that only with the ferroheme enzyme, and not with the ferriheme enzyme, can saturation of the allosteric site(s) evoke a conformational change reflected as an elevated $s_{20,w}$ (Poillon and Feigelson, 1971). Thus, presumably it is this allosterically induced taut configuration of the ferroheme tryptophan oxygenase which manifests enhanced affinity for the artificial ligand, carbon monoxide, and for its normal substrate–ligand, oxygen.

Acknowledgments

We are grateful to Myong Won Lee for her expert technical assistance in the purification of the enzyme. We thank Dr. William Blumberg, Bell Telephone Laboratories, and Dr. Jack Peisach, Albert Einstein School of Medicine, for their collaboration in the electron paramagnetic resonance studies. We thank Drs. Henry Jay Forman, Frank O. Brady, and William N. Poillon for their critical roles in the preparation of this manuscript.

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# Aspartate Transcarbamylases of *Citrobacter freundii**

Mary Sue Coleman† and Mary Ellen Jones‡

ABSTRACT: Two aspartate transcarbamylases (EC 2.1.3.2) which have different molecular weights can be separated from each other when an extract of *Citrobacter freundii* ATCC 8090 is subjected to gel filtration chromatography. Both enzymes have very similar, but not identical, kinetic characteristics and are inhibited by most nucleotides and by inorganic ortho- and pyrophosphate. They differ, however, in their response to ATP. ATP activates the large aspartate transcarbamylase but has little effect on, or is an inhibitor of, the small aspartate transcarbamylase when aspartate concentrations are below saturation. The larger enzyme can be converted to the smaller enzyme *in vitro*, suggesting that they possess a common subunit. The proportion of the two enzymes *in vivo* is not constant but varies such that the larger enzyme increases during logarithmic growth until it is the sole enzyme present in stationary phase of culture.

Aspartate transcarbamylases (ATCase)[1] derived from various bacterial species can be grouped into three distinct classes as a result of both the kinetic and gel filtration characteristics of the particular enzyme (Neumann and Jones, 1964; Bethell and Jones, 1969). Bethell and Jones (1969) found that most bacteria studied contained a single ATCase; however crude extracts of *Citrobacter freundii* ATCC 8090 and *Proteus vulgaris* ATCC 8427 contained both a class B (mol wt 300,000; ATCase activity that is inhibited by CTP) and a class C enzyme (mol wt 100,000; ATCase activity that is not affected by CTP) in nearly equal amounts. The latter author's preliminary study of the kinetic characteristics of the two *Citrobacter* enzymes showed that CTP inhibited only the class B ATCase at pH 7.0 which suggested that of these two ATCases only the larger one was under "feedback control."

At least two different possibilities would explain why *Citrobacter* should simultaneously possess significant amounts of two ATCases. It was possible that the two *Citrobacter* enzymes were chemically and physiologically distinct. In this case the enzymes would not share a common structural gene or a common polypeptide chain. A second possibility would be that the smaller *Citrobacter* ATCase contained a polypeptide(s)

* From the Department of Biochemistry, School of Medicine, University of North Carolina at Chapel Hill, North Carolina 27514. *Received September 18, 1970.* The investigation was supported by National Institutes of Health Grant HD-02148 and National Science Foundation Grant GB-7929. M. S. C. gratefully acknowledges support as a postdoctoral fellow of the National Institutes of General Medical Sciences (1 F02 6M 43, 405-01).

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[1] Abbreviations used are: carbamyl phosphate:L-aspartate carbamyltransferase (EC 2.1.3.2), ATCcase; carbamyl-L-aspartate, CAA; carbamyl phosphate, CAP; *p*-chloromercuribenzoate, *p*CMB.

also present in the larger regulated ATCase and that the gene for this "catalytic subunit" could either be transcribed independently of a gene for a regulatory subunit or that polymers of the smaller ATCase were regulated. In this case the two ATCases, one regulated and one nonregulated, would share a common structural gene.

This paper reports the separation of the two enzymes, studies on their kinetic characteristics, the *in vitro* conversion of the large ATCase (I) into the small ATCase (II), and some observations in the *in vivo* changes in the relative amounts of the two enzymes during culture. The present data suggest, but cannot prove, that the two enzymes may share a common subunit and therefore a common structural gene.

## Experimental Section

Inorganic salts, glucose, and sucrose were reagent grade and were obtained commercially. Tris [tris(hydroxymethyl)-aminomethane], L-aspartic acid, cytochrome *c* (beef fraction V), and catalase were purchased from Sigma Chemical Co., St. Louis, Mo. Nucleotides were obtained from Sigma or PL-Biochemicals, Inc., Milwaukee, Wis. Carbamyl phosphate was obtained from CalBiochem, Los Angeles, Calif., and carbamyl aspartate was from Nutritional Biochemicals Corp., Cleveland, Ohio.

