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EFFECTS OF pH AND SULFHYDRYL SPECIFIC REAGENTS ON 4-FUMARYLACETOACETATE FUMARYLHYDROLASE

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Summary

The pH-dependence of fumarylacetoacetase (4-fumarylacetoacetate fumarylhydrolase, EC 3.7.1.2) activity was studied in the pH range 6.25—8.50. After correction of the substrate concentration for enolate formation, the Michaelis constant was found to be pH independent in this range. Likewise, the K_i values for the competitive inhibitors chloride and fluoride were found to be independent of pH between 6.25—8.50. A bell-shaped curve described the log V vs. pH dependence, and ionization constants of 6.5 and 8.2 were calculated. Tentatively an imidazole group and a sulfhydryl group were assigned to the constants 6.5 and 8.2, respectively.

Both *p*-hydroxymercuribenzoate and 5,5′-dithiobis(2-nitrobenzoic acid) react with two sulfhydryl groups per subunit in the native protein and three sulfhydryl groups per subunit in the denatured protein. Substrate protects one sulfhydryl group in the native protein from reaction with 5,5′-dithiobis(2-nitrobenzoic acid). Substrate or the competitive inhibitor, fluoride, protect the enzyme from inactivation by *p*-hydroxymercuribenzoate. In addition *p*-hydroxymercuribenzoate shows saturation kinetics. Neither sulfhydryl inhibitor completely inactivates the enzyme. The enzyme is described as having three sulfhydryl groups per subunit, one of which is inaccessible to the sulfhydryl specific reagents when the protein is in the native state. One of the two accessible sulfhydryl groups is either near the active site or essential in maintaining the structure of the protein.

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Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PHMB, *p*-hydroxymercuribenzoate; Bistris, bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane.

Introduction

Fumarylacetoacetase (4-fumarylacetoacetate fumarylhydrolase, EC 3.7.1.2) is the enzyme at the terminal step of tyrosine catabolism and catalyzes the hydrolysis of a carbon-carbon bond in fumarylacetoacetate [1]. Recently a decreased activity of this enzyme has been suggested as the primary defect in hereditary tyrosinemia [2]. In rat liver this enzyme is localized in the cytoplasm [3].

The enzyme has been isolated from beef liver [4]. The protein has a molecular weight of about 86 000 made up of two identical subunits with three free sulfhydryl groups per subunit [5]. Preliminary reports of the pH dependence [5] and inactivation by sulfhydryl reagents [4] of fumarylacetoacetase have been made. A more detailed study of the effects of pH and sulfhydryl modification to further elucidate the structure and mechanism of this enzyme is described in the following account.

Experimental section

Materials. The enzyme, acetopyruvate and propionopyruvate were prepared as previously described [5]. Other materials were reagent grade and used without further purification.

Methods. Assays for the determination of K_m and V values for propionopyruvate were performed in triplicate at 25°C and the data analyzed as previously described [5]. The pK_a values from the log V vs. pH curve were determined using a non-linear least-squares program [6]. Errors are reported as standard deviations. The pH of each solution was measured with a Radiometer pH meter. If the pH changed by more than 0.1 units during the assay the data were rejected. Extinction coefficients for propionopyruvate at the various pH values were determined from the slopes of Beer-Lambert plots whose correlation constants were 0.96 or better. The pK_a of propionopyruvate was estimated to be 8.0 from a plot of absorbance vs. pH.

The determination of K_i values for chloride and fluoride as a function of pH was performed using the method of Braun and Schmidt [7]. Values of K_m previously obtained were used to calculate K_i values.

