A reappraisal of the diversity and class distribution of aspartate transcarbamoylases in Gram-negative bacteria

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Recently, the subunit composition of class A aspartate transcarbamoylases (ATCases) in fluorescent pseudomonads has been clarified. We present evidence that distribution of this type of ATCase may be more widespread than at first suspected. Bacterial ATCases exist in three forms: class A (molecular mass ~ 450–500 kDa); class B, typified by *Escherichia coli* ATCase (~ 300 kDa); and class C, typified by *Bacillus subtilis* ATCase (~ 100 kDa). Using gradient gel electrophoresis with activity-staining to scan bacterial sonicates, we report the existence of six more class A ATCases. We have purified one of these, *Acinetobacter calcoaceticus* ATCase, and found its subunit composition to be similar to that of the pseudomonad ATCases. Two of these ATCases come from bacteria outside the γ -subgroup of the Proteobacteria, one from the α -subgroup and one from *Deinococcus radiophilus*, a species phylogenetically remote from the Proteobacteria. Unexpectedly, three bacterial species, closely related to the fluorescent pseudomonads and acinetobacters, have ATCases of 100 kDa (class C). One of these, *Stenotrophomonas* (formerly *Xanthomonas*) *maltophilia* has been purified and found to be a homotrimer of 35 kDa polypeptide chains. We believe this is the first time that class C ATCases have been reported in Gram-negative bacteria. A distinctive cluster in the γ -3 subgroup of the Proteobacteria is formed by the enteric bacteria and their relatives. So far only class B ATCases have been reported in this group. The evolutionary implications of these findings are discussed.

Keywords: aspartate transcarbamoylase, molecular evolution, phylogeny

INTRODUCTION

The enzyme aspartate transcarbamoylase (ATCase; EC 2.1.3.2), which catalyses the first step unique to pyrimidine synthesis exists in a number of forms. The core catalytic component of all bacterial ATCases, however, is a homotrimer of approximately 34 kDa polypeptides. This arrangement has been shown in *Escherichia coli* (Ke *et al.*, 1984) and *Bacillus subtilis* (Brabson & Switzer, 1975), and is a consequence of the active site being shared between adjacent polypeptides (Robey & Schachman, 1985).

The most obvious feature of diversity in the bacterial ATCases is molecular size, which varies depending on whether or not the catalytic subunit is associated with other non-catalytic subunits, and the nature of these subunits. The earliest classification, by Bethell & Jones

(1969), proposed three classes of bacterial ATCase on the basis of size.

Class A ATCases are the largest and are found in the fluorescent pseudomonads. Recently, three of these enzymes have been purified (Bergh & Evans, 1993; Shepherdson & McPhail, 1993; Schurr *et al.*, 1995) and shown to have molecular masses in the region of 480 kDa. In addition to the 34 kDa polypeptide chains of the catalytic subunit, the molecules contain polypeptide chains of around 45 kDa. It is proposed that the 34 kDa chains form two catalytic trimers which associate with six of the larger chains. Dissociation of the enzyme into its separate subunits under conditions which retain activity has not been achieved (Bergh & Evans, 1993; Shepherdson & McPhail, 1993). It is not clear what function the 45 kDa chains serve, but derived amino acid sequence comparisons suggest that this polypeptide is a non-functional homologue of the next enzyme in the pathway, dihydroorotase (Schurr *et al.*, 1995).

Abbreviation: ATCase, aspartate transcarbamoylase.

Class B ATCases include those from the enteric bacteria of which the ATCase from *E. coli* has been extensively studied; the molecule has two catalytic trimers and three homodimers of 17 kDa chains which mediate very precise allosteric regulation of activity by binding nucleotides (reviewed by Kantrowitz & Lipscomb, 1990). The molecular mass of ATCases in this group is approximately 300 kDa. Dissociation of these ATCases into catalytic trimers and regulatory dimers with retention of enzyme activity is relatively easy (Gerhart & Holoubek, 1967).

