Inhibition of Fumarylacetoacetate Fumarylhydrolase by Monovalent Anions†

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ABSTRACT: Fumarylacetoacetate fumarylhydrolase is inhibited competitively by the monovalent anions bicarbonate, chloride, bromide, nitrate, perchlorate, thiocyanate, trichloroacetate, and fluoride in order of increasing effectiveness at pH 7.3 in 0.05 M phosphate buffer. One anion is bound per enzyme–inhibitor complex. All the anions bind at the same site. The inhibition is interpreted as the masking by the anions of a positively charged group in the active site of the enzyme which binds the diketo acid substrates in a productive complex. The order of effectiveness of inhibition by the anions generally follows the Hofmeister series.

The interaction of polar groups on protein molecules can markedly affect the catalytical function of enzymes (Steinhardt and Beychok, 1964). These effects seem especially marked when the substrate for the enzyme is itself ionic (Cheng *et al.*, 1971).

Fumarylacetoacetate fumarylhydrolase (EC 3.7.1.2) is an enzyme whose natural substrate, fumarylacetoacetate, is a dianion in the pH region where the enzyme is active, *i.e.*, pH 6–9 (Connors and Stotz, 1949). Any description of the mechanism of fumarylacetoacetate fumarylhydrolase will require determining the mode of substrate binding to this enzyme. There are at least four possible ways that a carboxyl anion may bind to a protein (Jenkins and D'Ari, 1966): (a) it may displace a preexisting buffer anion; (b) it may bind as an anion to an uncharged basic grouping such as a histidine residue; (c) it may bind to an uncharged potentially acidic group such as a phenolic hydroxyl; or (d) it may bind to a positive group unmasked by a conformational change of the protein.

This study investigates the inhibition of fumarylacetoacetate fumarylhydrolase by a number of monovalent anions. From these data it appears that the carboxyl anion of fumarylacetoacetate fumarylhydrolase substrates binds to the protein by the first of these alternatives.

Experimental Section

Materials. Fumarylacetoacetate fumarylhydrolase was purified according to the method of Hsiang *et al.* (1972) with minor modifications. The TEAE-cellulose chromatography was conducted with a linear gradient of 500 ml of Tris-hydrochloride buffer (0.2 M, pH 8.5, 1×10^{-4} M dithioerythritol) flowing into 500 ml of Tris-hydrochloride buffer (0.025 M, pH 8.5, 1×10^{-4} M dithioerythritol). The purified enzyme was stored in a 10% glycerol solution at −20°. All experiments were conducted with enzyme of a specific activity not lower than 36 units/mg of protein.

All inhibitors were reagent grade materials or were synthesized as previously described (Braun *et al.*, 1973).

Methods. The rate of hydrolysis of propionopyruvate was determined spectrophotometrically at 290 nm (Braun *et al.*, 1973) on a Gilford Model 220 absorbance recorder attached to a Beckman DU monochromator. The temperature of all assay systems was maintained at 30.0 ± 0.5° by circulating water from a thermostated bath through thermospacers at the end of the cell compartment. All reagents were preincubated in the 30.0 ± 0.5° thermostated bath.

Results

Fumarylacetoacetate fumarylhydrolase catalyzed hydrolysis of propionopyruvate (2×10^{-4} M) is inhibited by the added monovalent anions bicarbonate, chloride, bromide, nitrate, perchlorate, thiocyanate, trichloroacetate, and fluoride (Table I) at pH 7.3 in 0.05 M sodium phosphate buffer. This inhibition seems to be a property of inorganic monovalent anions since under the same conditions sodium sulfate, tetrasodium pyrophosphate, and EDTA in the concentration range 0.03–0.8 M have no effect on fumarylacetoacetate fumarylhydrolase. Also the enzyme is insensitive to the cation as sodium sulfate (0.03–0.8 M), ammonium sulfate (0.03–0.8 M), and potassium sulfate (0.03–0.4 M) had no effect on fumarylacetoacetate fumarylhydrolase catalyzed hydrolysis of propionopyruvate (2×10^{-4} M) in Tris-sulfate buffer (0.05 M, pH 7.3).

