Kinetics of Nucleotide Binding to Pyruvate Carboxylase†

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ABSTRACT: The kinetics of nucleotide binding to pyruvate carboxylase have been studied by measuring the fluorescence changes that occur on the binding and release of FTP and FDP, which are fluorescent formycin analogues of ATP and ADP. The rate constants and equilibrium binding constants for both MgFTP and MgFDP binding to pyruvate carboxylase have been determined. From the kinetics of displacement of MgFTP by MgATP and binding of MgFTP in the presence of MgATP, the rate constants of MgATP binding were estimated. A slow component to the fluorescence changes was seen to occur after the initial rapid, bimolecular binding step, when formycin nucleotides were mixed with the enzyme. HCO_3^- and pyruvate were shown to quench the fluorescence of enzyme-bound MgFTP, but did not affect the affinity of the enzyme for the nucleotide. Acetyl CoA reduced the affinity of the enzyme for both MgFDP and MgFTP by about 3-fold by decreasing the association rate constants (by 25%) and increasing the dissociation rate constants (by 2-fold). In the absence of Mg^{2+} a very rapid component to FTP binding was observed that was complete within about 3 ms, but no fast component was observed comparable to that seen in the presence of 4.5 mM $MgCl_2$. Increasing the [Mg^{2+}] gradually abolished this very fast component of the binding, while the amplitude of the fast component increased, although the rate constant for this component did not appear to be strongly dependent on [Mg^{2+}]. The rate constants of the slow component of Mg·formycin nucleotide binding did not appear to be dependent on nucleotide concentration. A corresponding slow component was also evident in the kinetics of MgATP displacement of the formycin nucleotides from the enzyme, indicating that it is not an artifact of formycin nucleotide binding.

Pyruvate carboxylase (EC 6.4.1.1) is a biotin-dependent enzyme that catalyzes the following reactions:

$$\text{MgATP} + HCO_3^- + \text{ENZ–biotin} \xrightleftharpoons{Mg^{2+},\ \text{acetyl CoA}} \text{MgADP} + \text{Pi} + \text{ENZ–biotin–}CO_2^- \quad (1)$$

$$\text{ENZ–biotin–}CO_2^- + \text{pyruvate} \rightleftharpoons \text{ENZ–biotin} + \text{oxaloacetate} \quad (2)$$

The steady-state kinetic mechanism of this and other biotin-dependent enzymes has been the subject of numerous studies [see Attwood and Keech (1984)], and the chemical mechanism has been and continues to be studied [see Knowles (1989)]. However, little has been done to study the kinetics of individual steps in the reactions catalyzed by pyruvate carboxylase and how these steps are affected by the cofactors acetyl CoA and Mg^{2+} (McGurk & Spivey, 1979; Attwood et al., 1984a).

Some time ago, it was noted that when FTP, a fluorescent analogue of ATP, bound to pyruvate carboxylase, its fluorescence was enhanced and that it was a competitive inhibitor of the chicken liver enzyme with respect to ATP (Attwood et al., 1984b). Moreover, the addition of the cofactors Mg^{2+} and acetyl CoA to solutions of enzyme and FTP was shown to induce changes in the FTP fluorescence (Attwood et al., 1984b).

In the current study we measured the kinetics of nucleotide binding to pyruvate carboxylase by monitoring the fluorescence changes that occurred on the binding of formycin nucleotides to the enzyme, using stopped-flow. In addition, we determined the effects of the cofactors Mg^{2+} and acetyl CoA on these kinetics. Finally, we also examined the effects of the other substrates, i.e., HCO_3^- and pyruvate, on nucleotide binding.

MATERIALS AND METHODS

Preparation of Chicken Liver Pyruvate Carboxylase. Chicken liver pyruvate carboxylase was prepared to a specific activity of 25 units/mg as described by Goss et al. (1979), except that a DEAE-Sepharose CL-6B column was used instead of a DEAE-Sephadex column. One unit of enzyme activity is defined as the amount of enzyme required to catalyze the formation of 1 μmol of oxaloacetate per minute at 30 °C in the presence of saturating substrates. The biotin content of the enzyme preparation was determined as described by Rylatt et al. (1977), and where concentrations of pyruvate carboxylase are quoted, this refers to the concentration of biotin and thus, since there is one biotin molecule per active site, to the concentration of enzyme active sites.

Preparation of Formycin Nucleotides. Formycin A 5′-monophosphate (FMP) was purchased from Sigma Chemical Co., and both FTP and FDP were prepared from this in

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[1] Abbreviations: FTP and FDP, formycin A 5′-tri- and di-phosphate; EDTA, ethylenediaminetetraacetic acid; ANS, 8-anilinonaphthalene-1-sulfonic acid.

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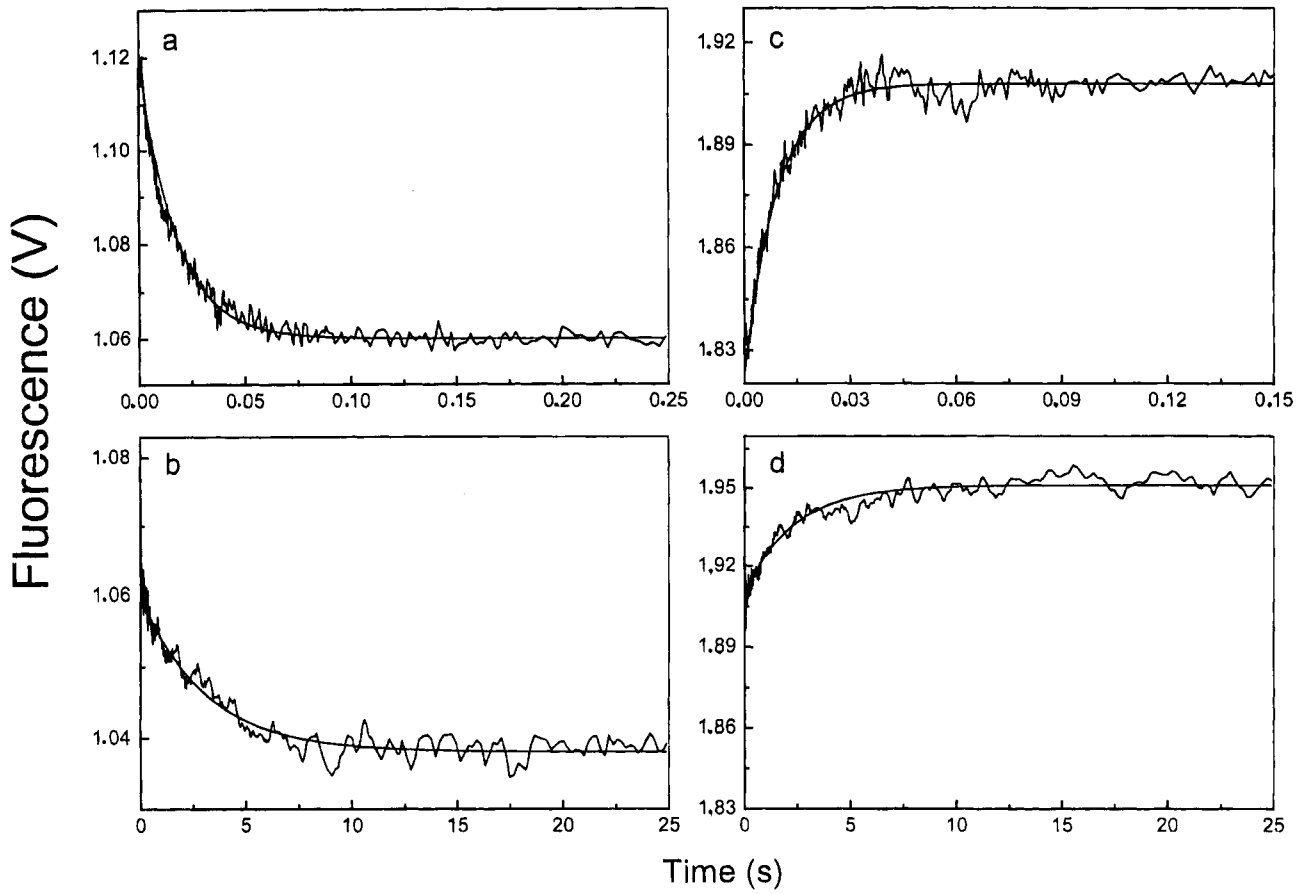