Class C ATCases are exemplified by *Bacillus subtilis* ATCase which consists of a catalytic trimer alone (Brabson & Switzer, 1975). All Gram-positive bacterial ATCases thus far examined have a molecular mass in the region of 100 kDa (Switzer & Quinn, 1993).

We have surveyed ATCases from 28 bacterial species, 12 of which have not been previously investigated and are reported here for the first time. We have classified them according to size. Most of the bacteria we have studied fall into the γ -3 subdivision of the Proteobacteria described by Woese (1987), and two are outside this group. We report the first purification of a class A ATCase which does not belong to the fluorescent pseudomonads, and a class C ATCase from a Gram-negative organism. On the basis of our findings, we suggest that class A ATCases are far more widely distributed than has been previously thought and propose that this structure may be the dominant form of ATCase in Gram-negative bacteria.

METHODS

Bacterial strains. Unless otherwise stated, the organisms were obtained from the National Collection of Industrial and Marine Bacteria (NCIMB; identification numbers are given in parentheses): *Xanthomonas campestris* (11803), *Stenotrophomonas* (previously *Xanthomonas*) *maltophilia* (9203), *Acinetobacter calcoaceticus* (11826), *Lysobacter enzymogenes* (9924), *Pseudomonas syringae* pv. *pisi* (race 7 isolate no. 1691 kindly provided by A. Vivian, this laboratory), *Azomonas agilis* (11693), *Azotobacter vinelandii* (12096), *Leuconothrix mucor* (2222), *Escherichia coli* DH5 α (13174), *Vibrio natriegens* (857), *Paracoccus denitrificans* (11627) and *Deinococcus radiophilus* (10648).

Culture conditions and preparation of extracts. All bacteria were grown to mid-exponential phase using culture conditions suggested in the NCIMB *Catalogue of Strains*. When sonicates were prepared, the cells were harvested by centrifugation, resuspended in Tris/HCl buffer (50 mM, pH 7.5) containing EDTA (1 mM) and dithiothreitol (1 mM), then disrupted with an MSE sonicator using a 6 mm diameter probe (4 $\times$ 30 s bursts with cooling in ice water between bursts). Sonicates were centrifuged for 2 h at 20000 g. On one occasion, 20% (w/v) PEG and 1 mM ZnCl₂ were included in the disruption medium of *S. maltophilia* cells, in an attempt to prevent possible subunit dissociation of the ATCase taking place.

Enzyme assay. ATCase activity was determined by measuring the amount of inorganic phosphate produced in the reaction, under conditions which minimize the spontaneous breakdown of carbamoyl phosphate, as described by Shepherdson & McPhail (1993). One unit of enzyme activity is defined as the amount of enzyme which catalyses the formation of 1 μ mol phosphate in 1 min. Protein was assayed by the method of Bradford (1976).

SDS-PAGE. In all cases, mini-slab gels (Atto Corporation) were used. The buffering conditions and gel composition were as described by Laemmli (1970).

Gradient electrophoresis under non-denaturing conditions.

Precast 4–20% (w/v) non-denaturing polyacrylamide gradient gels (Novex) were used to compare the size of ATCases present in different bacterial sonicates. Sonicates were suitably diluted with sample buffer so that the 10 μ l aliquots applied to each lane contained 0.01 units of ATCase activity. A mixture of molecular mass markers (Pharmacia, High Molecular Weight markers for electrophoresis under non-denaturing conditions) were run on the same gel. The buffering system was based on that described by Laemmli (1970) for SDS-PAGE except for the omission of SDS and reducing agent. Electrophoresis was carried out at 4 °C at a constant voltage of 125 V for 12 h. After the run the gels were activity-stained for ATCase (Grayson *et al.*, 1979).

