Ligand-Induced Conformational Transitions and Secondary Structure Composition of Chicken Liver Pyruvate Carboxylase

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Apparent conformational transitions induced in chicken liver pyruvate carboxylase by substrates, KHCO_3 and MgATP , and the allosteric effector, acetyl-CoA, were studied by using the fluorescent probe, 8-anilidonaphthalene-1-sulphonic acid and c.d. Fluorescence measurements were made with both conventional and stopped-flow spectrophotometers. Additions of acetyl-CoA and/or ATP to the enzyme-probe solutions quenched fluorescence of the probe by the following cumulative amounts regardless of the sequence of additions: acetyl-CoA, 10–13%; ATP, 21–24%; acetyl-CoA plus ATP, about 35%. Additions of KHCO_3 had no effect on the fluorescence. The rates of quenching by acetyl-CoA and MgATP (in the presence of acetyl-CoA) were too rapid to measure by stopped-flow kinetic methods, but kinetics of the MgATP effect (in the absence of acetyl-CoA) indicate three unimolecular transitions after the association step. The negligible effect of the probe on enzyme catalytic activity, a preservation of the near-u.v. c.d. effect of MgATP and acetyl-CoA in the presence of the probe and no observable unimolecular transitions after binding of the probe to the enzyme indicate that the probe had no deleterious effect on the enzyme. In contrast with results with 8-anilidonaphthalene-1-sulphonic acid, fluorescence of the ϵ -derivative of acetyl-CoA or ATP [fluorescent analogues; Secrist, Barrio, Leonard & Weber (1972) *Biochemistry* 11, 3499–3506] was not changed when either one was added to the enzyme. Secondary-structure composition of chicken liver pyruvate carboxylase estimated from the far-u.v. c.d. spectrum of the enzyme is 27% helix, 7% β -pleated sheet and 66% other structural types.

Binding of acetyl-CoA to chicken liver pyruvate carboxylase (EC 6.4.1.1) converts it from a nearly inactive into a fully active form, presumably owing to a conformational change (Utter *et al.*, 1975). The best additional evidence that acetyl-CoA induces a conformational change is that it accelerates enzyme inactivation by avidin, apparently because the biotin group becomes exposed when the enzyme binds acetyl-CoA. Conversely, the substrate ATP protects the enzyme from inactivation by avidin. Previous attempts to describe these presumed conformational changes by electron microscopy (Scrutton & Young, 1972), optical rotatory dispersion or protein fluorescence, however, have been unsuccessful (Utter *et al.*, 1975), and only small u.v. spectral changes are obtained (Frey & Utter, 1977).

The present report describes kinetic and equilibrium studies with the fluorescent probe 8-anilidonaphthalene-1-sulphonic acid and equilibrium c.d. measurements to characterize enzyme changes induced by MgATP and acetyl-CoA. Far-u.v. c.d. measurements also provide an estimate of the secondary structure of the enzyme.

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Experimental

Materials

Tris (Trizma base), phenylmethanesulphonyl fluoride, 5,5'-dithiobis-(2-nitrobenzoic acid), dithioerythritol, ATP, NADH, lithium salt of CoA, magnesium salt of 8-anilidonaphthalene-1-sulphonic acid (the fluorescent probe) and pig heart malate dehydrogenase (cat. no. 410-13) were from Sigma Chemical Co., St. Louis, MO, U.S.A.; EDTA and ultrapure $(\text{NH}_4)_2\text{SO}_4$ were from Schwarz/Mann, Orangeburg, NY, U.S.A.; oxaloacetate (sodium salt) was from Calbiochem, La Jolla, CA, U.S.A.; pyruvic acid and (+)-camphorsulphonic acid were from Aldrich, Milwaukee, WI, U.S.A.; citrate synthase was from Boehringer Mannheim, New York, NY, U.S.A.; crystalline bovine serum albumin was from Pentex, Kankakee, IL, U.S.A. Other chemicals were of the highest grades commercially available. Solutions were prepared in glass-distilled water.