FIGURE 1: Stopped-flow records of the changes in MgFTP fluorescence on (a and b) the binding of MgFTP to pyruvate carboxylase and (c and d) the displacement of MgFTP from pyruvate carboxylase by 2.5 mM MgATP. The reaction solutions contained 0.5 μM pyruvate carboxylase, 20 mM $NaHCO_3$, and 10 μM MgFTP. Panels a and c show the rapid components of the reactions, while panels b and d show the slow components of the same reactions. The curves shown overlaid on the data are nonlinear least squares regression fits to single-exponential reactions. The values of rate constants and amplitudes of reaction derived from these fits are as follows: (a) $k_{obs} = 112 \pm 7$ s^{-1}, % amplitude − 4.9; (b) $k_{obs} = 0.44 \pm 0.02$ s^{-1}, % amplitude = 2.2; (c) $k_{obs} = 65 \pm 3$ s^{-1}, % amplitude = 5.7; (d) $k_{obs} = 0.33 \pm 0.02$ s^{-1}, % amplitude = 2.1.

reactions using creatine kinase and adenylate kinase as described by Karlish et al. (1978). The individual formycin nucleotides were separated by chromatography on a Dowex-1-Cl column; unreacted FMP was eluted with 5 mM HCl, and the FDP and FTP were eluted by using a gradient of 0−0.2 M NaCl in 10 mM HCl. The elution positions of the formycin nucleotides correlated well with the elution positions of the corresponding adenine nucleotide standards when chromatographed in an identical manner on the same column. Concentrations of solutions of FTP and FDP were determined by absorbance measurements at 295 nm where the absorption coefficient is 10^4 M^{-1} cm^{-1} (Karlish et al., 1978).

Stopped-Flow Measurements. These fluorescence measurements were performed on a Hi-Tech SF61 stopped-flow instrument with an excitation wavelength of 310 nm, and fluorescence emission was measured at wavelengths above 360 nm by placing a 360-nm cut-on filter in front of the photomultiplier. These wavelengths were chosen to minimize the contribution to the measurements of intrinsic protein fluorescence. Data was collected as 768 12-bit data points, using the log time scale facility over 25 s so as to capture both rapid (millisecond) and slow (second) transients in one experiment. Each trace was stored digitally, and to obtain estimates of rate constants and amplitudes, at least four such traces were averaged and the averaged trace analyzed by nonlinear least squares regression. The fast (millisecond) and slow (second) components of the averaged traces were analyzed separately by fitting single- or double-exponential rate equations to each component of the data. Where standard errors are given of estimates of rate constants, these are the standard errors calculated from the nonlinear least squares regression analysis of the data. The percent amplitudes of the reaction components were calculated by expressing the amplitude of each component as a percentage of the final fluorescence at $t = \infty$ (both derived from the least squares regression analyses, in V). All experiments were performed at 20 °C in 0.1 M Tris-HCl at pH 7.8. In the experiments where the effect of increasing concentrations of Mg^{2+} on the kinetics of FTP binding to the enzyme were studied, a very rapid component of the kinetics occurred in the dead time of the stopped-flow machine. The amplitude of this component was calculated by subtracting from the final fluorescence recorded at $t = \infty$ the sum of the fluorescences of the enzyme alone, the FTP alone, and the fluorescence changes observed kinetically. This amplitude was then expressed as a percentage of the fluorescence at $t = \infty$.

Fluorescence Measurements. Fluorescence measurements were performed using an SLM fluorometer. The excitation wavelength was 310 nm, and emission spectra were recorded between 325 and 425 nm; all measurements were performed at 20 °C. Emission spectra of 0.5 μM pyruvate carboxylase

in 0.1 M Tris-HCl, pH 7.8, containing 4.5 mM $MgCl_2$ and 0.25 mM acetyl CoA were recorded in the presence or absence of 10 mM pyruvate or in the presence or absence of 20 mM added $NaHCO_3$. Similar spectra were obtained for identical solutions containing 10 μM FTP instead of pyruvate carboxylase, and finally spectra were obtained for identical solutions containing both 0.5 μM enzyme and 10 μM FTP. These spectra were stored in digital form. From the spectra of solutions containing both enzyme and FTP, the corresponding spectra of enzyme alone and FTP alone were subtracted to give spectra that represent the contribution of enzyme-bound FTP to the fluorescence spectra of solutions containing FTP and enzyme.

RESULTS AND DISCUSSION

Binding of MgFTP and MgFDP to Pyruvate Carboxylase. Figure 1a,b shows the averaged traces from six experiments, displayed on two different time scales, in which the kinetics of the increase in fluorescence associated with MgFTP binding to pyruvate carboxylase were measured by stopped-flow. Under these first-order conditions, the binding appeared to consist of two independent exponential components. Figure 1a,b shows that the fast component is essentially complete within 50 ms and the slow component is complete within about 10 s. The amplitude of the fast component is a little over twice that of the slow component. Panels c and d of Figure 1 show the fast and slow components, respectively, of the decrease in fluorescence that accompanies the displacement of MgFTP from the enzyme by an excess of MgATP. The time scales on which these two independent components occur are similar to the corresponding components of the binding process. The observed rate constants, but not the amplitudes, were independent of the MgATP concentration used; see Figure 5. Similar results were obtained in stopped-flow experiments to measure the kinetics of the binding and displacement of MgFDP, except that whereas the slow component of the binding gave good fits to a single-exponential process at all concentrations of MgFTP, with MgFDP this component was better described as two exponential processes (data not shown). The rate constants for the slow components of both MgFTP and MgFDP binding showed no strong dependence on formycin nucleotide concentration. The fraction of the total amplitude contributed by the slow component was also little affected by formycin nucleotide concentration. The mean values and standard deviations of the rate constant for the slow component of MgFTP binding over the range of [MgFTP] were 2.5−30 μM, and the ratios of the amplitude of the slow component to that of the fast component were 0.31 ± 0.04 s^{-1} and 0.37 ± 0.05, respectively. The values for MgFDP binding were 2.8 ± 0.9 and 0.43 ± 0.06 s^{-1} for the rate constants and 0.40 ± 0.17 and 0.42 ± 0.14 for the ratios of the amplitudes of the phases of the slow component to the corresponding amplitude of the fast component.

