Multiple chromatographic forms of ATP citrate lyase from rat liver

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ATP citrate lyase is shown to exist as multiple forms in extracts of rat liver. DEAE-Sephadex ion-exchange chromatography of liver supernatants reveals two peaks of activity. A minor, basic, component, comprising 14% of the recovered activity, is eluted without retention, whereas the major, acidic, form is eluted by a KCl gradient. Gel filtration of similar extracts shows the presence of a high- M_r form of ATP citrate lyase (M_r around 10^7) in addition to the tetrameric enzyme (M_r 4.1×10^5). This associated state, which represents 10% of the total activity, is unstable, breaking down to the tetramer, and appears to be disrupted by Mg^{2+} . The basic form changes in the partially purified state to give the acidic form. Most of the high- M_r enzyme is acidic in nature. No evidence could be found for an association of the enzyme with mitochondrial or microsomal membranes. ATP citrate lyase from rat brain also shows two peaks of activity on DEAE-Sephadex ion-exchange chromatography, but the activity is distributed between the peaks in almost equal proportions. However, only the tetrameric enzyme was observed on gel filtration.

ATP citrate lyase [ATP citrate (*pro-3S*)-lyase, EC 4.1.3.8] is a cytoplasmic enzyme that in liver and other tissues serves to produce acetyl groups, the precursors for lipogenesis, from citrate exported by mitochondria (Daikuhara *et al.*, 1968; Watson & Lowenstein, 1970). On the metabolic route from carbohydrate to lipid, the ATP citrate lyase reaction is the first enzymic step that is exclusively lipogenic. Because of this position it might be expected that ATP citrate lyase is a regulatory enzyme, particularly since the reaction catalysed is irreversible and consumes ATP. However, it is the next enzyme of the pathway, acetyl-CoA carboxylase, that is usually considered to be the regulatory enzyme of fatty acid biosynthesis.

Interest in the possibility that ATP citrate lyase is regulatory has been stimulated by the discovery that this enzyme is subject to reversible phosphorylation in hepatocytes and adipocytes (Alexander *et al.*, 1979; Ramakrishna & Benjamin, 1979). Both cyclic AMP-dependent and cyclic AMP-independent kinases are involved (Guy *et al.*, 1980; Alexander *et al.*, 1981; Ramakrishna & Benjamin, 1981; Ramakrishna *et al.*, 1981), and this phosphorylation is distinct from that which occurs as part of the reaction mechanism (Janski *et al.*, 1979; Linn & Srere, 1979). As yet it has not been possible to find

any regulatory significance of this modification of the enzyme (Ranganathan *et al.*, 1980; Guy *et al.*, 1981).

We have investigated the properties of ATP citrate lyase in rat tissues, and in the present paper provide evidence that the enzyme is not homogeneous but exists as several forms separable by column chromatography. This heterogeneity appears to be independent of the possible mitochondrial association reported for this enzyme (Ranganathan *et al.*, 1980; Janski & Cornell, 1980*a,b*).

Materials and methods

Materials

Malate dehydrogenase, substrates, cofactors, M_r -standard proteins, mercaptoethanol and dithiothreitol were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DEAE-Sephadex A-25, Sepharose 2B, Sepharose 6B and Blue Dextran 2000 were from Pharmacia (Uppsala, Sweden). $NaH^{14}CO_3$ (56 Ci/mol) was purchased from Amersham International (Amersham, Bucks., U.K.).

Animals

Adult female Wistar rats were obtained from the colony maintained at 21°C in the Department of Biochemistry, Royal Holloway College, on PRD

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diet (Labsure, Poole, Dorset, U.K.). Normal fed rats were allowed food and water *ad libitum*. For most experiments on liver extracts, animals were allowed water only for 48 h, and then re-fed for 72 h on the above diet with 5% (w/v) sucrose added to the drinking water. This procedure serves to induce the synthesis of liver ATP citrate lyase (Suzuki *et al.*, 1967).

