IONISATION CONSTANTS OF FUMARASE

by

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Knowledge of the chemical nature of groups in the enzyme molecule concerned with the enzyme catalysis is essential for a clear understanding of the mechanism of catalysis. Of the methods available for the identification of such groups the determination of the ionisation constant and heat of ionisation appears a very convenient method in the absence of any specific tests such as those applicable to sulphydryl groups or prosthetic groups. The recent theory of DIXON[1] and the preceding discussion by ALBERTY AND MASSEY[2] present methods by which the ionisation constant of groups at the active centre may be characterised. In this paper are presented data for fumarase obtained from both methods.

MATERIALS AND METHODS

Fumarase. The enzyme used in these experiments was twice recrystallised material obtained by the method of MASSEY[3] or by the method of FRIEDEN, BOCK AND ALBERTY[4]. Side by side experiments in this laboratory have shown that the material isolated by these two methods is identical in specific activity and kinetic properties.

Estimation of activity. The method of activity determination used was the spectrophotometric method of RACKER[5]. The reaction was followed with a Beckman DUR spectrophotometer, and the velocities reported here are initial velocities. Full details of the procedure have been published earlier[6,7]. Unless otherwise indicated the experiments were carried out at 25°.

NOMENCLATURE

Throughout this paper the terms acidic dissociation constant (pK_a) and basic dissociation constant (pK_b) are used to designate, respectively, those groups responsible for the acidic and basic regions of the pH-activity curves. It is fully appreciated that theoretically it is not possible to tell whether the groups giving rise to the acidic and basic sides of the pH activity curve are of the so-called acidic or basic types. In the case of fumarase the plots of maximum initial velocity (V) *vs* pH are symmetrical bell-shaped curves which can be represented mathematically with only two ionisation constants (*cf.*, Fig. 5). This indicates that the action of the enzyme is determined by only two ionisable groups in the range of pH 5–9.

In keeping with the nomenclature of the preceding article[2] the subscripts M and F will denote the pK in the presence of malate and fumarate respectively, while the subscripts P, A, T will denote that in the presence of a given amount of phosphate, *tris*-(hydroxymethyl)-aminomethane acetate or thiocyanate. In keeping with nomenclature already used a subscript placed to the right of E is used where the combination is occurring at the active centre while the subscript to the left of E is used when it is considered likely that the combination is not directly with the active centre. Thus pK_{aE} would designate the pK of group a in the free enzyme; pK_{aPE} would designate the pK of this group when phosphate is combined with the enzyme, pK_{aPEM} when both phosphate and malate were combined, etc. The term V in all cases employed here represents the maximum initial velocity calculated by extrapolating to infinite substrate concentration by the method of LINEWEAVER AND BURK[8] from data at sufficiently low substrate concentration to avoid activation by substrate. The subscripts used have the same significance as above.

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RESULTS

In a previous study of the effect of various anions on fumarase activity MASSEY[9] concluded that the effects of these anions, both activating and inhibiting, could be explained largely in terms of their effect on the ionisation constants of acidic and basic groups in the neighbourhood of the active centre. For example, it was concluded that a number of monovalent anions affected mainly an acidic ionisation constant, while a number of polyvalent anions affected mainly a basic ionisation constant. It was also concluded that the two substrates L-malate and fumarate affected the ionisation constants of these groups to different extents, the effect being mainly on the acidic dissociation constant.

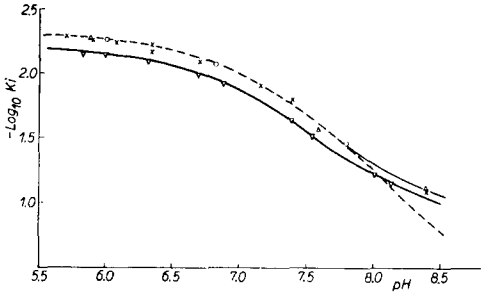

Fig. 1. The variation of K_i for thiocyanate with pH with L-malate as substrate. The broken line is the theoretical curve for a group in the protein with a pK of 7.0, calculated from a limiting value of $-\log_{10}K_i$ of 2.3 in the acid region.

