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ANION ACTIVATION OF MALEATE HYDRATASE

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SUMMARY

Maleate hydratase (D-malate hydro-lyase) has been found to have an absolute requirement for certain univalent anions. The relative effectiveness of the various anions in activating the hydratase can be ranked

$$Br^- > Cl^-, NO_3^- > I^- > SCN^- > formate^- > F^- > acetate^- = 0$$

Activation appears to be independent of pH in the range 6.0–9.0 and, at least in the case of Cl^-, is described by the expression

$$V/v = (1 + K_s/[S])\ (1 + K_a/[A])$$

where v is the initial velocity; V, the maximal initial velocity; $[S]$ substrate concentration; $[A]$, anion concentration; K_s, a Michaelis constant; and K_a, the apparent dissociation constant of enzyme with activating anion.

INTRODUCTION

Maleate hydratase (D-malate hydro-lyase), a soluble enzyme of rabbit kidney cortex[1] which catalyzes the hydration of maleate to D(+)-malate by *trans* addition to the double bond[2], has been known for some years, but only as a constituent of crude kidney homogenate or of a high-speed supernatant of such homogenates. In attempts to purify and further characterize this enzyme it was discovered that dialysis against 0.05 M sodium phosphate buffer (pH 7.4) led to a complete loss of activity. The activity of the dialyzed enzyme could be restored by the addition of protein-free ultrafiltrates of the original solution. The heat stability of the co-factor in ultrafiltrate and its behavior on passage through ion exchange columns indicated that an inorganic salt was responsible for enzyme reactivation. Subsequently, NaCl was found to restore completely the activity of dialyzed enzyme. Preliminary survey revealed similar activation of maleate hydratase by several univalent anions and indicated that these anions behaved kinetically as though they were substrates of the enzyme in a manner known only for some enzyme–divalent cation combinations[3].

The presence of an enzyme with such marked anion dependence in the kidney (which regulates the ionic environment) suggested that the function of the enzyme might be involved in some manner in the control of renal activity. Consequently a detailed study of anionic activation was begun.

METHODS AND MATERIALS

Protein was estimated by the method of GORNALL *et al.*[4] standardized with crystalline bovine albumin; D-malate, by the method of BRITTEN[5]. Crude maleate hydratase was prepared by homogenizing 100 g of decapsulated rabbit kidneys purchased frozen from Pel Freeze Biologicals, Rogers, Ark., in 250 ml of 0.15 M KCl containing $5 \cdot 10^{-4}$ M $Fe(NH_4SO_4)_2$ and $1.4 \cdot 10^{-2}$ M mercaptoethanol, followed by centrifugation at 23 000 $\times$ *g* for 30 min at 0°. The crude enzyme present in the supernatant was partially purified by $(NH_4)_2SO_4$ fractionation and selective heat denaturation. The supernatant was treated with 243 g $(NH_4)_2SO_4$, per l, equilibrated 0.5 h at 5°, and centrifuged at 23 000 $\times$ *g* for 20 min at 5°. The supernatant was treated with an additional 80 g $(NH_4)_2SO_4$ per l, followed by equilibration and centrifugation as before. The supernatant was discarded and the precipitate was taken up in a small volume of 0.02 M Tris–acetate buffer (pH 7.4) containing $5 \cdot 10^{-4}$ M $Fe(NH_4SO_4)_2$, and $1.4 \cdot 10^{-2}$ M mercaptoethanol. After exhaustive dialysis against that buffer, the protein solution was heated rapidly to 53–54°, held at that temperature for 3 min, cooled, and centrifuged at 30 000 $\times$ *g* for 30 min at 0°. In this manner an 8-fold increase in specific activity was obtained, as compared with the original supernatant.

Enzyme was assayed by incubating the protein in 1 ml of a solution containing sodium maleate (30 μmoles), Tris–acetate (30 μmoles, pH 7.4), NaCl (200 μmoles), $Fe(NH_4SO_4)_2$ (2.2 μmoles), sodium gluconate (2.2 μmoles), and mercaptoethanol (73 μmoles) for 15 min at 38°. The reaction was terminated by the addition of 0.3 ml of 0.33 M $K_3Fe(CN)_6$ in 1/3 M Tris–HCl (pH 8.6), and the resulting solution assayed for D-malate[5]. Specific activities were expressed in μmoles D-malate formed per min per mg protein. With each determination care was taken to insure that the activity was linear with respect to time and protein content. Ferrous ion and mercaptoethanol (a representative reducing agent) were included in all reactions to insure maximum enzymatic activity. Ferricyanide (a representative oxidizing agent), was chosen to terminate the reaction because it fitted naturally into the subsequent procedure for the analysis of D-malate.