Assays of enzyme activities

ATP citrate lyase activity was determined by a coupled spectrophotometric assay (Srere, 1959) by using a procedure modified from that of Szutowicz *et al.* (1974). The assay mixture contained 50 mM-Tris/HCl buffer, pH 7.8, 10 mM-MgCl₂, 100 mM-KCl, 5 mM-ATP, 0.2 mM-CoA, 20 mM-citrate, 0.15 mM-NADH, 10 mM-2-mercaptoethanol, 2 units of malate dehydrogenase and enzyme sample in a final volume of 1.0 ml. Assays were preincubated for 7 min at 37°C, and the reaction was started by the addition of CoA. The oxidation of NADH was monitored continuously at 340 nm in a Beckman 25 or 3600 spectrophotometer. Acetyl-CoA carboxylase (Inoue & Lowenstein, 1975), citrate synthase (Ochoa, 1955), fatty acid synthetase (Lynen, 1969), lactate dehydrogenase (Stolzenbach, 1966) and rotenone-insensitive NADH-cytochrome c reductase (Smoly *et al.*, 1971) activities were assayed by established methods. For all enzymes, 1 unit of activity is defined as the amount of enzyme required to transform 1 μ mol of substrate/min at 37°C.

Column chromatography

Ion-exchange chromatography was performed by the procedure of Hoffmann *et al.* (1979). Tissues were homogenized in 20 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-EDTA, 1 mM-dithiothreitol and 0.25 M-sucrose, in a Potter-Elvehjem homogenizer [liver 1:4 (w/v), brain 1:2 (w/v)]. Homogenates were centrifuged at 45 000 *g* for 30 min at 4°C, and the supernatants obtained were dialysed against 30 vol. of homogenizing buffer for 2 h. Dialysed supernatants were applied to DEAE-Sephadex A-25 columns (see Figure legends for dimensions) previously equilibrated with elution buffer, which was as above but without sucrose. After 2 bed volumes, a linear gradient of KCl (0–0.4 M) was applied in the elution buffer. Gel-filtration chromatography was performed in a similar way, except that, since omission of the dialysis did not appear to influence the resulting elution profile, supernatants were normally applied without dialysis. Samples (liver 5 ml, brain 10 ml) were applied to Sepharose columns (2.2 cm $\times$ 25 cm), which were then eluted with buffer without sucrose, and 2.5 ml fractions were collected. The void volumes of the columns were determined by the elution of Blue Dextran 2000. For both types of chromatography, fractions

were collected into tubes containing 0.125 vol. of 1 M-Tris/HCl buffer, pH 8.0, containing 10 mM-dithiothreitol.

Results

Ion-exchange chromatography

Anion-exchange chromatography on DEAE-Sephadex of liver extracts reveals two peaks of ATP citrate lyase activity (Fig. 1). The first peak is eluted without retention, whereas the second binds to the column, to be eluted by the salt gradient. For a series of five experiments the average recovery of activity in the first eluted peak was $14.2 \pm 1.6\%$ (mean $\pm$ S.E.M.). Our findings differ from those obtained by Hoffmann *et al.* (1979), who observed only a single peak of liver ATP citrate lyase activity, which was eluted with the salt gradient.

One possible interpretation of our observations would be that the non-retained peak is an artifact arising from overloading the ion-exchanger. If this were the case, decreasing the sample size should give rise to the loss of this first eluted peak. However, when the sample volume was decreased from 34 ml to 8 ml and with columns of similar bed volume (90 ml), there was no such change in the elution profile (results not shown). Hence this possibility can be excluded.

DEAE-Sephadex chromatography was performed under the same conditions on liver from animals starved and re-fed as described in the

Fig. 1. DEAE-Sephadex A-25 ion-exchange chromatography of ATP citrate lyase activity of crude extract from rat liver

Tissue supernatant (34 ml) from normal fed rats was applied to a 2.2 cm $\times$ 22 cm column of DEAE-Sephadex. ATP citrate lyase activity (●) was eluted with 20 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-EDTA and 1 mM-dithiothreitol, followed by a gradient of 0–0.4 M-KCl (○). Fractions (8 ml) were collected into 0.125 vol. of 1 M-Tris/HCl buffer, pH 8.0, containing 10 mM-dithiothreitol.