ATCase activity was determined by the colorimetric measurement of carbamyl aspartate (CAA) production (Prescott and Jones, 1969). Assay of the enzyme fractions were generally carried out at 30° for 5 or 10 min. Except when noted otherwise, the standard assay vessel contained 100 mM Tris-HCl buffer (pH 8.5), 47 mM aspartate (pH 8.5), 1 mM lithium carbamyl phosphate (CAP), and enzyme in a final volume of 1 ml. The reagents were preincubated for 1 min at 30° and the reaction was then initiated by the addition of enzyme. After 10 min, the enzymatic reaction was terminated by the addition of 1.0 ml of the antipyrine–sulfuric acid–oxime color assay mixture of Prescott and Jones (1969), unless otherwise noted. Enzyme fractions from G-200 Sephadex columns were used unless otherwise noted. The protein concentration in all extracts was determined by using the Oyama and Eagle modification of the method of Lowry (Oyama and Eagle, 1956). Catalase activity was analyzed by the method of Beers and Sizer (1952) and cytochrome *c* was measured by its absorption at 410 nm.

*Source of Citrobacter freundii.* A lyophilized culture of *C. freundii* ATCC 8090 was obtained from the American Type Culture Collection. Growth was initiated by placing the lyophilized pellet in 7 ml of Difco Nutrient Broth and incubating overnight at 37°. Stock cultures were maintained in slants of Difco agar. A culture was started in nutrient broth by transferring a small number of cells with a sterile loop from the slants to the broth and incubating for 18 hr at 37°. The liquid culture (1 ml) was diluted into 50 ml of Davis minimal medium (Ragland *et al.*, 1966) and shaken at 37° for 6 hr. The cultures were then transferred to 5 l. of fresh prewarmed minimal medium (1:100 dilution) and air was bubbled through the container or the culture was shaken at 37°. Cells[2] were harvested in middle-log (Klett reading of 135, filter 66) and late-log phase (Klett reading of 190–220, filter 66) by centrifugation of the medium in a Sorvall GSA at 10,000 rpm (16,300*g*) for 10 min. The cells were washed with 0.01 M potassium phosphate buffer (pH 7.5), drained, and frozen at −20°.

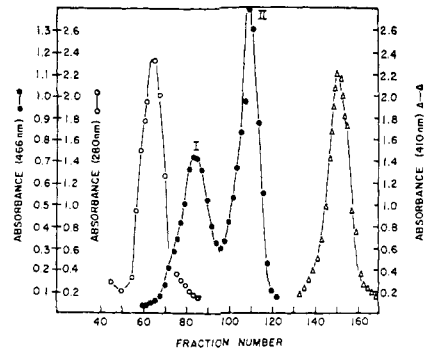


FIGURE 1: Elution pattern from Sephadex G-200 of *C. freundii* ATCase harvested in middle-log phase. A total of 88 mg of crude protein in 2 ml was applied to the column. (○) Excluded proteins; (●) ATCase activity; (△) cytochrome *c* absorption at 410 nm. The void volume was 163 ml and the elution volumes for peaks I and II were 213 and 275 ml, respectively.

*Preparation of the Extracts.* The cell paste was suspended in 0.01 M potassium phosphate buffer (pH 7.5), $5 \times 10^{-4}$ M mercaptoethanol, and $2 \times 10^{-5}$ M EDTA, in a homogenizer with a ground-glass pestle. The cells were ruptured in a French pressure cell at 12,000–16,000 psi. The cellular extract was centrifuged at 35,000 rpm for 3 hr in an Arden ultracentrifuge in a No. 40 Beckman rotor, after which the supernatant was dialyzed against the above buffer. Samples of the crude extract contained 40–50 mg of protein/ml. Crude extracts were dialyzed overnight against 0.01 M potassium phosphate buffer (pH 7.5), $5 \times 10^{-4}$ M mercaptoethanol, and $2 \times 10^{-5}$ M EDTA. The buffer was changed twice and its volume was 1000-fold that of the extract.

*Sephadex Column Preparation.* The method outlined by Bethell and Jones (1969) was used to swell Sephadex G-200 (bead form, Pharmacia, Lot No. 6471). The slurry was used to pack a 2.5-cm ascending flow Pharmacia column to a height of 95 cm. Flow rates of up to 30 ml/hr were obtained at 10 cm of water pressure and the columns could be used for several months without significantly slowing the flow rate. All columns were packed and used at 8°.

*Gel Filtration.* A 2-ml volume of the extract was applied to the bottom of the column. The void volume of each column was determined by measuring the absorption of large proteins in the crude extracts at 280 nm. Fractions of 2.5–3.5 ml were collected at 8° and assayed for ATCase activity immediately. Fractions containing enzyme (recovery of enzyme activity applied was 65 to 75%) were combined and concentrated to 4–5 ml in an Amicon ultrafiltration cell (Amicon, Scientific Systems Division, Lexington, Mass.) using a PM 10 Diaflo membrane. In experiments designed to convert ATCase I into ATCase II, the concentrate was reapplied to the bottom of the Sephadex G-200 column and rechromatographed at 8°. Recovery of ATCase activity applied was at least 60% when the activity of ATCase I and II peaks were summed.

[2] Purity and identity of the cultures was always checked by microscopic examination of Gram-stained preparations, and the original observation (Bethell, 1968; Bethell and Jones, 1969) that *Citrobacter* contained two ATCases was made on several extracts derived from cells grown from single colony isolates. The two ATCases are always observed in cultures grown to middle log, a result that we have observed repeatedly over a 2- to 3-year period with several different lots of *Citrobacter freundii* ATCC #8090 obtained from the American Type Culture Collection.