Figure 2 shows how the apparent first-order rate constants of the fast components of both MgFTP and MgFDP binding, obtained from data similar to those shown in Figure 1, vary with formycin nucleotide concentration. The upper limit on formycin nucleotide concentration was 30 μM since inner filter effects become significant beyond this, as does the increasing difficulty of measuring a relatively small change in fluorescence against a large background. As can be seen, both sets of data give reasonably linear plots. The second-order association rate constants (k_1) and the dissociation rate constants (k_{-1}) have been calculated from the slopes and intercepts, respectively. The dissociation rate constants are in reasonable agreement with those obtained from the experiments in which the formycin nucleotides were displaced from the enzyme with MgATP (Figure 1c and Table 1). From the values of the association and dissociation rate constants (using the values of the dissociation rate constants calculated from the graphs of k_{obs} versus [Mg·formycin nucleotide]), equilibrium dissociation constants (K_D) have been calculated for both MgFTP and MgFDP binding to the enzyme of 32 and 46 μM, respectively (Table 1). The absolute amplitudes changed with formycin nucleotide concentration in a way that was consistent with a $K_D \approx 50$ μM; i.e., the fraction of enzyme binding formycin nucleotide increased, but at the same time the background fluorescence due to free formycin nucleotide also increased.

The calculated association rate constants (k_1) for formycin nucleotide binding are a little lower than expected for a purely diffusion-controlled reaction but indicate that the rate of formycin nucleotide binding to the enzyme is substantially governed by the collision of the nucleotide with the enzyme.

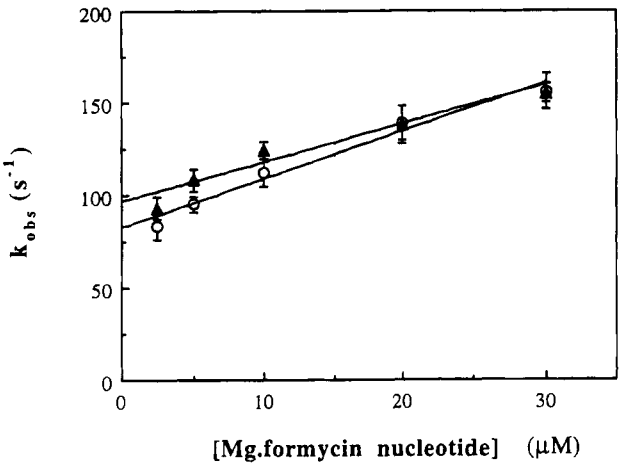

FIGURE 2: Variation of the observed first-order rate constant of the fast component of formycin nucleotide binding with [Mg·formycin nucleotide]: (○) MgFTP, (▲) MgFDP. The reactions were performed under conditions similar to those described in Figure 1, with the [enzyme] being 0.5 μM. The error bars represent the standard errors of the estimates of the rate constants derived from the nonlinear least squares regression analysis of the stopped-flow traces similar to that shown in Figure 1a. The lines represent linear least squares fits to the data, and the values of the association and dissociation rate constants derived from these fits are shown in Table 1.

Table 1: Kinetic Parameters of MgFTP and MgFTP Binding Derived from Stopped-Flow Experiments Similar to Those Described in Figure 1[a]

reactant in excess concn	k_1 (M^{-1} s^{-1})	k_{-1} (s^{-1})	k_{-1} (displacement by MgATP)	K_D (μM)
MgFTP	2.6 (±0.2) × 10^6	82 (±2)	65 (±3)	32
MgFDP	2.1 (±0.3) × 10^6	96 (±3)	87 (±3)	46
enzyme (MgFTP) (+acetyl CoA)	4.5 (±0.8) × 10^6	85 (±4)	79 (±10)	19
enzyme (MgFTP) (−acetyl CoA)	6.2 (±0.4) × 10^6	35 (±2)	46 (±3)	6

[a] The values of K_D for the enzyme·Mgformycin nucleotide complex were calculated as the k_{-1}/k_1 ratio using the value of k_{-1} obtained from the binding experiments and not that from the MgATP displacement experiments.

The equilibrium dissociation constant (K_D) for the enzyme·MgFTP complex of 32 μM is of the same order as that calculated from the competitive inhibition data of 50 μM (Attwood et al., 1984b). Although this value is lower than that reported for the enzyme·MgATP complex of 75 μM (Scrutton & Utter, 1965) or 96 μM (Frey & Utter, 1977), it is of the same order of magnitude and suggests that the binding of MgFTP to pyruvate carboxylase is similar to that of MgATP. In addition, the ratio of the K_Ds for MgFTP and MgFDP binding to the enzyme (1.43) is similar to the ratio of the K_ms for MgATP and MgADP (1.46) (Barden et al., 1972), suggesting that the relationship between the binding of the di- and triphosphates is the same.

In these experiments, the kinetics of binding of both MgFTP and MgFDP to the pyruvate carboxylase have been measured under conditions similar to those used for optimal assay of enzyme activity. In the case of both nucleotides, the binding has been shown to consist of two independent components. It was demonstrated that the kinetics of displacement of formycin nucleotides from the enzyme by MgATP similarly comprised two independent components. The slow component of the fluorescence increase observed on binding of formycin nucleotides generally seems to be too slow by comparison with the turnover number of the enzyme to occur in the normal catalytic cycle. A number of model reaction schemes were considered that might explain the existence of the slow component of the kinetics of formycin nucleotide binding including a slow, nucleotide-induced protein conformation change; the presence of two enzyme forms (or contaminating nucleotide binding protein) that bind nucleotides differently; or the presence of two forms of enzyme that are in slow equilibrium with each other, only one of which binds the nucleotide. None of these models satisfactorily explain the kinetic data obtained in this study, and the range of experiments that can be performed to further investigate the slow component is restricted by the range of formycin nucleotide concentrations that can be used owing to the inner filter effect, such that only partial saturation of the enzyme with MgFTP can be achieved. Such an investigation will have to be addressed when a fluorescently labeled form of the enzyme which reports nucleotide binding is available. In the subsequent parts of this paper, generally only the rapid components of the nucleotide binding kinetics will be considered.