Reagent preparations

Heavy-metal contaminants were extracted from analytical-reagent MgCl_2 with dithizon (Morrison & Uhr, 1966). Pyruvic acid was vacuum-distilled twice

and stored frozen as a 1 M solution. 8-Anilino-naphthalene-1-sulphonic acid was twice recrystallized from hot water, dried in a vacuum desiccator and stored in the dark at 5°C. Solution concentrations were determined spectrophotometrically at 350 nm, assuming $\epsilon = 4.95 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Daniel & Weber, 1966). Acetyl-CoA was prepared as described by Srere (1969), except that acetic anhydride was limited to 34% in molar excess of the initial lithium CoA to avoid acetylating the acetyl-CoA product (W. H. Frey & M. F. Utter, personal communication). The pH was adjusted just before use to the value required in measurements. Acetyl-CoA was assayed with citrate synthase, oxaloacetate and 5,5'-dithiobis-(2-nitrobenzoic acid) as described by Srere (1969). Trizma base was substituted for KHCO_3 in those preparations used to test the effect of HCO_3^- in fluorescence and c.d. experiments. ATP stock concentrations were calculated from spectrophotometric data, assuming $\epsilon = 15400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 259 nm (Bock *et al.*, 1956). Mg^{2+} concentrations were kept at least 4 mM in excess of ATP.

Substrate and fluorescent-probe solutions were prepared in buffers used for measurements and pH was adjusted if necessary. All buffers for fluorescence measurements were filtered to remove dust.

Enzyme preparations and assays

Malate dehydrogenase was collected from its $(\text{NH}_4)_2\text{SO}_4$ suspension by centrifugation and $(\text{NH}_4)_2\text{SO}_4$ removed by dialysing four or five times against 100 vol. of assay buffer (10 mM-potassium phosphate/1 mM-EDTA/8.58 mM- MgCl_2 /2 mM-dithioerythritol, pH 7.6). Enzyme concentration was determined by spectrophotometric assay at 340 nm with 0.1 mM-oxaloacetate and 0.2 mM-NADH at 25°C in the buffer specified above. Throughout this paper a unit of enzyme activity is that amount giving 1 μmol of product/min.

Pyruvate carboxylase was purified by the procedure of Scrutton & Fung (1972) from livers of 9–10-week-old Cornish $\times$ White Rock crossed chickens (usually starved for 36 h before death) and the purified enzyme was stored at 5°C as specified therein. A 1.5 h dialysis against 3 litres of 0.025 M-potassium phosphate buffer/2 mM-EDTA/5 mM-2-mercaptoethanol/2 μM -phenylmethanesulphonyl fluoride, pH 7.2, was substituted, however, for the Sephadex G-25 column (W. H. Frey & M. F. Utter, personal communication). The enzymic assay was started by addition of enzyme to the assay buffer specified above containing 10 mM-pyruvate, 2 mM-ATP, 15 mM- KHCO_3 , 0.1 mM-acetyl-CoA, 0.2 mM-NADH and approx. 5 units of malate dehydrogenase/ml; A_{340} was monitored. Protein concentrations were determined by the method of Kalckar (1947) and corrected (increased) by a factor of 2.0 (Taylor *et al.*, 1972), and occasionally by the

biuret method, with bovine serum albumin (Robinson & Hogden, 1940) as a standard. A_{280}/A_{260} ratios of 1.5 indicated the absence of protein-bound nucleotide. Enzyme molar concentrations were calculated by using a mol.wt. of 520000 for the native tetramer of subunits (Utter *et al.*, 1975). Specific activities for all fluorescence and c.d. measurements were 13.5–15.5 and 17.8–18.7 units/mg respectively.

Before use the purified enzyme was passed over a Sephadex G-50 (bead size 50–150 μm) column equilibrated at room temperature (approx. 25°C) with the assay buffer and eluted with the same buffer to remove sulphate, which inhibits the enzyme (Scrutton & Fung, 1972).

Fluorescence studies

Fluorescence titrations were made by ligand additions to enzyme or enzyme-probe solutions, and fluorescence corrections (maximum of 5%) were made for resulting dilutions. The assay buffer was used throughout, except as specified otherwise. The excitation and emission of enzyme-probe were 406 and 485 nm respectively, corresponding to uncorrected maxima on our Aminco instrument. The higher excitation-wavelength maximum than the approx. 370 nm reported for other protein-8-anilino-naphthalene-1-sulphonic acid complexes is undoubtedly due to the large increase in light intensity and photomultiplier sensitivity at 405 nm relative to lower wavelengths for both our Aminco spectrofluorimeter and stopped-flow instruments. All equilibrium and stopped-flow protein-probe fluorescence measurements were performed with enzyme that was more than 90% saturated with the probe. At least five repetitions of a stopped-flow reaction were obtained for each solution condition.