Materials and methods section. This dietary regimen gave rise to increases between 2- and 7-fold over the normal activity of 1.8 units/g wet wt. of liver. The same two-peak profile of ATP citrate lyase activity was obtained with no detectable change in the percentage of activity eluted as the non-retained peak ($17.7 \pm 3.6\%$, mean $\pm$ S.E.M., $n = 7$). Since the dietary manipulation did not alter the total protein content of the liver extracts, it was possible to compare the profiles for induced and normal fed rats, either by using the same total protein load but different ATP citrate lyase activities, or by using the same ATP citrate lyase activities but different amounts of total protein. This series of experiments (results not shown) produced the same proportion of non-retained activity throughout, consistent in each case with the above values. This is further evidence that the observed two-peak profile is not due to overloading of the ion-exchanger.

When 0.2 mM-phenylmethanesulphonyl fluoride was added to buffers used for homogenization, dialysis and chromatography, the two-peak ion-exchange profile obtained remained unchanged (results not shown). This proteolytic inhibitor protects liver acetyl-CoA carboxylase from proteolysis, which markedly affects the properties of that enzyme (Song & Kim, 1981). Thus neither of the observed ion-exchange peaks of liver ATP citrate lyase appears to be produced as an artifact by proteolytic degradation occurring during or after extraction.

When the dialysis period was extended to 24 h, both ion-exchange peaks were still observed. Similarly, extracts aged for 24 h at 5°C before the normal 2 h dialysis also contained both fractions of activity.

To test the possibility that the heterogeneity revealed here is due to mitochondrial-membrane binding, the chromatographic fractions were assayed for rotenone-insensitive cytochrome *c* reductase activity. Only a single peak of activity was found, and this was eluted with the salt gradient after the second peak of ATP citrate lyase activity (results not shown).

Ion-exchange chromatography of brain supernatants resulted in a similar two-peak elution profile of ATP citrate lyase activity. The only observable difference was that the first eluted peak contained a larger proportion of the total recovered activity, $39.9 \pm 7.4\%$ (mean $\pm$ S.E.M., $n = 3$). This result is significantly different from that for liver from both normal fed and induced rats at the $P = 0.05$ level for both Student's *t* and Mann-Whitney *U* tests.

Gel-filtration chromatography

Further studies of the heterogeneity of ATP citrate lyase were performed by gel-filtration chromatography on Sepharose 6B. As shown in Fig. 2,

Fig. 2. Gel filtration of rat liver extract on Sepharose 6B. Supernatant (5 ml) from liver of a starved and re-fed rat was applied to a 2.2 cm $\times$ 25 cm column of Sepharose 6B, which was eluted with 20 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-EDTA and 1 mM-dithiothreitol. ●, ATP citrate lyase activity; ○, lactate dehydrogenase activity. The arrow indicates the void volume (V_0).

there is a minor peak of activity eluted close to the void volume, followed by a major peak containing 90% of the recovered activity. Liver lactate dehydrogenase, used here as a cytoplasmic marker enzyme, shows no equivalent peak of activity at the void volume. This indicates that the high- M_r fraction of ATP citrate lyase is not due to entrapment of cytoplasm by membrane fragments present in the sample.

In a series of nine Sepharose 6B gel-filtration experiments, only one failed to show the presence of ATP citrate lyase activity at the void volume. In all other analyses, approx. 10% of the total recovered activity was present as the void-volume peak regardless of whether the animal had been normal fed, starved or starved and re-fed. In contrast with these results for liver, chromatography of brain extracts on Sepharose 6B always produced only a single peak of activity, corresponding to the major peak of the liver enzyme. This suggests that the high- M_r activity is not a methodological artifact, but arises either from the effect of some component of the liver extract, or from some specialized property of the liver enzyme itself.

The method of Andrews (1965) was used to estimate the M_r of the second-eluted and major peak of liver ATP citrate lyase obtained by gel filtration (Fig. 3). The result, M_r 4.1×10^5 , closely agrees with the value, M_r 4.4×10^5 , previously obtained by sedimentation equilibrium (Singh *et al.*, 1976). This is the first report of the use of gel-filtration chromatography in the estimation of the M_r of ATP citrate lyase, and it establishes that the major

Fig. 3. Estimation of M_r for the major peak of liver ATP citrate lyase activity by gel filtration on Sepharose 6B. Calibration standards (●): L, lactate dehydrogenase (M_r 134000); C, catalase (M_r 210000); P, pyruvate kinase (M_r 237000); F, ferritin (M_r 440000); T, thyroglobulin (M_r 669000). The continuous line was plotted by linear-regression analysis. Broken lines indicate the observed K_{av} value for the major ATP citrate lyase peak of liver (see Fig. 2) and the estimated $\log M_r$ value.

gel-filtration peak of the liver enzyme, and the only peak of the brain enzyme, correspond to the tetrameric enzyme molecule. The minor gel-filtration peak of the liver enzyme therefore represents some form of high- M_r association state, which, since it is eluted at the void volume of the column of Sepharose 6B, must be of minimum M_r 4×10^6 .

