Biochem. J. (1980) **189**, 653–654
Printed in Great Britain

Lack of deviation from Michaelis–Menten kinetics for pig heart fumarase

Bjørn ANDERSEN
Chemical Laboratory IV, H. C. Ørsted Institute, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen, Denmark

(*Received 27 May 1980*)

Studies of steady-state kinetics of fumarase in the usual substrate-concentration range from $0.1K_m$ to $10K_m$ and in the high substrate-concentration range from $10K_m$ to $200K_m$ are described. The purpose is to investigate reports of substrate inhibition and oscillatory kinetics. In the normal substrate-concentration range, no deviations from hyperbolic kinetics were found, and in the extended concentration range, up to more than 200 times the K_m value, no substrate inhibition was demonstrated. A discussion of the discrepancies between the mentioned reports of deviations from the hyperbolic kinetics and the present findings is given.

Several studies of the kinetics of pig heart fumarase have been concerned with deviations from the Michaelis–Menten kinetics for this enzyme. Various conclusions concerning the binding of substrate to the active centre were based on these findings. Alberty *et al.* (1954) reported substrate inhibition with malate concentrations from 33 to 100mM, as did Rajender & McCalloch (1967). These findings supported the idea of a two-point attachment of the substrate to the enzyme. More wide-ranging conclusions were drawn from the investigations of Crabbe & Bardsley (1976). Their findings, of kinetics in which the velocity curve descended from the hyperbolic form at substrate concentrations of $6K_m$ and, furthermore, oscillated, lead to the conclusion that a rate equation of at least fourth degree was required for the description and demanded a mixture of isoenzymes for its interpretation. A partial retraction from these results is found in a preliminary footnote in Hill *et al.* (1977). The present paper reports similar investigations on fumarase over a wide range of substrate concentrations with all relevant variables carefully maintained at a constant value. This time the reaction was found to follow the Michaelis–Menten kinetics closely over the full range. An explanation is proposed for the deviations mentioned above.

Materials, methods, calculations and results

In view of the sensitivity of both the enzyme and the substrate to pH and buffer concentration, a brief analysis of the conditions of Crabbe & Bardsley (1976) is relevant. Their procedure was to dissolve what was presumably malic acid (the text is not clear) in 0.2M-phosphate buffer, pH 7.0. In a buffer corresponding to this description, tentative concentrations of primary and secondary phosphate would be 0.020M and 0.080M respectively. Fig. 1 shows values of pH and of true substrate (secondary malate ion, M^{2-}) calculated on these premises when malic acid is added to the buffer in amounts shown on the abscissa. Apart from the obvious action of the released protons on the active centre, it is noteworthy that the linear augmentation of the real substrate (M^{2-}) is lost when the malic acid concentration exceeds 15mM for a value of pH near 6.3.

Repeating the kinetic experiment under such con-

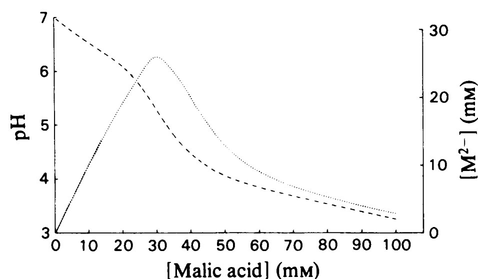

Fig. 1. *Calculated values of pH and malate ion (M^{2-}) when malic acid is added to 0.2*M*-phosphate buffer, pH 7.0*

The Figure shows experimental conditions giving rise to oscillatory deviations from hyperbolic kinetics (see the text). The linear augmentation of the real substrate (M^{2-}) is lost when the malic acid concentration exceeds 10–15mM. ——, Linear augmentation of M^{2-}; ----, pH; ...., actual concentration of M^{2-}.