Instrumentation

Assays were performed at 25°C in a Coleman 124 spectrophotometer, with a no. 801 scale expander and no. 165 recorder. Stopped-flow kinetic fluorescence measurements were made at 25°C in a Durrum-Gibson stopped-flow spectrophotometer equipped with a 1.82 cm-path-length cell, a Durrum fluorescence accessory no. 16400 and a xenon or tungsten light source. Excitation was at 406 nm with a 6 nm spectral bandwidth, and a Corning 3-72 filter used with the emission detector. The dead-time under conditions used in most experiments was approx. 4 ms. Investigation of equilibrium fluorescence changes induced by ligands on pyruvate carboxylase-probe, tests for stability of probe and enzyme-probe complex on prolonged irradiation, and enzyme-probe dissociation-constant determinations were performed at 25°C in a thermostatically controlled Aminco-Bowman spectrofluorimeter with a slit width of 3 mm. C.d. spectra were obtained on a Cary 61-CD

model spectropolarimeter at 25°C in 1mm- and 1cm-path-length cells for far- and near-u.v. regions respectively. The optical path was flushed with N₂, and spectra were obtained with a scan rate of 0.2 (far-u.v.) or 0.1 (near-u.v.) nm/s, filter period of 10s and constant spectral bandwidth of 2nm. The instrument was calibrated with an aqueous solution of (+)-camphorsulphonic acid with the use of the molar-ellipticity value determined by Cassim & Yang (1969).

Data analysis

Eqn. (1) (below) was analysed by weighted linear least squares, and other equations were fitted to data by the non-linear, direct-search minimizer STEPIT (Chandler, 1971) with problem-dependent 'driver routines'. The program for calculating protein secondary structures from c.d. data, previously described by Thompson *et al.* (1976), used the reference ellipticities of Chen *et al.* (1972). Standard deviations of the dependent variable at each data point i , $\sigma(y_i)$, provided weighting factors $w = [\sigma(y_i)]^{-2}$. For fluorescence data $\sigma(y_i)$ were estimated from the precision of reading photographic records as 2% of y_i . C.d. data from 243 to 204nm at 3nm intervals were used for analysis with an assumed $\sigma(y_i)$ of 5% of the mean residue ellipticities. Standard deviations of best-fit parameters were calculated by the usual linear approximation (Bevington, 1969), except for a few data for which a support plane method (Chandler *et al.*, 1972) was used (first row in Table 3). Systematic errors of approximately the same magnitude are likely, however, and thus total errors of the parameters are probably two to three times those obtained from least-squares analysis. Quality of fits were judged by the chi-square statistic (Hamilton, 1964). The chi-square probability is the probability of getting a worse fit, the expected (mean) value of which is 0.5 when chi-square equals the number of degrees of freedom.

Results and Discussion

Equilibrium enzyme-probe fluorescence

The addition of pyruvate carboxylase (0.64 μ M) to a solution of the probe, 8-anilino-naphthalene-1-sulphonic acid (0.32mM) in the standard buffer, resulted in approximately a 60-fold increase in fluorescence intensity of the probe and a characteristic blue shift from 515 to 485nm. Protein-probe fluorescence was markedly quenched (Table 1) by MgATP (21–24%) and acetyl-CoA (10–13%), with no significant shifts in either the excitation or emission wavelengths, suggesting that these ligands produce a pronounced alteration in the environment of the bound probe. The changes in relative fluorescence caused by MgATP and acetyl-CoA were additive (giving a sum of 31–35% quenching) and each change was independent of the sequence of

Table 1. Quenching of enzyme-probe fluorescence by ligands

Enzyme was 0.45–0.52 μ M and the probe (8-anilino-naphthalene-1-sulphonic acid) was 0.32mM; buffer contained 10mM-potassium phosphate, 1mM-EDTA, 8.58mM-MgCl₂ and 2mM-dithioerythritol at pH7.6 and 25°C. Excitation was at 406nm and emission at 485nm. The + denotes addition of a ligand to the previous solution of ligands specified in rows above it. Fluorescence was relative to that of the enzyme-probe complex at 100. Percentage quenching = $100 \times [(F_0 - F)/F_0]$, where F_0 and F are equilibrium fluorescence before and after the last ligand addition respectively. These quenchings were entirely independent of order of addition of the ligand.