The chromatographic properties of the liver enzyme were further investigated by gel filtration on Sepharose 2B (Fig. 4). Here the elution profile, which is typical of three experiments, shows a gradual increase in ATP citrate lyase activity from just after the void volume until a peak, comprising 90% of the activity and corresponding to the tetrameric M_r , is reached. The profile of the high- M_r enzyme activity suggests that it is not a single species but consists of a range of sizes around M_r 10^7 .

Column fractions were assayed for acetyl-CoA carboxylase and fatty acid synthetase activities. Neither of these two lipogenic enzymes was present in the fractions containing the minor, void-volume, peak of ATP citrate lyase, but were found as single chromatographic peaks corresponding to their accepted M_r values.

Sepharose 2B gel-filtration chromatography was also performed on liver extracts homogenized in the absence of sucrose. The sucrose-free extracts contained a higher activity, compared with normally prepared extracts, of the mitochondrial-matrix enzyme citrate synthase (0.011 and 0.001 unit/ml

Fig. 4. Gel filtration of rat liver extract on Sepharose 2B. Liver supernatant (5 ml) from a starved and re-fed rat was applied to a 2.2 cm $\times$ 25 cm column of Sepharose 2B. ●, ATP citrate lyase activity. The arrows indicate the void volume (V_0) and the elution of the standard proteins thyroglobulin, T (M_r 669000), and ferritin, F (M_r 440000). The broken line represents the separation of the two components of the activity and is drawn according to the symmetry of the major component.

respectively). However, this disruption of mitochondria in the absence of sucrose did not result in any appreciable increase in the proportion of the high- M_r ATP citrate lyase activity (results not shown).

Re-chromatography

To investigate the nature of the multiple forms of liver ATP citrate lyase, a series of re-chromatography experiments was performed in which the most active fractions of the various chromatographic peaks were subjected to a second chromatographic separation (Table 1). On re-chromatography of the first-eluted peak from DEAE-Sephadex, all the recovered activity was eluted only with

the salt gradient. These observations suggest that the basic, non-retained, form has changed into the more acidic and retained form.

This change in the elution characteristics is rapid, since it is seen to be complete even though the re-chromatography is performed immediately. Such behaviour is in contrast with the observation that crude liver supernatants, either dialysed or aged without dialysis for 24 h before application to the ion-exchange, retain both chromatographic peaks of activity.

When the major gel-filtration peak was re-run on Sepharose 6B, the activity was recovered at the same elution volume, indicating that the enzyme tetramer is stable. However, re-chromatography of the high- M_r enzyme (void-volume peak) on Sepharose

6B showed that this associated form is unstable, releasing the tetrameric enzyme. The re-chromatography on DEAE-Sephadex of the peaks obtained by gel filtration also yielded only the acidic activity, with the exception of a small amount of activity eluted as the basic form when the major peak from Sepharose 6B was used. As indicated in Table 1, the percentage recovery of activity from the second column was variable, but this can be accounted for by the availability of only small amounts of enzyme during such experiments, and also by the instability of ATP citrate lyase. The enzyme requires thiol protection in the form of dithiothreitol, both in the elution buffer and in the buffer into which the chromatographic fractions are collected (Cottam & Srere, 1969).

Table 1. *Re-chromatography of various chromatographic fractions of rat liver ATP citrate lyase*

Re-chromatography was performed with 20 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-EDTA and 1 mM-dithiothreitol, with a step gradient of 0.4 M-KCl to elute retained activity from the DEAE-Sephadex columns. Experimental conditions for individual re-runs on DEAE-Sephadex were as follows. DEAE-Sephadex basic peak sample (6 ml) applied to a 1.6 cm $\times$ 9 cm column, 1.6 ml fractions being collected; this run is typical of three. Sepharose 6B peak samples (2 ml) applied to 0.9 cm $\times$ 4.5 cm columns, 1 ml fractions being collected. Re-chromatography on Sepharose 6B involved 4.0 ml samples applied to 2.2 cm $\times$ 25 cm columns, 2.5 ml fractions being collected.