Effects of Acetyl CoA on MgFTP and MgFDP Binding to Pyruvate Carboxylase. In order to examine the effects of acetyl CoA on the kinetics of binding of formycin nucleotides, it was necessary to perform the measurements at high enzyme concentrations to avoid the complications arising from the inactivation and dissociation of the tetrameric enzyme that occur at low enzyme concentrations in the absence of acetyl CoA (below about 4 units/mL) (Attwood et al., 1993). Figure 3 shows the variation of apparent first-order rate constants for the fast component of MgFTP binding with enzyme concentration where the enzyme was in 5−30-fold excess of the MgFTP in the presence or absence of saturating acetyl CoA. Again the two sets of data are reasonably linear. The rate constants and dissociation constants derived from these plots are shown in Table 1. The dissociation rate constants are in reasonable agreement with those obtained by displacement of the MgFTP from the enzyme by MgATP. The dissociation rate constant obtained with the enzyme in excess in the presence of acetyl

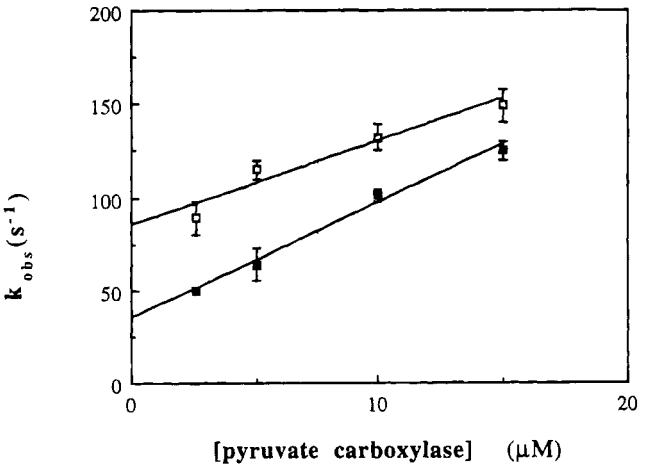

FIGURE 3: The variation of the observed first-order rate constant of the fast component of MgFTP binding with [enzyme], in the presence (□) or absence (■) of 250 μM acetyl CoA. The error bars represent the standard errors of the estimates of the rate constants derived from the nonlinear least squares regression analysis of the stopped-flow traces similar to that shown in Figure 1a. The lines represent linear least squares fits to the data, and the values of the association and dissociation rate constants derived from these fits are shown in Table 1.

CoA is similar to that obtained when MgFTP was in excess of the enzyme; however, the association rate constant obtained when the enzyme was in excess was significantly larger than that obtained when the MgFTP was in excess. It is, however, perhaps more meaningful to compare the data shown in Figure 3 and Table 1 obtained in the absence of acetyl CoA with those obtained in its presence with variable [enzyme]. It is clear that acetyl CoA has effects on the kinetics of MgFTP binding to pyruvate carboxylase: acetyl CoA reduces the size of k_1 by about a quarter and at the same time approximately doubles the size of k_{-1} with the result that the equilibrium dissociation constant in the absence of acetyl CoA is about 3 times lower than in its presence (Table 1). Thus it appears that, under these experimental conditions, acetyl CoA lowers the affinity of the active site of the enzyme for MgFTP. A similar effect of acetyl CoA was also observed on the displacement of MgFDP from the E·MgFDP complex by MgATP ($k_{-1} = 82 \pm 6$ s^{-1}, +acetyl CoA; $k_{-1} = 42 \pm 4$ s^{-1}, −acetyl CoA).

Thus, in summary, while the values of k_{-1} obtained both from these plots and from the MgATP displacement reactions (Table 1) were in reasonable agreement, the slopes of the plots (and the values of k_1 derived from them) were significantly different (but by less than a factor of 2). If a proportion of the enzyme present were inactive and unable to bind MgFTP, the biotin content of the enzyme solution would have given too high an estimate of effective enzyme concentration, but this would have led to an underestimate of the value of k_1, and there is no reason to believe that a significant proportion of the enzyme was inactive in this way. It is more difficult to account for an underestimate of enzyme concentration or an overestimate of k_1. There is no previously published evidence to suggest that there is more than one MgATP binding site per enzyme subunit and no reason, on the basis of our knowledge of the role of MgATP in the reaction mechanism, to propose the binding of two MgATP molecules per subunit. An error in [FTP] could also have produced the difference in measured values of k_1, but this is unlikely given that the [FTP] was always determined by

measurement of absorbance at 295 nm. A possible reason for the difference in the measured values of k_1 from the two sets of data is that the desalting procedure (Helmerhorst & Stokes, 1980) used to remove the sucrose present in the buffer used for storage of pyruvate carboxylase from the enzyme has not been totally effective and that the contaminating sucrose was affecting the kinetics of the reaction. From Helmerhorst & Stokes (1980) the amount of sucrose passing through the Sephadex G-25 would be expected to be less than 5%. However, in separate experiments, concentrations of up to 0.32 M, sucrose (20% of the concentration in the storage buffer) were shown to have no effect of the kinetics of MgFTP binding to pyruvate carboxylase. Another possibility is that there is some degree of negative cooperativity between the MgFTP binding sites on the enzymic tetramer, such that at higher degrees of occupancy of the sites k_1 decreases to an extent, while k_{-1} is relatively invariant. In the experiments where [enzyme] $\gg$ [MgFTP], the occupancies of the MgFTP binding sites varied between 2.8% and 1.1%, while in the set of experiments where [MgFTP] $\gg$ [enzyme], the occupancies varied between 14% and 48%. If this were the cause of the difference between the two sets of data, this would imply that the putative cooperativity manifested itself at low levels of saturation of the enzyme with MgFTP. In addition, there is no evidence from previous kinetic studies that such cooperativity exists with MgATP binding to the enzyme.

More important, however, is the comparison between the data obtained with excess enzyme in the presence and absence of acetyl CoA shown in Figure 3 and Table 1, where any effect of contaminating sucrose is likely to be the same under both sets of conditions. This clearly shows that acetyl CoA reduces the affinity of the enzyme for MgFTP and that this effect is mainly produced by the acetyl CoA-induced increase in k_{-1} as is evident from both the binding experiments and the MgATP displacement experiments (see Table 1). Hence the quenching of FTP fluorescence that Attwood et al. (1984b) observed on addition of acetyl CoA to a solution of enzyme and MgFTP can, at least in part, be explained by an induced dissociation of MgFTP from the enzyme. Ashman et al. (1972) found that acetyl CoA had no effect on the K_m for MgATP in the sheep kidney enzyme; there is however, no such data for the chicken liver enzyme. Acetyl CoA has been shown to induce conformational changes in the enzyme (Attwood et al., 1986, 1993), and it would appear that one result of these conformation changes is to markedly increase the rate of dissociation of MgFTP from the active site. This also appears to be the case for MgFDP.

