KINETICS OF THE MECHANISM OF ACTION OF FLAVIN PYRUVATE OXIDASE FROM AN ACETATE REQUIRING MUTANT OF *ESCHERICHIA COLI*

R.L. HOUGHTON and B.E.P. SWOBODA
Department of Molecular Sciences, University of Warwick, Coventry, CV4 7AL, England

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1. Introduction

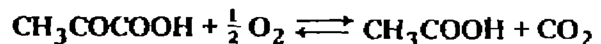
On the basis of kinetic analysis of the mode of action of flavoproteins [1] it has been proposed that a binary ping-pong bi-bi mechanism served as a general mechanism for several of these enzymes. In recent years, however, considerable evidence has been gathered by several authors [2–6] to suggest that in some cases a more ordered mechanism exists such as a Theorell–Chance or an ordered bi-bi mechanism. It has also been shown that in many of these cases the oxidised products acted as competitive inhibitors to the substrates of the reaction and that they often formed spectrally observable complexes with the enzyme itself.

The isolation of *N*-5 acyl derivatives of flavins [7] then prompted suggestions that flavins might conceivably function in a group transfer role as well as a redox one.

In the light of these factors the mode of action of pyruvate oxidase was studied by observing the effect of varying thiamine pyrophosphate (TPP), pyruvate and ferricyanide concentrations. In particular interest was given to the order of substrate and cofactor binding.

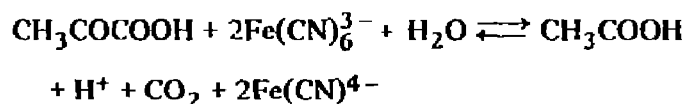
2. Materials and methods

Pyruvate oxidase, in the presence of a cytochrome b_1 containing fraction catalyses the oxidative decarboxylation of pyruvate to yield acetate and CO_2 [8] as products as shown in the basic reaction:



$$CH_3COCOOH + \frac{1}{2}O_2 \rightleftharpoons CH_3COOH + CO_2$$

If, however, oxygen was replaced by ferricyanide the stoichiometry became



$$CH_3COCOOH + 2Fe(CN)_6^{3-} + H_2O \rightleftharpoons CH_3COOH + H^+ + CO_2 + 2Fe(CN)^{4-}$$

For the reaction to proceed at a maximal rate the system requires to be preincubated with TPP, Mg^{2+}, pyruvate and a phospholipid or detergent activator for a period of 15 min before initiation of the assay with the acceptor, ferricyanide. Lag phases occurred in the kinetics if no preincubation was carried out but this aspect of the kinetics will be published elsewhere. Initial rates of ferricyanide reduction, in the above assay system, were shown to be linear with enzyme concentration in the range studied.

In all the ensuing studies the concentrations of Mg^{2+} and the activator sodium dodecyl sulphate (SDS) were held at 10 mM and 10^{-4} M, respectively. The concentrations of TPP, pyruvate and ferricyanide in the assay were held at 10^{-4} M, 50 mM and 4 mM, respectively, unless varied in the experiment. Assays were run in 0.1 M potassium phosphate buffer pH 6.0 at 25° by following the change in absorbance of the ferricyanide at 450 nm using a Gilford 2000 spectrophotometer. For ferricyanide concentrations < 2 mM the assay was monitored at 420 nm. The amount of ferricyanide reduced in the assay was

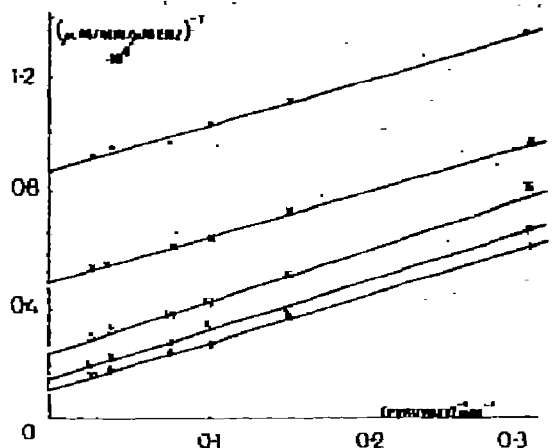


Fig. 1. Kinetic characteristics of pyruvate and ferricyanide. Lineweaver–Burk plots obtained with pyruvate as variable substrate at different ferricyanide levels. Assays contained 10^{-4} M SDS and preincubations were carried out for 15 min before initiating with the acceptor. The ferricyanide concentrations used were: 0.34 mM (•—•—•), 0.67 mM (X—X—X), 1.34 mM (▫—▫—▫), 2 mM (∘—∘—∘) and 4 mM (+—+—+). TPP and $MgCl_2$ were 10^{-4} M and 10 mM, respectively.