0306-3275/80/090653-02$01.50/1 © 1980 The Biochemical Society

Table 1. *Initial rate of formation of fumarate from L-malate*

The reaction took place in phosphate buffer (0.05 M), pH 7.0. The actual enzyme concentration was 0.323 μg/ml. The first 12 values of s and v were used to calculate $V_{max.}$ and K_m (see the text). $K_m = 1.54 \pm 0.04$ mM. These are single estimates in the concentration range 0.1–10.0 mM-M^{2-}. In the extended substrate-concentration range from 20 to 300 mM, no significant differences between experimentally found values of v and the calculated value of $V_{max.}$ could be demonstrated. The values of v at 300 mM-M^{2-} is the average of four determinations.

s ([M^{2-}]) (mM)	0.1	0.5	0.75	1.0	1.5	2.0	2.5	3.0	4.0	5.0	7.5	10.0	20	50	100	150	200	300
v (μM·min^{-1})	1.5	5.7	8.1	9.2	11.7	13.2	14.7	15.0	17.0	18.0	19.3	20.7	22.7	24.0	23.3	23.3	22.0	23.9
$V_{max.}$ calculated						23.6 ± 0.2												

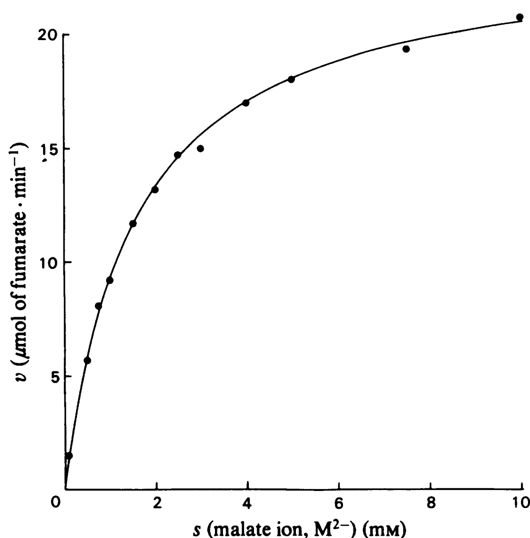

Fig. 2. *Initial velocity* (v) *versus concentration of malate* (s)

The experimental values are found in Table 1. $K_m = 1.54 \pm 0.04$ mM, $V_{max.} = 23.6 \pm 0.2\,\mu$M·min^{-1}. The calculated hyperbola and the experimental values are represented.

ditions gave rise to v-versus-s plots similar to those of Crabbe and Bardsley. In these experiments the pH was measured after each assay and a verification of the pH values in Fig. 1 was realized.

To verify the validity of the Michaelis–Menten kinetics for fumarase, a series of experiments was carried out in the range of 0–300 mM-M^{2-}. Phosphate buffer (0.05 M), pH 7.0, was used. The malic acid solution was neutralized to pH 7.0 before being added to the buffer. For each value of [M^{2-}] the pH was controlled before and after the experiment. A Beckman 35 recording spectrophotometer was used at 230 nm up to [M^{2-}] values of 2.0 mM and at 250 nm for higher values. The enzyme used was Boehringer crystalline pig heart fumarase with the following specifications: 10 mg of enzyme/ml in $(NH_4)_2SO_4$ solution; specific activity 350 units/mg. The given values of enzyme concentrations are based on dilution. Standard assays to check for any alteration in enzyme activity were run for each of the five assays. No significant difference in the measured K_m values was obtained with two different enzyme concentrations of 0.323 and 0.645 μg/ml.

Calculations of K_m and $V_{max.}$ are based on least-squares fitting of plots of s/v against s (Hanes plot). Table 1 gives all experimental values of v and s. Only the first 12 values of v and s (up to s values seven times the K_m value) were used to calculate K_m and $V_{max.}$. The hyperbola representing the Michaelis–Menten equation, based on the measured values of the two parameters, was then calculated. Fig. 2 shows the calculated hyperbola and the experimental values.

Conclusion

No deviations from the hyperbolic kinetics were found.

It is demonstrated in Table 1 that in the extended substrate concentration range from 20 to 300 mM-M^{2-} there are no significant differences between the experimentally found values of v and the calculated values of $V_{max.}$. It seems that the suppositions for the mechanism of reaction for fumarase based on substrate inhibition have to be reinvestigated. It should be borne in mind that the validity of information based on the Michaelis–Menten kinetics is strongly dependent on the implicit confidence in the experimental procedure.

References

Alberty, R. A., Massey, V., Frieden, C. & Fuhlbrigge, R. (1954) *J. Am. Chem. Soc.* **76**, 2485–2493

Crabbe, M. J. C. & Bardsley, G. (1976) *Biochem. J.* **157**, 333–337

Hill, C. M., Waight, R. D. & Bardsley, W. G. (1977) *Mol. Cell Biochem.* **15**, 173–177

Rajender, S. & McCalloch, R. J. (1967) *Arch. Biochem. Biophys.* **118**, 279–283