A Kinetic Analysis of Rabbit Muscle Pyruvate Kinase in the Reverse Direction
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For a complete kinetic analysis of an enzyme the reaction should be studied in both directions as only in this way can sufficient information about the enzyme complexes formed be obtained. In recent years several kinetic studies of the forward reaction of rabbit muscle pyruvate kinase have been made (Reynard et al., 1961; Ainsworth & MacFarlane, 1973) but no systematic studies of the reverse reaction have been reported. As a result of the unfavourable equilibrium constant of this reaction (McQuate & Utter, 1959; Krimskey, 1959) only a very small proportion of the substrates are converted into products at equilibrium. Kinetic studies of the reverse reaction have therefore been limited to conditions of high concentrations of both substrates (McQuate & Utter, 1959; Krimskey, 1959) such that sufficient products were formed to allow estimation by usual enzymic means. Consequently, an assay more sensitive than those already available was required to measure the reverse reaction of this enzyme and permit a full kinetic analysis.

An assay suitable for such studies has been developed and is based on the transfer of label from 32P-labelled ATP into phosphoenolpyruvate. The assay was carried out at 25°C in 1 ml of an incubation mixture containing 25 mm-Tris-HCl (pH 7.4) and 1 mm-EGTA [ethanedioxybis(ethylamine)tetra-acetate]. The substrates and products were added as their potassium salts and by adding KCl the final K+ concentration was maintained at 100 mm. Sufficient MgCl2 was added to give a free Mg2+ concentration of 0.5 mm; the amount required was calculated by using the dissociation constants given by MacFarlane & Ainsworth (1972). A known amount of previously purified labelled ATP was added such that the specific radioactivity of the ATP was 1–5 μCi/μmol. After thermal equilibration the reaction was initiated by addition of a small volume of enzyme (generally 10 μl).

Fig. 1. Double-reciprocal plot of enzyme rate as a function of ATP concentration

Conditions of the enzyme assay are given in the text with K+ total = 100 mm and Mg2+ free = 0.50 mm. The pyruvate concentrations used were: ○, 5 mm; □, 7 mm; ▲, 10 mm; ○, 20 mm; ○, 50 mm. The points are the experimental data and the lines are computer fits to the rate equation for competitive substrate inhibition by one substrate in a sequential Bi Bi reaction. The values of the constants obtained were: Km pyruvate = 4.87 ± 1.4 mm; Km ATP = 0.184 ± 0.034 mm; Kf pyruvate = 8.34 ± 3.2 mm; Kf ATP = 2.78 ± 0.87 mm; V = 0.0417 ± 0.0022 v-1 unit/v+1 unit.
After incubation, usually 5 min, the reaction was terminated by the addition of 50 μmol of trichloroacetic acid and the mixture kept in ice for 5 min. The solution was neutralized with 50 μmol of Tris base, and 0.5 μmol of phosphoenolpyruvate was added as carrier. A second incubation, after the addition of 100 μmol of glucose and 4 units of hexokinase, was continued for 20 min. The glucose 6-phosphate was readily separated from phosphoenol-pyruvate by chromatography on Dowex 1 (Cl− form). After neutralization with Tris base the phosphoenolpyruvate was hydrolysed by the addition of 1 μmol of mercuric acetate. The phosphate released was extracted into 2-methylpropan-1-ol as its phosphomolybdate complex. Samples of this extract were counted for radioactivity in a liquid-scintillation counter after being mixed with a suitable scintillant.

Under these conditions the overall recovery of phosphoenolpyruvate was greater than 97%, and the formation of phosphoenolpyruvate was linear with respect to time for 8 min with the lowest substrate concentrations used.

In order to normalize the rates measured in various experiments the rate in the reverse direction \( v_{-1} \) was expressed as a ratio of the activity of that found in the forward direction \( v_{+1} \) when assayed under standard conditions.

Initial-rate studies with ATP as the variable substrate (Fig. 1) shows substrate inhibition. A similar result was found in the forward direction with ADP (Jonson & Cleland, 1974). No such inhibition was seen with pyruvate as the variable substrate up to a concentration of 90 mM, but a secondary plot of the intercepts as a function of the reciprocal of the ATP concentration is linear, suggesting that the inhibition is competitive with respect to pyruvate.