All reactions for the titrations of protein sulfhydryl groups were performed on a Beckman ACTA MARK VI spectrophotometer. The cell compartment was maintained at 30°C for enzymatic activity assays and 25°C for spectrophotometric titrations. All reagents and substrate solutions were prepared freshly each day. 1.2 mg DTNB was dissolved in 1 ml of 50 mM acetate buffer, pH 5.0, and sealed with parafilm under a nitrogen atmosphere [8]. PHMB (1.9 mg) was dissolved in water plus 0.05 ml of 0.1 M sodium hydroxide to give a final volume of 10 ml. After centrifugation the concentration was checked spectrophotometrically at 232 nm by dilution in 50 mM phosphate buffer, pH 7.0. A molar extinction coefficient at $1.69 \cdot 10^4$ l/mol^{-1} · cm^{-1} was used to calculate the concentration of PHMB in solution [9], or the mercurial was titrated with glutathione [10]. Enzyme activity was determined using acetopyruvate or propionopyruvate and fumarylacetoacetase concentration determined using ϵ^{280} = 1.3 ml/mg^{-1} · cm^{-1} [5].

Accessible sulfhydryl groups of the native protein were determined with DTNB by the method of Ellman [11], and PHMB using the procedure of Boyer [12] with the modifications of Carne et al. [9]. The course of the reactions were followed at 412 nm in the case of DTNB and 250 nm in the case of PHMB. Titration of total sulfhydryl groups in the denatured enzyme was done as previously described with the following additions. Urea was added to a concentration of 8 M and the solution allowed to stir in a sealed vial under nitrogen for 10 min. After this time aliquots of DTNB or PHMB were added.

First-order-rate constants for inactivation by PHMB were determined from plots of the logarithm of percent activity vs. time. Reported rate constants are the average of three or four determinations. Enzyme in 1 ml of 0.1 M phosphate buffer (pH 7.3) was treated by adding PHMB (30 μl) to the stirred solution. Aliquots were removed from the reaction mixture at appropriate time intervals and tested for activity in a solution containing 0.2 mM propinopyruvate in 3 ml of 0.25 M phosphate (pH 7.3). Incubation under identical conditions without inhibitor served as a control. Protection against PHMB inactivation was studied by adding potentially protective substances to the enzyme solution before introduction of the mercurial.

Enzyme was inactivated with PHMB at 0°C to 20% of its original activity. The molecular weight of a sample of the inactivated enzyme was determined on a G-150 column (150 X 2 cm) as previously described [5].

Results

A series of buffers at various concentrations was tested for potential inhibition of enzyme activity using propionopyruvate as the substrate. The buffers Bistris sulfate at pH 6.5 and Tris-sulfate at pH 8.0 had no effect on enzyme activity over the concentration range 0.035—0.20 M. Bistris (0.1 M) was used to determine enzyme activities at pH values of 6.25, 6.50, 6.75, 7.00, 7.50 and Tris-sulfate was used to determine enzyme activities at pH values of 7.30, 7.70, 8.00, 8.30 and 8.50. In this pH range the enzyme was stable for at least 10 min.

The K_m and V values were determined as a function of pH from initial rates of the hydrolysis of propionopyruvate. This assay depends upon the decrease in absorbance due to the decrease in the concentration of the enol form of propionopyruvate. Extinction coefficients of propionopyruvate solutions were determined at each pH.

Values of the Michaelis constants were determined in two ways (Table I). In one case (K_m) the total concentration of substrate was used. This assumes that both the enolate and keto form of the substrate are bound and hydrolyzed by the enzyme. In the second case (K'_m) only the keto form of the substrate is assumed to be bound and hydrolyzed by the enzyme. The fraction of propionopyruvate in the keto form was calculated from the equation $[\text{keto form}]_0 = [\text{total substrate}]_0/1 + 10^{-8}$, where total substrate is the sum of both the keto and enolate forms and 8.0 is the pK_a value of the enolizable proton of propionopyruvate. A new least-squares analysis was performed to obtain the K'_m values listed in Table I. V values calculated from the plots using the substrate concentrations corrected for the enol content were within experimental error of those shown in Fig. 1. A plot of log V vs. pH is shown in Fig. 1. Assuming a

