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Purification and some kinetic properties of rat liver ATP citrate lyase

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1. A new purification procedure for rat liver ATP citrate lyase is described. The method reproducibly gives homogenous undegraded enzyme. 2. Steady-state kinetic analysis of ATP citrate lyase was complicated by the presence of ADP, a product of the reaction, in solutions of ATP. 3. The kinetic patterns observed were dependent on whether ADP was removed by the assay system. When assays were performed with a method in which ADP was removed, the results showed that the enzyme obeys a double-displacement mechanism with a phosphoenzyme intermediate. 4. This resolves a controversy between the results of previous kinetic studies and those of isotope-exchange and enzyme-labelling experiments.

ATP citrate lyase (EC 4.1.3.8) catalyses the first cytoplasmic step in the synthesis of long-chain fatty acids in mammalian tissues. It is now well known that the enzyme can be phosphorylated in liver or adipose tissue in response to adrenaline, glucagon or insulin (see, e.g., Alexander *et al.*, 1979; Janski *et al.*, 1979; Ramakrishna & Benjamin, 1979). These observations have caused considerable interest in the potential role of ATP citrate lyase in the regulation of fatty acid synthesis. ATP citrate lyase can be phosphorylated by at least two different protein kinases (Guy *et al.*, 1981; Ramakrishna *et al.*, 1981). Attempts to show that phosphorylation affects the activity of the enzyme have so far proved negative (e.g. Guy *et al.*, 1981; Ramakrishna *et al.*, 1983). However, no complete comparison of the kinetic properties of the phosphorylated and unphosphorylated forms of the enzyme has yet been reported. As an essential preliminary to such a study we investigated the kinetic mechanism of the unphosphorylated enzyme.

Previous workers have investigated the kinetic mechanism of ATP citrate lyase in several different ways and have obtained conflicting results. Steady-state kinetic analysis of the forward reaction suggested a sequential mechanism in which ATP bound first, followed by random-order equilibrium binding of citrate and CoA (Plowman & Cleland, 1967). Kinetic studies of the reverse reaction also suggested a sequential mechanism (Farrar & Plowman, 1971). On the other hand, the isotope-exchange studies of Plowman & Cleland (1967) suggested that the enzyme obeyed a double-displacement mechanism with a phosphoenzyme intermediate. Several groups have, in fact, been able to isolate such a species after incubation of ATP citrate lyase with [γ-^{32}P]ATP (Plowman & Cleland, 1967; Inoue *et al.*, 1968; Cottam & Srere, 1969; Walsh & Spector, 1969). There is thus a discrepancy between the results of steady-state kinetics experiments and those of isotope-exchange and enzyme-labelling studies.

The studies noted above were carried out before it became known that ATP citrate lyase is very susceptible to proteolysis during purification (see, e.g., Singh *et al.*, 1976; Linn & Srere, 1979). They are therefore open to the criticism that they were probably carried out using proteolytically degraded enzyme. We decided to investigate the kinetic mechanism of undegraded ATP citrate lyase. In the present paper we describe briefly a new method for the isolation of homogeneous undegraded ATP citrate lyase from rat liver. We present results that resolve the controversy noted above and demonstrate that the enzyme obeys a double-displacement mechanism.

Experimental

Materials

ATP, NADH, CoA, phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, malate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and β-galactosidase were from

Abbreviation used: SDS, sodium dodecyl sulphate.

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Boehringer Corp. (London), Lewes, Sussex, U.K. Bovine serum albumin, dithiothreitol and proteinase inhibitors were from Sigma (London) Chemical Co., Poole, Dorset, U.K. Haemocyanin and apoferritin were from C.P. Laboratories, Bishops Stortford, Herts., U.K. Fatty acid synthase was isolated from rabbit mammary gland (Hardie & Cohen, 1978). The *arom* multienzyme complex from *Neurospora crassa* was a gift from Dr. J. R. Coggins of this department. Affi-gel Blue was from Bio-Rad Laboratories, Watford, Herts., U.K. Ultrogels AcA 44 and A2 were from LKB Instruments, South Croydon, Surrey, U.K. All other materials were obtained as described previously (Borthwick *et al.*, 1984).