Plots of the reciprocal of the initial velocity *vs.* the reciprocal of the substrate concentration at a series of chloride concentrations were produced (Figure 1). All of the other inhibitory monovalent ions gave plots similar to that of chloride where the slope of the line varies with inhibitor concentration, and the y intercept remains constant. Families of lines for various inhibitor levels of the type found for fumarylacetoacetate fumarylhydrolase inhibition by monovalent anions indicate that competitive inhibition is present (Plowman, 1972). The three main types of competitive inhibition are: simple, hyperbolic, and parabolic (Cleland, 1963; Plowman, 1972). Analysis of these three types of competitive inhibition shows that a plot of v_0/v_i *vs.* $[A^-]$ should be fit by an equation of the form for simple competitive

$$\frac{v_0}{v_i} = 1 + \frac{K_m^{app}}{K_a([S_0] + K_m^{app})}[A^-] \quad (1)$$

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TABLE I: Inhibition of Fumarylacetoacetate Furmarylhydrolase Activity by Anions.

Inhibitor	Concn for 50% Inhibition (mM)[a]	K_a (mM)
Sodium bicarbonate	170	90
Sodium chloride	140	75
Sodium bromide	50	27
Sodium nitrate	12	6.5
Sodium perchlorate	6.0	3.3
Sodium thiocyanate	4.2	2.3
Sodium trichloroacetate	1.2	0.66
Sodium fluoride	1.2	0.61

[a] Propionopyruvate at 2×10^{-4} M in 0.05 M sodium phosphate buffer at pH 7.3.

for hyperbolic competitive

$$\frac{v_0}{v_i} = \frac{[S_0]}{K_m^{app} + [S_0]} + \frac{K_m^{app}\left(\frac{1 + a[A^-]}{1 + b[A^-]}\right)}{K_m^{app} + [S_0]} \quad (2)$$

and for parabolic competitive

$$\frac{v_0}{v_i} = \frac{[S_0]}{K_m^{app} + [S_0]} + \frac{K_m^{app}(1 + c[A^-] + d[A^-]^2)}{K_m^{app} + [S_0]} \quad (3)$$

where v_0 = initial velocity in the absence of inhibitor, v_i = initial velocity in the presence of inhibitor, K_m^{app} = dissociation constant of the enzyme–substrate complex, K_a = dissociation constant for the enzyme–inhibitor complex, $[S_0]$ = initial substrate concentration, $[A^-]$ = concentration of monovalent anion, and the constants a, b, c, and d are complex functions of constants. Inspection of eq 1–3 indicates that a plot of v_0/v_i *vs.* $[A^-]$ at constant substrate concentration will be linear only for simple competitive inhibition. When such plots were done for the monovalent anions, the function v_0/v_i was directly proportional to the anion concentration and the y intercept was unity. The monovalent anions appear to inhibit fumarylacetoacetate fumarylhydrolase by simple competitive inhibition as predicted by eq 1. Under the conditions of these determinations the y intercept as predicted by eq 2 and 3 should have been 0.34 calculated from $K_m^{app} = 3.9 \times 10^{-4}$ M for propionopyruvate at pH 7.3 in 0.05 M sodium phosphate buffer (Braun *et al.*, 1973). The inhibitor constants, K_a, can be calculated from the slopes of the v_0/v_i *vs.* $[A^-]$ plots (Table I).

The number of inhibitor molecules, r, in an enzyme–inhibitor complex is related to the various kinetic coefficients as follows (Ebersole *et al.*, 1944; Bergmann and Segal, 1954)

$$E + rA^- \rightleftharpoons EA_r^- \quad (4)$$

$$\log (v_0/v_i - 1) = r \log [A^-] + \log \left(\frac{K_m^{app}}{K_a(K_m^{app} + [S_0])}\right) \quad (5)$$

where v_0, v_i, K_m^{app}, K_a, $[S_0]$, and $[A^-]$ are as previously defined. The value of r is readily determined by plotting log $(v_0/v_i - 1)$ against log $[A^-]$ and estimating the slope of the resulting line. For each inhibitory anion the slope was 1 ± 0.2 (Table II).