TABLE I

K_m AND K'_m VALUES FOR PROPIONOPYRUVATE AND K_i VALUES FOR CHLORIDE AND FLUORIDE AS A FUNCTION OF pH

pH	$K_m \cdot 10^4$ (M)	$K'_m \cdot 10^4$ (M)	K_i (mM)	
			Chloride	Fluoride
6.25	2.3 ± 0.2	2.1 ± 0.2	56 ± 8	5.4 ± 0.9
6.50	2.8 ± 0.3	2.8 ± 0.2	50 ± 6	4.4 ± 0.6
6.75	3.3 ± 0.3	2.9 ± 0.2	66 ± 15	4.3 ± 0.4
7.00	3.8 ± 0.5	3.4 ± 0.5	51 ± 8	3.1 ± 0.4
7.30	4.2 ± 0.9	3.1 ± 0.6	53 ± 12	3.2 ± 0.7
7.50	4.5 ± 0.5	3.3 ± 0.7	43 ± 7	2.9 ± 0.3
7.70	4.7 ± 1.8	2.4 ± 0.9	55 ± 10	4.1 ± 0.5
8.00	5.6 ± 1.3	2.1 ± 0.4	59 ± 14	4.0 ± 1.0
8.30	5.6 ± 1.0	2.0 ± 0.4	59 ± 11	4.2 ± 0.7
8.50	6.0 ± 0.7	1.6 ± 0.7	59 ± 8	4.3 ± 0.5

bell-shaped curve, ionization constants at 6.55 and 8.19 were used to generate the solid line of Fig. 1.

Inhibition constants of chloride and fluoride were determined at the various pH values by the method of Braun and Schmidt [7]. At each pH the inhibition was found to be strictly competitive. The inhibition constants are listed in Table I.

The sulfhydryl groups of the enzyme were reacted with DTNB under conditions where the protein is denatured (8 M urea), where the protein is in its native form and where the protein is in the presence of the substrate acetopyruvate at a concentration 5-times its K_m value (Fig. 2). When the native enzyme is reacted with DTNB the activity decreased after 16 min to 20% of its original value. No precipitation of protein occurred during these assays.

The denatured enzyme in 8 M urea was also reacted with PHMB (Fig. 3). Treatment of the native enzyme with PHMB reduced the activity to about 22% of the original value (Fig. 4). Since the substrate, acetopyruvate, absorbed at 250 nm, it was impossible to do a PHMB titration of the native enzyme in the presence of the substrate at a concentration 5-times its K_m value.

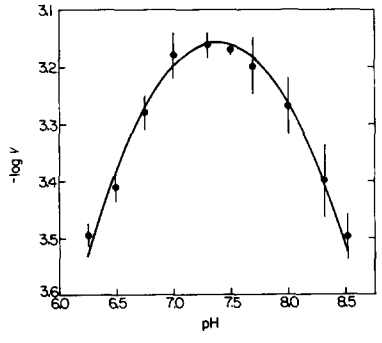
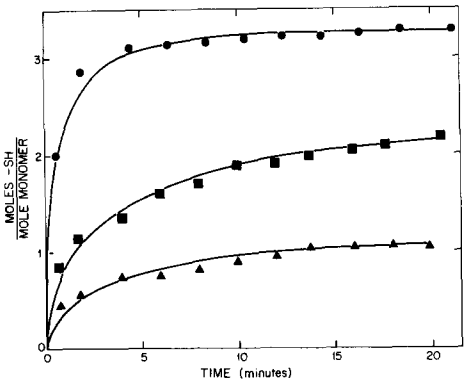


Fig. 1. Log V vs. pH assuming a pH-independent Michaelis constant.