The data has been analyzed in terms of the Michaelis–Menten equation in the double reciprocal form of LINEWEAVER–BURK[6]

$$V/v = K_m/[S] + 1 \qquad (1)$$

Since an equation of this form holds when either maleate or certain anions are considered "substrate" the following notation has been adopted for clarity.

When maleate is treated as substrate (in the presence of various concentrations of activating anions) Eqn. 1 is written as

$$v_{mal}\,(A^-)/v = K_{mal}\,(A^-)/[S] + 1 \qquad (2)$$

where [S] is [maleate] and A^- is the activating anion. When an anion is treated as substrate (at various maleate concentrations) Eqn. 1 is written as

$$v_A/v = K_A/[S] + 1 \tag{3}$$

where A may be Cl^-, Br^-, *etc.*, and [S], the concentration of Cl^-, Br^-, *etc.*, as the case may be.

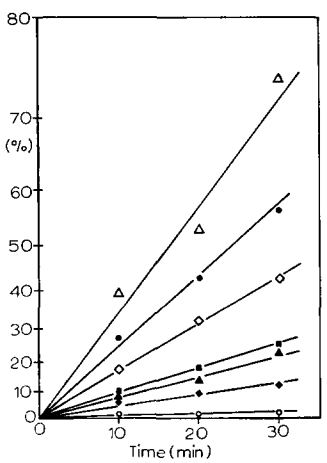
V is reserved for the hypothetical maximal initial velocity as both [maleate] and activating [anion] become infinite.

RESULTS

The effect of various sodium salts on the activity of maleate hydratase previously dialyzed against 0.02 M potassium phosphate buffer (pH 7.4) is shown in Fig. 1. Under the conditions chosen, the hydration proceeded as a first order reaction with the rate determined by the accompanying sodium salt. The salts examined were effective in reactivating the enzyme in the order.

$$Br^- > Cl^- > NO_3^- > I^- > SCN^- > \text{formate}^- > F^- > \text{acetate}^- = 0$$

In the absence of added activating anion or in the presence of sodium acetate no enzymatic activity could be detected even on prolonged incubation, although the enzyme remained potentially active in the assay system. No salt of a nonunivalent anion has been found to activate. Moreover, the accompanying cation appears to be



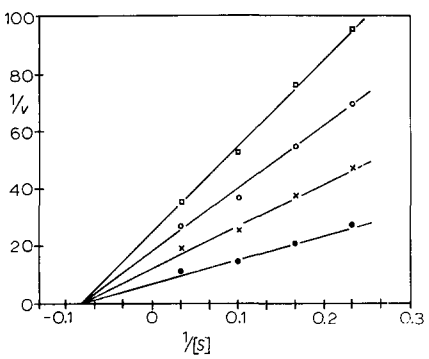


Fig. 1. Percentage conversion of maleate to D-malate. Incubation time in min. The incubation mixture contained potassium phosphate buffer (15.4 μmoles, pH 7.4), crude enzyme (23 mg protein), sodium maleate (30 μmoles) and 200 μmoles of either NaF (○), sodium formate (◆), NaSCN (▲), NaI (■), $NaNO_3$ (◇), NaCl (●), or NaBr (△) in 1 ml. Incubated at 30° with aliquots removed for the estimation of D-malate at the indicated times.

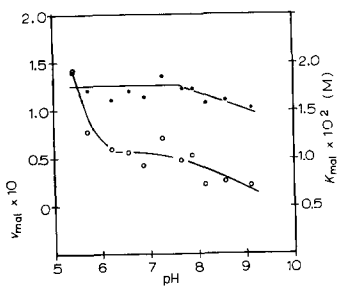
Fig. 2. Lineweaver–Burk plots of maleate hydratase activity at various concentrations of NaCl. v in μmoles D-malate formed per min per mg protein, maleate ([S]) in mM. The incubation mixture contained Tris–acetate buffer (100 μmoles, pH 7.4), partially purified enzyme (0.6 mg protein), the indicated amount of sodium maleate, and NaCl (either 200 μmoles (●), 66.7 μmoles (×), 40 μmoles (○), or 28.6 μmoles (□)) in 1 ml. Temp., 38°.

unimportant in the activation process. The chlorides of Na^+, K^+, Li^+, Rb^+, and NH_4^+ are equally effective; the acetates of Na^+, K^+, and NH_4^+ all failed to activate.