Additions (mM)	Relative fluorescence	Quenching (%)
ATP (4.0)	76	24
+KHCO ₃ (15.0)	76	0
+Acetyl-CoA (0.98)	68	10

addition of these two ligands and the presence of KHCO₃. Further additions of ligands produced no additional changes, as predicted from the known enzyme-ligand dissociation constants (14 and 100 μ M for acetyl-CoA and MgATP respectively; Frey & Utter, 1977). The effects of individual substrates and acetyl-CoA at pH7.0 (same standard buffer, except for 8.37mM-MgCl₂ and the pH) were similar to the above measurements at pH7.6; the dependence on order of addition and combinations of ligands was not tested at pH7.0, however.

Attempts to use the fluorescent ϵ -analogues of acetyl-CoA or ATP (Secrist *et al.*, 1972) as probes of enzyme conformational changes were unsuccessful. Their fluorescences were not affected by addition of the enzyme alone or of the enzyme with ligands.

Significance of the ligand-induced fluorescence changes

The following experiments and considerations attempt to establish the molecular basis of the ligand-induced fluorescence changes and whether the probe causes any changes in the enzyme's conformation or activity.

(1) *Fluorescence changes not caused by self-absorbance, ligand-probe interactions, changes in ionic strength or Mg²⁺ concentration, or collisional quenching.* None of the added species in Table 1 had an absorption in either the excitation or emission region, thus excluding an inner-filter mode of quenching, nor did these species have an effect on the fluorescence of the probe in the absence of enzyme. Since increasing ionic strength (by addition of up to 0.072M-KCl) in the absence of ligands or in the presence of acetyl-CoA did not change the fluorescence of enzyme-bound probe, the fluorescence quenchings were not ascribed to ionic-strength changes. Nor could changes in Mg²⁺ concentration resulting from ATP

additions have caused the fluorescence changes, since increasing Mg^{2+} to compensate for ATP did not alter the results.

A collisional ('dynamic') quenching mechanism appears to be excluded for the following reasons: (a) increasing MgATP or acetyl-CoA concentrations caused no further fluorescence quenching as stated above; dynamic quenching, however, should increase without bounds (Parker, 1968), except for quenching of special cases not likely to occur in our experiments; even then, the quenching would not change in accordance with that caused by static quenching as found (see below); (b) neither MgATP nor acetyl-CoA at concentrations causing maximum quenching of the enzyme-probe complex quenched the fluorescence of the free probe; (c) the extents of quenching of enzyme-probe by MgATP are in excellent agreement with the extents of MgATP binding to enzyme-probe, calculated from the dissociation constant of enzyme-MgATP (Frey & Utter, 1977), as described below; although subsaturating acetyl-CoA concentrations were not tested, the nearly maximum quenching by acetyl-CoA concentrations of $50\mu M$ (see Table 2) and above are also consistent with its dissociation constant of $14\mu M$ (highly co-operative binding; Frey & Utter, 1977); (d) the maximum dynamic quenching calculated from the maximum bimolecular collision constant, $k = 6.5 \times 10^9 M^{-1} \cdot s^{-1}$, the life-time of the enzyme-probe excited state, τ_E approx. 2.7 ns (Stryer, 1965) and ligand concentrations (Parker, 1968) are 0.2% with the 0.1 mM-MgATP used (Table 3) and 0.1% with the 0.05 mM-acetyl-CoA used (Table 2). Thus the maximum possible collisional quenching is nearly two orders of magnitude lower than the observed quenching. The τ_E for this enzyme-probe complex is calculated from its fluorescence relative to that of the free probe and the lifetimes and quantum yields of other protein complexes with 8-anilino-naphthalene-1-sulphonic acid (Stryer, 1965). The only assumption in this calculation is that life-times and quantum yields are proportional, which is probably accurate within a factor of two and surely accurate within an order of magnitude (Scott *et al.*, 1970).