Gel filtration. A Pharmacia Hiload Superdex 16/60 column was used to determine the molecular mass of ATCase in sonicates. The column was loaded with 0.5 ml of sonicate containing approximately 5 units ATCase ml⁻¹. The eluting buffer was Tris/HCl (50 mM, pH 7.5) containing NaCl (0.1 M). The column was calibrated with thyroglobulin (666 kDa), apoferritin (440 kDa), *E. coli* ATCase (310 kDa), catalase (250 kDa), glucose-6-phosphate dehydrogenase (150 kDa) and hexokinase (100 kDa).

Purification of *Ac. calcoaceticus* and *S. maltophilia* ATCases.

In both cases the growth conditions, harvesting and sonication of the cells were the same. Cells were grown at 30 °C in Nutrient Broth (Oxoid) in 1 l conical flasks containing 350 ml medium. Each flask was inoculated with an overnight culture (50 ml). The cells were harvested at mid-exponential phase (after about 6 h). Sonication and centrifugation were performed as described above.

***S. maltophilia*.** Solid ammonium sulphate was added to the supernatant (slowly, with stirring, at room temperature). The pellet obtained after centrifugation of the 45–65% saturated fraction was collected; the pellet was stored overnight at 4 °C. Subsequently, the pellet was dissolved in Tris/HCl (20 mM, pH 7.5) and adjusted to an ammonium sulphate concentration of 1.7 M before chromatography on a Pharmacia Phenyl Sepharose column (HR 5/5) using a linear gradient over the range 1.7–0.0 M ammonium sulphate in Tris/HCl (0.1 M, pH 7.5) over 30 ml. Active fractions from the column were desalted (Pharmacia Fast Desalting column HR 10/10) and rechromatographed on a Pharmacia Mono Q column which was eluted with a linear salt gradient over the range 0–1 M NaCl in Tris/HCl (20 mM, pH 8.5) over 40 ml. Active fractions were separately desalted, concentrated (Microcon 10, Amicon) and electrophoresed on a 7.5% polyacrylamide gel with a 4% stacking gel under non-denaturing conditions. The gel was first stained for ATCase activity and the opalescent white bands nicked with a blade before staining the gel with Coomassie Blue. The fraction showing no protein contaminants in the region of the enzyme was electrophoresed once more under non-denaturing conditions after loading into a single lane running across the width of the gel. After activity staining, the white band was excised, and the protein electroeluted from the gel fragments (Biotrap, Schleicher & Schuell). After concentration, the eluate was electrophoresed by SDS-PAGE using a 15% gel and a 4% stacking gel and stained with Coomassie Blue.

***Ac. calcoaceticus*.** The sonicate was diluted 1:1 with deionized water and applied to a Pharmacia Mono Q column in 2 ml aliquots (approx. 25 mg protein). Column elution was achieved with a linear gradient over the range 0–1 M NaCl in Bis Tris

Propane (20 mM, pH 8.0). Active fractions were pooled and dialysed overnight at 4 °C against 1.7 M ammonium sulphate, 50 mM Tris/HCl, 1 mM dithiothreitol, adjusted to pH 7.4. The dialysate was rechromatographed on a Pharmacia Phenyl Sepharose column (HR5/5) eluted with a linear gradient over the range 1.7–0.0 M ammonium sulphate. Buffering and additives were as described for the dialysing medium. Active fractions from the column were pooled and concentrated to 0.5 ml by centrifugal concentration (Microcon 10, Amicon). The concentrate was applied to a gel filtration column (Hiload Superdex 16/60, Pharmacia) equilibrated with 50 mM Tris/HCl, 0.1 M NaCl, 0.05 % sodium azide, pH 7.4. Active fractions were concentrated and analysed by PAGE carried out under non-denaturing conditions as described above. The gels were stained for ATCase activity and for protein as above. Fractions in which only one band was present which stained for both protein and enzyme activity were assessed by SDS-PAGE.

Chemicals. Chemicals were purchased from Sigma unless otherwise stated.