Cl^-, because of its physiological importance, was the anion initially selected for more detailed studies. After verifying that the initial rate of maleate hydration was a linear function of protein concentration in the presence of a constant Cl^- concentration, the initial rate was studied as a function of substrate concentration at several Cl^- concentrations. Typical results are given in Fig. 2 in the double reciprocal LINEWEAVER–BURK[6] plots. The maximum initial velocity, v_m (Cl^-) is a function of Cl^-, while the apparent Michaelis–Menten constant, K_m (Cl^-), is not.

Since variations of $v_{mal}(Cl^-)$ and $K_{mal}(Cl^-)$ with pH yield information about the ionizing groups involved in enzyme–substrate interaction[7,8] these constants were determined in the presence of 0.2 M Cl^- at a pH ranging from 5.5 to 9.0. As can be seen in Fig. 3, $v_{mal}(Cl^-)$ was found to be essentially independent of pH. Similarly, $K_{mal}(Cl^-)$ varied little except at values below 6.2 and so in the range where a large fraction of maleate (pKa_2 6.6) would be expected to be in the univalent form. In separate experiments, it was established that the enzyme is unstable at higher pH's. The loss under the conditions of the reported experiment amounted to 15–20% at pH 9. This instability probably accounts for the tendency of $v_{mal}(Cl^-)$ to decline at higher pH.

The lack of a simple dependence of the kinetic parameters on the species of activating anion is disclosed in Fig. 4. Initial inspection of these Lineweaver–Burk plots for maleate hydratase in the presence of 0.2 M Br^-, Cl^-, or NO_3^- might suggest that a common $v_{mal}(A^-)$ was present. However, the differences between the Cl^- and Br^- curves depicted are consistently repeatable and it would appear that no common point exists on the Lineweaver–Burk plots of maleate hydratase in the presence of



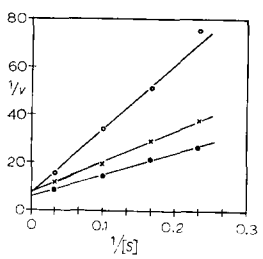
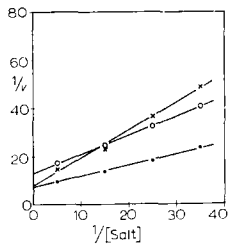


Fig. 3. Variation of v_{mal} (●), and K_{mal} (○), with pH. v_{mal} in μmoles D-malate formed per min per mg protein. Constants were determined by the method of least squares from Lineweaver–Burk plots of maleate hydratase activity buffered at the indicated pH. For all determinations the incubation mixture contained NaCl (200 μmoles), $Fe(NH_4SO_4)_2$ (2.2 μmoles), sodium gluconate (2.2 μmoles), mercaptoethanol (7.2 μmoles), Tris–acetate buffer of appropriate pH (100 μmoles), partially purified enzyme (0.17 mg protein and sodium maleate (either 30 μmoles, 10 μmoles, 6 μmoles or 4.29 μmoles) in 1 ml. Incubated at 38° for 27 min.

Fig. 4. Variation in Lineweaver–Burk plots of maleate hydratase activity in the presence of various anions. v in μmoles D-malate formed per min per mg protein, [S] in mM. The incubation mixture contained $Fe(NH_4SO_4)_2$ (2.2 μmoles), sodium gluconate (2.2 μmoles), mercaptoethanol (7.2 μmoles), Tris–acetate buffer (100 μmoles, pH 7.4), partially purified enzyme (0.31 mg protein, the indicated amounts of sodium maleate and 200 μmoles of either NaCl (×), NaBr (●), or $NaNO_3$ (○) in 1 ml. Incubated 21 min at 38°.