(2) *Dissociation constant of the enzyme-probe complex.* Data obtained from fluorescence titrations of pyruvate carboxylase with the probe in the presence and in the absence of MgATP or acetyl-CoA are shown in Fig. 1. Estimates were obtained for the apparent dissociation constant, K_d , for the enzyme-probe complex and the fluorescence maximum, F_{max} . Least-squares values of K_d and F_{max} , and their standard deviations were determined by eqn. (1):

$$F = \frac{-K_d F}{[probe]} + F_{max}. \quad (1)$$

The linear plots obtained (Fig. 1) indicate that a single dissociation constant is being evaluated. K_d

values of $19.9 \pm 0.4\mu M$, $19.5 \pm 0.1\mu M$ and $19.2 \pm 0.2\mu M$ were obtained in the absence of substrates and in the presence of MgATP and acetyl-CoA respectively. These results indicate that MgATP and acetyl-CoA do not produce any significant change in the affinity of the enzyme for the fluorophore. This finding suggests that the observed decrease in F_{max} produced by MgATP and acetyl-CoA is caused by an induced change in enzyme conformation, which either decreases the number of hydrophobic binding sites for the probe, or alters the polarity of the environment of enzyme-bound probe, or both. A direct interaction between the ligands and enzyme-bound probe might quench the probe's fluorescence without involving a change of enzyme conformation. This would seem unlikely, however, since such a direct interaction would most likely alter the probe or MgATP dissociation constants.

(3) *Nativeness of the enzyme-probe complex.* Enzyme activation (Kapoor, 1974) as well as kinetic inhibition (Fan *et al.*, 1974) by 8-anilino-naphthalene-1-sulphonic acid have been reported for other enzyme systems. Solutions of pyruvate carboxylase (0.43 – $1.28\mu M$) and this probe (0.46 – $7.68\mu M$) were incubated for 15 min and samples assayed in the presence or in the absence (controls) of 2.3 – $92.1\mu M$ -probe. No effects on enzyme activity could be observed over this wide range of assay conditions. Lack of inhibition indicates that the probe is not competing for the active site or causing detectable enzyme inactivation.

Fig. 1. Determination of the pyruvate carboxylase-probe dissociation constant

Increasing concentrations of the probe (8-anilino-naphthalene-1-sulphonic acid) were added to a solution of $0.72\mu M$ -pyruvate carboxylase in buffer (as specified in Table 1) in the absence of acetyl-CoA and MgATP (○), in the presence of 0.1 mM -acetyl-CoA (Δ) and in the presence of 4.0 mM -ATP (●). Corrections were made for dilution by additions of the probe. Excitation and emission were at 406 and 485 nm respectively.

The enzyme-probe interaction was further studied by measuring the rate of binding of the probe to pyruvate carboxylase with stopped-flow methods. Transitions after the bimolecular binding step would indicate that the enzyme is converted into a non-native conformation. For stopped-flow measurements, the probe (0.021–0.32 mM after mixing) was present in one syringe and enzyme (0.011–0.49 μ M after mixing) in the other. The enzyme-probe complex-formation was complete within the 'dead-time' of the instrument (approx. 4 ms). No evidence of subsequent transitions after the bimolecular binding step was observed. C.d. measurements (see below) also failed to show any evidence of probe-induced changes of the enzyme.

Kinetics of ligand-induced enzyme-probe fluorescence changes

Stopped-flow measurements were made to quantify the rates of acetyl-CoA- and MgATP-induced enzyme-probe fluorescence quenching. Table 2 summarizes the results of these studies. The quenchings by acetyl-CoA or by MgATP in the presence of acetyl-CoA were complete within the 4 ms 'dead-time' of the stopped-flow instrument whether or not saturating HCO_3^- was added. Equilibrium values of quenchings agreed with those in non-stopped-flow measurements. Typical progress curves for the MgATP fluorescence quenching in the absence of acetyl-CoA are shown in Fig. 2 for three enzyme and

Table 2. *Stopped-flow results*

The probe is 8-anilidonaphthalene-1-sulphonic acid. Conditions were the same as specified in Table 1. Contents given are the concentrations after mixing the two drive syringes. Percentage quenching is as in Table 1. Reactions were complete within the 4 ms instrument dead-time, except that of the last row, for which see Fig. 2.