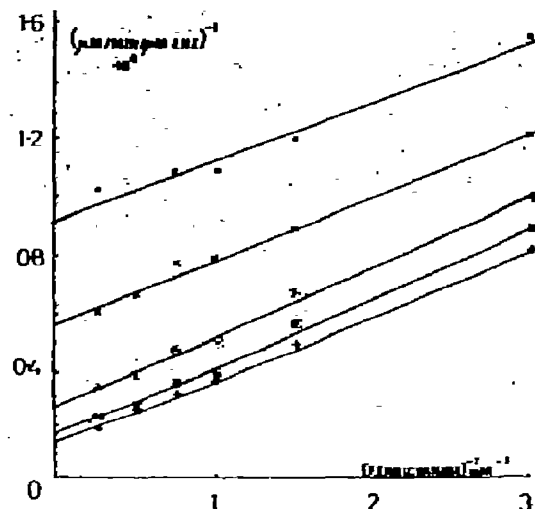


Fig. 2. Kinetic characteristics of pyruvate and ferricyanide. Lineweaver–Burk plots obtained with ferricyanide as variable substrate at different pyruvate levels. Assays contained 10^{-4} M SDS and preincubations were carried out for 15 min before initiating with the acceptor. The pyruvate concentrations were: 2 mM (•—•—•), 3.34 mM (X—X—X), 6.67 mM (∘—∘—∘), 13.34 mM (▫—▫—▫) and 26.7 mM (+—+—+). TPP and $MgCl_2$ were 10^{-4} M and 10 mM, respectively.

estimated using an ϵ_{mM}^{450} of 0.218 cm^{-1} mM^{-1} and an ϵ_{mM}^{420} of 1.076 cm^{-1} mM^{-1}. Enzyme concentrations were determined from the flavin content assuming 4 moles FAD bound per molecular weight of 265,000 [8]. The flavin concentration was estimated using an ϵ_{mM}^{438} of 14.6 cm^{-1} mM^{-1} per FAD. Each assay contained 5 μg of the flavoprotein.

The flavoprotein was isolated from an acetate requiring mutant of *Escherichia coli* (191–6) and shown to be > 90% pure by SDS polyacrylamide gel electrophoresis and ultracentrifugation [9].

TPP and sodium pyruvate were obtained from the Sigma Chemical Co., potassium ferricyanide (analytical grade) was from Fisons Ltd. and the sodium dodecyl sulphate was the specially pure grade supplied by British Drug Houses. All other compounds were of analytical grade and were also obtained from British Drug Houses.

3. Results and discussion

The effect of varying the concentration of pyruvate and ferricyanide are shown in figs. 1 and 2. In both cases the plots remain parallel at both high and low concentrations of ferricyanide and pyruvate. This indicates that neither the substrate nor the acceptor function as inhibitors in the concentration ranges used, namely 0.34–4 mM for ferricyanide and 3.34–40 mM for pyruvate. In both cases the K_m vs V_m plots obtained from figs. 1 and 2 were shown to pass through the origin suggesting the likely absence of a ternary complex involving substrate and acceptor.

No evidence was obtained to suggest that the reaction rate was a function of $[\text{ferricyanide}]^2$, thus suggesting that the reoxidation of the flavin occurs via discrete one electron processes.

The effect of varying pyruvate concentrations at different TPP levels and vice versa are shown in figs. 3 and 4. In both cases the lines are seen to converge indicating that interactions occur between the cofactor and the substrate. An increase in pyruvate concentration gave rise to an apparent decrease in the K_m for TPP and a concomitant change in V_m. In the cases of pyruvate an increase in the TPP concentration produces a similar change in the kinetic parameters although the small variations in V_m lie within the experimental error of the procedure used.

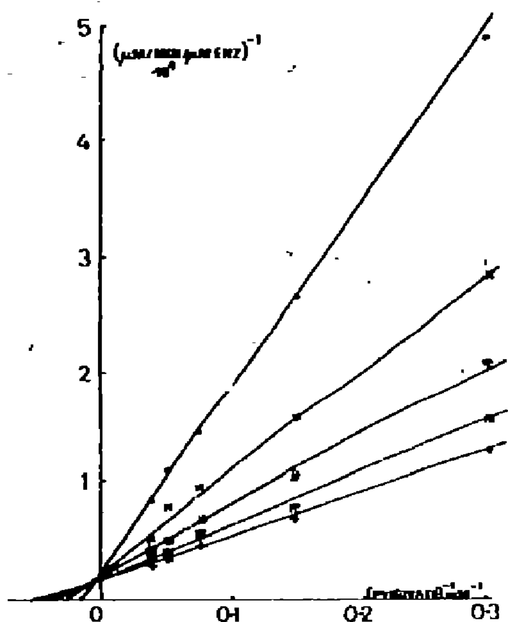


Fig. 3. Kinetic characteristics of pyruvate and TPP. Lineweaver–Burk plots obtained with pyruvate as variable substrate at different TPP levels. Assays contained 10^{-4} M SDS and preincubations were carried out for 15 min before initiating with acceptor. The TPP concentrations used were: 5 μM (●—●—●), 10 μM (X—X—X), 20 μM (⊖—⊖—⊖), 50 μM (▫—▫—▫) and 100 μM (+—+—+). Ferricyanide and $MgCl_2$ were held at 4 mM and 10 mM, respectively.

