamine and water. The experimental points are those values calculated from the data in Figures 1–3. △, Methyl glutarate; ○, glutaramate; ◇, *p*-methylphenyl glutarate.

[1] Although the data in Figures 1–3 is too limited for a detailed kinetic analysis, the rate equations (Chung *et al.*, 1970) for the total reaction:

$$V = \frac{V_T(A)}{K_{AT} + A\frac{(1 + (B)/K_B)}{(1 + (B)/K_{IB})}}$$

and hydroxaminolysis

$$V = \frac{V_T(A)(B)}{K_{AT}(B) + K_B(A) + K_{AH}K_B + (A)(B)}$$

both reduce to $V = V_T(A)/[K_{AT} + (A)]$ at infinite hydroxylamine. Thus at infinite hydroxylamine, the observed equality of rates for the total reaction, and the hydroxaminolysis reaction is predicted from the rate equations.

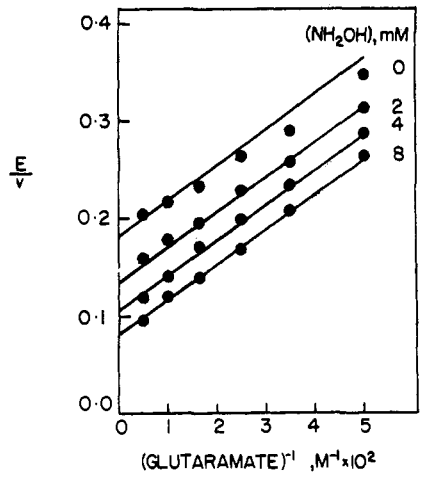


FIGURE 5: Uncompetitive action by hydroxylamine for the rate of glutaramate disappearance. Reaction mixtures were identical with Figure 1 except that glutaramate and hydroxylamine were added as shown in the figure. The rate of glutaramate disappearance was measured by ammonia release. E/V expressed as 1/μmole of glutaramate utilized per min per mg of protein.

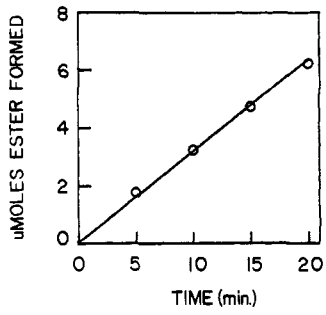


FIGURE 6: Methanolysis of glutaramate. Reaction mixtures contained 50 mM potassium phosphate buffer (pH 7.0), 20 mM glutaramate, 2.0 M methanol, and 88 μg of enzyme in a final volume of 1.5 ml. The reaction mixture was incubated at 30°, and at the time periods indicated, 0.2-ml aliquots were withdrawn and assayed for ester formation as described in Methods. Control experiments in which enzyme was omitted or boiled enzyme was used, showed no ester formation.

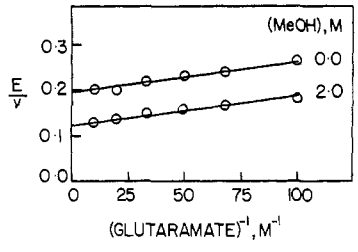


FIGURE 7: Uncompetitive activation by methanol for the rate of glutaramate disappearance. Reaction mixtures identical with Figure 5 except that methanol was used in place of hydroxylamine. The rate of glutaramate disappearance was measured by ammonia release. E/V expressed as 1/μmole of glutaramate utilized per min per mg of protein.

prompted us to determine if we could measure alcoholysis of the amide substrate. Using methanol as the acyl acceptor, the alcoholysis of glutaramate was detected by measuring the appearance of ester with alkaline hydroxylamine (see Methods). A typical experiment is shown in Figure 6 in which

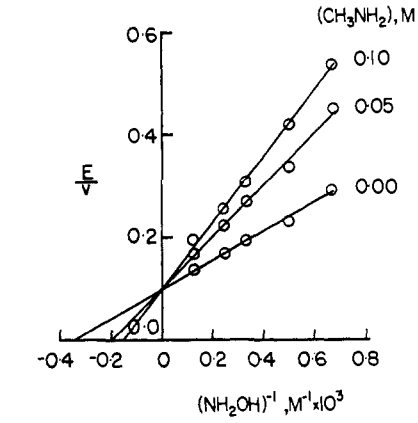


FIGURE 8: Competitive inhibition by methylamine with respect to hydroxamine. Reaction mixtures contained 50 mM Tris-hydrochloride buffer (pH 8.5), 10 mM glutaramate, 40 μg of enzyme, and hydroxylamine and methylamine as indicated in a final volume of 1.5 ml. Hydroxamate formation measured as described in Methods. E/V expressed as 1/μmole of glutaryl hydroxamate formed per min per mg of protein.

