

Interaction of formycin A-5'-triphosphate with pyruvate carboxylase

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Formycin triphosphate (FTP), a fluorescent analogue of ATP, is a competitive inhibitor of chicken liver pyruvate carboxylase with respect to ATP. The chicken liver enzyme is unable to utilise FTP as a substrate at a measurable rate, but FTP is a poor substrate for the sheep liver enzyme. When FTP binds to the enzyme, its fluorescence is enhanced and in this way the formation of enzyme-FTP complexes can be monitored. Using this property of FTP, the effect of Mg^{2+} and acetyl-CoA on the binding of nucleoside triphosphates to the chicken liver enzyme was examined. Mg^{2+} was found to enhance the binding of FTP whilst acetyl-CoA reduced the fluorescence intensity of a mixture of Mg^{2+} , enzyme and FTP. Most probably, this was caused by a conformational change in the enzyme which changed the environment of the fluorophore.

Pyruvate carboxylase Formycin triphosphate

1. INTRODUCTION

Formycin 5'-triphosphate (FTP) is a fluorescent analogue of ATP which on binding to enzyme active sites exhibits enhanced fluorescence [1,2]. This property of FTP has led to its use as a tool in the investigation of nucleoside triphosphate binding mechanisms in a number of enzymes [2,3].

MgATP is a substrate in the reactions catalysed by pyruvate carboxylase (EC 6.4.1.1) i.e.,



MgATP is a substrate of the first partial reaction (i) in which the biotin prosthetic group is carboxylated and in which Mg^{2+} and acetyl-CoA are activators [4,5]. In various kinetic experiments, Mg^{2+} has been found to lower the K_m for MgATP [6] and MgATP has been found to enhance the binding of Mg^{2+} [7]. The fluorescent properties of

FTP provide a means to directly observe nucleoside triphosphate binding to pyruvate carboxylase and to examine the effects of Mg^{2+} and acetyl-CoA on this process.

2. MATERIALS AND METHODS

Formycin A-5'-triphosphate was supplied as the tetralithium salt by Calbiochem-Behring, LaJolla, CA. This was then converted to the sodium salt by

ion exchange chromatography. The purity of the product was checked by thin layer chromatography and FTP concentrations determined from absorbance measurements at 295 nm, where the absorption coefficient is $10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [3]. Solutions of $MgCl_2$ were prepared from spectroscopically pure Mg and constant boiling HCl.

Chicken liver pyruvate carboxylase was purified as in [8] to a specific activity of 30 units $\cdot \text{mg}^{-1}$ pro-

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tein (1 unit of enzymic activity is defined as the amount of enzyme required to catalyse the formation of $1 \mu\text{mol}$ oxaloacetate $\cdot \text{min}^{-1}$ at 30°C in the presence of saturating concentrations of substrates and effectors). Sheep liver pyruvate carboxylase was purified to a specific activity of $13 \text{ units} \cdot \text{mg}^{-1}$ protein [8].

All of the kinetic experiments were performed using the spectrophotometric assay of enzymic activity [9] and the lines shown on the plot of v^{-1} vs $[S]^{-1}$ are derived from computer fits of hyperbolae to the raw data (vs $[S]$), using a non-linear least-squares regression program [10].

All measurements in the fluorescence experiments were made on a Perkin Elmer 3000 Fluorescence Spectrometer [3] using an excitation

wavelength of 310 nm and the enzyme concentration was determined by assay of the biotin content of the enzyme solution [11]. The concentration of enzyme used was $12 \mu\text{M}$, which refers to the biotin concentration and thus to the concentration of enzyme active sites. The concentration of FTP used was $6 \mu\text{M}$ in all fluorometry experiments. Mg^{2+} was added in the form of small volumes of concentrated MgCl_2 solutions such that in the titration where the concentration of Mg^{2+} in the fluorescence cuvette was gradually increased, the final volume of MgCl_2 solutions added was less than 3% of the initial volume of the solution in the cuvette. The end-point of the titration was determined by the addition of excess MgCl_2 to a final concentration of 1.35 mM .