Contents		Quenching (%)
Syringe 1	Syringe 2	
0.48 μ M-Enzyme, 0.32 mM-probe	0.10 mM-Acetyl-CoA	11
0.48 μ M-Enzyme, 0.32 mM-probe	0.10 mM-Acetyl-CoA, 4.0 mM-ATP	35
0.48 μ M-Enzyme, 0.32 mM-probe, 0.10 mM-acetyl-CoA	4.0 mM-ATP	35
0.51–1.65 μ M-Enzyme, 0.32 mM-probe	0.1–4.0 mM-ATP	11–24

Fig. 2. Progress curves for the reaction of pyruvate carboxylase-probe with MgATP for various enzyme and MgATP concentrations

The probe (8-anilidonaphthalene-1-sulphonic acid) concentration was 0.32 mM. Enzyme and ATP concentrations were respectively: 0.51–0.55 μ M and 4.0 mM, or 1.0 μ M and 4.0 mM, or 1.65 μ M and 4.0 mM, all shown by ○; 0.51–0.55 μ M and 0.34 mM, Δ; 0.51–0.55 μ M and 0.10 mM, ●. Buffer and other conditions were the same as given in Table 1.

three ATP concentrations from 0.1 to 4.0 mM (the enzyme-MgATP dissociation constant is approx. 0.10 mM; Frey & Utter, 1977). Measurements were performed under pseudo-first-order conditions, $[MgATP] \gg [enzyme]$. Each curve possesses three phases. The slower phases are not due to photodecomposition of the enzyme-bound or free probe, since irradiation for over 15 min produced no detectable fluorescence changes. With the stopped-flow drive pressures used, only an estimated 0.4% quenching occurred within the instrument 'dead-time'. Thus the fluorescence at 4 ms was taken to be the initial fluorescence. That the complete progress curve was observed was also substantiated by comparison of the equilibrium magnitude of quenching observed in the Aminco-Bowman fluorimeter with stopped-flow records. When pyruvate carboxylase-probe was mixed in the stopped-flow instrument with buffer (no ATP), no change in fluorescence was observed, indicating no perturbation of the enzyme-probe fluorescence after the 'dead-time' because of dilution or flow stress on the complex. Specific activities of the enzyme before and after stopped-flow experiments (sample from stopped-flow cuvette) differed by 10% or less, further indicating that the stopped-flow system induced no irreversible effects on the enzyme.

Progress curves in Fig. 2 were represented by a sum of exponentials. No less than three exponentials were required to give a reasonable fit to data by the chi-square criterion for goodness of fit. A satisfactory fit (χ^2 probability ≥ 0.5) to eqn. (2) was obtained, where F_0 and F_t are fluorescences observed initially and at time t respectively; A_1 , A_2 and A_3 are pre-exponential or amplitude parameters; and λ_1 , λ_2 and λ_3 are exponential or time parameters:

$$(F_0 - F_t)/F_0 = A_1 + A_2 + A_3 - (A_1)e^{-\lambda_1 t} - (A_2)e^{-\lambda_2 t} - (A_3)e^{-\lambda_3 t} \quad (2)$$

The fitted values of these parameters are tabulated in Table 3. Systematic errors of two or three times the

random error are probable. Within expected errors, amplitude factors show no apparent dependence on enzyme concentration, but a substantial ATP-dependence. In rows 1 and 2 of Table 3 the extents of quenching by MgATP, Δq , are less than the maximum quenching, Δq_{max} , in subsequent rows. The sum of amplitudes compared with maximum values give $\theta_F = \Delta q/\Delta q_{max} = 0.51$ and 0.78 for 0.10 mM- and 0.340 mM-MgATP respectively. These values may be compared with the fractional saturation (θ) of the enzyme-probe with MgATP obtained by using $\theta = [MgATP]/(1 + K[MgATP])$ and the known dissociation constant of MgATP from the enzyme complex ($K = 96 \mu M$; Frey & Utter, 1977). The calculated θ values are 0.50 and 0.77 for 0.10 mM- and 0.340 mM-MgATP respectively. These are in excellent agreement with the θ_F values, indicating that quenching is proportional to the extent of MgATP binding to its active enzyme site.