Source of sample	Units applied	Re-chromatography on DEAE-Sephadex		Recovery of activity (%)
		Units recovered as basic peak	Units recovered as acidic peak	
DEAE-Sephadex basic peak	0.31	0.00	0.21	68
Sepharose 6B void-volume peak	0.15	0.00	0.15	100
Sepharose 6B major peak	1.20	0.013	0.63	54
Re-chromatography on Sepharose 6B				
		Units recovered as void-volume peak	Units recovered as major peak	
Sepharose 6B major peak	3.1	0.00	2.6	84
Sepharose 6B void-volume peak	0.37	0.00	0.36	97

Table 2. *Effect of high-speed centrifugation on liver ATP citrate lyase elution profile on DEAE-Sephadex ion-exchange chromatography*

Livers from two starved and re-fed rats were homogenized as described in the Materials and methods section, and the homogenates were mixed. Samples were then centrifuged as indicated for 90 min and the supernatants were dialysed against 50 vol. of 20 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-EDTA, 1 mM-dithiothreitol and 0.25 M-sucrose. Samples (1.5 ml) were applied to 0.9 cm $\times$ 4.5 cm columns of DEAE-Sephadex. The columns were eluted with buffer without sucrose, and after 3 bed volumes a step gradient of 0.4 M-KCl was applied. Fractions (1 ml) were collected. The values shown are averages for duplicate columns, and the results are typical of three experiments.

	Units applied	Activity recovered as basic peak		Activity recovered as acidic peak		Total recovered activity	
		(units)	(% of applied activity)	(units)	(% of applied activity)	(units)	(% of applied activity)
Control supernatant (45 000 g)	1.2	0.235	19	0.89	73	1.125	92
High-speed supernatant (150 000 g)	0.93	0.46	49	0.53	57	0.99	106
	-0.27	+0.225		-0.36		-0.135	

Effect of high-speed centrifugation on elution profiles of the liver enzyme

A further series of chromatographic analyses was performed on liver supernatants prepared by centrifugation at 150 000g for 90 min, in place of the 45 000g for 30 min normally employed. Sepharose 6B gel-filtration chromatography of this high-speed supernatant (Fig. 5) revealed the presence of only the tetrameric enzyme. A control sample obtained from the same homogenate but centrifuged simultaneously at 45 000g for 90 min was found to contain both major and minor peaks of activity, and in proportions similar to those observed in Fig. 2 (results not shown). The effect of centrifugation on the ion-exchange profile is shown in Table 2. Both ion-exchange peaks are still present in the high-speed supernatant, even though the high- M_r component is absent.

Corresponding to the removal of the high- M_r peak there is a decrease in the total activity of the high-speed supernatant (Table 2). It is of some interest to determine the nature of this lost activity. There is in fact a loss of a similar magnitude from the acidic peak, suggesting that the high- M_r enzyme activity normally resides there.

However, an anomalous increase in activity is apparent in the basic peak. This increase was found

Fig. 5. Effect of high-speed centrifugation of liver extracts on the elution of ATP citrate lyase activity from Sepharose 6B

Liver homogenate from starved and re-fed rats was centrifuged at 150 000g for 90 min at 4°C. Supernatant (5 ml) was used in a chromatographic separation performed under conditions identical with those described in Fig. 2 legend.

in all experiments and, as shown in Table 2, cannot be accounted for by variations in the recovery of activity from the columns. Such an unexpected change in the activity of this peak could mask the loss of enzyme. Thus the possibility that some high- M_r enzyme activity is basic in nature cannot be excluded.