Purification of rat liver ATP citrate lyase

Male or female Wistar rats (120–200g) were starved for 24h and then re-fed on a fat-free diet (BP Nutrition, Stepfield, Essex, U.K.) for 48h. Unless stated otherwise, all buffers used in the purification contained 1mM-EDTA, 0.1% 2-mercaptoethanol, 2mM-benzamidine hydrochloride, 200μg of lima bean trypsin inhibitor/ml, and 20μg of each of antipain, leupeptin and aprotinin/ml. All steps were carried out at 0–4°C.

Livers from six rats were rinsed and then homogenized in 3vol. of 0.25M-sucrose/20mM-potassium phosphate/10mM-potassium citrate, pH7.0. A particle-free supernatant was prepared by centrifugation at 20000*g* for 10min and then at 105000*g* for 2h (Step 1). The proteins that precipitated between 30% and 40% saturation with $(NH_4)_2SO_4$ were collected; the pH was maintained at 7.3–7.4 during this operation. The precipitate was dissolved in 10mM-potassium phosphate, pH7.0, and equilibrated in this buffer by gel filtration on Sephadex G-25 (Step 2). The enzyme was then loaded onto a column (2.2cm × 16cm) of DEAE-cellulose equilibrated in this buffer. The column was washed with the buffer and developed with a 300ml linear gradient of 10–150mM-potassium phosphate, pH7.0. Fractions containing ATP citrate lyase, but not fatty acid synthase, were pooled (Step 3) and applied to a column (1.6cm × 10cm) of Affi-gel Blue equilibrated in 150mM-potassium phosphate, pH7.0. The column was washed with this buffer and then with 250ml of this buffer containing 5mM-ATP and 0.25mM-CoA. The enzyme was specifically eluted as a broad peak with the second wash (Step 4). The enzyme was concentrated to 2ml by vacuum dialysis. Minor contaminants of low M_r were removed by gel filtration on a column (1.6cm × 50cm) of Ultrogel AcA 44 equilibrated in 100mM-potassium phosphate/10mM-potassium citrate, pH7.0. The enzyme was dialysed into 100mM-triethanolamine/HCl, pH7.8, containing 0.4mM-benzamidine hydrochloride and 40% (v/v) glycerol, but no other additions, and stored at −20°C.

Assay methods

During the purification, ATP citrate lyase was assayed as described by Linn & Srere (1979). The protein concentrations of crude fractions were determined by the method of Bradford (1976). The protein concentration of pure ATP citrate lyase was determined by measurements of A_{280}, with the absorbance coefficient reported by Singh *et al.* (1976).

Fatty acid synthase, β-galactosidase and pyruvate kinase were assayed by standard methods (Carey & Dils, 1970; Wallenfels *et al.*, 1959; Valentine & Tanaka, 1966). ADP was assayed by the method of Adam (1963).

One unit of enzyme activity is defined as the amount required to catalyse the formation of 1μmol of product/min.

Polyacrylamide gel electrophoresis and estimation of M_r values

Non-denaturing gels (3%) were run by the procedure of Davis (1964); SDS/4% polyacrylamide gels were run as described by Weber & Osborn (1969). The marker proteins and their M_r values were fatty acid synthase (250000), the *N. crassa arom* complex (165000), β-galactosidase (118000), bovine serum albumin (68000) and glyceraldehyde-3-phosphate dehydrogenase (36000). The native M_r of ATP citrate lyase was estimated by analytical gel filtration on a column of Ultrogel A2 (1.6cm × 30cm) equilibrated in 50mM-potassium phosphate/20mM-potassium citrate, pH7.0. The marker proteins and their M_r values were haemocyanin (3×10^6), β-galactosidase (520000) apoferritin (440000) and pyruvate kinase (237000).

Kinetic studies

ATP citrate lyase was assayed by using two different coupled assays. In each case the assay mixture (1ml) contained 50mM-Mops (4-morpholinepropanesulphonic acid)/NaOH, pH7.0, 0.25M-KCl, 15mM-2-mercaptoethanol, 0.05mM-NADH and 0.424mM free Mg^{2+}. The amounts of ATP, magnesium acetate and potassium citrate required to give 0.424mM free Mg^{2+} and the indicated concentrations of MgATP and Mg-citrate were determined as described by Plowman & Cleland (1967).