× 0.033 M phosphate 25° 0.087 M malate
△ 0.033 M tris-acetate 25° 0.087 M malate
○ 0.033 M phosphate 25° 0.001 M malate
▽ 0.033 M phosphate 35° 0.087 M malate

According to the theory of DIXON[1] it is possible to determine pK values for the enzyme and the enzyme-inhibitor complex from the variation of inhibition constants with pH. Fig. 1 shows the variation of $-\log_{10}K_i$ with pH for thiocyanate (which is a non-competitive inhibitor of fumarase) under various conditions with L-malate as substrate. From Dixon's theory these results show a pK of some group in the enzyme under the influence of malate at pH 7.0, either in the presence of 0.033 M phosphate or in 0.033 M tris-acetate. Furthermore the slope of the plot shows that the combination of enzyme and thiocyanate results in a gain of net positive charge or loss of net negative charge[1]. This is consistent with the acid-weakening or base-strengthening effect on an ionising group expected of the presence of an anion[2,9] either in the immediate proximity of the ionising group or by combination with a neighbouring positively charged group which influences the ionisation of the activating group. As it has been shown previously[9] that thiocyanate causes a displacement toward the alkaline of the acid branch of the pH-activity curve this pK determined may be identified tentatively as pK_{aPEM}. It is interesting to note that pK_{aPEM} is identical with pK_{aAEM} showing as suggested previously that phosphate has no effect on pK_{aM}.

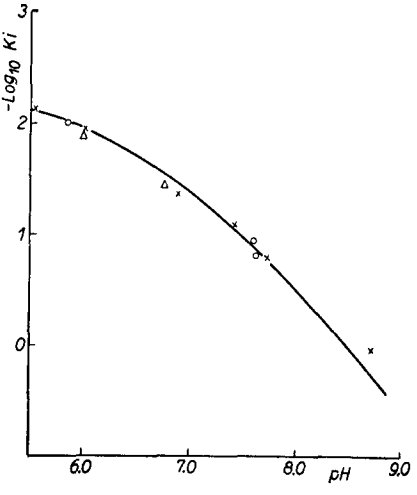

Fig. 2. The variation of K_i with pH for fumarate as substrate. All determinations at 25°.

× 0.033 M phosphate 0.0167 M fumarate
△ 0.033 M phosphate 0.00125 M fumarate
○ 0.033 M tris-acetate 0.0167 M fumarate

Fig. 2 shows similar data for fumarate. Here the accuracy of the determination of pK_a is not so good as with malate, owing to the low pH's required to evaluate pK_a. However the value of pK_{aEF} is in the region of pH 6.0. Again pK_{aPEF} is the same

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as pK_{aAEF} but much lower than for pK_{aPEM} or pK_{aAEM}. This is again consistent with the previous conclusion[9] that malate and fumarate affect to different extents the ionisation of the group responsible for the acid side of the pH-activity curve.

Fig. 1 also shows the effect of temperature on pK_{aPEM}. Within experimental error there is no change in pK_{aPEM} from 25° to 35°. From data of this sort it would be possible to detect a change in pK of 0.1. Hence it may be concluded that the heat of ionisation of this particular group is less than 4500 cals/mole. The possible significance of this observation will be considered in the discussion.

If thiocyanate causes a shift of pK_a to more alkaline values then it should be possible also to evaluate pK_{aPTEM}, etc. From Figs. 1 and 2 there is no good evidence of a pK_{aTEM} or pK_{aTEF} in the range of pH investigated, although some of the experimental points at higher pH values do lie above the theoretical curve for pK_{aPEM} and pK_{aAEM} of 7.0. However the reason why pK_{aTEM} and pK_{aTEF} does not show up clearly is probably because of the difficulty of determining inhibition constants at high pH values, because a change over from inhibition to activation occurs as the pH is raised. Fig. 3 illustrates this effect. At pH values close to where the transition from inhibition to activation occurs the effect of thiocyanate can be to activate or inhibit according to the concentration used. Fig. 4 shows this effect. Accordingly the determination of the inhibition constant for thiocyanate becomes uncertain under such conditions.