Effects of Pyruvate and Added HCO_3^- on the Binding of MgFTP to Pyruvate Carboxylase. Experiments similar to those used to obtain the data shown in Figure 2, performed in the presence of 10 mM pyruvate or 20 mM added HCO_3^-, showed that these substrates of pyruvate carboxylase had no effect on the kinetics of binding of MgFTP to the enzyme (data not shown); thus neither substrate affected the K_D of the enzyme for MgFTP. Figure 4a shows that pyruvate induces a marked reduction in fluorescence of enzyme-bound MgFTP. Since pyruvate does not change the K_D of the enzyme·MgFTP complex, the effect is not simply due to dissociation of MgFTP from the enzyme resulting in a reduction in the concentration of enzyme·MgFTP. The effect

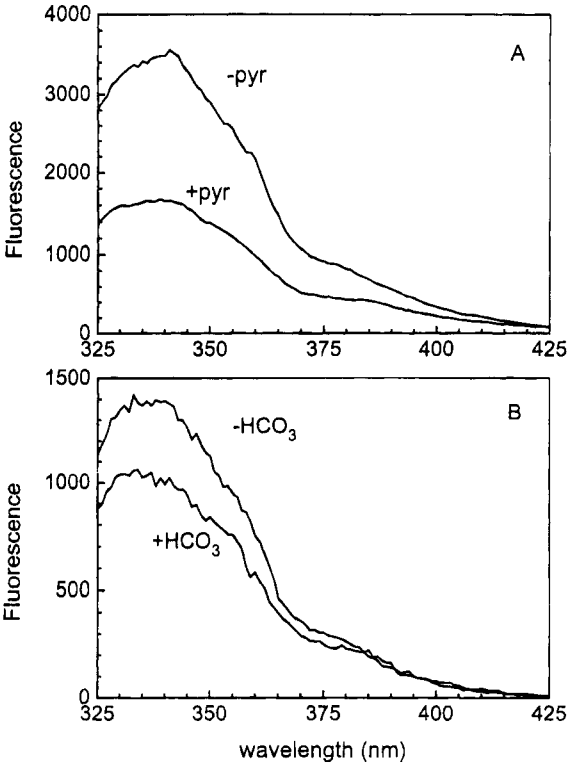

FIGURE 4: Fluorescence emission spectra of enzyme-bound MgFTP in the presence or absence of (a) 10 mM pyruvate or (b) 20 mM $NaHCO_3$. The solutions contained 0.5 μM enzyme and 10 μM MgFTP. The excitation wavelength was 310 nm, and the spectra were recorded at 20 °C. The spectra presented were derived as described in Materials and Methods.

must therefore result from a pyruvate-induced quenching of fluorescence of enzyme-bound MgFTP. Figure 4b shows that HCO_3^- has a qualitatively similar, but quantitatively smaller, effect on enzyme·MgFTP fluorescence. For the same reasons as with pyruvate, this effect must be due to a HCO_3^--induced quenching of enzyme-bound MgFTP. This quenching may be due to direct interaction between MgFTP and HCO_3^- or pyruvate in the enzyme active site, although this seems likely for pyruvate, which is considered to bind at a site that is spatially separate from the nucleotide binding site [see Attwood and Keech (1984)]. Alternatively, the quenching may be due to substrate-induced conformational changes in the enzyme active site that change the environment of the enzyme-bound MgFTP. There is evidence that pyruvate is capable of inducing such changes (Attwood et al., 1986), whereas no such evidence exists for HCO_3^-.

The effect of pyruvate and HCO_3^- is different from that of acetyl CoA, which affects the rate of equilibrium MgFTP binding. The data for pyruvate are consistent with the steady-state data, which suggest that pyruvate has little effect on the K_m for MgATP (McClure et al., 1971). The lack of effect of HCO_3^- on the rate of equilibrium nucleotide binding appears to contradict the conclusion of McClure et al. (1971) that HCO_3^- and MgATP mutually enhance each other's binding to the enzyme and to be more in agreement with the conclusions of Warren and Tipton (1974) and Attwood and Graneri (1992) that MgATP binds first, followed by HCO_3^-.

Effects of MgATP Concentration on the Displacement of MgFTP from Pyruvate Carboxylase and MgFTP Binding in the Presence of MgATP. Figure 5a shows the effects of

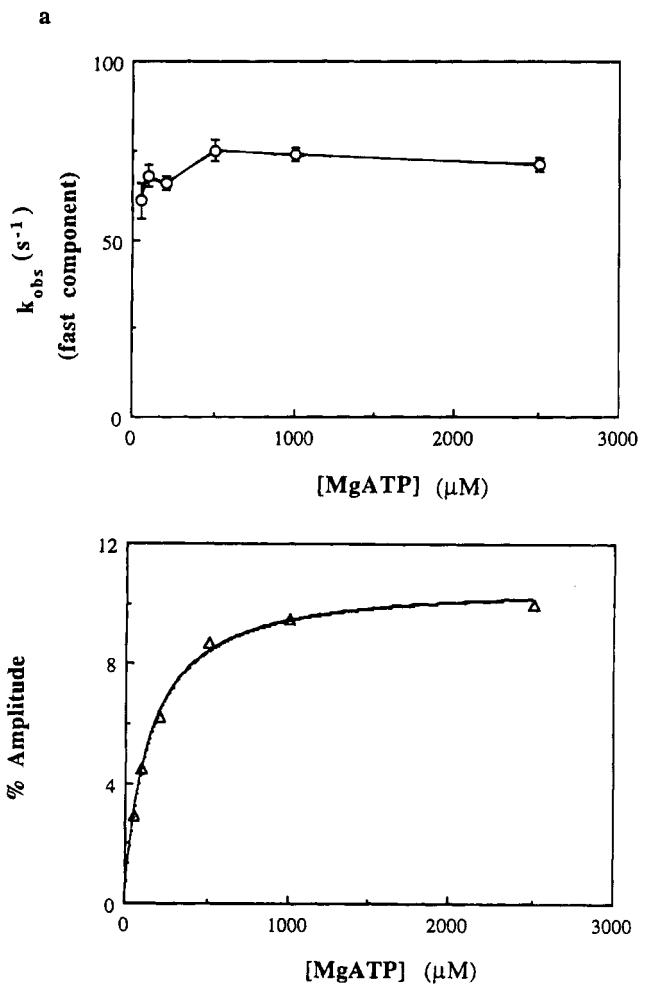

FIGURE 5: Effect of increasing [MgATP] on the rate constant and amplitude of the reaction in which displacement of MgFTP from the enzyme occurs. Panel a shows the variation in the rate constant with [MgATP] for the fast component of the reaction, and the error bars represent the standard errors on the estimates of the rate constants derived from the nonlinear least squares regression analysis of stopped-flow traces similar to those shown in Figure 1c. Panel b shows the variation in the percent amplitude of the fast component of the reaction with [MgATP]. The percent amplitudes were calculated as described in Materials and Methods. The solid line drawn through the data represents a nonlinear least squares fit to the equation % amplitude = $100F/(1 + K_A(1 + K_D/[MgFTP])/[MgATP])$ where F is the ratio of the change in fluorescence of the enzyme + MgFTP on addition of MgATP to the final fluorescence at the end of the reaction, K_A is the dissociation constant of the enzyme·MgATP complex, and K_D is the dissociation constant of the enzyme·MgFTP complex. The reaction solutions contained 20 mM $NaHCO_3$, 1.0 μM enzyme, and 10 μM MgFTP.

increasing [MgATP] on the rate constants and amplitude of the fast component of the displacement of MgFTP from pyruvate carboxylase. The observed first-order rate constant shows little dependence on [MgATP] in the range used. These results indicate that, in the fast component of the observed reaction, binding of MgATP is not rate-limiting over the range of [MgATP] used. Examination of the amplitude of the reaction shown in Figure 5b shows that the dependence on the amplitude of the fast component of MgFTP displacement on [MgATP] is a typical saturation curve. From the regression analysis of the amplitudes of the fast component as described in the legend to Figure 5, a value of the K_D for MgATP binding to the enzyme of 103 μM was obtained.