TABLE III: Maximal Velocities for Acyl-Transfer Reactions.

Acyl Donor	mM	Acyl Acceptor	K_m (mM)	V_{max} (μmoles/min per mg of Protein)
Glutaramate	20	NH_2OH	3.7	15.1
	20	CH_3NH_2[a]	43[b]	14.5
	20	MeOH	5000	14.2
Methyl glutarate	20	NH_2OH	4.1	12.6
	20	CH_3NH_2[a]	30[b]	13.2
p-Methylphenyl glutarate	5	NH_2OH	1.0	2.8
	5	CH_3NH_2[a]	55[b]	2.5

[a] The reaction was measured at pH 8.5. Unpublished results showed that both the hydrolysis and hydroxaminolysis are independent of pH over the range of 6.5–8.5. [b] These values are for total methylamine, for the free-base form of methylamine, K_m is $\sim 10^2$ times smaller than the value listed above.

alcoholysis was measured over a 20-min time period. Since ammonia (pK = 9.2) is a much stronger nucleophile than methanol (pK = 15.5), it would be expected that alcoholysis of an amide would be difficult to demonstrate because of the effective competition for the acyl group by small concentrations of ammonia. The ability to observe alcoholysis in the present case can most probably be accounted for by two factors: first, initial velocities were measured so that the amount of ammonia produced was quite small, and secondly, the reaction was conducted at pH 7.0, a pH at which the free base form of ammonia is approximately 0.5% of the total ammonia concentration.

As shown for the reaction in the presence of hydroxylamine, uncompetitive activation for V(total) was observed in the presence of 2 M methanol (Figure 7). No alcoholysis was observed using ethanol, 2-chloroethanol, and 2,2,2-trifluoroethanol at concentrations from 0.2 to 2.0 M. At concentrations greater

than 2 M, these alcohols caused irreversible inactivation of the enzyme.

The reaction of methylamine with substrates of the ω-amidase has previously been demonstrated (Hersh, 1971a), however, maximal velocities were not determined. Table III lists the maximal velocities for this reaction, along with the maximal velocities for hydroxamate formation and methanolysis. (The latter reaction was only determined with glutaramate as the acyl donor.) As is apparent from this table, these reactions have identical maximal velocities for a particular acyl donor.

Methylamine was found to inhibit the hydroxaminolysis of glutaramate. This inhibition was found to be competitive with respect to glutaramate (Figure 8). That methylamine reacts in the free-base form is evidenced by the observation that at pH 7.0 methylamine (0.2 M) has no effect on the hydroxaminolysis reaction.

Discussion

Several kinetic criteria are presented in this paper which strongly support the contention that reactions catalyzed by rat liver ω-amidase proceed by a two-step mechanism involving an acyl-enzyme intermediate. These include the following: (a) demonstration that the total reaction rate increases with increasing concentrations of hydroxylamine for the two most reactive glutarate derivatives, glutaramate, and methyl glutarate, but remains constant for the less reactive substrate, *p*-methylphenyl glutarate; (b) demonstration of identical partitioning ratios between hydroxylamine and water for the amide, the methyl ester, and the *p*-methylphenyl ester of glutarate; (c) demonstration of uncompetitive activation by hydroxylamine and methanol for the rate of glutaramate utilization; and (d) demonstration of identical maximal velocities for acyl transfer to hydroxylamine and methylamine.

The effect of an added nucleophile on the total reaction rate has been previously discussed by Greenzaid and Jencks (1971), and, as described in the text, is best accounted for in terms of an acyl-enzyme intermediate.

The demonstration that an acyl group derived from chemically different donor molecules is partitioned identically between two nucleophiles has been used previously to support an acyl-enzyme mechanism for chymotrypsin (Epand and Wilson, 1963, 1965), glutaminase (Hartman, 1968), and 5-hydroxy-*N*-methylpyroglutamate synthetase (Hersh, 1971b). Although a direct transfer from the donor molecule to the acceptor could proceed at a constant ratio of rates (Jencks, 1969), this situation appears unlikely since the ester and amide of acetate, which are chemically similar to the substrates used in this study, differ in their nonenzymatic partitioning between hydroxylamine and water by a factor of 10^4 (Hartman, 1968).

Uncompetitive activation of a reaction represents a special case of Ping-Pong kinetics (Cleland, 1963), and requires that the reaction be sequential, that the first product be released before the second substrate reacts, and that the enzyme exist in two forms. Although this kinetic pattern does not, in itself, require a covalent enzyme–substrate intermediate, in the present case no other intermediate would logically fit these criteria.