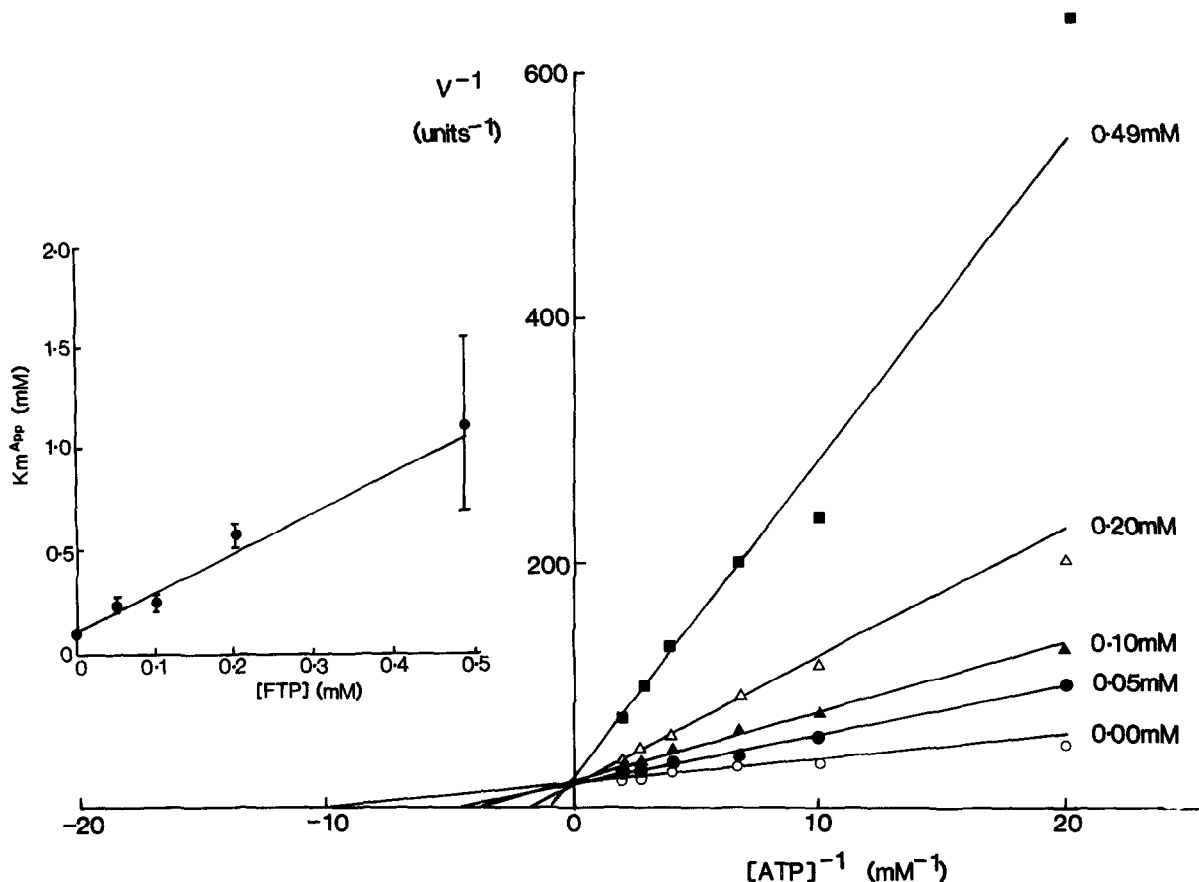


Fig. 1. Inhibition of chicken liver pyruvate carboxylase by MgFTP and MgATP as the varied inhibitor-substrate pair. The velocities are expressed as units of enzymic activity and the concentrations of MgFTP as mM. The reactions were performed at pH 7.8 and 30°C . Inset is a graph of the apparent K_m values vs [FTP] derived from the primary plots. The value of K_i derived from this graph is $50 \mu\text{M}$.