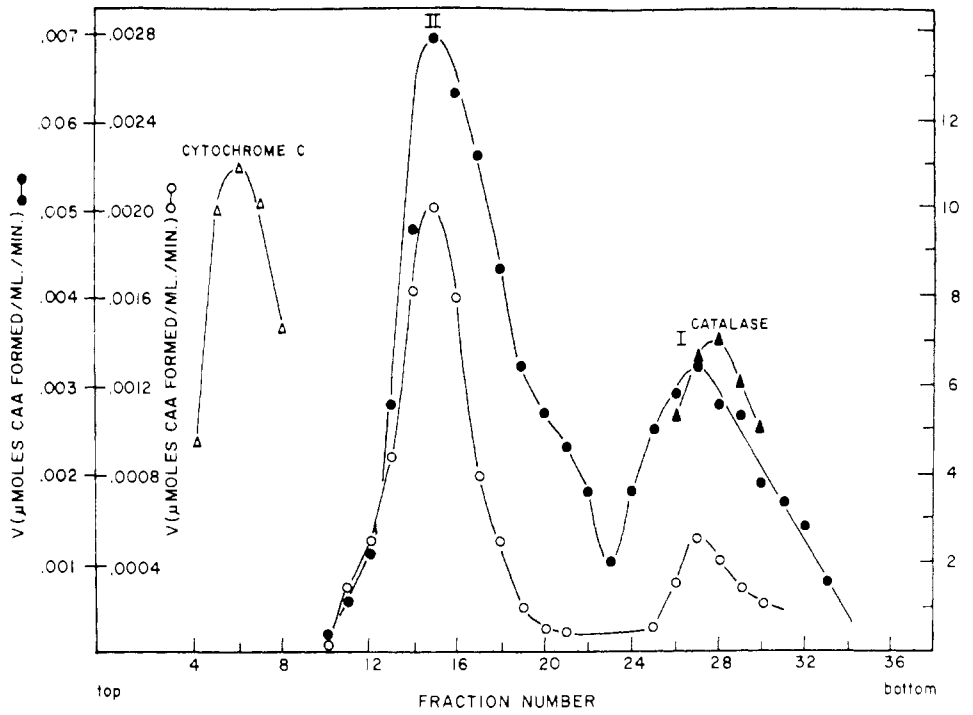


FIGURE 2: Sucrose density gradient profiles of the ATCase activity in a crude extract from bacteria grown to middle log (●) and in the combined eluates containing ATCase I and ATCase II from a Sephadex G-200 column (○). Crude extracts (4.2 mg of protein in 0.1 ml) or a mixture (0.1 ml) of ATCase I (0.081 mg of protein) and ATCase II (0.26 mg of protein) from Sephadex G-200 (see Figure 1) were used. ATCase activity was determined as described in the Experimental Section by the addition of a standard volume of each fraction to the 1-ml assay volume. The velocity is the CAA formed per minute per aliquot of enzyme in the 1-ml assay volume. Commercial catalase and cytochrome *c* were the standards.

*Sucrose Density Gradient Sedimentation.* Both the crude extracts and samples of the two enzymes isolated by gel filtration were layered on top of exponential isokinetic sucrose gradients prepared and analyzed by the method described by McCarty *et al.* (1968). The gradients were prepared in a buffer solution containing 0.01 M $KH_2PO_4$ (pH 7.5), 0.05 M KCl, $5 \times 10^{-4}$ M mercaptoethanol, and $2 \times 10^{-5}$ M EDTA. Cellulose nitrate tubes for the SW rotor (Beckman Instruments) were filled to 11.2 ml with the gradient which had an initial sucrose concentration of 5% (w/v) and a final concentration of 25% (w/v).

For experiments involving crude extracts of *C. freundii*, 0.1 ml of the extract was mixed with 0.05 ml (2000–10,000 Sigma units) of catalase and layered onto the gradient. For experiments in which fractions collected from Sephadex G-200 columns were recombined, 0.05-ml portions of the large and small enzymes were mixed; 0.05 ml of catalase was added, and the mixture was then layered onto the gradients. Solid cytochrome *c* was added to the samples to give a visible red color. Centrifugations were performed for 13 hr at 35,000 rpm at 5° in the No. 41 rotor of the Beckman-Spinco Model L-2 65 ultracentrifuge. Fractions were collected and assayed for ATCase and catalase activity and for cytochrome *c*.

## Results

A representative elution pattern for *C. freundii* ATCase activity from a Sephadex G-200 column is shown in Figure 1. For this experiment the *C. freundii* was harvested during the middle-log phase (Klett reading of 135, filter 66). The elution profile is similar to those of Bethell and Jones (1969) which show that crude extracts of this organism[2] have significant amounts of both a large (I) and a small (II) form of the enzyme.