The observation that the reaction of a series of nucleophiles with a particular acyl donor proceed at identical maximal velocities is also consistent with the proposed mechanism. It is difficult to imagine how direct nucleophilic attact by compounds of such widely different nucleophilic reactivity as hydroxylamine, methylamine, and methanol, could proceed with the same rate constants. A slow isomerization of the enzyme, or enzyme–substrate complex, could give such a result; however, the observation that acyl transfer proceeds more rapidly than hydrolysis rules out this possibility. On the other hand, this observation is consistent with the acyl-enzyme mechanism, in which acylation of the enzyme is the rate-determining step in acyl-transfer reactions.

Unfortunately, the fact that acylation is rate determining for acyl group transfer reactions does not permit an unambiguous assignment of a nucleophilic binding site to the enzyme. The observation that ethanol and its derivatives do not react with the acyl-enzyme, even though the p*K* of trifluoroethanol is 2 p*K* units less than methanol, and that methylamine but not ethylamine is an inhibitor of α-ketoglutaramate hydrolysis (Hersh, 1971a) implies that there is a nucleophilic binding site on the enzyme which cannot accommodate molecules larger than methanol or methylamine.

Acknowledgments

I thank Drs. J. E. Folk and William P. Jencks for many helpful discussions and Mrs. Judy Gravina for her skilled technical assistance. I also extend my gratitude to Dr. J. E. Folk for performing the computer analysis.

References

Braunstein, A. E., and T'ing-Sen, H. (1960a), *Biokhimiya 25*, 758.

Braunstein, A. E., and T'ing-Sen, H. (1960b), *Biochim. Biophys. Acta 44*, 187.

Chung, S. I., Shrager, R. I., and Folk, J. E. (1970), *J. Biol. Chem. 245*, 6424.

Cleland, W. W. (1963), *Biochim. Biophys. Acta 67*, 104, 173.

Cooper, A. J. L., and Meister, A. (1972), *Biochemistry 11*, 661.

Epand, R. M., and Wilson, I. B. (1963), *J. Biol. Chem. 238*, 1718.

Epand, R. M., and Wilson, I. B. (1965), *J. Biol. Chem. 240*, 1104.

Errera, M. (1949), *J. Biol. Chem. 178*, 483.

Errera, M., and Greenstein, J. P. (1949), *J. Biol. Chem. 178*, 495.

Gaetjens, E., and Morawitz, H. (1960), *J. Amer. Chem. Soc. 82*, 5328.

Greenstein, J. P., and Price, V. E. (1949), *J. Biol. Chem. 178*, 695.

Greenzaid, P., and Jencks, W. P. (1971), *Biochemistry 10*, 1210.

Hartman, S. C. (1968), *J. Biol. Chem. 243*, 853.

Hersh, L. B. (1970), *J. Biol. Chem. 245*, 3526.

Hersh, L. B. (1971a), *Biochemistry 10*, 2884.

Hersh, L. B. (1971b), *J. Biol. Chem. 246*, 6803.

Hestrin, S. (1949), *J. Biol. Chem. 180*, 249.

Jencks, W. P. (1969), Catalysis in Chemistry and Enzymology, New York, N. Y., McGraw-Hill, p 53.

Knot, G. D., and Reace, D. K. (1971), Modelab User Documentation, DCRT Report, National Institutes of Health.

Lipmann, F., and Tuttle, L. C. (1945), *J. Biol. Chem. 159*, 21.

Meister, A. (1953), *J. Biol. Chem. 200*, 573.

Meister, A. (1954), *J. Biol. Chem. 210*, 17.

Meister, A., Levintow, L., Greenfield, R. E., and Abendschein, P. A. (1955), *J. Biol. Chem. 215*, 441.

Meister, A., Sobert, H. A., Tice, S. V., and Fraser, P. E. (1953), *J. Biol. Chem. 197*, 319.
Meister, A., and Tice, S. V. (1950), *J. Biol. Chem. 187*, 173.
Su, S., Alkizati, L., and Chaykin, S. (1969), *J. Biol. Chem. 244*, 2956.
Yoshida, T. (1967), *Bitamin 35*, 227.