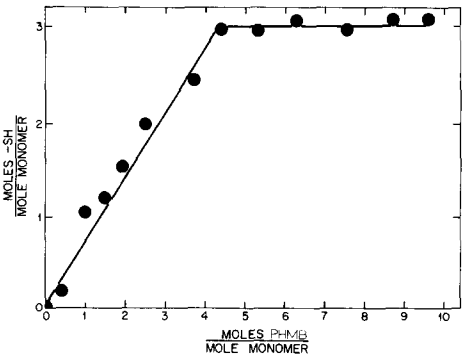
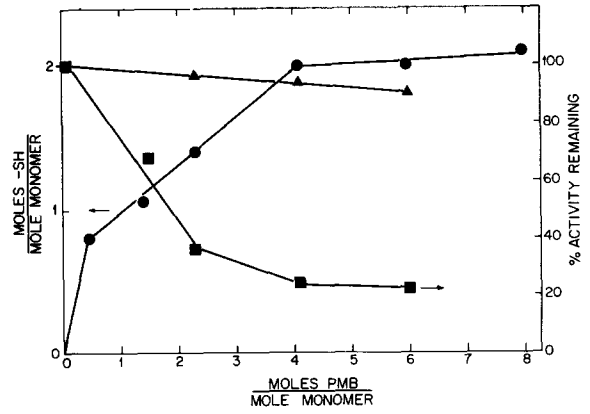


Fig. 2. Determination of accessible sulfhydryl groups by reaction with DTNB. DTNB, 0.1 mM; fumarylacetoacetate fumarylhydrolase, 0.146 mg/ml; EDTA, 10 mM; sodium phosphate, 0.1 M (pH 8.0). ▲———▲, with acetopyruvate, $2.3 \cdot 10^{-3}$ M; ■———■, no additions; ●———●, with 8 M urea.

Fig. 3. Determination of total sulfhydryl groups by PHMB titration. Sodium phosphate, 50 mM (pH 7.0); urea, 8 M; fumarylacetoacetate fumarylhydrolase, 0.031 mg/ml.

The activity of fumarylacetoacetase is inhibited in a first-order manner by PHMB (Fig. 5) in 0.1 M sodium phosphate (pH 7.3). The rate of inactivation is slower by a factor of 2.4 when the treatment is performed in the presence of the substrate propionopyruvate at a concentration about equal to its K_m value (Fig. 5).

A possible reaction scheme for the inactivation of the enzyme by PHMB is the formation of a dissociable complex (E · PHMB) which may be converted to a covalently inactivated form of the enzyme (E — PHMB) as E + PHMB $\underset{\rightarrow}{K_i}$ E ·



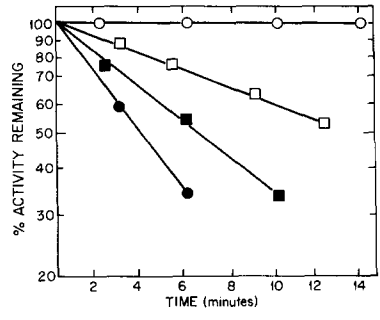


Fig. 4. Determination of accessible sulfhydryl groups by PHMB titration. Sodium phosphate, 50 mM (pH 7.0), fumarylacetoacetate fumarylhydrolase, 0.031 mg/ml. ●———●, mol-SH/mol subunit; ■———■, percent remaining activity; ▲———▲, percent remaining activity in the absence of PHMB.

Fig. 5. Inactivation of fumarylacetoacetate fumarylhydrolase by PHMB in 0.1 M sodium phosphate (pH = 7.3). ○———○, 0.0 mM PHMB/0.0 mM propionopyruvate; □———□, 0.025 mM PHMB/0.4 mM propionopyruvate; ■———■, 0.025 mM PHMB/0.0 mM propionopyruvate; ●———●, 0.037 mM PHMB/0.0 mM propionopyruvate.