RESULTS

Estimation of molecular mass of ATCases by PAGE on gradient gels under non-denaturing conditions

Pretreated sonicates were electrophoresed for 12 h at 125 V. After this time, there was no further migration as each molecular species had reached its limiting pore size in the gel (Arcus, 1970). The opaque white bands which appeared after activity staining the gels for ATCase are shown in Fig. 1. The ATCases fall into three migration groups which correspond approximately to 100 kDa: (*X. campestris*, *S. maltophilia* and *Lys. enzymogenes*), 300 kDa (*E. coli*) and 450–500 kDa (*Ac. calcoaceticus*, *Leu. mucor*, *Azomonas agilis*, *Azotobacter vinelandii*, *D. radiophilus*, *Ps. syringae* and *Para. denitrificans*).

Two activity staining bands were always visible on the gel from the *Azomonas agilis* (lane 8) and *D. radiophilus* (lane 6)

Fig. 1. PAGE of bacterial sonicates under non-denaturing conditions on 4–20% gradient gels. Lanes: 1, *X. campestris*; 2, *S. maltophilia*; 3, *Lys. enzymogenes*; 4, *Para. denitrificans*; 5, *Ps. syringae*; 6, *D. radiophilus*; 7, *Azotobacter vinelandii*; 8, *Azomonas agilis*; 9, *Leu. mucor*; 10, *Ac. calcoaceticus*; 11, *E. coli*.

Table 1. Estimation of molecular mass by gel filtration

See Methods for details of procedure.

Organism	Molecular mass (kDa)
<i>V. natriegens</i>	280
<i>D. radiophilus</i>	500
<i>Ac. calcoaceticus</i>	480
<i>Ps. syringae</i>	490
<i>Lys. enzymogenes</i>	120
<i>S. maltophilia</i>	112
<i>X. campestris</i>	126

extracts. No explanation can be offered for this but they appear to be due to genuine ATCase activity, since there were no bands on the control gel where aspartate was omitted from the activity stain. The ATCase from *V. natriegens* falls into the same molecular mass band as *E. coli*, but was not included on the gel in Fig. 1 because it stained much more slowly than the other enzymes. The relationship between molecular mass and migration distance departs from strict linearity below 140 kDa, which means obtaining an accurate estimate of molecular mass for proteins is not possible in this region of the gel.

The sonicate from *S. maltophilia* cells which had been extracted in the presence of 20 % PEG and 1 mM ZnCl₂ was electrophoresed and treated as described. After activity staining, the apparent molecular mass of the enzyme was unchanged.

At least three separate batches of each species were cultured and analysed.

Estimation of molecular mass of ATCases by gel filtration

To confirm some of the results obtained from the gradient gels, molecular mass determinations were carried out by gel filtration. The results are shown in Table 1. The estimates obtained from gel filtration agreed with the molecular mass banding pattern shown on the gradient gels. The error of the gel filtration procedure was assessed at approximately $\pm 5\%$, so it is not possible to attribute significant differences to molecular mass values of ATCases in the region of 100 kDa.

Purification of the ATCases from *S. maltophilia* and *Ac. calcoaceticus*

The purification procedures for both enzymes were performed on at least three occasions.

***S. maltophilia*.** It would be reasonable to assume that the ATCases found to have a molecular mass value in the region of 100 kDa would have the structure of the basic catalytic trimer as shown for the Gram-positive organism *Bacillus subtilis* (Brabson & Switzer, 1975). To test this assumption, the ATCase from *S. maltophilia* was purified (the stages in the purification are described in Table 2). The overall purification after the chromatographic steps

Table 2. Purification of ATCase from *S. maltophilia*

Step	Total activity (units)	Specific activity [units (mg protein) ⁻¹]	Purification factor (-fold)	Yield (%)
1. Sonicate*	321	0.26	1	100
2. 45–65% ammonium sulphate pellet	194	1.1	4	60
3. Hydrophobic interaction chromatography (Phenyl Sepharose)	120	13	12.2	37
4. Anion exchange (Mono Q) (most active fraction)	6.8	83	319	2

* Prepared from 6 l of culture.