Sucrose density gradient centrifugation of the crude bacterial extracts or of the mixtures of fractions I and II from a Sephadex column fractionation permit estimation of the molecular weights of the large and small forms of the enzyme (Figure 2). In a given crude extract the ratio of the activity of the large enzyme to that of the small enzyme is the same, within experimental error, whether the ratio was obtained from a gel filtration or a sucrose density gradient centrifugation. The second curve in Figure 2 shows the distribution of a mixture of enzymes I and II that had first been separated from one another by gel filtration and then recombined prior to the sucrose gradient fractionation. The two peaks at ATCase activity occur in the same tube whether crude extract or fractions from the Sephadex columns were used. Sedimentation constants and molecular weights for the ATCases were calculated, Table I, by the formula of Martin and Ames (1960; see also McCarty *et al.*, 1968). The molecular weights for the two ATCases calculated using catalase as the standard were 250,000 and 93,000. With cytochrome *c* as the standard, molecular weights were 124,000 and 49,000, respectively. This discrepancy is probably due to the different $f/f_0$ values of the two standard proteins (Siegel and Monty, 1966). The numerical values obtained from these experiments as well as the earlier study of the $K_D$ values for these two ATCases from the Sephadex G-200 columns (Bethell and Jones, 1969) indicate that the small enzyme as a maximum is one-half the size of the larger enzyme.

The effect of pH on the activity of the two ATCases at 30 and 37° is shown in Figure 3. At 30° the two enzymes have very similar pH-dependence curves with a large maximum at

TABLE I: Molecular Weights of Small and Large Aspartate Transcarbamylase from *C. freundii* Derived from Sucrose Gradient Sedimentation.[a]

| Experiment | Large (I)[b] | Small (II)[b] | Large (I)[c] | Small (II)[c] |
|---|---|---|---|---|
| 1 | 236,000 | 93,600 | | |
| | (10.8)[d] | (5.9) | | |
| 2 | 225,000 | 93,600 | | |
| | (10.5) | (5.9) | | |
| 3 | 265,000 | 114,000[e] | 118,000 | 46,100 |
| | (11.8) | (6.7) | (8.4) | (4.6) |
| 4 | 265,000 | 92,100 | 130,000 | 52,100 |
| | (11.8) | (6.4) | (8.8) | (5.0) |

[a] Proteins from a crude extract of *Citrobacter freundii* plus pure cytochrome *c* and catalase were layered on a 5–25% sucrose gradient as described in the Experimental Section. Fractions were tested for ATCase activity, catalase activity, and absorption at 410 nm. [b] The figures listed were calculated using the known molecular weight (250,000) and the sedimentation constant (11.3) of catalase and the sedimentation constants for the two ATCases estimated from the sucrose gradient. [c] The values listed were calculated using the known molecular weight (12,384) and the sedimentation constant of cytochrome *c* (1.9) and sedimentation constants for the two ATCases estimated from the sucrose gradient. [d] Sedimentation constants, given in parentheses, are the values estimated from the sucrose gradient using catalase or cytochrome *c* as reference standards, as indicated. [e] This value was not used to obtain the mol wt value for ATCase II quoted in the text. Experimentally this value means that peak enzyme activity was observed one tube more distant from the catalase peak than it was for the other 3 experiments.

pH 8.5 and a second, smaller maximum at pH 7.75. For the small ATCase II the pH curve at 37° is very similar to the cruve at 30°, although the activity peak around pH 7.75 has become markedly broader. However at 37° the curve for ATCase I is changed markedly from that observed at 30° for there is a single optimum at pH 8.5. Since the maximum optimal pH was always 8.5 for both enzymes, since the two enzymes have a similar pH curve at 30°, and since the rate of the chemical decomposition of CAP is lower at 30° than 37°, most studies were carried out at pH 8.5 and 30°.

The aspartate saturation curves for enzymes I and II are shown in Figure 4. Both curves are hyperbolic and mild substrate inhibition is evident. The Lineweaver–Burk plots are similar for the two enzymes and the apparent $K_m$ for aspartate is $2.2 \times 10^{-2}$ M when the lower curve of the Lineweaver–Burk plot is used for the determination.

In Figure 5 representative carbamyl phosphate saturation curves for enzymes I and II are shown along with double-reciprocal plots for both. The apparent $K_m$ at 30° is $6.9 \times 10^{-5}$ M for the large enzyme and $3.9 \times 10^{-5}$ M for the small enzyme. Both CAP saturation curves are hyperbolic, at the CAP concentration studied, and there is no evidence of inhibition by CAP at high concentration levels.

The effect of *p*-chloromercuribenzoate (*p*CMB) on the large and small ATCases was examined by incubating the enzymes with $1 \times 10^{-7}$ to $1 \times 10^{-3}$ M *p*CMB for 30 min at

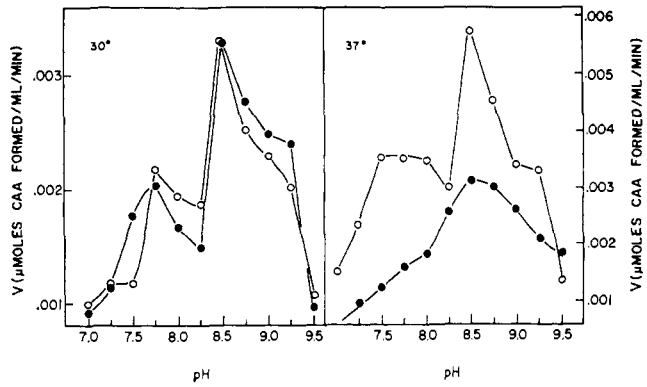


FIGURE 3: The effect of pH on the activity of the ATCases from *C. freundii*. The incubation mixture contained the standard quantities of substrates and Tris buffer (see Experimental Section) but the Tris buffer and aspartate were carefully adjusted to the pH indicated on the abscissa while held at either 30 or 37°. Protein added was 6.7 μg for ATCase I and 15.6 μg for ATCase II at 30°. At 37°, the protein added was 10.1 μg at ATCase I or 31.2 μg of ATCase II. The symbols used in both halves of the figure are: (●) large ATCase; (○) small ATCase II.

