Pig heart fumarase really does exhibit negative kinetic co-operativity at a constant ionic strength

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The kinetics of the action of fumarase on L-malate and fumarate were investigated at constant ionic strength. This was done to evaluate reports that fumarase follows simple Michaelis–Menten kinetics. However, when pH, buffer concentration and ionic strength are all maintained at constant values, the Lineweaver–Burk plots exhibit pronounced downward curvature, characteristic of negative kinetic co-operativity.

INTRODUCTION

Previous studies by Andersen (1980) and Wharton & Szawelski (1982) have indicated that the fumarase-catalysed interconversion of L-malate and fumarate does not deviate from simple Michaelis–Menten kinetics. This conclusion seems to be at odds with the studies by Alberty and co-workers (Alberty & Bock, 1953; Alberty *et al.*, 1954; Taraszka & Alberty, 1964) and the more recent study by Bardsley *et al.* (1980). Although Andersen (1980) and Wharton & Szawelski (1982) were careful to prepare substrate solutions of the correct pH, neither of these studies appears to have maintained a constant ionic strength as the substrate concentration was increased. From the results obtained by Frieden *et al.* (1957) and Alberty & Hammes (1958) it is known that some of the fumarase kinetic parameters are quite sensitive to ionic strength, as might be expected for the reaction of a dianionic substrate. In order to resolve this discrepancy, we have performed experiments similar to those of Andersen (1980) where the ionic strength increased with increasing substrate concentration, and others where the ionic strength was maintained at a constant value by the addition of an inert electrolyte.

MATERIALS AND METHODS

Pig heart fumarase (L-malate hydro-lyase, EC 4.2.1.2) was dialysed several times against 10 mM-sodium phosphate buffer, pH 7.3. Stock L-malate and fumarate solutions were neutralized to pH 7.0 before being added to the miniature motor-stirred 1 cm reaction cell at 25 °C. The pH was measured before and after the reaction to ensure that there were no significant pH changes. The ionic strength was maintained constant as the substrate concentration varied by the addition of NaCl or NaNO₃ as indicated. The reaction was started by injecting a small volume of stock enzyme solution into the stirred reaction cell. The reaction was followed at 250 nm by monitoring either the appearance or the disappearance of the fumarate absorption ($\epsilon = 1.45 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). Initial-velocity measurements were made on a Shimadzu 260 spectrophotometer and were computed from first-derivative time-course plots extrapolated back to zero time. Only the first 2% of the L-malate reaction and the

first 8% of the fumarate reaction were followed, to avoid errors due to build-up of product. The L-malate concentrations ranged from 0.7 to 14.3 mM and the fumarate concentrations from 0.33 to 12.5 mM. Slow decreases in the stock enzyme activity upon storage were corrected for.

RESULTS AND DISCUSSION

Lineweaver–Burk plots for both L-malate (Fig. 1) and fumarate (Fig. 2) both exhibit downward curvature when the ionic strength is held constant. However, when the ionic strength is allowed to increase from 0.102 to 0.132 as the L-malate concentration is increased (Fig. 1) an essentially straight-line plot is obtained. The last result is similar to that obtained by Andersen (1980) and Wharton & Szawelski (1982), and it leads to incorrect conclusions about the enzyme mechanism. The change in v due to the change in ionic strength is just sufficient to eliminate the curvature of the Lineweaver–Burk plot. For the fumarate reaction the term $V_{\text{max}}/(K_s \cdot [E]_t)$ has been reported (Alberty & Hammes, 1958) to decrease by about 70% for a similar change in ionic strength. When NaNO₃ was used instead of NaCl (Fig. 1) to make up the ionic strength no significant change in v was observed, indicating that the curved plots of Figs. 1 and 2 are not due to a specific ion effect. However, there is a strong dependence of the kinetics on the phosphate buffer concentration, as has been previously described (Alberty & Bock, 1953; Alberty *et al.*, 1954).

The downward curvatures of the Lineweaver–Burk plots at constant ionic strength are characteristic of negative co-operativity (Segel, 1975; Neet, 1980). This can occur when the binding of a substrate molecule to the enzyme results in a decrease in affinity for the remaining vacant sites. Fumarase is a tetramer (Beeckmans & Kanarek, 1977), and it is not surprising that its kinetics exhibit co-operativity.

