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Adenosine Triphosphate: Glutamine Synthetase Adenylyltransferase of *Escherichia coli*: Two Active Molecular Forms

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Abstract. Two active forms of purified ATP:glutamine synthetase adenylyltransferase from *Escherichia coli* are apparent on polyacrylamide gel electrophoresis at pH 8. The slower migrating component, which is identical to the P_I-protein fraction of the glutamine synthetase deadenylylating enzyme system, has $s_{20,w} \simeq 5.1$ S and a molecular weight of about 130,000. The more rapidly migrating adenylyltransferase component has $s_{20,w} \simeq 4.0$ S and a molecular weight of about 70,000. During storage at 4°C, the larger adenylyltransferase component (P_I) converts to the smaller active unit with a concomitant loss of both P_I deadenylylating activity and soluble protein. It is concluded that the low-molecular weight form of the adenylyltransferase is a subunit of the deadenylylating P_I-protein.

The ATP:glutamine synthetase adenylyltransferase (ATase) is a part of an elaborate system for the regulation of glutamine synthetase activity in *Escherichia coli*.[1–6] The adenylyltransferase catalyzes the covalent attachment of a 5′-adenylyl group to each of the 12 subunits of glutamine synthetase[2,3,5–8] and thereby markedly changes the catalytic and regulatory characteristics of the synthetase.[1–10] The 5′-adenylyl group is esterified in phosphodiester linkage to the phenolic hydroxyl moiety of a specific tyrosyl residue of the subunit polypeptide chain.[11,12] In *E. coli*, there exists also a complex deadenylylating enzyme system that specifically catalyzes the removal of 5′-adenylyl groups from glutamine synthetase.[5,13,14]

Ebner *et al.*[15] recently described the purification and some properties of ATase from *E. coli* B, whereas an ATase of low molecular weight was isolated from *E. coli* W in this laboratory[16]. Except for its smaller size, many characteristics of the enzyme from the W strain were similar to those of the enzyme studied in Holzer's laboratory.[6,15,17] The studies of Anderson *et al.*[18] and the present report show that ATase activity can be associated with the P_I-protein fraction of the deadenylylating enzyme system. This communication gives information on the relationship of the two active molecular forms of ATase obtained during the purification of this enzyme.

Materials and Methods. Three different ATase preparations were used: (*1*) Fractions from the hydroxyapatite column described in Fig. 3 of ref. 18, containing >400 units ATase per mg protein, were combined and dialyzed against 10 mM potassium

phosphate–1.0 mM 2-mercaptoethanol buffer (pH 7.6). This preparation contained 681 units (as against 22 units in the crude extract) of ATase and 1210 units of P_I-DA activity per mg protein. (*2*) A second ATase preparation was isolated by preparative disc gel electrophoresis. The initial purification steps were as previously reported[19]; after 2 months storage at 4°C, the ATase was further purified by gel filtration through Agarose and chromatography on hydroxyapatite.[18] The recovered ATase activity was concentrated in an Amicon ultrafiltration cell using an XM-50 Diaflow membrane (Amicon Corp.), and dialyzed against 20 mM Tris·HCl buffer (pH 7.6) containing 1 mM 2-mercaptoethanol. Dithiothreitol (10 mM), 20% glycerol, and tracking dye were added to the dialyzed ATase fraction (118 mg protein, 470 units/mg) before applying it to a 7.5% polyacrylamide separating gel (7 cm) with stacking gel (3 cm) in a Canalco Preparative Electrophoresis apparatus with column PD-2/320. The gel system of Ornstein and Davis[20] and pH 8.3 Tris–glycine electrode buffer (with 1 mM dithiothreitol added) were prepared according to the Canalco Manual. Electrophoresis was at 4°C with a current of 3 mA; protein was eluted with the above Tris·HCl buffer applied initially at a rate of 1 ml/min. After the tracking dye had migrated through the gel column (32 hr), fractions (2 ml) were collected. The ATase was eluted in two widely separated peaks of activity: the first (fractions 24–38), and the second (fractions 60–104), contained 25 and 75% of the ATase activity, respectively. Fractions 81–98 (the more slowly migrating ATase), were pooled, concentrated as above, and dialyzed against buffer (pH 7.6) containing 0.01 M sodium phosphate–0.1 M NaCl–1 mM 2-mercaptoethanol and 0.1 mM EDTA prior to the analyses reported below. (*3*) Purified P_I-protein was that obtained from purification of the deadenylylating (DA) preparation by gel filtration and chromatography on DE32 cellulose as described.[18] The P_I-protein contained 2336 units of ATase and 2290 units of P_I-DA activity per mg protein.