30° (Figure 6). The two enzymes show equal sensitivity to this agent.

*Effect of Nucleotides.* Both enzymes are affected by a variety of nucleotide and phosphate compounds (Table II). The most marked effects can be summarized as follows. (1) Inhibitors (or activators) are phosphate compounds for cytosine and

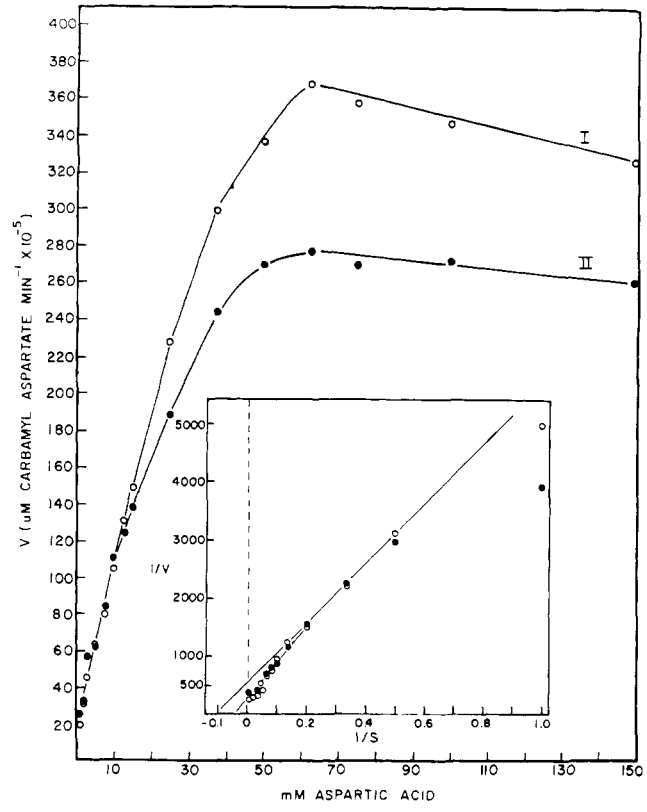


FIGURE 4: Aspartate saturation curves for *C. freundii* ATCase I and II. Assay conditions were as described in the Experimental Section. Protein concentrations were 4.3 μg of ATCase I or 6.87 μg of ATCase II. The insert shows the double-reciprocal plots for both substrate saturation curves: (○) large enzyme; (●) small enzyme.

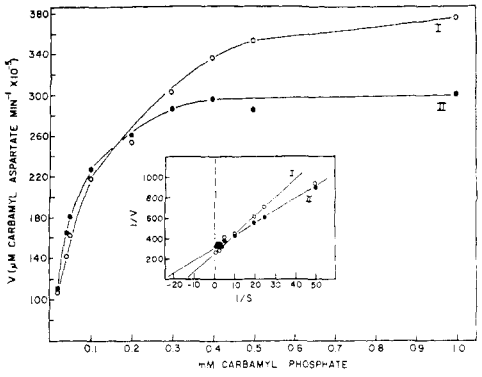


FIGURE 5: Carbamyl phosphate saturation curves for *C. freundii* ATCase I and II at 30°. The incubation mixtures were the same as Figure 4 except that the aspartate (47.5 mM) was constant and CAP was varied. The double-reciprocal plots are also shown (insert): (○) large enzyme; (●) small enzyme.

cytidine have essentially no effect under the condition studied. (2) The effect of various phosphate compounds depends on which substrate is limiting. (a) When carbamyl phosphate is the limiting substrate, all di- and trinucleotides as well as $PP_i$ and $P_i$ (a product of the reaction) are good inhibitors of both enzymes, while CMP and ribose phosphate are less effective inhibitors. (b) When aspartate is the limiting substrate, several phosphate derivatives are still slightly inhibitory, *i.e.*, CTP, CDP, GTP, UTP, and $PP_i$, while CMP, $P_i$, and ribose-phosphate have essentially no effect. ATP now activates ATCase I but has little or no effect on ATCase II.

The most dramatic of these results is the distinctly different effect of ATP on the large and small ATCases of *C. freundii* when CAP concentrations are saturating and the aspartate concentration is near the $K_m$ value. Figure 7 quantitates this effect and shows that the ATP activation of ATCase I is apparent at 0.2 mM ATP, although full activation is not obtained until the ATP concentration is 5 mM where a 2.5-fold activation is observed.