In method 1, the production of oxaloacetate was linked to the oxidation of NADH and each assay contained 2.5 units of malate dehydrogenase. In method 2 the production of ADP was linked to the oxidation of NADH and each assay contained

1mM-phosphoenolpyruvate, 7units of pyruvate kinase and 7units of lactate dehydrogenase. For both methods reactions were carried out at 37°C and the rate of change of A_{340} was measured by using a chart recorder set at a full scale of 0.025 absorbance units. All solutions were passed through Millipore filters (0.22μm) before use. Assays were performed in duplicate or triplicate. Where replicate assays gave identical results only a single point is shown. Double-reciprocal plots were analysed by a weighted least-mean-squares procedure as described by Meek & Nimmo (1983).

Results and discussion

Purification and characterization of ATP citrate lyase

The purification procedure is described in the Experimental section and the results are summarized in Table 1. Our method differs from some others that have been published recently (Linn & Srere, 1979; Guy *et al.*, 1980) in that we deliberately avoided the use of fractionation with poly(ethylene glycol); such treatment has been shown to alter irreversibly the kinetic and physical properties of such enzymes as phosphofructokinase (Reinhart, 1980) and mammary gland acetyl-CoA carboxylase (B. Houston & H. G. Nimmo, unpublished work). Hoffman *et al.* (1979) also used dye-ligand chromatography to isolate ATP citrate lyase, and their procedure gave a much higher yield (50–70%) than does ours; however, the final specific activity that they obtained is considerably lower than our value. Our procedure is very reproducible and the final specific activity (13.6 ± 1.1 units/mg, $n = 13$) is at least comparable with the values reported by other groups (Linn & Srere, 1979; Redshaw & Loten, 1981; Singh *et al.*, 1976).

The purified enzyme was homogeneous by the criteria of denaturing and non-denaturing polyacrylamide-gel electrophoresis (Fig. 1). Only one protein-staining band was detectable on gels loaded with up to 20μg of protein. This indicates that, unlike many earlier preparations of ATP citrate lyase (see, e.g., Singh *et al.*, 1976) our purified enzyme was not proteolytically degraded. The values of the native and subunit M_r of the purified enzyme were estimated to be 500000 ± 20000 ($n = 3$) and 123000 ± 2000 ($n = 5$) respectively. These values are in agreement with those of previous workers (Singh *et al.*, 1976; Alexander *et al.*, 1979).

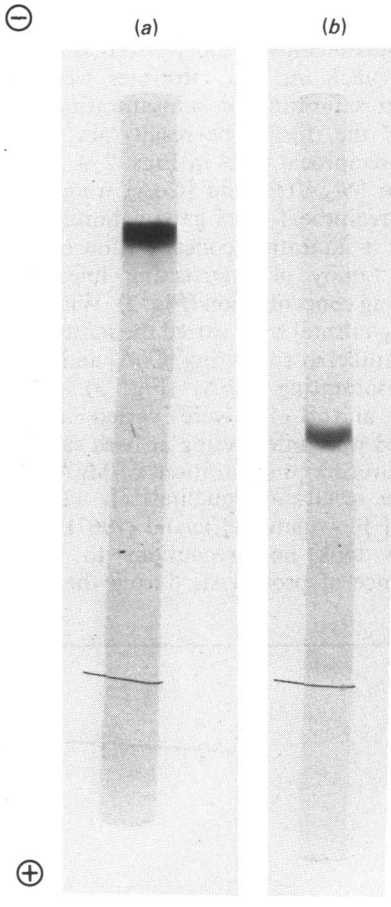

Fig. 1. *Polyacrylamide-gel electrophoresis of purified ATP citrate lyase*

(*a*) A 3% non-denaturing gel was loaded with 10μg of protein; (*b*) a 4% SDS gel was loaded with 6μg of protein.

Table 1. *Purification of ATP citrate lyase from rat liver*

Step	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
1 105000*g* supernatant	129	2400	380	0.14	1	100
2 30–40% $(NH_4)_2SO_4$ precipitate	8	380	344	0.9	6.4	90
3 DEAE-cellulose	125	54	241	4.4	32	63
4 Affi-gel Blue	230	9.4	103	10.9	78	27
5 Ultrogel AcA 44	4.6	2.8	40	13.6	97	11

Kinetic studies

Plowman & Cleland (1967) showed that the substrates of ATP citrate lyase were MgATP and Mg-citrate rather than the uncomplexed species. Our assays were carried out at a fixed concentration of free Mg^{2+} and the substrate concentrations shown in the Figures refer to the complexed species. Our initial experiments were carried out with assay method 1, in which the product oxaloacetate is removed by malate dehydrogenase. Three sets of initial velocity studies were carried out; in each set two substrates were varied at constant saturating or sub-saturating concentrations of the third. The results are displayed as double-reciprocal plots in Figs. 2–4.