Protein determinations[21] and enzyme activities (ATase, P_I-DA, and glutamate-activated [^{32}P]PP_i-ATP exchange activities) were assayed as described previously.[18] ATase is activated by L-glutamine, ATP, and $MgCl_2$: P_I-DA by P_{II}-protein, α-ketoglutarate, UTP, ATP, P_i, and $MnCl_2$.

Sedimentation studies: Sedimentation velocity studies were conducted at 40,000 rpm (4–7°C) with a double sector (aluminum-filled epon) capillary type synthetic boundary cell. Viscosity and density measurements, instrument calibrations, and calculations of the data were as described.[22] Sedimentation coefficients were corrected to values in water at 20°C. Rayleigh optics and the meniscus depletion method,[23] using short liquid columns and 0.16–0.35 mg/ml protein concentrations, were employed for sedimentation equilibrium studies at 4–5°C.

Polyacrylamide gel electrophoresis: Analytical gel electrophoresis of ATase fractions at pH 8 and gel fractionations were as described.[18] Gel electrophoresis in 0.1% sodium dodecyl sulfate at pH 7.2 was used.[24] The P_I-protein fraction was concentrated by negative dialysis against 0.01 M sodium phosphate at pH 7.2, then 45 μg of the P_I-protein in 0.12 ml was incubated for 30 min at 37°C with 0.8% sodium dodecyl sulfate and 10 mM dithiothreitol before electrophoresis. Gels were stained with Coomassie Blue; bovine serum albumin, rabbit muscle aldolase, and myoglobin were used as standards for polypeptide chain molecular weights.[24]

Results. Partial resolution of two forms of ATase during rechromatography on hydroxyapatite: When ATase was further purified by a second chromatography on hydroxyapatite, the complex elution profile illustrated in Fig. 1 was observed. A major peak of ATase activity, containing also P_I-DA activity, emerged first. There was considerable ATase activity, however, in later fractions that had little P_I-DA activity. The combined fractions 22–34 of the P_I-ATase peak of Fig. 1, although 6-fold more purified in ATase activity, had lost about 50% of the P_I-DA activity during this rechromatography under conditions identical to those used only 4 days before. Between the first[18] and second

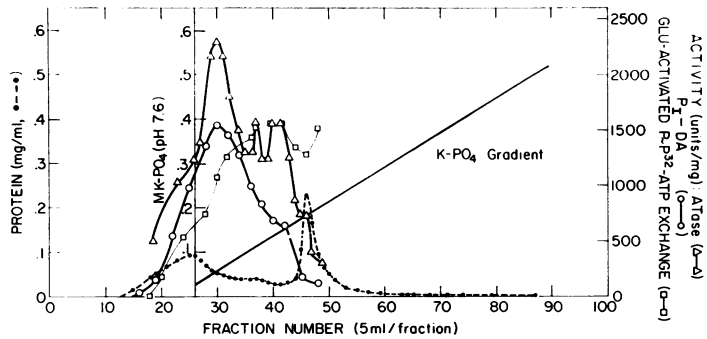

FIG. 1. Second chromatography of ATase on hydroxyapatite at pH 7.6. The active ATase fractions from the initial hydroxyapatite column step[18] [124 mg of preparation (1)] were rechromatographed 4-days later under conditions identical to the first chromatography. Enzyme assays and units of activities were as in Table 1; [^{32}P]PP$_i$-ATP exchange expressed as nmol [^{32}P]ATP/30 min at pH 7.6, 37°C. Protein concentrations were estimated from 280 nm/260 nm absorbancy ratios.[21] Pooled fractions 22–34 (used for the experiments reported in Figs. 2 and 3) had 4015 units/mg of ATase, 658 units/mg of P$_I$-DA activity, and a protein concentration of 0.26 mg/ml.

chromatographic separations on hydroxyapatite there appeared to have been some conversion to a form that retains ATase activity but is without P$_I$-DA activity.