Fig. 2. SDS-PAGE of aspartate transcarbamoylase from *S. maltophilia* (lane 2). Lanes 1 and 3 are molecular mass markers.

was 320-fold. At this stage PAGE under non-denaturing conditions, followed by activity staining and then by protein staining, showed that the enzyme constituted about one-third of the total protein in the sample (not shown). Excision and elution of the gel strip containing the enzyme activity, followed by SDS-PAGE gave a single band corresponding to 35 kDa (Fig. 2). This information taken together with an estimated molecular mass of 112 kDa suggests a trimeric structure for the enzyme.

A. calcoaceticus. The reasons for purifying this enzyme were similar. It has been shown that three fluorescent pseudomonads have ATCases with molecular mass values in the 480 kDa region and are constructed from polypeptides of around 34 and 45 kDa (Bergh & Evans, 1993;

Shepherdson & McPhail, 1993; Schurr *et al.*, 1995). It therefore seemed likely that bacterial ATCases from outside the pseudomonad group which have similar high molecular mass values would have the same structure. Purification of *A. calcoaceticus* ATCase was undertaken to test this presumption. The enzyme was purified 780-fold (Table 3). SDS-PAGE on the purified protein showed the molecule to contain polypeptides of 44 and 36 kDa (Fig. 3), thus confirming the similarity of this enzyme with the ATCases of the fluorescent pseudomonads.

DISCUSSION

Our findings, and those of others, on the distribution of the different classes of bacterial ATCase are summarized in Table 4. It shows clearly that class A ATCases (molecular mass ~ 450 kDa) are not confined to the fluorescent pseudomonads, but appear to be much more widely distributed. Enzymes of this type are now known to be present in nine species (see Table 4). It may be useful to view these results in a phylogenetic context. Within the γ subdivision of the Proteobacteria, Woese *et al.* (1985) have defined three subgroupings, γ -1, γ -2 and γ -3 on the basis of 16S rRNA partial sequence comparisons. They have further sought to define clusters of closely related organisms within these groups. One such cluster in the γ -3 group is the fluorescent pseudomonads, the acinetobacters, the xanthomonads, *S. maltophilia* and lysobacters. It might therefore be expected that ATCases from all these organisms would share the class A structure. This is almost certainly so for *Ac. calcoaceticus* ATCase which shows strong similarity to the ATCases of the fluorescent pseudomonads, both in the molecular mass of the polypeptide chains composing the subunits, and in the molecular mass of the holoenzyme. Unexpectedly however, *X. campestris*, *S. maltophilia* and *Lys. enzymogenes*, had ATCases of approximately 100 kDa, suggesting that only the catalytic trimer was present. Purification of the *S. maltophilia* ATCase confirmed that only 35 kDa polypeptide chains were present in the molecule. An explanation for this could be that the catalytic and non-

Table 3. Purification of ATCase from *Ac. calcoaceticus*

Step	Total activity (units)	Specific activity [units (mg protein) ⁻¹]	Purification factor (-fold)	Yield (%)
1. Sonicate*	40	0.4	1	100
2. Anion exchange (Mono Q)	22	37	92	55
3. Hydrophobic interaction chromatography (Phenyl Sepharose)	18	197	492	45
4. Gel filtration	8	310	775	20

* Prepared from 1 l of culture.