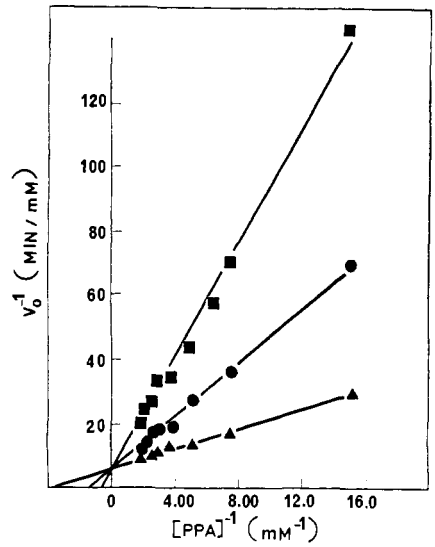

FIGURE 1: Competitive inhibition by sodium chloride of fumarylacetoacetate fumarylhydrolase catalyzed hydrolysis of propionopyruvate. The standard assay procedure was used. Each cuvette (1.0-cm light path, 3.0-ml volume) contained 0.05 M sodium phosphate buffer at pH 7.3, and propionopyruvate as indicated: (■) 0.2 M sodium chloride; (●) 0.05 M sodium chloride; (▲) 0.0 M sodium chloride.

Mixed inhibition studies were carried out to determine if the various anions inhibit by combining at an identical site on the enzyme or at independent, noninteracting sites. In the case of simple competitive inhibition anions A^- and B^- will act additively if they bind at an identical site (eq 6).

$$v_i = \frac{v_0}{[K_m^{app}/(K_m^{app} + [S_0])][([A^-]/K_a) + ([B^-]/K_b)] + 1} \quad (6)$$

where v_i, v_0, K_m^{app}, K_a, $[S_0]$, and $[A^-]$ are as previously defined while $[B^-]$ = concentration of the second anion and K_b = dissociation constant of the EB^- complex. For independent, noninteracting sites the activity remaining after anion A^- and B^- inhibition can be represented by

$$v_i = \frac{v_0}{[K_m^{app}/(K_m^{app} + [S_0])][([A^-]/K_a) + ([B^-]/K_b) + ([A^-][B^-]/K_aK_b)] + 1} \quad (7)$$

Mixed inhibition studies were performed with chloride, bromide, fluoride, and thiocyanate. The enzyme activities expected in the presence of various mixtures of anions were calculated for identical and independent sites according to eq 6 and 7, respectively. The calculated activities were compared with the enzymatic activities actually observed (Table III).

A number of structural analogs of fumarylacetoacetate fumarylhydrolase substrates were tested as potential inhibitors (Table IV). The most potent inhibitors of the hydrolase appear to be α-keto acids.

Discussion

The poorly understood effect which anions play in enzymatic catalysis, where the substrates themselves are anionic as in fumarase (Massey, 1953), malic dehydrogenase (Weim-

TABLE II: r Values of Inhibition of Fumarylacetoacetate Fumarylhydrolase by Various Anions.

Inhibitor	r[a]
Sodium bicarbonate	0.8
Sodium chloride	0.8
Sodium bromide	1.2
Sodium nitrate	1.0
Sodium perchlorate	0.9
Sodium thiocyanate	1.2
Sodium trichloroacetate	0.8
Sodium fluoride	1.1

[a] r value as defined in eq 5.

berg, 1967), carbonic anhydrase (Coleman, 1967), succinic dehydrogenase (Zeylemaker *et al.*, 1970), and aspartate transaminase (Cheng *et al.*, 1971), has recently become an area of study for the enzymologist (Cheng and Martinez-Carrion, 1972). Fumarylacetoacetate fumarylhydrolase is an enzyme which falls in this category and any mechanism postulated for this enzyme must explain its inhibition by monovalent anions (Table I).