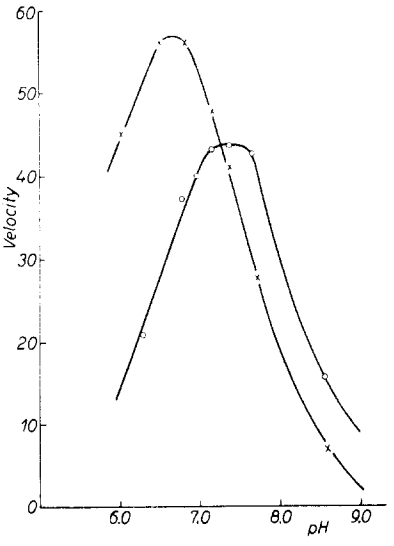

Fig. 3. The effect of 0.027 M KCNS on the pH-activity curve for 0.0167 M fumarate as substrate in the presence of 0.033 M phosphate 25°.
× fumarate alone
○ fumarate + thiocyanate

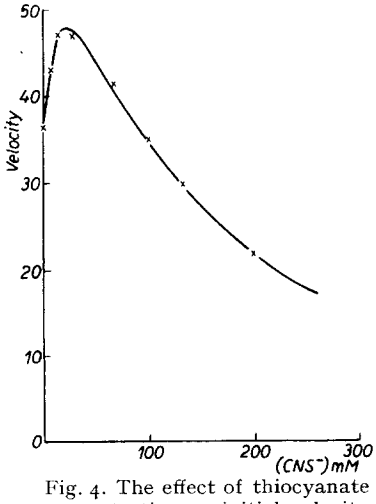

Fig. 4. The effect of thiocyanate concentration on initial velocity at pH 7.7. Sodium fumarate concentration 0.0167 M, phosphate concentration 0.033 M.

As an independent check on these results pK_a and pK_b can also be determined from the variation of maximum initial velocity with pH. Fig. 5 shows a plot of V_F and V_M *vs* pH at 0.033 M phosphate at 25°. For both substrates, at this and other phosphate concentrations, symmetrical bell-shaped pH-maximum initial velocity curves are obtained. From equation (6) of the preceding paper[2] pK_{aPEM} and pK_{aPEF} are calculated as 7.2 and 5.7, respectively. These values are in good agreement with the values of pK_{aPEM} of 7.0 and pK_{aPEF} of 6.0 obtained from the thiocyanate inhibitor data. The values of pK_{bPEM} and pK_{bPEF} obtained from Fig. 5 are 9.0 and 7.7, respectively.

A series of plots such as shown in Fig. 5 have been obtained at other phosphate concentrations[7] and the values of pK_a and pK_b obtained from these plots are summarised in Table I. Also shown in Table I are the values obtained by extrapolation of V_F to zero phosphate concentration.

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Fig. 6 shows the pH-variation of V_F obtained by extrapolation to zero phosphate concentration. In the case of malate as substrate it was not possible to obtain an accurate extrapolation to zero phosphate since at all pH values the activation by phosphate is almost complete at concentrations of about 0.005 M. However, with fumarate as substrate the activation is not complete until considerably higher concentrations of phosphate are reached.

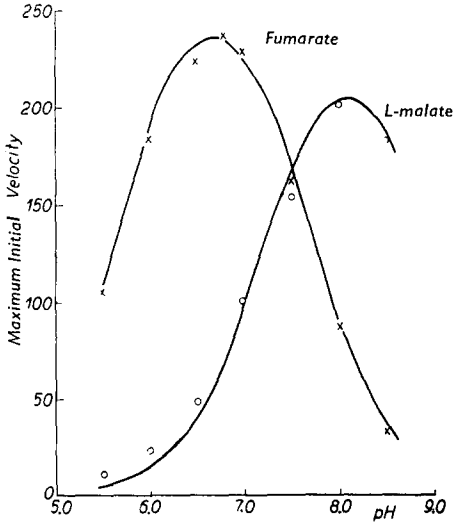

Fig. 5. The effect of pH on the maximum initial velocities for fumarate and L-malate at 0.033 M phosphate and 25°. The maximum initial velocities were obtained by extrapolation to infinite substrate concentration by the method of LINEWEAVER AND BURK[8]. The solid line is the theoretical curve obtained from equation (6) of the preceding paper using the pK values of Table I.

Fig. 6. The variation with pH of the maximum initial velocity for fumarate as substratein the absence of phosphate at 25°. The points shown on the graph were obtained by double extrapolation, extrapolation to infinite fumarate concentration over the whole pH-range at five different phosphate concentrations, followed by extrapolation to zero phosphate concentration.

The data shown in Table I indicate that both pK_{aEM} and pK_{aEF} are independent of phosphate concentration, whereas pK_{bEF} and probably pK_{bEM} are increased in the presence of phosphate. This conclusion is in full agreement with that from the thiocyanate inhibition data, where it is shown that both pK_{aEF} and pK_{aEM} are independent of phosphate.