3. RESULTS AND DISCUSSION

The results shown in fig.1 demonstrate that MgFTP is a competitive inhibitor of chicken liver pyruvate carboxylase with respect to MgATP, with an apparent K_I of $50\ \mu\text{M}$ (calculated from replot of K_m app. vs [FTP], see inset Fig. 1). The rate of turnover of MgFTP by the chicken enzyme was negligible. However, the sheep liver enzyme was able to utilise MgFTP at a significant rate as is demonstrated in fig. 2 and is therefore a poor substrate for this enzyme. The V_{\max} for MgATP is approximately 23 times higher than that for MgFTP, however, the K_m for MgFTP ($50\ \mu\text{M}$) is 3.5 times lower than that for MgATP ($175\ \mu\text{M}$). Thus, although the apparent dissociation constants of the enzyme-MgFTP complexes of the sheep and chicken enzymes are the same, it is clear that the

sheep enzyme is much better able to utilise MgFTP as a substrate.

In order to examine FTP binding alone, the subsequent experiments were performed using the chicken liver enzyme. In fig. 3a spectra 1a-3a represent the fluorescence emission spectra of a mixture of enzyme and FTP to which consecutive additions of Mg^{2+} and acetyl-CoA have been made and from which the fluorescence spectrum of the enzyme alone has been subtracted. Spectra 4a-6a represent fluorescence emission spectra of FTP to which consecutive additions of Mg^{2+} and acetyl-CoA have been made and from which the buffer baseline spectrum has been subtracted.

Comparing spectra 4a and 5a it can be seen that the addition of excess Mg^{2+} to a solution of FTP reduces its fluorescence, indicating that the fluorescence yield of MgFTP is lower than that of

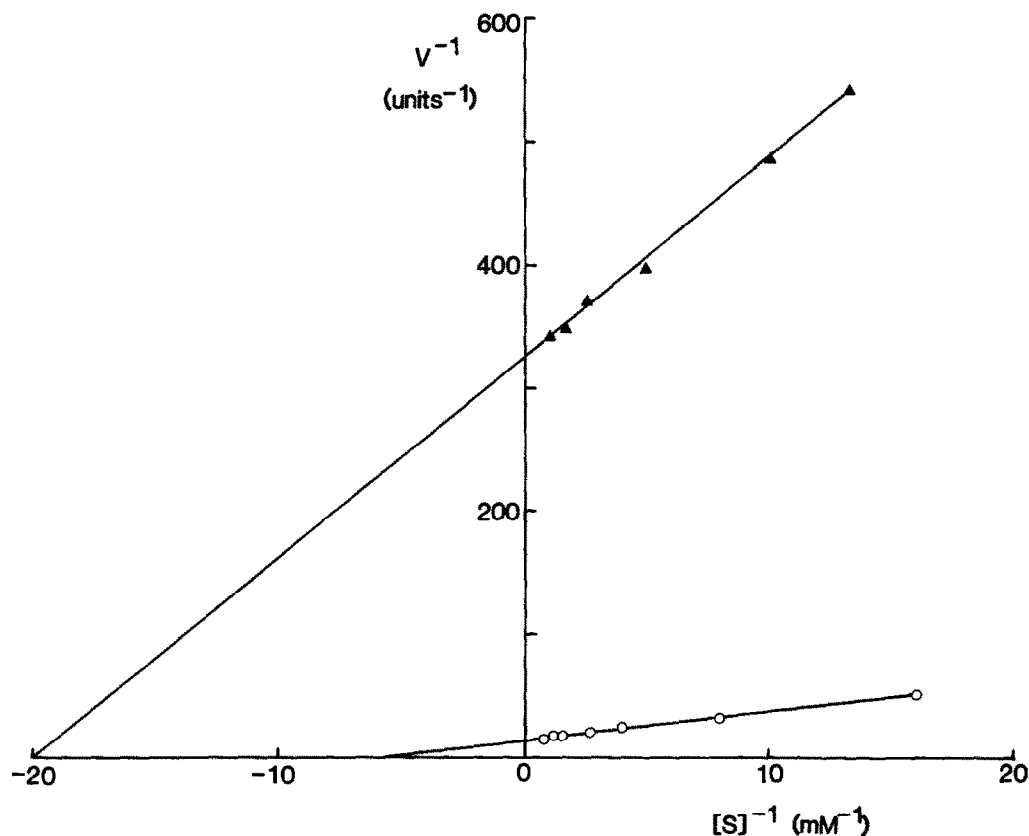
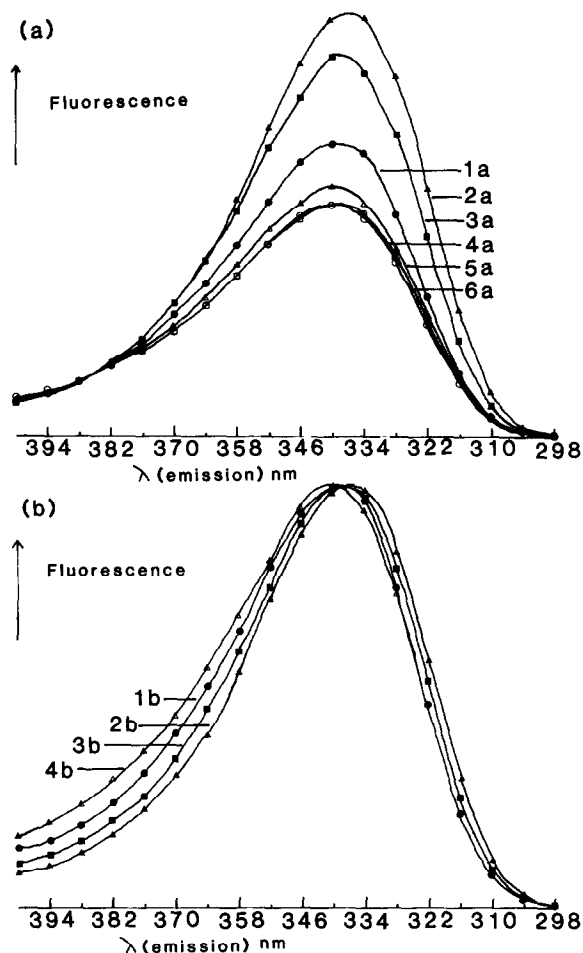