The mechanism by which an inorganic monovalent anion can inhibit the association of the enzyme with its substrate is not clear. One possibility might be that increasing salt concentrations may merely cause a change in ionic strength which in turn causes a change in the concentration of active enzyme. Clearly for fumarylacetoacetate fumarylhydrolase this is not the case since over a wide range of sulfate, pyrophosphate, and EDTA concentrations (0.03–0.8 M) no effect on fumarylacetoacetate fumarylhydrolase activity was detectable.

Another possibility would be that increasing salt concentration may alter the keto–enol equilibrium of the substrate propionopyruvate to a form which is not bound by fumarylacetoacetate fumarylhydrolase. Solutions of propionopyruvate at the concentrations and pH used in this study did not exhibit any differences in absorption at 290 nm in either the presence or absence of any of the monovalent anions.

A much more likely case in our opinion is that the inorganic monovalent anion is competing with propionopyruvate for the same site on the enzyme molecule. This possibility is not usually considered due to the common beliefs (Jenkins and D'Ari, 1966) that a structure comparable to the substrate is a requirement for competitive inhibition and that all positive charges on proteins are neutralized intramolecularly by carboxyl groups. In fact some of the monovalent anions are better fumarylacetoacetate fumarylhydrolase inhibitors (Table I) than are the organic anions (Table IV) which are related structurally to fumarylacetoacetate fumarylhydrolase substrates.

All of the monovalent anions appear to bind at the same site on the enzyme (Table III). This binding site is probably identical with the binding site for the carboxylate anion of the diketo acid substrates of fumarylacetoacetate fumarylhydrolase. The fumarylacetoacetate fumarylhydrolase substrates when forming enzyme–substrate complexes must displace the buffer anion which normally is bound at this active site. The masking by inorganic anions of binding sites in enzymes whose biological substrates are anionic is probably much more prevalent than previously suspected.

The biological substrate for fumarylacetoacetate fumarylhydrolase is the dicarboxylic acid fumarylacetoacetate. The enzyme may have two binding sites for this acid (Hsiang *et al.*, 1972). Inhibition of the hydrolysis of the unnatural substrate propionopyruvate (a monovalent anion) seems to depend upon the binding of a monovalent anion at only one site (Table II). This does not eliminate the possibility that anions may bind at both cationic binding sites of fumarylacetoacetate fumarylhydrolase since propionopyruvate may

TABLE III: Inhibition of Fumarylacetoacetate Fumarylhydrolase by Mixtures of Anions.[a]

Inhibitors	v_i (mM/min) Calcd for Identical Sites	v_i (mM/min) Calcd for Independent Sites	v_i (mM/min) Exptl
NaCl (0.1 M)	0.0109		0.0098
NaBr (0.05 M)	0.0098		0.0096
NaF (0.002 M)	0.0075		0.0085
NaSCN (0.004 M)	0.0100		0.0100
NaCl (0.1 M) + NaBr (0.05 M)	0.0075	0.0053	0.0076
NaCl (0.1 M) + NaF (0.002 M)	0.0061	0.0049	0.0063
NaCl (0.1 M) + NaSCN (0.004 M)	0.0077	0.0055	0.0071
NaBr (0.05 M) + NaF (0.002 M)	0.0056	0.0032	0.0060
NaBr (0.05 M) + NaSCN (0.004 M)	0.0070	0.0046	0.0071
NaF (0.002 M) + NaSCN (0.004 M)	0.0057	0.0033	0.0060

[a] Propionopyruvate at 2×10^{-4} M in 0.05 M sodium phosphate at pH 7.3.