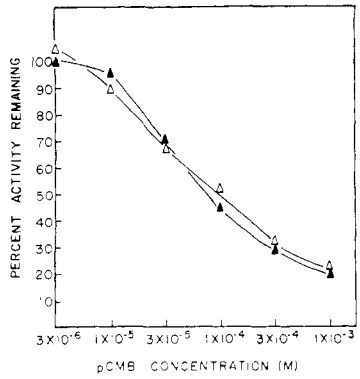


FIGURE 6: The effect of *p*-chloromercuribenzoate on small and large enzyme fractions. A stock solution of $10^{-3}$ M *p*CMB was prepared in 0.01 M potassium phosphate buffer (pH 7.4) and diluted in the same buffer. Enzyme (10 μl) was added to 0.5 ml of each *p*CMB buffer mixture and incubated for 30 min at 30°. The concentration of *p*CMB shown on the abscissa is that used during the preincubation. Each mixture was then diluted 1 to 3 with 0.01 M potassium phosphate buffer for the enzymatic assay and 0.1 ml of the dilution was used for assay. The amount of protein from the large enzyme fractions was 1.13 μg and from the small enzyme fractions was 1.9 μg. The standard assay described in the Experimental Section was used: (△) large enzyme; (▲) small enzyme.

TABLE II: Effect of Nucleotides and Phosphate Compounds on the Sephadex G-200 Eluates Containing *C. freundii* Large (I) or Small (II) ATCase with Limiting Concentrations of CAP or Aspartate.

| | % Inhibition[a] | | | |
|---|---|---|---|---|
| | CAP (1 mM) Aspartate (15 mM) | | CAP (0.05 mM) Aspartate (47 mM) | |
| Addn (5 mM) | ATCase I | ATCase II | ATCase I | ATCase II |
| ATP | −98[b] | 1 | 52 | 62 |
| GTP | 26 | 19 | 67 | 72 |
| UTP | 12 | 35 | 74 | 64 |
| CTP | 34 | 31 | 51 | 52 |
| CDP | 28 | 48 | 56 | 64 |
| CMP | 15 | 10 | 21 | 37 |
| Cytidine | −3 | −7 | −2 | −5 |
| Cytosine | −3 | −7 | −5 | −6 |
| Ribose-$PO_4$ | −2 | 1 | 19 | 19 |
| $PP_i$ | 45 | 47 | 73 | 83 |
| $P_i$ | 4 | 7 | 43 | 61 |

[a] All incubations were carried out as described in the Experimental Section except that the substrate concentrations were changed as indicated above and 5 mM of the nucleoside, nucleotide, or phosphate compounds indicated was also added. Protein concentrations were 11.3 μg of ATCase I (large) or 14.7 μg of ATCase II (small). [b] Negative numbers indicate stimulation of enzymatic activity.

*In Vivo Changes in the ATCase I/II Ratio.* If the bacteria were harvested in late-log rather than in middle-log phase (Figure 1) a single peak of ATCase activity (Figure 8, closed circles) was seen. This peak was eluted with the volume of buffer expected for the large ATCase (I) of Figure 1 and the ATCase was activated by ATP.

The difference in distribution of enzyme activities in middle- and late-log phases of bacterial growth suggested that the proportion of the two enzymes changes during the growth of the bacteria. It seemed that the effect of 10 mM ATP on the ATCase activity of dialyzed crude extracts could be used as a rough measure of the ratio of ATCase I and II since this amount of ATP activates ATCase I and inhibits ATCase II (see Figure 7). For this experiment crude extracts were prepared from bacteria collected at 30-min intervals from early-log phase (Klett reading of 19) to stationary phase (Klett reading of 220). The ATCase activity of extracts prepared from cells harvested before mid-log culture (Klett reading of 80) was inhibited (50–20%) by ATP. The ATCase of late-log to stationary cultures was activated (20–80%) by ATP. This change is what one would expect if the small ATCase II is the only ATCase present in early-log and if the large ATCase is formed in increasing amounts from some time point near middle-log (Figure 1) until it is the only ATCase present at stationary phase (Figure 8, closed circles).

*In Vitro Conversion of ATCase I to ATCase II.* When the central fractions (tubes 50–65) of peak I of Figure 8 (closed circles) are combined, concentrated by ultrafiltration, layered under the column, and eluted again, a new profile of ATCase activity, shown by the open circles in Figure 8, is obtained. There are now two peaks of ATCase activity.