Negative co-operativity can usually be accommodated (Alberty & Bock, 1953; Taraszka & Alberty, 1964; Segel, 1975; Neet, 1980; Bardsley *et al.*, 1980) by an equation of the general form:

$$v = \frac{c[S] + d[S]^2}{1 + e[S] + f[S]^2} \quad (1)$$

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Fig. 1. Lineweaver-Burk plots (v^{-1} versus $[L\text{-malate}]^{-1}$) for the fumarase-catalysed reaction

All data are at pH 7.0 and refer to a fumarase concentration of 32 ng/ml. The top two plots were obtained at a constant ionic strength of 0.140 with a constant sodium phosphate buffer concentration of 9.3 mM (○) and 46.3 mM (△) respectively. The corresponding symbols ● and ▲ indicate that NaNO_3 was used instead of NaCl to make up the ionic strength. The bottom plot (□) was obtained at a constant phosphate buffer concentration of 50 mM, but with the ionic strength increasing from 0.102 to 0.132 as the $[L\text{-malate}]$ was increased, resulting in an essentially straight-line plot. The continuous lines are calculated by using the best-fit parameters of Table 1 in eqn. (3).

where c , d , e and f are constants that are functions of rate and equilibrium constants for a particular mechanism. One mechanism among a number that were tried that

Fig. 2. Lineweaver-Burk plots (v^{-1} versus $[fumarate]^{-1}$) for the fumarase-catalysed reaction

All data are at pH 7.0 at a constant ionic strength of 0.140 and refer to a fumarase concentration of 32 ng/ml. The plots were obtained at constant sodium phosphate buffer concentrations of 9.3 mM (○), 23 mM (□, broken line) and 47 mM (△). The lines are calculated by using the best-fit parameters of Table 1 in eqn. (3).

satisfactorily accommodates the substrate concentration dependence of v is (Segel, 1975):

Table 1. Best-fit parameters to eqn. (3) for the fumarase-catalysed reaction of L-malate and fumarate

Fumarase concentration was 32 ng/ml at pH 7.0, the temperature 25 °C and the ionic strength 0.140.

Substrate	$k_p \cdot [E]_t$ ($\mu\text{M} \cdot \text{s}^{-1}$)	K_s (mM)	a	b	[Phosphate] (mM)
L-Malate*	0.37 (0.35)	0.32 (0.26)	53 (32)	1.2 (1)	9.3
L-Malate	0.47	0.11†	9100†	240†	46.3
Fumarate*	2.9 (3.3)	0.30 (0.39)	13 (49)	0.79 (1)	9.3
Fumarate	1.7	0.21	340†	45†	23.2
Fumarate*	2.4 (3.2)	0.68 (1.00)	2.0 (2.0)	1.3 (1)	46.6

* These data sets also gave satisfactory three-parameter fits with $b = 1$. The three-parameter fit values are given in parentheses beside the four-parameter fit values.

† Standard errors of these parameters greatly exceed the parameter values.

which yields with the fast-equilibrium assumption:

$$v = \frac{2 \cdot k_p \cdot [E]_t \cdot \frac{[S]}{K_s} + 2 \cdot k_p \cdot [E]_t \cdot b \cdot \frac{[S]^2}{a \cdot K_s^2}}{1 + \frac{2 \cdot [S]}{K_s} + \frac{[S]^2}{a \cdot K_s^2}} \quad (3)$$

which is of a form similar to eqn. (1).

Negative co-operativity in this mechanism results when $a > 1$, i.e. when the second substrate dissociation constant, $a \cdot K_s$, is larger than the first, K_s . From eqn. (3), $V_{\max} = 2 \cdot b \cdot k_p \cdot [E]_t$. The calculated lines of Figs. 1 and 2 were obtained from four-parameter non-linear least-squares analyses of the data in eqn. (3) assuming a constant error in v . For three of the data sets satisfactory three-parameter fits with $b = 1$ were obtained. The best-fit parameters obtained are given in Table 1. The values of a and b , the factors by which K_s and k_p respectively are modified by the binding of a second substrate molecule, are not well defined for all of the data sets, owing both to the degree of curvature of some of the plots and to how close K_s is to the experimental range of substrate concentrations. It is clear from Figs. 1 and 2 that the kinetics depend upon the phosphate buffer concentration, and hence the parameters of eqn. (3) are themselves functions of buffer concentrations. It should be emphasized that a number of other reaction schemes may equally well be accommodated by eqn. (1). However, mechanism (2) does provide a useful set of parameters for comparative purposes. Fumarase isoenzyme heterogeneity (Penner & Cohen, 1971) could also lead to curved Lineweaver-Burk plots.

In summary, these results illustrate the importance of maintaining a constant ionic strength when studying enzyme reactions of ionic species, and indicate that fumarase exhibits negative kinetic co-operativity.

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