PHMB k_3E — PHMB. By analogy with Kitz and Wilson [13] the apparent first-order-rate constant for inactivation (k_{app}) is related to K_i, k_3, and the concentration of PHMB by

$$\frac{1}{k_{app}} = \frac{1}{k_3} + \frac{K_I}{k_3} \frac{1}{[PHMB]}$$

A linear plot ($r = 0.95$) of $1/k_{app}$ vs. 1/[PHMB] was obtained at 0.1 M phosphate (pH 7.3) over a concentration range of 0.012—0.14 mM for PHMB (8 concentrations). Values of k_3 and K_i were estimated to be 0.37 min^{-1} and 0.041 M, respectively. In the presence of a competitive inhibitor of the enzyme the rate of inactivation was decreased. Fluoride (0.20 M) was added to the reaction mixture and k_{app} values were determined again as a function of [PHMB]. The double-reciprocal plot was linear ($r = 0.90$, five concentrations of PHMB) and values of k_3 and K_i were 0.35 min^{-1} and 0.1 M, respectively. Fluoride is a known competitive inhibitor of fumarylacetoacetase [7].

The PHMB inhibition of the enzyme can be partially reversed. When cysteine was added in a final concentration of 8.0 mM to an enzyme sample, which had been reduced to 20% of its orginal activity with 0.135 mM PHMB in 0.1 M sodium phosphate (pH 7.3), the enzyme regained 74% of its control activity in 2 min. Longer incubation times did not further increase activity. When PHMB-treated enzyme (80% inhibited) was passed through a calibrated G-150 column, a single symmetrical peak of material absorbing at 280 nm was detected. The elution volume of the mercurial-treated enzyme corresponded to a molecular weight of 78 000. Untreated enzyme had an elution volume which corresponded to a molecular weight of 87 000.

Discussion

In the study of kinetic parameters as a function of pH, one must consider the ionization states of the substrate. Propionopyruvate can be expected to have pK_a values in the range of 2.6—3.2 for the carboxylic acid and 7.6—8.5 for the formation of the enolate anion [14,15]. In the pH range (6.25—8.5) of this study the carboxylic acid group of propionopyruvate will be completely ionized and, therefore, have no effect on the rate. Accumulation of the enolate form of the substrate should become appreciable, however, at the higher pH values studied. Inspection of K_m values determined (Table I, column 1) shows an increase of K_m with increased pH. This increase in K_m may be the result of the formation of the enolate form of propionopyruvate which may not bind and/or be a substrate of the enzyme. From investigations of the nonenzymatic hydrolysis of β-diketones in basic solution, it appears that only the diketo form is susceptible to cleavage [16—18]. It is likely that, by analogy with chemical reactions the keto form is the substrate for fumarylacetoacetase; this assumption is also in accord with results shown in Table I. After the substrate concentrations are corrected to represent only the diketo form, new kinetic constants (K'_m) were calculated from new Lineweaver-Burk plots (Table I, column 2). This method for correction of substrate concentration assumes the pK_a value of the substrate is that determined in solution and no shift in pK_a occurs upon binding to the enzyme. Also any inhibition of the enzyme by the enolate of the substrate is not taken into account.

Assuming that the active form of the substrate is the diketo acid, an analysis of the pH rate profile of the enzyme was made. The K'_m values (Table I, column 2) are constant in the pH range studied. As a result of this pH independent behavior of K'_m, plots of log V vs. pH became equivalent to log V/K'_m vs. pH for determining the empirical pK_a values necessary to generate the bell-shaped curve (Fig. 1). The pK_A and pK_B values determined from the data of Fig. 1 are 6.55 and 8.19, respectively.

The interpretation of empirical pK values determined from studies such as this is complicated. These empirical values may not be simple ionization constants but may contain in addition to various ionization constants some rate constant ratios of unknown magnitude [19]. Also the pK values of amino acid side chains may be shifted according to their environment. This appears to be the case for acetoacetate decarboxylase where the pK of the active site lysine is shifted to pK 6 [19]. As Knowles [20] has discussed, other assumptions (several difficult to test experimentally) must be fulfilled before the empirically determined ionization constants may be assigned to specific amino acid side chains. Bearing these complications in mind, tentative assignments of an imidazole group of histidine to the pK of 6.5 and the sulfhydryl group of cysteine to the pK of 8.2 [21] shall be made. As discussed later, there is additional evidence that a sulfhydryl group affects the activity of this enzyme.