No apparent enzyme- or MgATP-dependence is shown in the time constants. From the latter result it follows that the bimolecular addition of MgATP to enzyme (E) was complete within the dead-time of the instrument (4 ms), since the pseudo-first-order rate constant of the bimolecular reaction of eqn. (3) should depend on MgATP concentration. Similarly we argue that if the bimolecular step was observed, the ratio of initial velocities with 4.0 mM- and 0.1 mM-MgATP $[(\Delta F/\Delta t_{4.0 \text{ mM}})/(\Delta F/\Delta t_{0.1 \text{ mM}})]$ should be 40 with constant enzyme-probe concentration; experimentally this ratio was 2. If the bimolecular reaction was complete within the 4 ms dead-time, it also follows that addition of MgATP to enzyme-probe does not itself effectively change the fluorescence of bound probe, since the total fluorescence change observed kinetically agreed quantitatively with the change between initial and final equilibrium states. Thus the three kinetic phases of fluorescence change observed in Fig. 2 must result from subsequent unimolecular changes.

Table 3. Kinetic parameters for MgATP-induced changes in enzyme-probe fluorescence

Best-fit constants of eqn. (2) and their s.d. values are shown. About 110 data points per full-time reaction curve of Fig. 2 were used for analyses. Conditions not specified below were the same as given in Table 1. The s.d. errors in the first row of data were calculated from the more accurate support plane method rather than the linear approximation used for the remaining data (see the Experimental section). Thus these + errors need not equal the - errors.

Enzyme (μM)	ATP (mM)	Parameters					
		$10^2 \times A_1$	$10^2 \times A_2$	$10^2 \times A_3$	$10^{-1} \times \lambda_1$ (s $^{-1}$)	$10 \times \lambda_2$ (s $^{-1}$)	$10^2 \times \lambda_3$ (s $^{-1}$)
0.51-0.55	0.10	+0.04	+0.08	+0.1	+0.03	+0.5	+0.09
		3.4	3.8	4.4	1.5	5.5	1.1
		-0.05	-0.2	-0.08	-0.03	-0.2	-0.05
0.51-0.55	0.34	5.1 ± 0.1	5.7 ± 0.2	6.9 ± 0.2	1.8 ± 0.06	6.0 ± 0.4	1.1 ± 0.07
0.51-0.55	4.0	6.8 ± 0.08	7.2 ± 0.2	8.6 ± 0.2	1.7 ± 0.03	5.3 ± 0.3	1.1 ± 0.07
1.01	4.0	6.8 ± 0.08	7.3 ± 0.2	8.5 ± 0.2	1.7 ± 0.03	5.2 ± 0.3	1.1 ± 0.06
1.65	4.0	6.9 ± 0.03	7.2 ± 0.07	8.5 ± 0.08	1.6 ± 0.02	5.1 ± 0.1	1.1 ± 0.03

The series reactions of eqn. (3):

where E is enzyme and X is various E-probe-MgATP forms, are considered the most reasonable mechanism to explain the data. Mechanisms with parallel reaction steps may also be written, but are very unlikely. This is mainly because, with the nearly saturating concentrations of the probe and MgATP used, the parallel reaction mechanisms require more than one enzyme-probe or enzyme-MgATP dissociation constant or three mutually exclusive probe-binding sites with the same binding constants and fluorescence emissivities. The latter is both hard to imagine and hard to reconcile with the three vastly different rates of ATP-induced reactions, and only one dissociation constant was observed for either the enzyme-probe (Fig. 1) or enzyme-MgATP complex (Frey & Utter, 1977).

By assuming the bimolecular step in eqn. (3) to be in rapid equilibrium with the next reaction, the differential equations describing the coupled reactions of eqn. (3) were integrated (McGurk, 1976; Hammes & Schimmel, 1970). The resulting equations are compatible with the data represented by eqn. (2), if reasonable assumptions are made about the negligible quantitative contribution of a few terms relative to the majority of terms (McGurk, 1976). Since the derived equation contains 11 unknown constants (six rate and one equilibrium constant, and four emissivities of the five E-probe complexes), further quantitative analysis of these data seems impractical.

Summary and implications of the kinetic results

The kinetic results are summarized in Scheme 1, based on the mechanism of eqn. (3). All the transitions are virtually complete in 4ms, except for the ones marked 'Slow'. This implies first-order rate constants of above $6 \times 10^3 \text{ s}^{-1}$. Since full enzymic activity was also obtained within 4ms when acetyl-CoA and

pyruvate were stopped-flow-mixed against enzyme and other substrates, or when ATP was mixed against enzyme, acetyl-CoA and substrates (F. C. North & H. O. Spivey, unpublished work), it is quite likely that the conformational transitions monitored by fluorescence are simultaneous with enzyme activation.