Heat inactivation: Results illustrated in Fig. 2 suggest that the combined fractions 22–34 (Fig. 1) contain two forms of ATase that differ in heat stability. Plots of the logarithm of relative activities versus the reciprocal of absolute temperature are shown in Fig. 2*B*. The linear relationship observed for the decrease in P$_I$-DA activity indicates the presence of probably only one protein with DA activity; the nonlinear relationship observed for the inactivation of ATase suggests the presence of more than one protein with ATase activity. Heating the purified P$_I$-protein under the same conditions produced symmetrical and parallel losses in both DA and ATase activities with increasing temperature.[18] The data of Fig. 2 suggest that the P$_I$-ATase is more sensitive to heat inactivation than is the ATase without P$_I$-DA activity.

Separation of the two active forms of ATase by polyacrylamide gel electrophoresis: As is shown in Fig. 3, electrophoresis of the combined ATase frac-

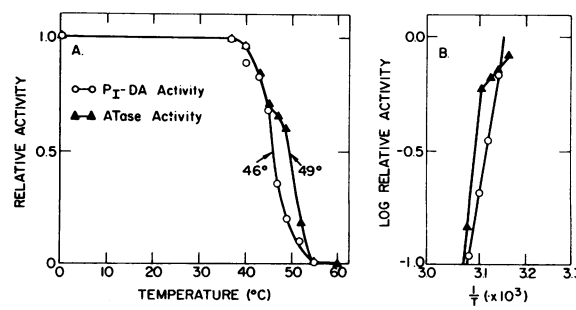

FIG. 2. Heat inactivation of mixed ATase forms. Purified ATase from Fig. 1 was used after dialysis against 15 mM potassium phosphate, pH 7.2. Aliquots (0.1 ml, containing 260 μg protein) were heated in the same bath, at the indicated temperatures, for 10 min and then iced. Enzyme assays were performed 1–5 hr from the time of heating. In *A*, relative activity = activity with heated sample/activity with unheated preparation. In *B*, the data of *A* are replotted as the logarithm of the relative activity versus the reciprocal of the absolute temperature.

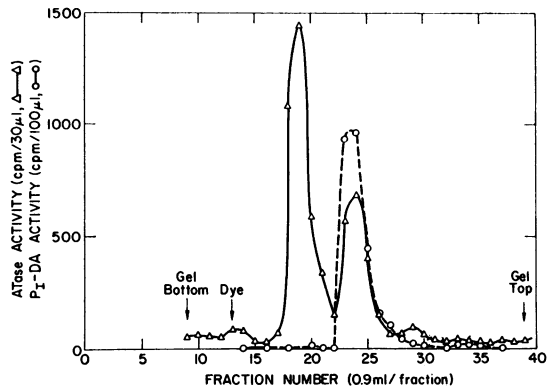

FIG. 3. Analytical polyacrylamide gel electrophoresis at pH 8. Electrophoresis of 180 μg of the column eluate shown in Fig. 1. Migration is to the anode; the gel was fractionated from bottom to top. The bromphenol blue position is indicated.

tions, 22–34 (Fig. 1), resulted in nearly complete separation of two peaks of ATase activity, only one of which is associated with P_I-DA activity. The fast-migrating ATase corresponds to the low molecular weight form previously isolated in this laboratory,[16] which lacks P_I-DA activity and possesses negligible glutamate-activated PP_i-ATP exchange activity. Exact correspondence of P_I-DA activity with the slower moving ATase identifies this as the P_I-protein component of the deadenylylating enzyme system.[18]

A conversion of P_I-ATase to the fast migrating ATase: The slow migrating ATase peak (P_I-ATase) was isolated by preparative disc gel electrophoresis. During storage at 4°C, analyses by analytical disc gel electrophoresis indicated that there was a conversion of the slow to fast electrophoretic component. Fig. 4*A* and *B* show the ATase activity distribution in gels prepared after 45 and 85 days of storage of this P_I-ATase fraction at 4°C. With the loss of the peak at fraction 19 that occurred in 85 days, there was a corresponding increase in the activity peak at fraction 13. Comparing the patterns of Fig. 4 to Fig. 3, the peak at fraction 19 can be designated the P_I-ATase activity peak. The assay results in Table 1 show that there was no net loss of ATase units in the sample from 45–85 days at 4°C. Thus, aging at 4°C causes a