When [MgATP] and [CoA] were varied, the double-reciprocal plots gave a family of parallel lines at a saturating concentration of Mg-citrate and a family of intersecting lines at a sub-saturating concentration (Fig. 2). When [MgATP] and [Mg-citrate] were varied the patterns obtained were parallel at saturating [CoA] and intersecting at sub-saturating [CoA] (Fig. 3). When [Mg-citrate] and [CoA] were varied, the patterns obtained were intersecting at both saturating and sub-saturating concentrations of MgATP (Fig. 4).

These results are qualitatively identical with those of Plowman & Cleland (1967). Since these workers took no precautions to prevent the occurrence of proteolysis during the isolation of ATP citrate lyase, this suggests that proteolysis does not cause a gross change in the kinetic mechanism of the enzyme. As discussed by Plowman & Cleland (1967), the patterns shown in Figs. 2–4 imply a sequential mechanism in which MgATP binds to the enzyme first and this is followed by random-order binding of Mg-citrate and CoA. They do not appear to be consistent with a double-displacement mechanism involving a phosphoenzyme intermediate. For such a mechanism, parallel patterns should be obtained for the plots shown in Figs. 2 and 3, irrespective of the concentrations of Mg-citrate (Fig. 2) or CoA (Fig. 3).

It is, however, well known that commercially available ATP can be contaminated with ADP. Indeed, we found by direct assays that our ATP solutions contained considerable amounts (up to 10%) of ADP. Thus ADP was present in all the assays shown in Figs. 2–4. It is therefore invalid to rule out a double-displacement mechanism with a phosphoenzyme intermediate on the basis of these results; for such a mechanism, the presence of ADP would render the release of ADP during the formation of the phosphoenzyme reversible. This in turn would cause the double-reciprocal plots shown in Figs. 2 and 3 to be intersecting rather than parallel provided that the concentration of the non-varied substrate was sub-saturating (Cleland, 1970), as indeed is observed.

We therefore repeated the experiments shown in

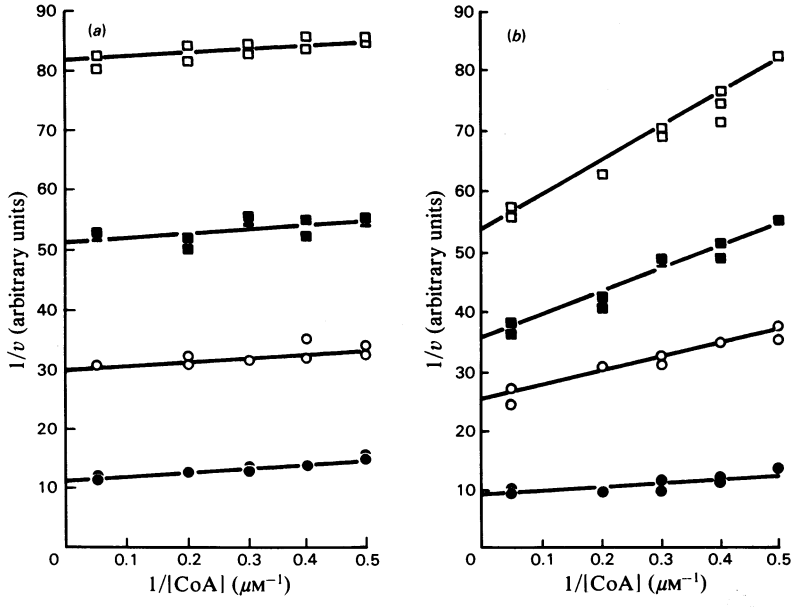

Fig. 2. *Initial-velocity studies with CoA and MgATP as the varied substrates*
Assays were performed with method 1 (see the Experimental section). The concentrations of Mg-citrate were (*a*) 9.0mM and (*b*) 0.15mM. The concentrations of MgATP were: 400μM (●), 76μM (○), 40μM (■) and 20μM (□).