Whether the MgATP-induced enzyme transitions in the presence of acetyl-CoA are the same as in its absence, except for the rates, is uncertain. In favour of their similarity, however, are (a) the fact that the relative fluorescence changes ($\Delta F/F_0$) with and without acetyl-CoA are the same within experimental error, (b) acetyl-CoA has little effect on the K_m values of the substrate, and the latter have little effect on the activation constant and Hill coefficient of acetyl-CoA (Utter *et al.*, 1975), and (c) experiments with the enzyme from other species where the properties of both free enzyme and enzyme-acetyl-CoA complex can be observed; these experiments suggest that the fundamental mechanism of enzyme action is not altered by acetyl-CoA, though the rates are (Utter *et al.*, 1975).

With the avian enzyme, acetyl-CoA activation of the enzyme is thought to be nearly independent of binding of substrates; binding of acetyl-CoA is considered to release the immobilized biotin to permit transfer of its bound CO_2 from the nucleotide site to the oxo acid site (Utter *et al.*, 1975). The above fluorescence results, however, show that acetyl-CoA also affects the rate of response of enzyme to binding MgATP.

Advantages of the 8-anilidonaphthalene-1-sulphonic acid probe

A useful spectroscopic probe should not significantly alter the properties of the enzyme. Three separate criteria (enzyme activity, c.d. and fluorescence change on mixing 8-anilidonaphthalene-1-sulphonic acid and enzyme) fail to reveal any changes in enzyme properties or conformation caused by this probe, though such effects are known with other

Scheme 1. Summary of kinetic results assuming the mechanism of eqn. (3)
E is the enzyme and X_1 etc. are various E-MgATP conformations.

enzymes. Also our results indicate that the ligands are inducing conformational changes and not simple competitive displacement of the probe. An additional advantage of this probe is the magnitude of its response to acetyl-CoA, which is about four times that of other optical properties. The presence of acetyl-CoA- and MgATP-induced conformational changes of this enzyme are indicated by their effects on rates of avidin inactivation of the enzyme, among numerous other criteria (Frey & Utter, 1977). The advantages of fluorescent probes lie in their ability to monitor continuously the conformational changes and the possibility of obtaining geometric relationships among probe, allosteric and active sites (Radda & Dwek, 1972). The fact that previous attempts to use fluorescence (Utter *et al.*, 1975), and that our attempts with ϵ -(fluorescent) ATP and acetyl-CoA derivatives failed, emphasizes the value of trying numerous fluorescence probes. We hope that these results will encourage additional experiments to exploit the potential of these and similar probes.

Circular dichroism

C.d. measurements were made to characterize conformational changes of the enzyme further, test for effects of the probe on the enzyme, and provide estimates of its secondary structure. The probe produced no changes in the far- or near-u.v. c.d. spectra, which are shown in Fig. 3. Both acetyl-CoA and MgATP caused small changes in the near-u.v. region, but no detectable effects on the far-u.v. spectrum. Since free acetyl-CoA and MgATP have small c.d.

activity, it is not certain whether their effects on the enzyme's spectra are due to enhancement by the enzyme of the bound ligand's optical activity (extrinsic effects) or to conformational changes of the enzyme.

The fitted values for secondary structure types were $27.3 \pm 0.6\%$ helix and $6.6 \pm 0.9\%$ β -pleated sheet. The experimental spectrum with fitted points is shown in Fig. 3. The chi-square value for the fitted curve was 33, compared with an expected value of 12, indicating only a fair fit (χ^2 probability < 0.1). To improve chi-square would require the standard deviation to be greater than 5% of $[\theta]_A$, which is an unrealistic estimate. Inexactness in the model probably accounts for the poor quality of fit. The helical estimate will be high or low according to how well the average helical lengths of pyruvate carboxylase agree with the overall helical lengths of the chosen reference proteins (Chen *et al.*, 1974).

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Fig. 3. (a) Far- and (b) near-u.v. c.d. spectra of pyruvate carboxylase

Buffer was 10mm-potassium phosphate/8.58mm-MgCl₂, pH 7.6 at 25°C. Enzyme concentrations were 0.2mg/ml (a) and 3.0mg/ml (b). Fitted values were for far-u.v. spectrum, obtained by using the empirical method of Chen *et al.* (1972) (○); —, experimental values.

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