Effect of Mg^{2+} ions on the gel-filtration profile of the liver enzyme

The effect of additional salts on the Sepharose 6B gel-filtration profile of liver supernatants (45 000g)

Fig. 6. Effects of Mg^{2+} and Na^+ ions on the elution of rat liver ATP citrate lyase activity from Sepharose 6B Rat liver supernatants were prepared from starved and re-fed rats as described in the Materials and methods section except that all buffers contained either (a) 20 mM-NaCl or (b) 10 mM- $MgCl_2$. Supernatants (5 ml) were applied to 2.2 cm $\times$ 25 cm columns of Sepharose 6B, which were eluted with buffers containing the respective added chloride salt. ●, ATP citrate lyase activity. The absorbance at 280 nm (○) was measured at 1:9 (v/v) dilution of fractions in distilled water. The arrow indicates the void volume (V_0).

was tested by experiments in which either 10mM-MgCl₂ or 20mM-NaCl was present in both homogenization and elution buffers. Comparison of Fig. 6(a) with Fig. 2 shows that NaCl at this concentration is without apparent effect. However, MgCl₂ has caused the removal of the high- M_r peak of activity (Fig. 6b). It is also seen that loss has occurred of an A_{280} peak, which in Fig. 6(a) is co-eluted with this high- M_r enzyme. Since Mg²⁺ ions do not cause any inhibition of ATP citrate lyase under our assay conditions, and since no ATP citrate lyase activity can be detected in resuspended pellets from liver homogenized in the presence of Mg²⁺, these observations suggest that the high- M_r component is being disrupted to release tetrameric enzyme.

Discussion

We present here evidence that ATP citrate lyase in crude liver extracts is heterogeneous in both size and ionic charge. Anion-exchange chromatography reveals the presence of a minor, basic, peak comprising 14% of the total recovered activity. The remaining activity binds to DEAE-Sephadex under the conditions employed and is eluted by a KCl gradient. Our demonstration of two ion-exchange peaks in crude tissue extracts contrasts with the results obtained by Hoffmann *et al.* (1979), who in their original use of this method found only a single, acidic, peak of liver ATP citrate lyase.

Gel-filtration chromatography also reveals ATP citrate lyase heterogeneity in crude liver extracts. Approx. 10% exists in an associated high- M_r state (M_r around 10⁷), whereas the remaining activity exists as the tetrameric enzyme (M_r 4.1 × 10⁵).

It may be seen that the minor peak of ion-exchange chromatography and the minor component on gel filtration are present in similar proportions, namely 10–15%. Moreover, on re-chromatography both these minor peaks change, since they are eluted as the major form of the activity. The simplest interpretation of the heterogeneity of the liver enzyme is therefore that there would be two forms, one basic and of high M_r , and the other acidic, tetrameric, enzyme. We published such an explanation in a preliminary account of this work (Corrigan & Rider, 1981). However, the observation reported in the present paper that both ion-exchange peaks remain when the high- M_r activity has been removed by high-speed centrifugation renders such a simple account untenable. In this experiment there is evidence for acidic tetrameric, basic tetrameric and acidic high- M_r forms of the enzyme. However, the existence of basic high- M_r enzyme activity cannot at present be excluded.

Our findings indicate that the heterogeneity of brain ATP citrate lyase is less complex than that found in liver. In brain extracts, both the ion-

exchange peaks of activity are found, albeit in near-equal proportions. However, on gel filtration only tetrameric enzyme is found, the high- M_r material being absent. Thus brain appears to contain only two forms of the enzyme, acidic and basic, both of which are tetrameric. This demonstration that for the brain enzyme ion-exchange heterogeneity occurs in the absence of size heterogeneity supports our view that for the liver enzyme the two types of heterogeneity are independent of each other.

Prolonged dialysis or aging of crude liver extracts indicates that both ion-exchange forms persist in otherwise untreated supernatants. However, re-chromatography of the ion-exchange fractions establishes that the basic form of the liver enzyme changes rapidly in the partially purified condition to give the acidic peak. Similarly, re-chromatography of the high- M_r enzyme activity shows that this form is also unstable and disintegrates to the tetrameric state. These results suggest that the observed heterogeneity of ATP citrate lyase does not arise from the existence of true isoenzymes, but is due to alternative states in which the enzyme can exist.