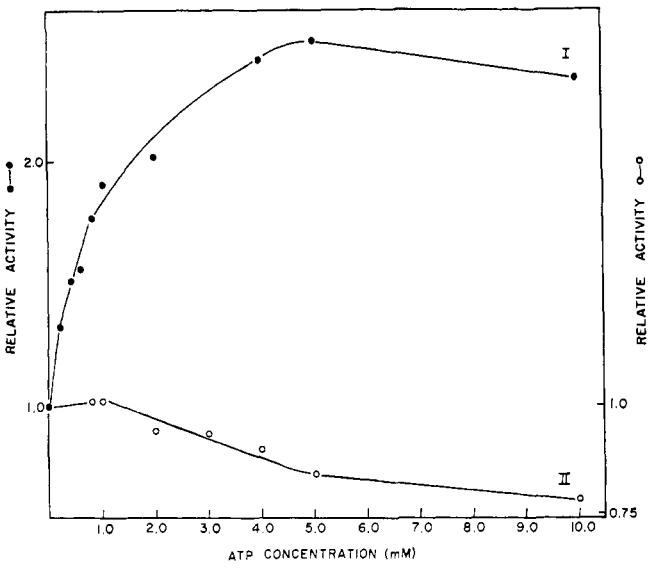


FIGURE 7: Effect of ATP on ATCase I and ATCase II at a low aspartate concentration. Assay conditions were those described in the Experimental Section except that the aspartate concentration was 15 mM. Protein concentrations were 17 μg of ATCase I or 14.7 μg of ATCase II. Incubation time was 10 min at 30°. The relative velocity of 1.0 for the small ATCase II is equivalent to 0.0033 μmole of CAA produced per min and the relative velocity of 1.0 for the large ATCase I is 0.0018 μmole of CAA produced per min. (●) Large enzyme; (○) small enzyme.

The first peak emerges at the position of the single peak obtained on the initial gel filtration of this late-log crude extract while a new minor peak emerges in a volume typical of peak II in Figure 1. Dilution of the large ATCase (peak I), which occurs during gel filtration and rechromatography, can therefore produce the small ATCase (peak II). When the procedure is repeated, thereby diluting the large enzyme again, an increasingly greater proportion of the small enzyme (peak II) has been obtained. In fact, as much as 30% of the original peak of large enzyme (solid circles in Figure 8) has been converted to the small enzyme in this manner. The new ATCase (peak II) activity is inhibited by 5 mM ATP.

The conversion of large enzyme to small enzyme was also observed when the ATCase I and II fractions were combined and subjected to sucrose density gradient centrifugation of

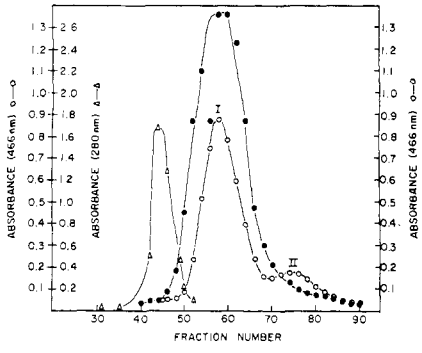


FIGURE 8: Elution pattern of *C. freundii* ATCase harvested in late-log phase from Sephadex G-200. The elution pattern of 2 ml (92 mg of protein) of crude extracts shows a single peak of ATCase activity (●). Fractions 50 to 65 of this peak (10.2 mg of protein in 94 ml) were combined, concentrated to 6.0 ml, and then reapplied to the column and eluted (○). The void volume was 154 ml and the elution volume for peaks I and II were 203 and 259 ml, respectively.

Figure 2. Of the total ATCase activity recovered from the sucrose gradient, 85% sedimented as the small enzyme (II) while only 15% sedimented as the large enzyme (I). This distribution of activity indicates the conversion of nearly half of the large enzyme applied to the gradient to the small enzyme since 100% of the enzyme activity applied was recovered and since 36% of the total ATCase activity of the sample applied was large enzyme.

If the crude extract from either the middle- or late-log bacteria was held at 0° for 3, 5, or 10 days before Sephadex chromatography, the distribution of the ATCase activity of the extract remained as illustrated in Figure 1 or Figure 8, respectively. These control experiments indicate that the two peaks are not artifacts due to proteolysis or ageing of the extract, and therefore they may be representative of the enzyme activity as it occurs in the cell.

## Discussion

These studies were initiated because *Citrobacter freundii* cells were shown by Bethell and Jones (1969) to possess nearly equal amounts of two ATCases. It seemed important to inquire if these two enzymes were completely distinct in protein structure and physiological function (perhaps one being an anabolic and the other a catabolic enzyme as is common for many enzymes in bacteria), or if they represented two chemically related enzymes. The results presented here suggest that the two enzymes may share a common subunit. The most direct evidence we have on this point is the conversion of the large ATCase I (mol wt *ca.* 250,000) to the small ATCase II (mol wt *ca.* 93,000) *in vitro* by repeated gel filtration of ATCase I. Studies with pure enzymes will be necessary to establish that this is indeed the case.

We have attempted to reconvert ATCase II to ATCase I by concentrating the proteins in the peak containing ATCase II. If this experiment had produced ATCase I it would have indicated that the reaction was reversible and also that only proteins in the 90,000–100,000 molecular weight fraction of the Sephadex column were required to produce ATCase I. The experiments were unsuccessful; however, in view of the extremely small amount of ATCase II which was available to us, we do not consider the experiments conclusive at this time.

Conditions that may favor the conversion of ATCase I to ATCase II are the continued dilution of the protein on chromatography, and purification of the ATCases from other proteins. Ammonium sulfate fractionation of crude late-log extracts yields only purified ATCase I, so it would seem that dilution rather purification might cause the conversion of ATCase I to ATCase II. As mentioned in the results section, storage of the same extracts at 0° for 10 days does not result in formation of ATCase II, thereby excluding time (as well as proteolysis and ageing) as the sole factor in the process of conversion.