Fig. 2. Velocity response of sheep liver pyruvate carboxylase to varying concentrations of (▲) MgFTP and (○) MgATP at 30°C and pH 8.4. Velocities are expressed as units of enzyme activity.



free FTP. Addition of 100 μ M acetyl-CoA, however, has only a small effect on the fluorescence emission spectrum of MgFTP (see spectra 4a and 6a).

It can be seen from spectra 1a and 5a that when FTP was added to the enzyme in the absence of Mg^{2+} , some of the FTP bound, resulting in a general increase in fluorescence. Addition of Mg^{2+} to a final concentration of 5 mM resulted in a large enhancement of fluorescence (spectrum 2a), the value of the fluorescence at 330 nm being 210% of that of FTP + Mg^{2+} in the absence of enzyme (spectrum 4a). Addition of acetyl-CoA to a final concentration of 100 μ M resulted in an appreciable quenching of fluorescence (spectrum 3a).

In order to examine the effects of the addition of Mg^{2+} and acetyl-CoA on the environment of the

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Fig. 3(a). Fluorescence emission spectra of various combinations of FTP, chicken liver pyruvate carboxylase, Mg^{2+} and acetyl-CoA at 30°C in 0.1 M Tris-Cl buffer pH 7.8, with an excitation wavelength of 310 nm. Spectra 1a-3a are spectra of enzyme and FTP to which consecutive additions of Mg^{2+} and acetyl-CoA have been made and from which the spectrum of the enzyme alone in buffer has been subtracted: 1a (●), enzyme (12 μ M) + FTP (6 μ M); 2a (▲), enzyme (12 μ M) + FTP (6 μ M) + $MgCl_2$ (5 mM); 3a (■) enzyme (12 μ M) + FTP (6 μ M) + $MgCl_2$ (5 mM) + acetyl-CoA (100 μ M). Spectra 4a-6a are spectra of FTP to which consecutive additions of Mg^{2+} and acetyl-CoA have been made and from which the buffer baseline spectrum has been subtracted: 4a (○), FTP (6 μ M); 5a (Δ), FTP (6 μ M) + $MgCl_2$ (10 mM); 6a (□), FTP (6 μ M) + $MgCl_2$ (10 mM) + Acetyl-CoA (100 μ M).