The experimental data were fitted to the rate equation for competitive substrate inhibition in a sequential mechanism, derived by assuming that the binding constant of the inhibitor for the enzyme is the same as that for the enzyme-substrate complex, by using the hyperbolic least-squares regression method of Cleland (1967) to give the values of the kinetic constants.

One significant feature of these results was the finding that the ratio of the maximal

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\frac{v_{max}}{v_{max} - v_{-1}} = \frac{1}{1 + \frac{[Pyruvate]}{K_{p,app}}} + \frac{1}{1 + \frac{[Phosphoenolpyruvate]}{K_{p,app}}}
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Fig. 2. Double-reciprocal plot showing the competitive inhibition between phosphoenolpyruvate and pyruvate

Conditions of assay as in Fig. 1 except ATP_\text{total} was 0.251 mM in all lines. The phosphoenolpyruvate concentrations used were: ○, 0.0 m; □, 0.0174 mM; ▲, 0.0348 mM; ■, 0.0522 mM; ●, 0.0696 mM. The lines are the result of fitting the experimental data to the rate equation for parabolic competitive inhibition by using hyperbolic regression analysis. The values of the apparent constants were: \( K_{p,pyruvate} = 6.35 \pm 1.0 \text{mM} \); \( K_{p,app} = 0.251 \pm 0.97 \text{mM} \); \( K_{p,app} = 0.000530 \pm 0.000010 \text{mM} \); \( V = 0.0144 \pm 0.000071 v_{-1} \) unit/\( v_{+1} \) unit, where PEP is phosphoenolpyruvate.
velocities in the forward and reverse directions was 54.5:1, somewhat lower than that previously reported (McQuate & Utter, 1959).

Product-inhibition experiments with a non-saturating concentration of the constant substrate showed that in every case competitive inhibition occurs. This is consistent with a rapid-equilibrium random Bi Bi mechanism for the reverse reaction, a similar mechanism to that proposed for the forward reaction (Reynard et al., 1961; Ainsworth & MacFarlane, 1973). Analysis of the slope replots showed that there was linear competitive inhibition between ATP and both products. When pyruvate is the variable substrate, however, parabolic competitive inhibition occurred with both products. The experimental data for each of these plots were fitted to the relevant rate equation by the least-squares method of Cleland (1967) and apparent constants were obtained. Fig. 2 shows the experimental data with pyruvate as the variable substrate and phosphoenol-pyruvate as the product inhibitor.

Parabolic slope effects are normally the result of multiple combinations of the inhibitor with the enzyme. It is curious that the parabolic effects were only seen with one substrate and not both, as would be the case if complexes of the form enzyme–product–product occurred in a rapid equilibrium mechanism. One explanation is that the binding of pyruvate is not at complete thermodynamic equilibrium, and that the reaction possesses some non-rapid-equilibrium random Bi Bi character. Isotopic-exchange experiments at equilibrium should confirm whether or not the enzyme really does catalyse a rapid equilibrium mechanism.


Enzyme Inhibition by Sodium Alkyl Sulphates

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The irreversible denaturation of proteins by anionic detergents, particularly sodium dodecyl sulphate, has been well documented (Tanford, 1968, 1970). As the concentration of detergent in a protein solution is increased, the protein undergoes a relatively sudden and irreversible transition from the native to the denatured state. The nature of both protein and detergent affect the concentration at which denaturation takes place, and it has been suggested that the more hydrophobic the non-polar region of the detergent, the lower the concentration required to cause denaturation (Decker & Foster, 1966).

In this study, the effects of a variety of sodium alkyl sulphates, esterified at C-1, on two enzyme activities present in the digestive juices of the snail Helix pomatia (supplied by Industrie Biologique Française, Gennevilliers, France) are reported. Glucuronidase activity was measured by the method of Fishman et al. (1948), which uses phenolphthalein glucuronide as substrate; sulphatase activity was assessed by the same method, by substituting only the dipotassium salt of phenolphthalein disulphate as substrate.

Results are shown graphically in Figs. 1 and 2. The pattern of inactivation of the enzymes by the detergents was irreversible and, where it occurred, resembled certain physical changes (in colligative properties and electrical conductivity) which take place in anionic detergent solutions alone in the neighbourhood of the critical micelle concentration of the detergent (Preston, 1948). However, the detergent concentrations causing enzyme inhibition proved to be considerably below the critical micellar concentrations.