In principle, the rate of MgATP binding can be measured by observing the competitive effect of MgATP on the rate of MgFTP binding. If the enzyme is mixed with MgFTP and a varying concentration of MgATP, the observed rate of MgFTP binding will depend on [MgATP] and the rate constants controlling MgATP binding compared to those of MgFTP binding:

$$E + MgFTP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}MgFTP$$

$$E + MgATP \underset{k_{-1a}}{\overset{k_{1a}}{\rightleftharpoons}} E{\cdot}MgATP$$

If both [MgATP] and [MgFTP] ≫ [E], then three situations can be envisaged:

(1) If $(k_{1a}[MgATP] + k_{-1a}) \gg (k_1[MgFTP] + k_{-1})$, then MgATP will rapidly equilibrate with the enzyme and then be displaced by MgFTP to a final equilibrium position with an observed rate constant $k_{obs} = k_1[MgFTP]/(1 + [MgATP]/K_{MgATP}) + k_{-1}$, where $K_{MgATP} = k_{-1a}/k_{1a}$.

(2) If $(k_{1a}[MgATP] + k_{-1a}) \ll (k_1[MgFTP] + k_{-1})$, then MgFTP will rapidly equilibrate with the enzyme and then be displaced by MgATP to a final equilibrium position with a $k_{obs} = k_{1a}[MgATP]/(1 + [MgFTP]/K_{MgFTP}) + k_{-1a}$, where $K_{MgFTP} = k_{-1}/k_1$.

(3) If $k_{-1} = k_{-1a}$, then $k_{obs} = k_{1a}[MgATP] + k_1[MgFTP] + k_{-1}$, i.e., a linear function of both [MgATP] and[MgFTP]. All other cases are complex and do not give a simple dependence of k_{obs} on nucleotide concentration.

In experiments in which 1 μM enzyme was mixed with 10 μM MgFTP and MgATP varied from 0 to 500 μM (in the presence of 250 μM acetyl CoA, 20 mM HCO_3^-, and 4.5 mM free Mg^{2+}), the observed rate constant of the fast component of FTP binding showed no marked dependence on [MgATP] (k_{obs} decreased from 118 ± 5 to 101 ± 12 s^{-1} as [MgATP] was increased from 0 to 500 μM), while the amplitude of the reaction decreased markedly from 8.9 to 1.9%. Premixing the MgATP with the enzyme before mixing with MgFTP gave rate and amplitude data identical to that above. The fact that in the experiments where the enzyme was co-mixed with MgATP and MgFTP we observed MgFTP binding and not displacement eliminates the possibility that MgATP binding is much slower than MgFTP binding. As [MgATP] was varied over a large range with little change in the observed rate of MgFTP binding, this eliminates a simple dependence on [MgATP] unless the association rate constant was very small, and we have already discounted this possibility. The data are compatible with situation (1) in which MgATP binds rapidly to the enzyme and is displaced by MgFTP binding. Under the conditions of the experiment, in the absence of MgATP, k_{obs} is dominated by k_{-1} and so little change in k_{obs} is seen as [MgATP] is increased.

The lack of dependence on [MgATP] of the observed rate constant for the displacement of MgFTP from the enzyme, at least at 100 μM MgATP and above, suggests that the rate-limiting step in the reaction is the dissociation of the MgFTP from the enzyme and not the binding of MgATP. Thus, the pseudo-first-order rate constant for MgATP binding at 100 μM MgATP must be $\gg 70\ s^{-1} = (k_{1a} \times 10^{-4} + k_{-1a})\ s^{-1}$. The calculated value of K_D for the enzyme·MgATP complex of 103 μM is similar to previously obtained values (Scrutton