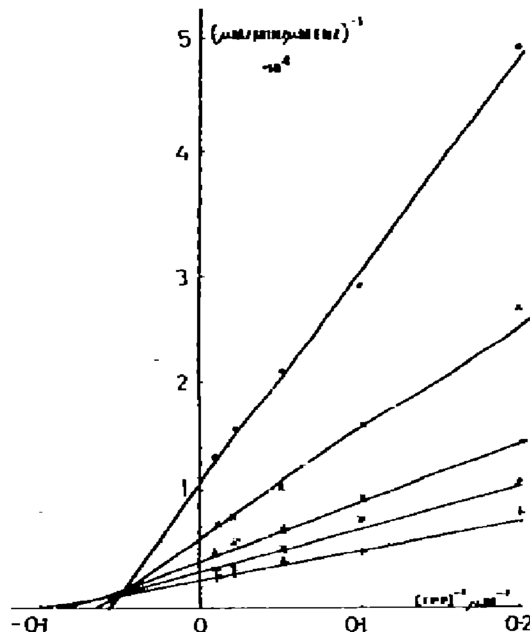


Fig. 4. Kinetic characteristics for TPP and pyruvate. Lineweaver–Burk plots obtained with TPP as variable substrate at different pyruvate levels. Assays contained 10^{-4} M SDS and preincubations were carried out for 15 min, before initiating with acceptor. The pyruvate concentrations used were: 3.34 mM (●—●—●), 6.67 mM (X—X—X), 13.4 mM (⊖—⊖—⊖), 20 mM (▫—▫—▫), 26.7 mM (+—+—+). Ferricyanide and $MgCl_2$ were held at 4 mM and 10 mM, respectively.

On the basis of the kinetic data obtained it would appear that pyruvate oxidase functions via an ordered mechanism without a ternary complex involving ferricyanide and pyruvate. In terms of the Dalziel notation [10] a minimum equation required to explain the data would then be of the form

$$\frac{e_0}{v} = \phi_0 + \frac{\phi_1}{A} + \frac{\phi_2}{S}\left(1 + \frac{\phi_3}{TPP}\right)$$

in which ϕ_0, ϕ_1, ϕ_2 and ϕ_3 are complex constants consisting of several rate constants, and A and S are the ferricyanide and pyruvate concentrations, respectively. v is the initial rate of the reaction and e_0 the initial enzyme concentration. Values for the ϕ constants can be evaluated from the Lineweaver–Burk plots and from secondary plots involving slopes and intercepts from the primary plots. The values for the constants obtained are listed in table 1. Since the catalytic activity e_0/v was plotted as a function of the variables, the ϕ values have been normalised with respect to enzyme concentration.

In the notation used ϕ_1/ϕ_0 and ϕ_2/ϕ_0 represent the K_m values for ferricyanide and pyruvate, respectively, at infinite TPP concentration. Values obtained were 2 mM and 11 mM. ϕ_0^{-1} represents the reaction velocity at infinite substrate and acceptor concentrations and was estimated as 87,000 μM substrate/min/μM enzyme. ϕ_3 (52.7 μM) is the dissociation constant for the enzyme–TPP complex.

The question arises as to what basic mechanism will display this sort of rate equation. As the order of product release is not easily distinguishable one can propose two possible mechanisms. Both of these

Table 1
Kinetic constants for pyruvate oxidase.

ϕ_0 (sec)	ϕ_1 (M·sec)	ϕ_2 (M·sec)	ϕ_3 (M)
6.84×10^{-4}	1.35×10^{-6}	7.27×10^{-6}	5.27×10^{-5}

require an ordered binding of TPP and pyruvate to the flavoprotein. The pyruvate, presumably attached to the TPP, is then decarboxylated to yield an α-hydroxy ethyl TPP intermediate which is then transformed to a 2-acyl TPP intermediate with concomitant reduction of the flavin. The diversity of product release can now occur in that one can get hydrolysis of the 2-acyl intermediate followed by re-oxidation of the flavin or vice versa. The overall rate equations obtained are indistinguishable. Evidence for the involvement of an α-hydroxyethyl intermediate in the catalysis was indicated from the finding [11] that it was capable of reducing the enzyme bound flavin.

In such mechanisms one would expect acetate to be a competitive inhibitor for either pyruvate or ferricyanide depending on the order of product release. However, extensive studies with acetate showed the absence of any appreciable inhibition indicating either the low affinity of the enzyme for acetate or the existence of another mechanism. The low affinity postulate might be expected on the basis of model studies and theoretical considerations of the mode of action of TPP, since it has been shown that TPP does not attack because of the strongly anionic properties of the carboxyl group [12].

Another mechanism which would explain the data, including the absence of acetate inhibition would be one in which *N*-5 acetyl 1,5 dihydro-FAD [7] is postulated as an intermediate. The initial steps of the mechanism as far as the formation of α-hydroxyethyl TPP are the same but at this point group transfer would occur yielding $-FADHCOCH_3$. This flavin derivative would then break down in the presence of ferricyanide to reform the oxidised flavin.

At present, therefore, one cannot differentiate between the mechanisms any further using kinetic methods. Isolation of intermediates will, however, narrow the possibilities.

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