Isolation and Properties of Luciferase, a Non-Heme Peroxidase, from the Bioluminescent Earthworm, *Diplocardia longa*†

Ronald Bellisario,‡ Terry E. Spencer, and Milton J. Cormier*

ABSTRACT: It has been demonstrated that *in vivo* luminescence from the bioluminescent earthworm, *Diplocardia longa*, arises from the coelomic fluid exuded by the worm following mechanical or electrical stimulation. The blue-green luminescence has an unstructured peak emission at 19,700 cm^{-1} (507 nm) with a half-width of 3070 cm^{-1} (78 nm). The source of the luminescence has been shown to be cells present in the coelomic fluid of the worms; these coelomic cells, about 40 μ in diameter, have been isolated by sucrose gradient centrifugation. Luciferase has been isolated in highly purified form from the coelomic cells of *D. longa* and has been shown to have an approximate molecular weight of 300,000 and a frictional ratio (f/f_0) of 2.10 indicating that it is highly asymmetric. Data from sodium dodecyl sulfate gel electrophoresis indicates that luciferase is composed of three pairs of nonidentical subunits of molecular weight 71,000, 58,000, and 14,500. The absorption and fluorescence spectra of luciferase are those of a typical protein with no evidence of any ultraviolet or visible absorbing prosthetic groups. Luciferase is inhibited by a variety of metal-binding agents such as potassium cyanide, *o*-phenanthroline, and sodium diethyldithiocarbamate, but the activity of luciferase could not be correlated with the metal content of the protein. The enzyme is also irreversably inactivated by H_2O_2 and kinetic data suggest that one molecule of H_2O_2 per luciferase site is required for this inactivation. During the light reaction, therefore, luciferase does not exhibit normal enzymatic catalysis and "turnover."

It has been previously demonstrated that luciferin, luciferase, and H_2O_2 are required for *in vitro* light emission from the earthworm, *Diplocardia longa* (Cormier *et al.*, 1966; Bellisario and Cormier, 1971). These requirements for luminescence are expressed in the following reaction scheme

$$\text{luciferin} + H_2O_2 \xrightarrow{\text{luciferase}} \text{light (max} = 507 \text{ nm)} + \text{products}$$

Thus, the *Diplocardia* luminescent system belongs to a class of peroxidative bioluminescent reactions in which H_2O_2 is utilized for light emission instead of molecular oxygen (Cormier and Totter, 1968; Hastings, 1968).

We present evidence that luciferase and luciferin are located within the coelomic cells of this bioluminescent worm. *Diplocardia* luciferase has been isolated in the present study in order to determine the physical properties of a luciferase of the peroxidative type. Furthermore, we show that *Diplocardia* luciferase is a non-heme protein and exhibits no activity toward a number of typical peroxidase substrates such as guaiacol, benzidine, or pyrogallol. In addition, luciferase is irreversibly inactivated by H_2O_2 and kinetic data indicates that luciferase does not exhibit normal enzymatic catalysis and "turnover."

Materials and Methods

The following materials were purchased from the suppliers indicated: crystalline bovine serum albumin and crystalline egg albumin (Pentex); twice crystallized catalase (Worthington); fumarase (Nutritional Biochemicals); glutamic dehydrogenase (Boeringer); fibrinogen (Mann); lysozyme and twice crystallized catalase (Sigma); Sephadex gels and Blue Dextran 2000 (Pharmacia); acrylamide, Agarose 1.5 m gel, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylenediamine (California Biochemicals); sodium lauryl sulfate (Mann); Sepraphore III cellulose acetate electrophoresis strips and Tris-barbital (pH 8.8) buffer (Gelman); riboflavin and Bromophenol Blue (Eastman); Coomassie Blue (Colab Laboratories); DEAE-cellulose (Whatman); hydroxylapatite (Serva); hydrogen peroxide (Baker); succinyl peroxide and ethyl hydroperoxide (K & K Labs); dimethylbenzyl hydroperoxide (Matheson, Coleman & Bell); *o*-phenanthroline (G. F. Smith Co.); sodium diethyldithiocarbamate (Eastman); 8-hydroxyquinoline, benzidine, *o*-dianisidine, guaiacol (Sigma). All other chemicals used were from commercial sources and are the highest quality available.

Isolation of Coelomic Cells. Coelomic fluid from *D. longa* was collected by electrically stimulating two worms with a manually operated magneto generator in 30 ml of an earthworm Ringer solution (Block *et al.*, 1964) which was made hypertonic with 0.1 M KCl and 0.1 M NaCl. Following this stimulus, the coelomic fluid was exuded from the mouth and dorsal pores of the worms and had a milky appearance due to the presence of numerous cells suspended in the fluid. All following operations were carried out at 4°.

When the worms ceased exuding coelomic fluid, they were washed and homogenized in 60 ml of 0.1 M sodium borate

† From the Department of Biochemistry, University of Georgia, Athens, Georgia 30601. *Received December 16, 1971.* This work was supported in part by grants from the U. S. Atomic Energy Commission [AT (40-1)-2741, AT (30-1)-3401, and AT (40-1)-3974] and the National Science Foundation (GB-7400X1). M. J. C. is a Career Development awardee (20K3-GM-3331-07) of the U. S. Public Health Service.

‡ In partial fulfillment of the requirements for the degree of Doctor of Philosophy.

* To whom correspondence should be addressed.