Studies elsewhere on hepatocytes with the detergents digitonin and Kyro EOB have indicated that 25% of the ATP citrate lyase activity is either bound to mitochondrial membranes (Janski & Cornell, 1980a) or located in the mitochondrial matrix (Janski & Cornell, 1980b). Also, it has been shown that 10% of the activity of purified liver ATP citrate lyase binds to mitochondrial preparations (Ranganathan *et al.*, 1980). The integrity of the mitochondria in our preparations is preserved by the presence of iso-osmotic sucrose. Therefore any enzyme bound to mitochondria will have been removed from the supernatants by the centrifugation normally employed. Nonetheless, we have sought to determine whether either the ion-exchange or the gel-filtration heterogeneity could represent ATP citrate lyase attached to fragments of mitochondrial outer membrane stripped off during homogenization. Rotenone-insensitive cytochrome *c* reductase was employed during ion-exchange chromatography as a marker for the mitochondrial outer membrane. As neither of the peaks of ATP citrate lyase is co-eluted with this enzyme, the ion-exchange heterogeneity appears to be independent of any possible mitochondrial binding. Since rotenone-insensitive NADH-cytochrome *c* reductase is also present on liver microsomal membranes (Sottocasa *et al.*, 1967), the possibility of binding to microsomal membranes may be similarly excluded. Furthermore, the persistence of both ion-exchange peaks in supernatants prepared by centrifugation at 150 000 *g* for 90 min (Table 2) and at 100 000 *g* for 60 min (results not shown) also rules out the possibility that the ion-exchange heterogeneity is due to membrane binding.

Gel-filtration chromatography indicates a component of very high M_r , and therefore particular attention to the possibility of mitochondrial binding must be paid here. The fragmentation of mitochondria caused by the removal of sucrose from the homogenizing medium should result in greater yields of mitochondrial membranes in our supernatants. However, no increase in the proportion of high- M_r ATP citrate lyase was detected. Moreover, in subcellular-fractionation studies we have been unable to detect ATP citrate lyase in mitochondrial fractions (results not shown), although this could be due to the instability under the conditions employed of the putative mitochondrial association. Further doubt is cast on the possibility that the high- M_r enzyme activity is bound to mitochondrial membranes by our observations on the effect of Mg^{2+} ions. Janski & Cornell (1980a) have shown that 10mM- Mg^{2+} stabilized the association of ATP citrate lyase with mitochondrial membranes in a manner similar to its effect on the binding of hexokinase, another ATP-utilizing enzyme, to the mitochondrial outer membrane (Rose & Warms, 1967). In the present work, however, we find that 10mM- Mg^{2+} appears to disrupt our high- M_r form, liberating the tetrameric enzyme. Finally, binding to membranes, whether mitochondrial or microsomal, is unlikely, since the high- M_r fraction was present in unchanged proportions when the routine centrifugation conditions were replaced by 100 000g for 60 min (results not shown).

It could be that the high- M_r enzyme activity is due to self-association of ATP citrate lyase. Previously, traces of octameric enzyme have been detected in purified preparations of the mammary-gland enzyme, which is otherwise tetrameric (Guy *et al.*, 1981). The possibility that ATP citrate lyase exists in liver as a highly polymerized form in addition to the tetramer thus requires further investigation.

An alternative explanation is that the high- M_r form involves the association of ATP citrate lyase with other cell constituents. The enzyme catalysing the next step in lipogenesis, acetyl-CoA carboxylase, undergoes reversible polymerization in the presence of citrate as part of its regulation, and linear chains of similar size to the high- M_r ATP citrate lyase are produced (Lane *et al.*, 1974). Under the chromatographic conditions employed in the present work, acetyl-CoA carboxylase does not polymerize and therefore is not co-eluted with the associated form of ATP citrate lyase.

In the context of the possible association of ATP citrate lyase with other proteins, it is noteworthy that acetyl-CoA carboxylase has been found to bind to a specific kinase (Lent & Kim, 1982). This protein has been shown to be self-associating as well as capable of binding to the carboxylase. One might speculate that our observations could be explained by the

existence of a similar protein binding to ATP citrate lyase.

We have shown in the present work that liver ATP citrate lyase exists in crude extracts in at least three alternative states separable by column chromatography. We conclude that this heterogeneity is not due to binding to mitochondria, nor to co-polymerization with the other lipogenic enzymes, acetyl-CoA carboxylase and fatty acid synthetase. Currently, there is considerable interest in the possible involvement of ATP citrate lyase in the short-term regulation of lipogenesis. In particular, although the enzyme is subject to complex reversible phosphorylation, apparently under hormonal influence (Guy *et al.*, 1980; Alexander *et al.*, 1981; Ramakrishna & Benjamin, 1981; Ramakrishna *et al.*, 1981), no progress in finding the regulatory significance of this modification has yet been made (Janski *et al.*, 1979; Ranganathan *et al.*, 1980; Guy *et al.*, 1981). Future work on the regulatory importance of this enzyme should take into account the evidence given in the present paper that ATP citrate lyase appears to exist in various states, some of which are unstable on partial purification.