From the apparent molecular weights, ATCase I is larger than ATCase II by the nonintegral factor of 2.7, suggesting that ATCase I could contain a protein (mol wt *ca.* 60,000) not present in ATCase II. However, since these molecular weights are only approximations, it is possible that the large ATCase is a dimer or trimer of the small ATCase, particularly if the $f/f_0$ values for these two enzymes are quite different. A close similarity between the two enzymes is also suggested by their $K_m$ values for aspartate and CAP, and their pH curves at 30°, which are nearly analogous. This result could be observed if only one form of the enzyme, or an equilibrium mixture of the two forms, existed at the low protein concentrations used for

assay. Neither possibility explains the differences in the pH curves at 37°, or why ATP activates only ATCase I. [One might wish however to see if the addition of ATP to concentrated ATCase II might aid its conversion to ATCase I, or whether the addition of substrates to ATCase I might speed its conversion to ATCase II.]

Both enzymes are markedly inhibited by all di- and trinucleotides if the CAP concentration is near the $K_m$ value, *ca.* $4 \times 10^{-5}$ M. $PP_i$ and $P_i$, a product of the reaction, also inhibit strongly under these conditions. It is possible that the di- and trinucleotides are good inhibitors because their terminal phosphates can fit the site where $PP_i$ binds; the reason then that CMP and ribose phosphate inhibit poorly might be that they have only one ionizable phosphate group, but unlike orthophosphate, a good inhibitor, the are undesirably bulky. Other portions of the nucleotide structure apparently do not bind to the enzyme since cytidine and cytosine are not inhibitors.

The relative lack of specificity for the nucleotide base with *Citrobacter* ATCases I and II is more marked than for other ATCases; however, *Escherichia coli* ATCase is also inhibited by all nucleotides including ATP when the CAP concentration is sufficiently low and nucleotide concentrations are 1 mM or higher (Bethell, 1968) and *Pseudomonas fluorescens* ATCase is also inhibited by all nucleotides (L. B. Adair and M. E. Jones, unpublished results). The metabolic significance of this "low-specificity" nucleotide effect, in contrast to the "high-specificity" of the classic CTP inhibition of *E. coli* ATCase (Gerhart and Pardee, 1962; Bethell and Jones, 1969), is not known.

With *Citrobacter* ATCase I there is a large difference in the effect of nucleotides, particularly ATP, depending on whether CAP or aspartate is limiting. This difference highlights the fact that nucleotide control can be quite different *in vivo* depending not only on which substrate is limiting, but also, in this case, on the ratio of the two ATCases which changed during culture.

As far as we know this is the first report in which there is an *in vivo* change from a small ATCase to a second larger which apparently contains the small enzyme as a subunit. Pulse-labeling of ATCase II at a point in the cell culture before ATCase I is present, and measurements to see if incorporation of the pulse-labeled ATCase II into ATCase I occurs as the culture ages would demonstrate if ATCase II is directly converted to ATCase I *in vivo*.

Results directly analogous to those obtained here would occur in *E. coli* if the message for the catalytic subunit polypeptide is not on the same mRNA strand as the message for the regulatory subunit polypeptide and if the gene for the catalytic subunit is transcribed first. No studies have been reported on the kinetic characteristics or the size of *E. coli* ATCase during cell culture nor is it known, although it is assumed, that the gene for the regulatory subunit is adjacent (or near) to the gene for the catalytic subunit. *E. coli* ATCase however differs from ATCase I of *Citrobacter* in that it dissociates into its component subunits only after chemical treatment or mild denaturation (Gerhart and Pardee, 1962; Gerhart and Schachamn, 1965).

There may be some resemblance between these two *Citrobacter* ATCases and the ATCase of yeast (Lue and Kaplan, 1969). Purified yeast ATCase can have a molecular weight, as judged by Sephadex G-200 chromatography, of $6 \times 10^5$, $3 \times 10^5$, or $1.4 \times 10^5$. The largest enzyme also has carbamyl phosphate synthetase activity and can bind UTP, which inhibits both enzyme activities.[3] Dilution of the largest enzyme converts it to the smaller ATCases of $3 \times 10^5$ or $1.4 \times 10^5$ molecular weight units (Lue and Kaplan, 1969, 1970; Kaplan and Messer, 1969). The smallest enzyme has only ATCase activity which is no longer inhibited by UTP. There is some kinetic evidence that two forms of enzyme can exist *in vivo* (Kaplan *et al.*, 1969). Therefore in both *Citrobacter* and yeast the feedback inhibition or activation properties of ATCase may depend on the molecular size of the ATCase. In both cases *in vitro* dilution of the larger molecular weight ATCase results in its conversion to an ATCase that is smaller. In both organisms the size of the enzyme within the cell may depend on growth conditions.

We are anxious to extend these studies to understand the exact relationship between ATCase I and II protein structure, to understand why ATCase II appears in larger amounts during the early growth of *C. freundii* while only ATCase I occurs in the late phase of growth, and to see if ATCase II is converted directly to ATCase I *in vivo*.

Acknowledgment

We are grateful for the technical help of Hattie M. Edwards with many of the experiments reported here.

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[3] We have not measured the carbamyl phosphate synthetase activity of the fractions obtained in experiments like those of Figures 1 and 8, so the possibility exists that synthetase activity is present in one or both of the two *Citrobacter* ATCases.