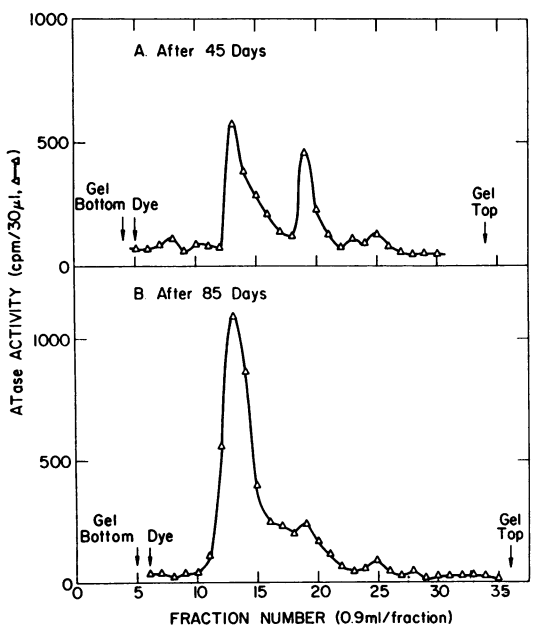

FIG. 4. Loss of P_I-ATase upon storage at 4°C. ATase activity from analytical polyacrylamide gels (pH 8) after electrophoresis of combined preparative disc gel fractions 81–98 stored at 4°C for (*A*) 45 days and (*B*) 85 days. 0.2 ml of the sample was applied to the gel.

TABLE 1. *Properties of different adenylyltransferase preparations.*

Preparation	Days stored at 4°C	% of total ATase[a] Fast	% of total ATase[a] Slow	Soluble protein mg/ml	$s_{20,w}$ (S)	Activity, units/ml[b] ATase	Activity, units/ml[b] P_I-DA
P_I-ATase[c]	10	0	100	2.8	...	6200	...
"	35	...	...	...	4.7[d]	...	1466
"	45	65	35	...	...	9610	...
"	85	80	20	1.3	4.0[e]	9300	266
P_I-protein[f]	...	0	100	1.7	5.1	1480	~1000
ATase[g]	~180	100	0	0.7	4.1 ± 0.2[h]	7000	0
Crude ATase[i]	~60	...	...	~20	5.1 ± 0.2[h]	1800	...

[a] Estimated from measurements of area under peaks of fast and slow migrating ATase forms on electrophoresis (see Fig. 4).

[b] Units expressed as[18]: ATase = nmoles [^{14}C]AMP incorporated into unadenylylated glutamine synthetase at pH 7.8 per 15 min at 37°C; P_I-DA = pmoles[^{14}C]AMP removed from [^{14}C] adenylylated glutamine synthetase in the presence of purified P_{II}-protein at pH 7.2 per min at 37°C.

[c] This P_I-ATase consisted of the slow moving material (Fractions 81–98) obtained by preparative disc gel electrophoresis.

[d] From symmetrical Schlieren boundaries, after 40 min sedimentation, that gave values of 4.6 S and 4.8 S, at protein concentrations of 1.4 and 0.7 mg/ml., respectively.

[e] Schlieren peak was asymmetric; sedimentation coefficient of the slower main compound.

[f] Purified P_I-protein concentrated by negative dialysis against 80 mM KPO_4, pH 7.2.

[g] 500-fold purified ATase isolated after prolonged storage at 4°C.

[h] Sedimentation coefficients estimated by sucrose density gradient centrifugation,[25] rabbit muscle aldolase and bovine hemoglobin markers, in a 5–20% sucrose gradient containing 20 mM Tris·HCl (pH 8.0) and 1.0 mM 2-mercaptoethanol.

[i] Obtained by acid ammonium sulfate precipitation at an early step in enzyme purification.[19]

conversion of the P_I-ATase to an electrophoretically faster migrating ATase component.