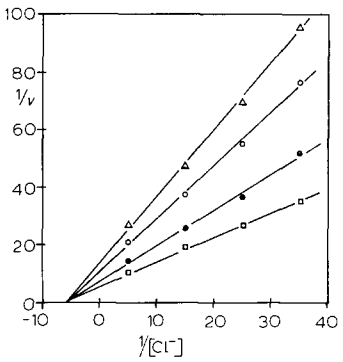


Fig. 5. Variation in Lineweaver–Burk plots of maleate hydratase activity with various salts as "substrate" in the presence of constant maleate. v in μmoles D-malate per min per mg protein, [Salt] in M. The incubation mixture contained $Fe(NH_4SO_4)_2$ (2.2 μmoles), sodium gluconate (2.2 μmoles), mercaptoethanol (7.2 μmoles), Tris–acetate buffer (100 μmoles, pH 7.4), partially purified enzyme (0.31 mg protein), sodium maleate (30 μmoles, and the indicated amounts of either NaCl (×), NaBr (●), or $NaNO_3$ (○) in 1 ml. Incubated 21 min at 38°.

Fig. 6. Lineweaver–Burk plots of maleate hydratase activity with Cl^- treated as "substrate" at several concentrations of maleate. v in μmoles D-malate per min per mg protein, $[Cl^-]$ in M NaCl. The incubation mixture contained Tris–acetate buffer (100 μmoles, pH 7.4), partially purified enzyme (0.6 mg protein) the indicated amount of NaCl and sodium maleate (either 4.29 μmoles (△), 6 μmoles (○), 10 μmoles (●), or 30 μmoles (□)) in 1 ml incubated at 38°.

equimolar concentrations of the various activators.

Many of the expressions relating initial velocity to substrate and activator concentrations derived for cases of enzyme activation predict that plots of the reciprocal of initial velocity *versus* the reciprocal of activator concentration in the presence of constant substrate concentration will be linear[3,6,9]. That this is the case for anion activators of maleate hydratase is shown in Fig. 5 which depicts the effect of varying concentrations of Cl^-, Br^-, or NO_3^- on v with maleate at constant concentration. In this figure it should be noted that the NO_3^- line crosses the Cl^- line. This indicates that at low concentrations the activating effect of NO_3^- will be greater than that of Cl^-, while at higher concentrations Cl^- will be the better activator. Thus, the order of effectiveness of anion activators given earlier is concentration dependent.

Since a pH dependence of anion activators has been noted in many instances[10–12] the relative activation effect of 0.2 M anions (sodium salts of Br^-, Cl^-, NO_3^-, SCN^-, formate$^-$, F^-, and acetate$^-$) was determined over the pH range 5.4–9.0. The order observed was the same over the entire pH range, except in the case of SCN^- where a marked decrease in activation was noted with decreased pH. This appeared to be explained by an equally marked instability of the enzyme at lower pH in the presence of that anion. No great instability was found in the presence of the other anions. In general, the order of anion activation at a constant concentration was independent of pH.

In Fig. 5 no common point in the family of curves is apparent. However, a simple relationship between one of the kinetic parameters does emerge if only one anion is examined. Fig. 6 gives the curves obtained on plotting the reciprocal of the

initial velocity against the reciprocal of the Cl^- concentration at several different concentrations of maleate. These curves share a common intercept on the $1/[S]$ axis, giving a pattern similar to that already seen in Fig. 2.

The constants K_{Cl^-} and v_{Cl^-} obtained from the treatment of maleate hydratase with Cl^- as "substrate" were examined over the pH range 5.4–9.0 as done earlier for the $K_{mal}(Cl^-)$ and $v_{mal}(Cl^-)$ in Fig. 3. The data, plotted as before, appeared very similar to Fig. 3. v_{Cl^-} was constant over the pH range and averaged 0.14 μmole D-malate per min per mg protein. K_{Cl^-} was a constant 0.12 M except at pH's below 6.1, where it increased with decreasing pH.

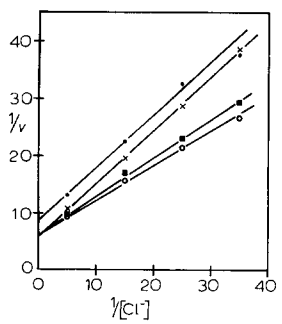


Fig. 7. Influence of various anions on the Lineweaver–Burk plots of maleate hydratase activity with NaCl treated as "substrate". v in μmoles D-malate per min per mg protein, $[Cl^-]$ in M NaCl. The incubation mixture contained sodium maleate (30 μmoles), $Fe(NH_4SO_4)_2$ (2.2 μmoles), sodium gluconate (2.2 μmoles), mercaptoethanol (7.2 μmoles), partially purified enzyme (0.29 mg protein), Tris–acetate buffer (100 μmoles, pH 7.4), and the indicated amounts of NaCl alone (○), or with either NaF (200 μmoles) (●), sodium acetate (200 μmoles) (■), or NaCN (9.5 μmoles (×).