Fig 3(b). Spectra 1b-4b are the corresponding spectra from fig. 3a (1a-4a) normalised to the peak fluorescence of spectrum 2a. In all spectra shown in Figs. 3a and b the symbols represent points calculated from the original spectra and the solid lines are hand-drawn between these points.

enzyme-bound FTP molecule, in fig. 3b spectra 1a-4a from fig. 3a are shown, normalised to the same peak fluorescence as spectrum 2a. By comparing spectra 4b, 1b and 2b it can be seen that in going from FTP in solution, to some FTP bound to the enzyme and finally to maximum binding of FTP to the enzyme in the presence of excess Mg^{2+} , the fluorescence spectrum of FTP undergoes a blue shift. This indicates that the environment of the bound FTP is less polar than that of FTP in solution, the enhanced fluorescence of bound FTP largely being due to a reduction in quenching caused by interaction with the water solvent. Addition of acetyl-CoA appears to cause the FTP to be exposed to a more polar environment since the emission spectrum is red-shifted (spectrum 3b).

A titration was performed as described in section 2 in which the concentration of Mg^{2+} in a solution of enzyme and FTP was gradually raised and the enhancement of fluorescence monitored at 330 nm. The half-maximal increase in fluorescence occurred at concentration of 'free' Mg^{2+} ($[Mg^{2+}]_{total} - [MgFTP]$) of about 110 μ M. This is over 7 times higher than the dissociation constant

of MgATP [7] and therefore, presumably, of MgFTP. Thus the enhancement of fluorescence is caused by the interaction between the Mg^{2+} and the enzyme. This fluorescence enhancement may result either from a change in the environment of the MgFTP in the enzyme active site brought about by a conformation change induced by Mg^{2+} binding or from increased binding of FTP. From kinetic experiments, authors in [7] found that Mg^{2+} increased the apparent binding constant for MgATP and obtained a value for the apparent dissociation constant of the Mg^{2+} -enzyme complex of $150 \pm 40 \mu M$ which is in reasonable agreement with the value observed in the present titration experiment. Thus it appears that the enhanced fluorescence observed at high $[Mg^{2+}]$ is caused by increased binding of MgFTP to the enzyme and the stimulatory effect of Mg^{2+} on enzyme activity can be accounted for mainly by its enhancement of nucleoside triphosphate binding to the enzyme. The binding of Mg^{2+} to the enzyme might change the enzyme's affinity for MgFTP and MgATP by inducing a conformational change as has been suggested in [6].

The effect of the addition of a saturating concentration of acetyl-CoA to the solution of enzyme, MgFTP and 5 mM Mg^{2+} was to reduce the fluorescence at 330 nm from 210% (spectrum 2a) to about 177% (spectrum 3a) of that of Mg^{2+} and MgFTP alone (spectrum 5a). Since acetyl-CoA does not have a significant effect on the fluorescence of MgFTP and Mg^{2+} alone (see spectrum 6a) it would appear that the binding of acetyl-CoA to the enzyme either increases the dissociation constant of the enzyme-MgFTP complex, or by inducing a conformational change affects the environment of the fluorophore. Authors in [12] found that acetyl-CoA had no effect on the K_m for MgATP, and previous studies have revealed that acetyl-CoA induced only small changes in the conformation of pyruvate carboxylase [13]. However, in recent electron microscopic studies [14-16], acetyl-CoA has been shown to produce significant effects on the quaternary structure of this enzymic tetramer. It is more likely, therefore, that the effect of acetyl-CoA observed in the present experiments is produced by a conformational change in the enzyme which exposes the bound MgFTP to a more polar environment (see spectrum 3b), thus increasing fluorescence quenching. However, as

MgFTP does not appear to be a substrate of the chicken enzyme, changes in MgFTP binding induced by acetyl CoA cannot be ruled out entirely.

Here, we have been able to directly examine the effects of the activators Mg^{2+} and acetyl-CoA on the interaction of the enzyme with MgFTP. In this way we have been able to localise the stimulatory effect of Mg^{2+} on enzymic activity to its enhancement of nucleoside triphosphate binding. As the sheep liver enzyme can utilise FTP as a substrate at a significant rate, this may provide opportunities to examine nucleotide binding and release during the catalytic cycle in greater detail.

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