Table 1 also shows that the ATase activity of the fraction first isolated had a lower activity than that observed after storage at 4°C. There is also a loss of soluble protein during the storage at 4°C. Both effects have been observed with other ATase preparations. As indicated in Table 1, there is concomitant loss of P_I-DA activity with the increase in ATase units and loss of soluble protein.

Molecular parameters: Table 1 also summarizes results obtained in sedimentation velocity studies. The purified P_I-protein[18] has $s_{20,w}$ = 5.1 S. The mixture of ATase forms after 35 days storage at 4°C had a symmetrical Schlieren boundary with $s_{20,w}$ = 4.7 S. After 92 days at 4°C, $s_{20,w}$ was 4.0 S for the slower sedimenting component, which constituted most of the mass of the sample. In this case, the Schlieren boundary was asymmetric for the about 0.1:0.9 mixture. A value of 4.7 S for the unresolved mixture suggests a weight distribution of 0.36 (4.0 S) + 0.64 (5.1 S).

Results from a study to be published elsewhere[16] also are shown in Table 1. The electrophoretically fast migrating ATase that was previously isolated[16] had $s_{20,w}$ = 4 S as measured by sucrose density centrifugation;[25] this ATase form sedimented at about the same velocity as bovine hemoglobin. For a spherical globular protein, 4.1 S corresponds to a molecular weight of about 68,000. An enzyme fraction from an early step in the purification procedure (acid-ammonium sulfate step 4[19]) sedimented in a sucrose gradient at about 5.1 S.

The mixture of ATase forms (Table 1) present after 37 days at 4°C had an apparent weight average molecular weight of about 94,000 by sedimentation-equilibrium measurements (assuming $\bar{v}$ = 0.73 ml/g for both ATase forms). If

this mixture was about 60% ATase with a molecular weight of about 70,000, the calculated molecular weight of the P_I-ATase form is about 130,000.

When purified P_I-protein[18] was treated with 1% sodium dodecyl sulfate and then subjected to polyacrylamide electrophoresis in detergent,[24] two equally intense bands were visible after staining. The two bands corresponded to molecular weights of about 70,000 and 60,000. The 4.1S ATase had an apparent molecular weight of about 69,000 as measured by gel electrophoresis (unpublished data). The sum of the subunit molecular weights of the P_I-protein is ~130,000. If the electrophoretically fast migrating ATase is a subunit of the P_I-protein, then the subunit required for deadenylylating activity has a molecular weight of about 60,000. Thus, the P_I-protein appears to be composed of two polypeptide chains of unequal size, with the slightly larger unit being the ATase subunit. The formation of a precipitate when the 5.1S P_I-ATase unit is converted to the 4.0S ATase suggests that once the P_I-ATase molecules are dissociated, the deadenylylating subunits have a high tendency to aggregate and precipitate from solution, whereas the ATase subunits remain in solution as monomeric units.

The about 130,000 molecular weight extrapolated here for the P_I-protein agrees with the molecular weights of 120,000[6] and 145,000[15] reported for the ATase isolated and characterized in another laboratory.[4-6, 15, 17] The P_I-protein appears to be quite asymmetric since a particle of 130,000 molecular weight and $s_{20,w}$ of about 5.1 S would have a rather high frictional coefficient; the calculated value for $D_{20,w}$ is $\simeq 3.5 \times 10^{-7}$ cm^2 sec^{-1}. The smaller ATase unit, of about 69,000 molecular weight and $s_{20,w} \simeq 4.0$ S, has a calculated $D_{20,w}$ of $\simeq 5.2 \times 10^{-7}$ cm^{2}sec^{-1}. Sedimentation equilibrium studies with purified fractions of P_I[18] and the 4.0S ATase are in progress.

Interaction between the different protein components: In ATase assays, there was no evidence of interaction between the P_I-protein and the low-molecular weight ATase form. Mixtures of the two gave additive ATase activities. Also, addition of the purified P_{II}-protein[18] to the P_I-protein (as in the deadenylylating assay system) had no influence on the ATase activity of P_I, nor did P_{II} affect the ATase activity of the small ATase unit. In contrast, the addition of equal quantities of the low-molecular-weight ATase, which itself has no deadenylylating activity, to the P_I-protein inhibited P_I-DA activity 22%, indicating that the small ATase does interact with the P_I-protein. This was evident also in sedimentation velocity experiments in which the 4.0S ATase and the 5.1S P_I-ATase components were somewhat resolved only when the mass ratio of 5.1 S:4.0 S was about 0.1 : 0.9.