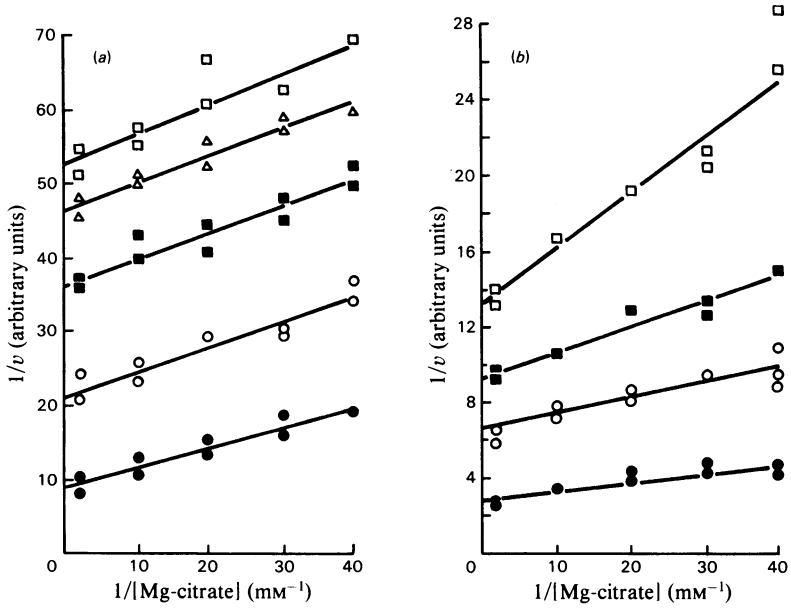

Fig. 3. *Initial-velocity studies with Mg-citrate and MgATP as the varied substrates*
Assays were performed with method 1. The concentrations of CoA were (*a*) 200 μM and (*b*) 10 μM. The concentrations of MgATP were: 400 μM (●), 76 μM (○), 40 μM (■), 26 μM (△) and 20 μM (□).

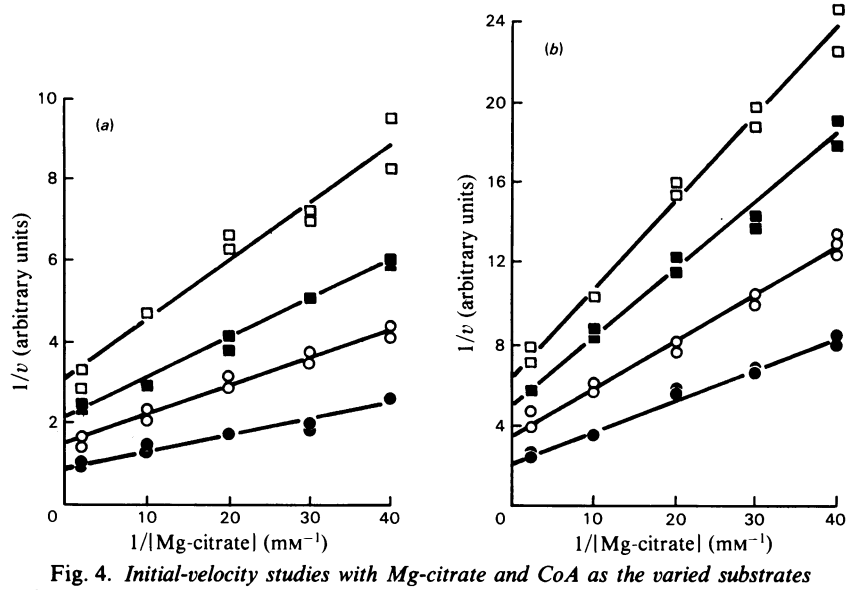

Fig. 4. *Initial-velocity studies with Mg-citrate and CoA as the varied substrates*
Assays were performed with method 1. The concentrations of MgATP were (*a*) 17.3 mM and (*b*) 200 μM. The concentrations of CoA were: 20 μM (●), 5 μM (○), 3 μM (■) and 1 μM (□).

Figs. 2–4 using assay method 2, in which ADP is removed by the actions of pyruvate kinase and lactate dehydrogenase; this ensures that initial velocities are measured under conditions in which ADP is absent. As shown in Fig. 5, patterns of parallel lines were obtained either when [MgATP] and [CoA] were varied at a subsaturating concentration of Mg-citrate or when [MgATP] and [Mg-citrate] were varied at a sub-saturating concentration of CoA. This is precisely the behaviour