TABLE I

pK VALUES AT DIFFERENT PHOSPHATE CONCENTRATIONS

(*phos*) mM	pK_{aEM}	pK_{bEM}	pK_{aEF}	pK_{bEF}
0	—	—	5.8	7.2
5	7.3	8.5	5.9	7.3
15	7.1	8.3	5.8	7.6
33.3	7.2	9.0	5.7	7.7
60	7.3	8.5	5.7	7.7
133.3	7.3	8.5	5.9	7.7

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DISCUSSION

Two independent methods have yielded substantially the same values of pK_{aEF} and pK_{aEM}. The results also indicate that pK_{aEM} and pK_{aEF} are independent of phosphate concentration, whereas pK_{bEF} and possibly pK_{bEM} increase in the presence of phosphate. Also the results suggest that pK_{aEM} and pK_{aEF} are increased in the presence of thiocyanate. Fig. 3 also suggests that pK_{bEF} is increased in the presence of thiocyanate. Since pK_{aEF} and pK_{bEF} are lower than pK_{aEM} and pK_{bEM} it seems likely that fumarate and malate also increase the values of pK_a and pK_b in the free enzyme, and that malate is more effective in producing these shifts in pK_a and pK_b than is fumarate.

Theoretically, it is possible to determine the values pK_{aE} and pK_{bE} uninfluenced by substrate by plotting V/K_m *vs* pH (ALBERTY AND MASSEY[2]). Such data is not yet available for fumarase, but if the foregoing conclusions are correct it would be predicted that $pK_{aE} \leqslant 5.7$ and $pK_{bE} \leqslant 7.2$. While these values are uncertain it is not possible to speculate too seriously on the nature of the ionising groups *a* and *b*. The evidence that group *a* has a $pK \leqslant 5.7$ and a heat of ionisation of < 4500 cals/mole suggests that it is a carboxylic group. Its identity with an imidazole group is unlikely as this group has a heat of ionisation of $\geqslant 6900$ cals/mole[10].

However, this conclusion is not unequivocal as the $\triangle$ H of < 4500 cals is for the group *a* under the influence of L-malate and phosphate. The $\triangle$ H might be different when these anions were not present.

The pK of group *b* is consistent with that of an imidazole group. The effect of temperature on pK_b should provide evidence of this possibility.

The increase of both pK_a and pK_b in the presence of various anions is consistent with an acid-weakening or base-strengthening effect which would be expected of the proximity of an anion or its combination with a neighbouring positively charged group in the protein capable of influencing the ionisation of the particular activating group. It is of considerable interest to note that phosphate affects only pK_b. It is therefore concluded that phosphate must combine with a positively charged group in the protein so located that its effect is to influence mainly the ionisation of group *b*. The effect of other anions such as substrate or thiocyanate could be (i) to combine with the same group as that with which phosphate combines *plus* another group whose ionisation could affect the ionisation of group *a* or (ii) to combine with a group capable of influencing the ionisation of both *a* and *b*.

ACKNOWLEDGEMENTS

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SUMMARY

1. Values for ionisation constants of groups concerned with the catalytic action of fumarase have been determined by two independent methods.

2. The ionisation constants of these groups have been shown to be increased by the addition of various anions, including the substrates. It is considered that this effect is largely responsible for the activating and inhibiting action of these anions at various pH values.

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RÉSUMÉ

1. Les valeurs des constantes d'ionisation des groupes qui interviennent dans l'activité catalytique de la fumarase ont été déterminées par deux méthodes indépendantes.

2. Les constantes d'ionisation de ces groupes sont augmentées par l'addition de divers anions, parmi lesquels les substrats. Les auteurs considèrent que ce phénomène est en grande partie responsable de l'activation ou de l'inhibition par ces anions aux divers pH.

ZUSAMMENFASSUNG

1. Es wurden Werte für die Ionisationskonstanten von Gruppen, die an der katalytischen Wirkung der Fumarase beteiligt sind, durch zwei von einander unabhängige Methoden bestimmt.

2. Die Ionisationskonstanten dieser Gruppen werden, wie gezeigt wurde, beim Zusatz verschiedener Anionen, einschliesslich Substrats grösser. Es wird angenommen, dass dieser Effekt weitgehend für die Aktivierungs- und Hemmungswirkung dieser Anionen bei verschiedenen pH-Werten verantwortlich ist.

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