Selective inhibition of ATase activity by *p*-chloromercuriphenyl sulfonate: The small ATase subunit (70,000 daltons) is completely inhibited by 2 hr incubation with 10^{-4} M PMPS in 0.1 M Tris·HCl buffer at pH 8.5 at 0°C (unpublished data). Under these same conditions, the ATase activity of purified P_I-protein[18] is inhibited 91%, whereas neither P_I-DA activity nor the glutamate-activated PP_i-ATP exchange activity is affected.

Discussion. Our results show that adenylyltransferase normally exists as a high molecular weight (about 130,000) form, which during storage at 4°C can be converted slowly to a lower molecular weight (about 70,000) form having a

somewhat greater ATase activity. The large ATase molecule is probably identical to that isolated by Holzer and co-workers.[4-6,15,17] The small ATase molecule is probably identical to the one isolated in this laboratory;[16] it may correspond to a faster migrating component detected by Ebner *et al.*[15] during electrophoresis of various purified ATase preparations. The low molecular weight ATase[16] has about the same kinetic characteristics as the large ATase[6] and also catalyzes the reverse reaction reported by Mantel and Holzer[26]; however, the catalytic potential of the small ATase is somewhat greater than that of the large enzyme.

The results presented here and in the report of Anderson *et al.*[18] show that the larger ATase molecule is identical with the P_I-protein which, together with a second protein component (P_{II}-protein), comprises the deadenylylation enzyme system. The P_I-protein appears to be a multifunctional complex composed of two dissimilar subunits of unequal size: an ATase subunit and a slightly smaller subunit of about 60,000 daltons that is needed for deadenylylating activity. The sedimentation coefficient of 5.1 S measured for the P_I-protein of about 130,000 molecular weight suggests that this complex is somewhat asymmetric. Perhaps the P_{II}-protein component of the deadenylylating enzyme system[18] is the missing symmetry unit. Upon aging at 4°C, the ATase subunit dissociates from the P_I-protein complex in an active monomeric form, whereas the DA activity is lost. The loss of soluble protein during storage at 4°C possibly is due to aggregation of monomeric DA subunits that then precipitate from solution. The P_I-protein complex may be added to a rapidly growing list of isolated multienzyne aggregates[27].

The fact that dissociation of the P_I-protein leads to loss of DA-activity suggests that the ATase subunit, in its presumed role as an adenylyl group carrier, could be directly involved in both adenylylation and deadenylylation reactions. In this case the DA subunit, together with the P_{II}-protein component of the DA system, would be concerned with regulation and catalysis of a phosphorolytic cleavage of the, as yet hypothetical, adenylyl-ATase complex to yield ADP. Alternatively, the ATase subunit may not have a direct catalytic role in deadenylylation but its specific interaction with the DA subunit could be necessary to stabilize the active conformation of the DA subunit. The fact that inactivation of the P_I-ATase activity by PMPS did not result in loss of P_I-DA activity suggests that the ATase may not have a catalytic role in the deadenylylation reaction. However, the results obtained with PMPS could be misleading if, for example, the effect of PMPS involves modification of the effector site for glutamine, which is required for ATase activity, but not for DA-activity.

Even though a perfect correspondence between the glutamate-dependent PP_i-ATP exchange activity and P_I-DA activity has not been observed[18] (see also Fig. 1), the possibility that this exchange activity is a property of the P_I-protein has not been excluded. If so, the fact that PMPS does not affect the exchange activity of P_I-preparations might suggest that the exchange activity is a function of the DA-subunit.

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Abbreviations: ATase or adenylyltransferase, ATP:glutamine synthetase adenylyltransferase; P_I-DA activity, activity of protein fraction P_I in the deadenylylating assay system which contains added protein fraction P_{II}; P_I-ATase, adenylyltransferase activity associated with the P_I-protein fraction of the deadenylylating enzyme system; PMPS, *p*-chloromercuriphenylsulfonate.

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