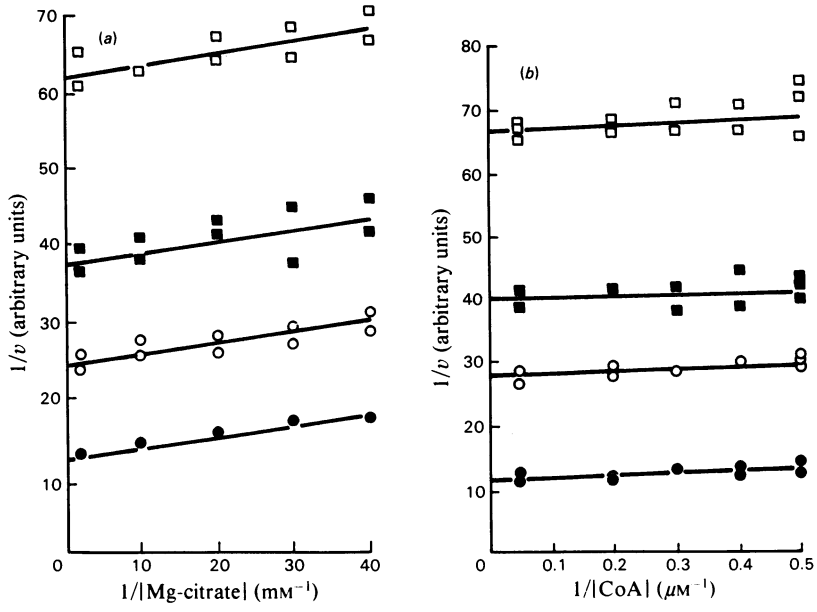

Fig. 5. *Initial-velocity studies in the absence of ADP*

Assay method 2 was used. (*a*) Mg-citrate was varied at a CoA concentration of 10 μM; (*b*) CoA was varied at a Mg-citrate concentration of 0.15 mM. The concentrations of MgATP were: 40 μM (●), 76 μM (○), 40 μM (■) and 20 μM (□).

expected of a double-displacement mechanism involving a phosphoenzyme intermediate. Thus the results of steady-state kinetics experiments carried out in the absence of ADP are in agreement with those of isotope-exchange and enzyme-labelling experiments.

We therefore attribute the fact that the kinetic plots shown in Figs. 2(*b*) and 3(*b*) are intersecting rather than parallel to the presence of ADP in the assays. Plowman & Cleland (1967) obtained patterns similar to those shown in Figs. 2 and 3. They took no special steps to remove ADP from solutions of ATP and they assayed ATP citrate lyase by a direct spectrophotometric assay in which ADP was not removed. It therefore seems likely that their results were also caused by the adventitious presence of ADP in the assays. Farrar & Plowman (1971) studied the kinetics of the enzyme in the reverse direction and concluded that its mechanism was sequential. However, it is very difficult to interpret the patterns that they obtained because it is quite likely that the acetyl-CoA used was contaminated with free CoA. As with the forward reaction, the presence of a product during the measurement of initial velocities could affect the kinetic pattern obtained for a double-displacement mechanism.

Plowman & Cleland (1967) concluded from results analogous with those shown in Figs. 2 and 3 that the order of binding of CoA and Mg-citrate must be random. This conclusion is still valid, even though the assays contained ADP, and clearly applies also to our undegraded enzyme. Thus our results show that in the forward direction ATP citrate lyase obeys a double-displacement mechanism. The formation of a phosphoenzyme intermediate and release of ADP is followed by random order binding of CoA and Mg-citrate. Our results do not pertain to events downstream of the formation of the phosphoenzyme·CoA·Mg-citrate complex. However, we have now developed the framework necessary to analyse the effects of phosphorylation on the kinetic properties of ATP citrate lyase. That analysis, and numerical values for the kinetic parameters of the enzyme, will be presented in a further paper.

Contamination of ATP with ADP may have caused anomalies in kinetic studies of other enzymes. One possible example concerns the controversy surrounding the kinetic mechanism of acetyl-CoA carboxylase. Hashimoto & Numa (1971) suggested a double-displacement mechanism on the basis of kinetic studies in which they employed a coupled assay based on the removal of ADP. This mechanism is consistent with much that is known about the structure and the chemical mechanism of the enzyme (see, e.g., Lane *et al.*, 1974). However, also on the basis of steady-state kinetics, Beaty & Lane (1982) proposed a sequential mechanism involving a quaternary complex.

They used a [^{14}C]bicarbonate fixation assay and they took no special steps to remove ADP from ATP. Using arguments similar to those advanced above for ATP citrate lyase, the discrepancy between these two studies could be explained simply by postulating the presence of ADP in the assays carried out by Beaty & Lane (1982).

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