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References

- Alexander, M. C., Kowaloff, E. M., Witters, L. A., Dennihy, D. T. & Avruch, J. (1979) *J. Biol. Chem.* **254**, 8052–8056
- Alexander, M. C., Palmer, J. L., Pointer, R. H., Koumjian, L. & Avruch, J. (1981) *Biochim. Biophys. Acta* **674**, 37–47
- Andrews, P. (1965) *Biochem. J.* **96**, 595–606
- Corrigan, A. P. & Rider, C. C. (1981) *Biochem. Soc. Trans.* **9**, 557–558
- Cottam, G. L. & Srere, P. A. (1969) *Arch. Biochem. Biophys.* **130**, 304–311
- Daikuhara, Y., Tsunemi, T. & Takeda, Y. (1968) *Biochim. Biophys. Acta* **158**, 51–61
- Guy, P. S., Cohen, P. & Hardie, D. G. (1980) *FEBS Lett.* **109**, 205–208
- Guy, P. S., Cohen, P. & Hardie, D. G. (1981) *Eur. J. Biochem.* **114**, 399–405
- Hoffmann, G. E., Schiessl, J. & Weiss, L. (1979) *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 1445–1451
- Inoue, H. & Lowenstein, J. M. (1975) *Methods Enzymol.* **35B**, 3–11
- Janski, A. M. & Cornell, N. W. (1980a) *Biochem. Biophys. Res. Commun.* **92**, 305–312
- Janski, A. M. & Cornell, N. W. (1980b) *Biochem. J.* **186**, 423–429

- Janski, A. M., Srere, P. A., Cornell, N. W. & Veech, R. L. (1979) *J. Biol. Chem.* **254**, 9365–9368
- Lane, M. D., Moss, J. & Polakis, S. E. (1974) *Curr. Top. Cell. Regul.* **8**, 139–195
- Lent, B. & Kim, K.-H. (1982) *J. Biol. Chem.* **257**, 1897–1901
- Linn, T. C. & Srere, P. A. (1979) *J. Biol. Chem.* **254**, 1691–1698
- Lynen, F. (1969) *Methods Enzymol.* **14**, 17–33
- Ochoa, S. (1955) *Methods Enzymol.* **1**, 685–694
- Ramakrishna, S. & Benjamin, W. B. (1979) *J. Biol. Chem.* **254**, 9232–9236
- Ramakrishna, S. & Benjamin, W. B. (1981) *FEBS Lett.* **124**, 140–144
- Ramakrishna, S., Pucci, D. L. & Benjamin, W. B. (1981) *J. Biol. Chem.* **256**, 10213–10216
- Ranganathan, N. S., Srere, P. A. & Linn, T. C. (1980) *Arch. Biochem. Biophys.* **204**, 52–58
- Rose, I. A. & Warms, J. V. B. (1967) *J. Biol. Chem.* **242**, 1635–1645
- Singh, M., Richards, E. G., Mukherjee, A. & Srere, P. A. (1976) *J. Biol. Chem.* **251**, 5242–5250
- Smoly, J. M., Wakabayashi, T., Addink, A. D. F. & Green, D. E. (1971) *Arch. Biochem. Biophys.* **143**, 6–21
- Song, C. S. & Kim, K.-H. (1981) *J. Biol. Chem.* **256**, 7786–7788
- Sottocasa, G. L., Kuylenstierna, B., Ernster, L. & Bergstrand, A. (1967) *J. Cell Biol.* **32**, 415–424
- Srere, P. A. (1959) *J. Biol. Chem.* **234**, 2544–2547
- Stolzenbach, F. (1966) *Methods Enzymol.* **9**, 278–288
- Suzuki, F., Fukunishi, K., Daikuhara, Y. & Takeda, Y. (1967) *J. Biochem. (Tokyo)* **62**, 170–178
- Szutowicz, A., Stepien, M. & Angielski, S. (1974) *J. Neurochem.* **22**, 85–91
- Watson, J. A. & Lowenstein, J. M. (1970) *J. Biol. Chem.* **245**, 5993–6002