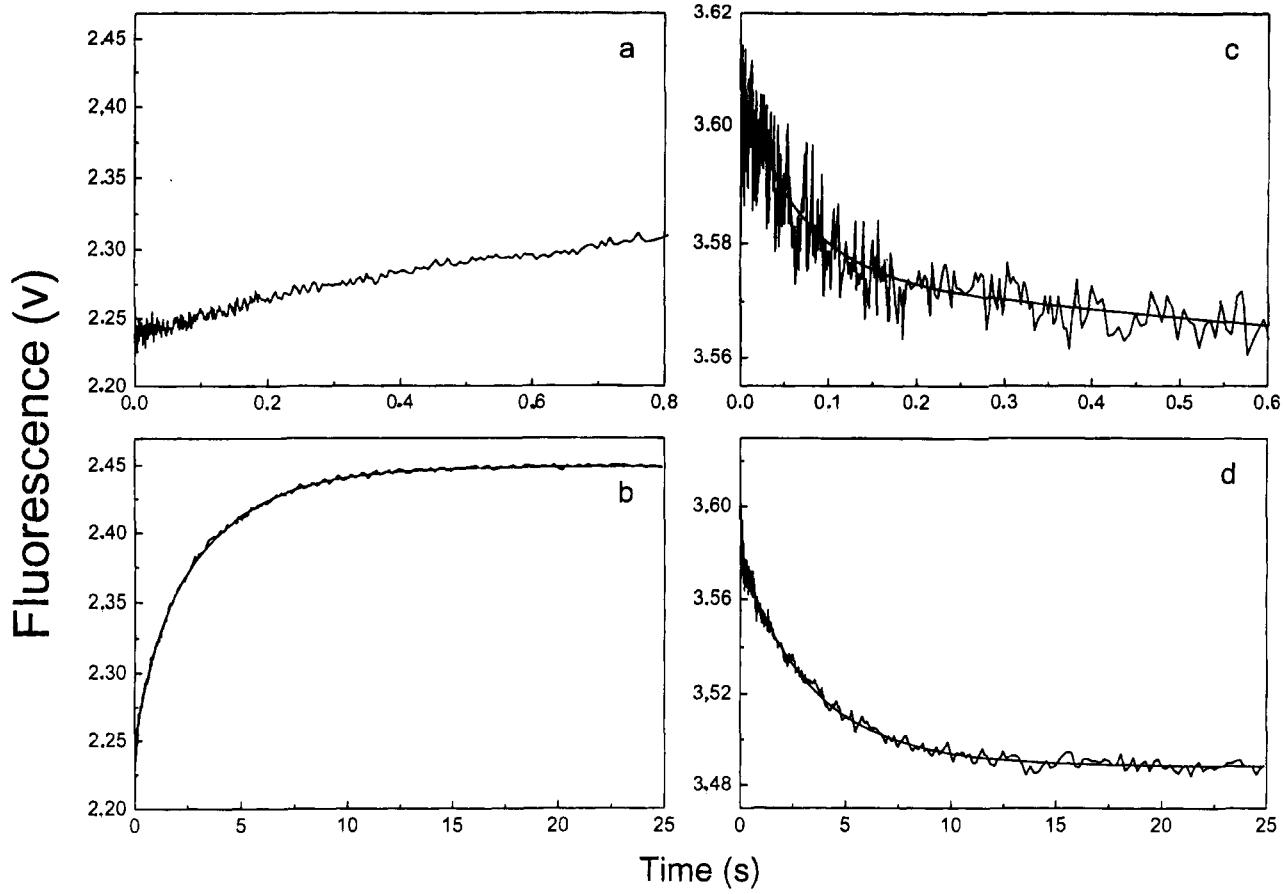

FIGURE 6: Stopped-flow records of the changes in FTP fluorescence on (a and b) the binding of FTP to pyruvate carboxylase and (c and d) the displacement of FTP from pyruvate carboxylase by 2.5 mM ATP. The reaction solutions contained 0.5 μM pyruvate carboxylase, 20 mM $NaHCO_3$, 10 μM FTP, and no added $MgCl_2$. Panel a shows the time course of the reaction in which the fast component of MgFTP binding in the presence of 4.5 mM $MgCl_2$ is normally seen (see Figure 1a). Panel b shows the slow component of the binding reaction. Panels c and d show the fast and slow components, respectively, of the ATP displacement reaction. The curves shown overlaid on the data are nonlinear least squares regression fits to either single (c) or double (b and d) exponential reactions. The values of rate constants and amplitudes of reaction derived from these fits are as follows: (b) $k_1 = 2.3 \pm 0.2$ s^{-1}, % amplitude = 1.6, $k_2 = 0.31 \pm 0.01$ s^{-1}, % amplitude = 7.0; (c) $k_{obs} = 13 \pm 4$ s^{-1}, % amplitude = 0.8; (d) $k_{obs} = 0.28 \pm 0.01$ s^{-1}, % amplitude = 2.6.

& Utter, 1965; Frey & Utter, 1977), thus $k_{-1a} = 1.03 \times 10^{-4} \times k_{1a}$. Therefore, k_{1a} must be $\gg 3.5 \times 10^5$ M^{-1} s^{-1} and $k_{-1a} \gg 35$ s^{-1}. When MgATP was present in the MgFTP syringe prior to the reaction with the enzyme, the fast component of the binding was still an apparently first-order process, with no signs of a slower displacement of MgFTP later in the reaction. This, coupled with the fact that the observed rate constant for this component was the same whether or not the MgATP had been premixed with the enzyme, suggests that the binding of MgATP is rapid compared to that of MgFTP. If this were the case, the observed rate constant for MgFTP binding in the presence of MgATP would be a function of [MgATP] as follows:

$$k_{obs} = k_1[\text{MgFTP}]/(1 + [\text{MgATP}]/K_{\text{MgATP}}) + k_{-1}$$

Thus as the concentration of MgATP increases from zero toward infinity, k_{obs} should decrease from the value obtained in the absence of MgATP toward the limiting value of k_{-1}. On the basis of the values of k_1 and k_{-1} shown in Table 1, the expected limiting values of k_{obs} should be 108 s^{-1} at 0 μM MgATP and 82 s^{-1} at infinite MgATP. Therefore, a large variation in k_{obs} would not be expected from this reaction model. Computer simulations of MgFTP binding in the presence of MgATP, in which the values of the binding (k_{1a}) and dissociation (k_{-1a}) rate constants for MgATP binding were varied but maintained in a ratio so as to give $K_{\text{MgATP}} = 100$ μM, showed that at certain values of these rate constants there was little difference in k_{obs} between 0 and 500 μM MgATP. When $k_{1a} = 6 \times 10^6$ M^{-1} s^{-1} and $k_{-1a} = 600$ s^{-1}, k_{obs} was calculated to be the same at both 0 and 500 μM MgATP. Thus rate constants for MgATP binding similar to these would be consistent with the data from both the MgATP displacement experiments and the MgFTP binding experiments. In their studies on the effects of MgATP binding on the fluorescence of enzyme-bound 8-anilinonaphthalene-1-sulfonic acid, McGurk and Spivey (1979) concluded that the bimolecular step involved in MgATP binding was complete within 4 ms, even with as low as 100 μM MgATP. Our results are consistent with this conclusion, since at 100 μM MgATP with $k_{1a} = 6 \times 10^6$ M^{-1} s^{-1} and $k_{-1a} = 600$ s^{-1}, the observed pseudo-first-order rate constant for MgATP binding would be 1200 s^{-1} ($t_{1/2} = 0.6$ ms).

FTP Binding in the Absence of Added $MgCl_2$. Stopped-flow experiments were performed in which the kinetics of FTP binding and displacement by ATP were measured in the absence of added Mg^{2+}. Figure 6a,b shows the results of the FTP binding experiments. Figure 6a shows that there is no fast component of the increase in fluorescence comparable to that seen when $MgCl_2$ was present at 4.5 mM

(see Figure 1). The slow component is present on the same time scale as in the presence of 4.5 mM $MgCl_2$ but is better described here by two exponential processes. Measurement of the fluorescence due to FTP alone and that of the enzyme alone (see Materials and Methods) showed that a very rapid increase in fluorescence had occurred in the dead-time of the stopped-flow instrument on mixing enzyme with FTP under the conditions described in Figure 6a. This will be discussed further in the subsequent section.

Figure 6c,d shows the kinetics of the decrease in fluorescence on displacement of FTP from the enzyme by ATP with no added $MgCl_2$ present. Here, a fast and a slow component are visible. Both components give good fits to single-exponential processes, and the observed first-order rate constant of the fast component of 13 s^{-1} was approximately 20% of that measured in the presence of 4.5 mM $MgCl_2$ (see Figure 1c). The rate constant for the slow component (0.28 s^{-1}) was similar to that of the slow component in the presence of 4.5 mM $MgCl_2$ (see Figure 1d).

Effects of [$MgCl_2$] on the Kinetics of FTP Binding to Pyruvate Carboxylase. As described in the previous section, when no added $MgCl_2$ was present, there was no fast component to the kinetics of FTP binding comparable to that seen in the presence of 4500 μM $MgCl_2$. Instead there was a very rapid component that occurred within the dead time of the stopped-flow instrument (1.5 ms). As the concentration of added $MgCl_2$ was increased, the amplitude of this very rapid component declined and a fast component became apparent, with rate constants that were similar to that observed for the fast component of MgFTP binding in the presence of 4500 μM $MgCl_2$ (see Figure 2). Figure 7 shows that this fast component became apparent at 110 μM $MgCl_2$ and above. Figure 7a shows that the observed first-order rate constant for this component showed no strong dependence on the concentration of $MgCl_2$ between 100 and 4510 μM $MgCl_2$. Figure 7b shows that, in going from 0 μM $MgCl_2$ (in the presence of 10 mM EDTA) to 20 μM added $MgCl_2$ (i.e., stoichiometric with FTP), the amplitude of the very rapid component of FTP binding increased; but above this concentration of $MgCl_2$, the amplitude decreased, and at 4510 μM $MgCl_2$ this component of the kinetics of FTP binding was completely abolished. Increasing [$MgCl_2$] above 110 μM resulted in an increase in the amplitude of the fast component of FTP binding. From the data showing the change in amplitude of the very rapid component of FTP binding with [$MgCl_2$], an estimate of the [$MgCl_2$] required to produce the half-maximal decrease in this amplitude is of the order of 300 μM.