The pH-independence of the Michaelis constant may indicate that this constant is equal to the thermodynamic dissociation constant of the enzyme-substrate complex. Cornish-Bowden [22] has shown this to be the case for a number of reasonable mechanisms. If the thermodynamic dissociation constant is unaffected by pH, it is possible that the ionization of group(s) on the enzyme, responsible for binding substrate, have ionization constants outside the pH range of 6.25—8.50. As suggested by Hsiang et al. [4] the enzyme probably has two positively charged binding groups in the active site. Groups positively charged above pH 8.5 could be an α-amino nitrogen or the side chains of either lysine or arginine.

The pH independence of the K_i values of chloride and fluoride supports the suggestion of Braun and Schmidt [7] that these anions, which act as competitive inhibitors, compete with substrates for the same binding site on the enzyme. Both substrate and anion binding are independent of pH in the range 6.25—8.50 which suggests a similar binding site for both species.

There appear to be three free sulfhydryl groups per monomer. Reaction of denatured protein with either DTNB (Fig. 2) or PHMB (Fig. 3) was in agreement as to the number of total sulfhydryl groups per monomer. Reaction of the native protein with either sulfhydryl reagent showed only a total of two sulfhydryl groups per monomer (Figs. 2 and 4). Apparently one sulfhydryl group per monomer is inaccessible to either DTNB or PHMB in the native protein in agreement with earlier results [5].

Modification of one of the two accessible sulfhydryl groups decreases the activity of the enzyme. Both DTNB and PHMB reduce the activity of the enzyme to about 20% of the original value. In the case of reaction of enzyme with DTNB in the presence of a substrate at a concentration of 5-times its K_m value, only one of the two accessible sulfhydryl groups is free to react (Fig. 2). Apparently interactions of substrate at the active site decrease the reactivity of

one sulfhydryl group. A similar decrease in reactivity of a sulfhydryl group by interactions at the active site is seen with the PHMB modification. The rate of inactivation of the enzyme with PHMB is decreased in the presence of a substrate (Fig. 5). The irreversible inhibition of the enzyme shows saturation kinetics, which can be explained by the formation of a reversible enzyme-mercurial complex before covalent attachment of the mercurial to the enzyme although other explanations would give similar kinetic behavior [23]. Fluoride acts as a competitive inhibitor to PHMB-inactivation. This could arise because fluoride binds competitively at the active site of the enzyme.

Inhibition of enzyme activity by reagents may proceed by a variety of mechanisms [21]. These include:

(a) The modified group is at the active site and is functional in substrate binding or catalysis,

(b) The modified group is vicinal to the active center. Thus, the reagent introduces a new structure onto the enzyme which is near enough to modify the reactions at the active site, or

(c) Reactions of the modified group alter the enzyme structure.

Since neither DTNB or PHMB completely inactivate the enzyme, it appears unlikely that the sulfhydryl group modified is in the active site. Either the critical sulfhydryl group is near the active site or its modification alters the structure of the enzyme, thus decreasing the activity. If such a conformational change occurs, it is not in the nature of dissociation into subunits as has been reported for a number of PHMB-treated enzymes [24—29]. Determination by gel filtration of the molecular weight of PHMB-treated enzyme gave a value of 78 000. This value is within experimental error of the molecular weight of 86 000 ± 10 000 of the untreated enzyme. If dissociation into subunits had occurred, the expected molecular weight would be 40 000—43 000 [5].

Fumarylacetoacetase is a protein with three sulfhydryl groups per monomer of 40 000. One of these sulfhydryl groups is not accessible to reaction with either DTNB or PHMB. Of the two accessible sulfhydryl groups, modification of one decreases activity. This critical sulfhydryl group is probably either near the active site or has an essential role in the enzyme structure. The role of a critical sulfhydryl group is implicated from pH studies.

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