The similarity of the double reciprocal plot with activators treated as "substrate" to the classical Lineweaver–Burk plot suggested a study of the interactions of univalent anions, with the hope of demonstrating "competitive inhibition" at the activator site. Fig. 7 depicts such a study. Here the effects of acetate, F^- and CN^- are shown on the Cl^- activated enzyme. The data may be interpreted as evidence that CN^- is relatively powerful, and acetate, a weak competitive inhibitor of Cl^- at the activating site. The action of F^-, while inhibitory, is not simply competitive but is better classified as mixed[13].

DISCUSSION

Anion activation has been noted with a number of enzymes[10–12], but has usually been reported in terms of a change in the relationship between activity and pH. To our knowledge anion activation has never been found to be an absolute requirement. In the case of maleate hydratase reported here, anion activation is unique in that the presence of some activator is required for the expression of any enzyme activity. This activation has a simple mathematical description valid over the entire range of anion concentrations tested which predicts zero activity in the absence of activating anions. Moreover the activation is independent of pH within the limits of enzyme stability. In its details this example of anion activation appears

most closely allied to the enzyme activation noted with some divalent cations and discussed theoretically by ALBERTY[7] and DIXON AND WEBB[9]. For example, Dixon and Webb have considered the case in which an activator acts by combining with the enzyme independently of the substrate, and assuming that only the enzyme–activator–substrate complex is required for product formation, derived the expression

$$k[E]/v = (1 + K_s/[S])\,(1 + K_a/[A]) \tag{4}$$

where v is the initial velocity; $[E]$, the enzyme concentration; k, the rate constant of product formation from active complex; $[S]$, the substrate concentration; $[A]$, the activator concentration; K_s a Michaelis constant; and K_a the apparent dissociation constant of enzyme with activator. Application of this to the present case leads to the identifications

$$K_{\mathrm{mal}}\,(A) = K_s,\ K_{A^-} = K_a$$
$$v_{\mathrm{mal}}\,(A^-) = k[E]/(1 + K_a/[A]),\ v_{A^-} = k[E]/(1 + K_s/[S])$$

These expressions would predict that Lineweaver–Burk plots at different concentrations of a single activating anion would have a common apparent Michaelis–Menten constant but differ in their $1/v$ intercepts as is found in Fig. 2. The symmetry of the kinetic expression in S and A demands that Lineweaver–Burk plots with activator as "substrate" should have the same general form. In the case of maleate hydratase given in Fig. 6, this too is found. Further the true maximal initial velocity, V, should be obtained by extrapolation of $1/v$ intercepts either of Fig. 2 to infinite activating anion concentration or of Fig. 6 to infinite maleate concentration. If this is done the result in each case is approx. 0.28 μmole/min per mg protein. The apparent equality is further justification for the application of Eqn. 4 to maleate hydratase–anion–maleate interactions.

The description provided by the expression above is not completely successful, however. For example, it predicts that Lineweaver–Burk plots with different activators should share a common $K_{\mathrm{mal}}(A^-)$, contrary to what is observed in Fig. 4. Similarly the kinetic expression would predict that the Lineweaver–Burk plots with different activator anions treated as "substrate" at constant maleate concentration should share a common constant v_{A^-}, contrary to what is observed in Fig. 5. Some complications accounting in part for these discrepancies are disclosed in the behavior of various anions on Cl^- activation given in Fig. 7. The difference in the competitive behavior of CN^- and F^- implies that anions influence the enzyme in at least two ways. Since the kinetic expression given earlier is derived allowing only one enzyme–anion interaction, other enzyme–anion interactions necessarily would lead to deviations from the derived expression.

The natural function of maleate hydratase in the rabbit kidney is presently unknown. However, the demonstration of an absolute requirement for anion activation raises interesting physiological possibilities. Such an anion-activated enzyme would allow the anionic environment to influence cellular metabolism in a direct manner and, conversely, might play a role in controlling the distribution of anion in cellular compartments.

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