As shown in Figure 6, both FTP and ATP are capable of binding to pyruvate carboxylase in the absence of added $MgCl_2$; however, the kinetics of binding and dissociation under these circumstances are quite different from those in the presence of 4.5 mM $MgCl_2$. The binding kinetics display a very fast component that occurs within the dead time of the stopped-flow instrument followed by a biphasic slow component with rate constants similar to those observed with MgFDP binding in the presence of 4.5 mM $MgCl_2$. The amplitude of the very fast component of the binding was lower in the presence of EDTA than when the concentration of added $MgCl_2$ was equal to the concentration of FTP, suggesting that MgFTP binds more tightly to the enzyme than FTP. The half-maximal abolition of the very fast component at about 300 μM $MgCl_2$ suggests that this effect

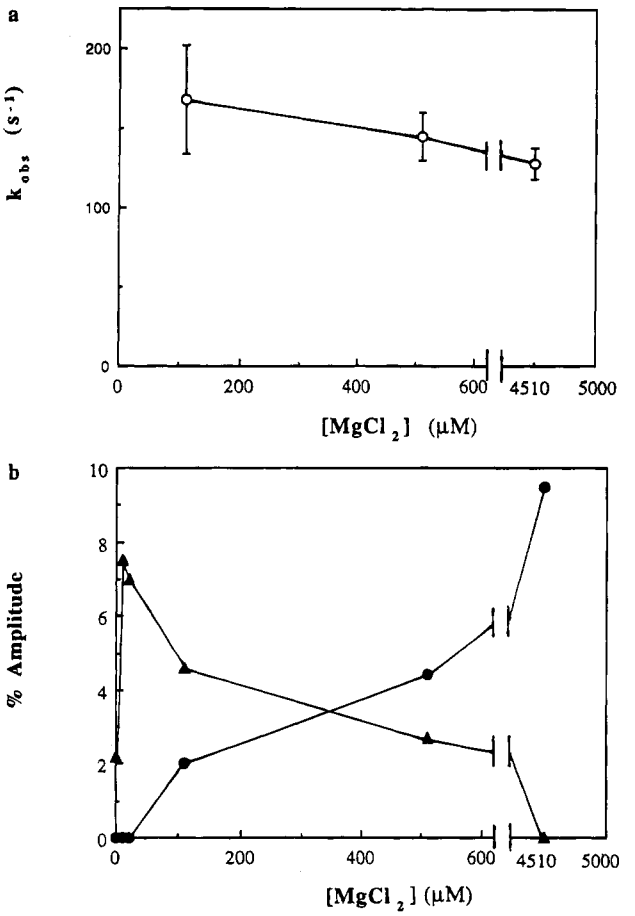

FIGURE 7: Effects of [$MgCl_2$] on (a) the observed first-order rate constant of the fast component of MgFTP binding to pyruvate carboxylase and (b) the percent amplitudes of the very fast (▲) and the fast (●) components of the binding reaction of FTP or MgFTP. Note that the values of the percent amplitudes shown at 0 $MgCl_2$ were obtained in reaction solutions containing 10 mM EDTA. In general, the reaction mixtures contained 0.5 μM pyruvate carboxylase, 20 mM $NaHCO_3$, and 10 μM FTP.

is due to an interaction of the Mg^{2+} with the enzyme, since this value is over 20 times the dissociation constant for MgFTP, assuming it to be similar to that for MgATP (Barden & Scrutton, 1974). Over the range of [$MgCl_2$] from 110 to 4510 μM, the observed rate constant for MgFTP binding in the fast component appeared not to change significantly given the errors on the estimates of these values. These results suggest that MgFTP is binding in a kinetically independent way to two forms of the enzyme, as shown in Figure 8. On mixing the enzyme with MgFTP and Mg^{2+}, an equilibrium is rapidly formed (in the dead time of the stopped-flow instrument) between the free enzyme (E), the magnesium•enzyme complex (E_{Mg}), and the MgFTP•enzyme complex (E•MgFTP). A slower reaction in which MgFTP binds to E_{Mg} then follows. The very rapid component of the increase in fluorescence therefore corresponds to the formation of E•MgFTP (or E•FTP in the total absence of Mg^{2+}), and the fast component corresponds to the formation of E_{Mg}•MgFTP. Thus, as the concentration of [$MgCl_2$] increases, a smaller proportion of the enzyme is present in the form of E•MgFTP in the rapid equilibrium formed between E, E_{Mg}, and E•MgFTP prior to binding of MgFTP to E_{Mg} and hence the amplitude of the very rapid component decreases. It is generally agreed that, in the catalytic cycle of pyruvate carboxylase, Mg^{2+} binds to the enzyme at equilibrium prior

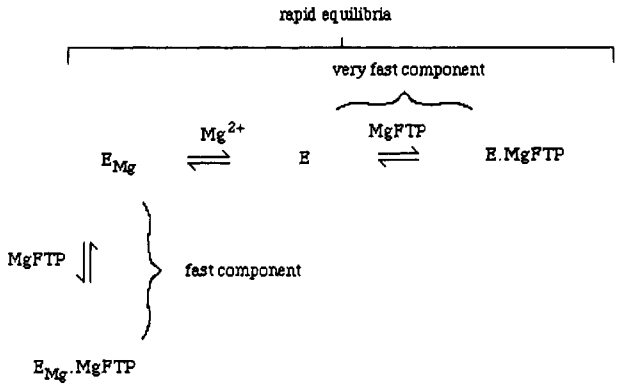

FIGURE 8: Proposed reaction model for the binding of MgFTP to pyruvate carboxylase in the presence of Mg^{2+}. It is proposed that the equilibria between the enzyme (E) and Mg^{2+} and MgFTP are rapidly established (within the dead time of the stopped-flow instrument) and that the binding of MgFTP to E constitutes the very rapid component of the binding reaction. The subsequent slower binding of MgFTP to E_{Mg} constitutes the fast component of the binding reaction.

to the binding of MgATP (McClure et al., 1971; Warren & Tipton, 1974; Attwood & Graneri, 1992). The model proposed in Figure 8 is not at variance with this if one considers that E·MgFTP and E·FTP are not catalytically viable complexes, and thus the fast component of the reaction seen at high [$MgCl_2$] represents the normal binding of the nucleotide in the catalytic cycle.

In summary, we have shown that MgFTP and MgFDP bind to pyruvate carboxylase in a similar manner to the corresponding adenine nucleotides. Thus the fluorescence changes that accompany the binding of the formycin nucleotides to the enzyme can be used to study nucleotide binding in general to the enzyme. We have made use of these fluorescence changes to measure the kinetics of nucleotide binding and study the effects of the cofactors acetyl CoA and Mg^{2+} and of the substrates pyruvate and HCO_3^- on this binding. In addition, we have been able to estimate the rate constants of MgATP binding to the enzyme. There is a component to the binding of both the magnesium-complexed formycin nucleotides and the free nucleotides that is very slow by comparison with the turnover number of the enzyme. The lack of dependence of the kinetics of this component on nucleotide concentration suggests the occurence of an essentially unimolecular reaction. None of the simple reaction models considered satisfactorily explain the data, and further investigation of this facet of formycin nucleotide binding was limited by the experimentally useful range of formycin nucleotide concentrations that could be employed. Further study of this slow component of nucleotide binding will require the use of an experimental system in which saturating concentrations of nucleotide can be used.

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