TABLE IV: Inhibition of Fumarylacetoacetate Fumarylhydrolase Activity by Substrate Analogs.[a]

Inhibitor[b]	% Inhibition
Dihydroxytartrate	100
Oxalacetate	90
Pyruvate	50
α-Ketobutyrate	50
α-Ketoglutarate	50
Acetoacetate	30
4-Ethyl oxalacetate	30
Ketomalonate	0
3-Benzoylpropionate	40
4-Ketopimelate	10
4-Acetylbutyrate	0
Levulinate	0
2,4-Pentanedione	0
Fumarate	0
Monoethyl malonate	0
Monoethyl succinate	0
Benzoylformate	0

[a] The inhibition studies were carried out in 0.025 M sodium phosphate buffer (pH 7.3) using 1×10^{-4} M sodium propionopyruvate as the substrate. [b] 1×10^{-3} M.

be oriented properly for catalysis when it is bound at only one of these sites.

Another possible explanation exists for the inhibition of fumarylacetoacetate fumarylhydrolase by anions. A mutual exclusion between anion and substrate from the active center occurs but without the sharing of a common subsite. This appears to be the case for aspartate transaminase where the anion binds to an active center histidine (Cheng and Martinez-Carrion, 1972). Photooxidation of the active center histidine abolishes anion binding but has no effect on substrate binding. The data for fumarylacetoacetate fumarylhydrolase allow no choice between this possibility and the alternative possibility where anion competes with substrate for the same active center subsite.

The studies of specific effects of anions on colloidal systems, in aqueous media, date back to Hofmeister (1888), who measured the effects of various salts on the solubility of egg albumin. Series of anions arranged in order of ability to modify some observable function and similar in sequence to the series of anions found by Hofmeister are usually referred to as the Hofmeister or Lyotropic series (Alexander, 1937). Comparable series have been obtained on the basis of the effects of salts on such diverse phenomena as the surface tension, compressibility, and viscosity of water (Traube, 1910; Bingham, 1941; Randles, 1957), the electrophoretic mobility of proteins (Longsworth and Jacobsen, 1949), and the molecular weight (Wolff, 1962), urea sensitivity (Rajagopalan *et al.*, 1961), and activity (Massey, 1953; Walaas and Walaas, 1956; Richards and Rutter, 1961; Fridovich, 1963) of enzymes. The general order of the Hofmeister series for the anions is (Mysels, 1959): F^-, IO_3^-, $H_2PO_4^-$, BrO_3^-, Cl^-, ClO_3^-, Br^-, NO_3^-, ClO_4^-, I^-, and SCN^-. The series probably represents the order of hydration of the ions (West *et al.*, 1966; von Hippel and Schleich, 1969). This study supports this idea since the more capable of hydration the anion was, the less efficient was its ability to bind at the active site of the enzyme. It may be that poorly hydrated anions are bound more strongly, and thus are better inhibitors, either because their hydrated radii are less than the strongly hydrated anions and can fit into the binding pocket of the protein more easily, or because the binding involves a dehydration which is more facile with poorly hydrated anions (Taylor and Kuntz, 1972). Of the anions tested in our study, only fluoride did not follow the conventional order of the Hofmeister series (Table I). Instead of being the least effective it was the most inhibiting. This may not be too surprising since there are many deviations from the Hofmeister series. In a related series in which anions were rated as to their absorbability on acid alumina columns F^- was displaced to the other end of the Hofmeister series and was about as effective as SCN^- (Jirgensons and Straumanis, 1962).

Acetoacetate decarboxylase has also been found to be inhibited by monovalent anions and not by divalent anions (Fridovich, 1963). Although the monovalent anions were noncompetitive inhibitors of acetoacetate decarboxylase, the order of affinity of the ions chloride, bromide, nitrate, perchlorate, and thiocyanate were the same for the hydrolase and the decarboxylase. Trichloroacetate and fluoride are, however, much more effective inhibitors of fumarylacetoacetate fumarylhydrolase than of acetoacetate decarboxylase. The affinities of the various monovalent anions for the sensitive site on the decarboxylase roughly correlated with the negativity of the entropy of inhibition. This was interpreted to indicate that water hydrogen bond formation made a large contribution to the stability of the anion–site complex. Other factors influencing the stability were thought to be polarizability of the anion and site or field intensity of the cationic binding site. An exact correlation between the order of affinities of the anions with different proteins is probably not to be expected due to differences in cationic binding sites on these proteins.

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