Fig. 3. SDS-PAGE of the stages in the purification of ATCase from *Ac. calcoaceticus*. Lanes: 1, sonicate; 2, after anion exchange chromatography; 3, after Phenyl Sepharose chromatography; 4, the purified enzyme after gel filtration; 5 and 6, high- and low-molecular-mass markers, respectively.

catalytic subunits dissociate very readily in these species, although we regard this as unlikely in view of the known stable binding of the two types of subunit in the fluorescent pseudomonads (Bergh & Evans, 1993; Shepherdson & McPhail, 1993). We have tried to exclude the possibility that the 100 kDa enzymes identified in this study are dissociation products of class A or class B ATCases. We have not been able to detect the presence of class B non-catalytic subunits (genes or polypeptides) in *S. maltophilia* using Southern or Western blotting (results not shown). A similar approach to the class A enzymes is complicated by the homology of the 45 kDa gene with the dihydroorotase gene (Schurr *et al.*, 1995). In testing for the expression of the 45 kDa subunit, we were handicapped by polyclonal antisera raised to *Ac. calcoaceticus*

ATCase proving to be very species-specific. A further indication that *S. maltophilia* ATCase has not dissociated during extraction is the observation that the molecular mass is unaltered under conditions designed to preserve the integrity of the enzyme.

Another possibility, which cannot be discounted, is that the 100 kDa enzyme is the dissociation product of some hitherto unrecognized form of ATCase.

As far as we are aware, ATCases of the class C type (molecular mass ~ 100 kDa) have not previously been reported in any Gram-negative bacteria, although they seem to be the only type to have been observed in Gram-positive bacteria (see Table 4).

From RNA/DNA hybridization studies, *Azomonas agilis* and *Azotobacter vinelandii* both appear to be related to Group 1 pseudomonads (DeSmedt *et al.*, 1980). Evidence from Byng *et al.*, (1986) based on the pattern of enzymes involved in aromatic biosynthesis, suggest that they are closest to Group 1a. We found both these organisms had class A ATCases. Interestingly, so did *Para. denitrificans* and *D. radiophilus*, the former species being from the α -subgroup of the Proteobacteria, and the latter, being phylogenetically distant from the Proteobacteria (Woese, 1987).

The enterobacters, vibrios and photobacteria form another well-defined grouping within the γ -3 subgroup (Woese *et al.*, 1985). Wild *et al.* (1980) have determined the molecular mass of many ATCases from the Enterobacteriaceae, and found all of them to be of the class B (molecular mass ~ 300 kDa) variety, typified by *E. coli* ATCase. We have found *V. natriegens* ATCase to be of the same type.

From the present state of evidence, it seems that class B ATCases may be confined to the enteric bacteria and their close relatives. The comparatively widespread distribution of class A ATCases, in contrast to the apparently restricted distribution of class B ATCases, makes it tempting to consider the possibility that the ATCases of the type found in the enterobacteria and their relatives, are an offshoot from the main evolutionary pathway and that those on the main pathway are the class A type.

Table 4. Distribution of the three classes of bacterial ATCase

Organism	Class A (~ 450 kDa)*	Class B (~ 300 kDa)*	Class C (~ 100 kDa)*	Source/ reference†
Gram-negative				
<i>Aeromonas hydrophila</i> ‡		+		(1)
<i>Citrobacter diversus</i> ‡		+		(1)
<i>Enterobacter aerogenes</i> ‡		+		(1)
<i>Enterobacter (Serratia) liquefaciens</i> ‡		+		(1)
<i>Erwinia carnegieana</i> ‡		+		(1)
<i>Erwinia herbicola</i> ‡		+		(1)
<i>Escherichia coli</i> ‡		+		(1,2)
<i>Proteus vulgaris</i> ‡		+		(1)
<i>Salmonella typhimurium</i> ‡		+		(1)
<i>Serratia marcescens</i> ‡		+		(1)
<i>Shigella flexneri</i> ‡		+		(1)
<i>Yersinia enterocolitica</i> ‡		+		(1)
<i>Vibrio natriegens</i> ‡		+		(2)
<i>Pseudomonas fluorescens</i> §	+			(3)
<i>Pseudomonas syringae</i>	+			(2)
<i>Pseudomonas putida</i> ¶	+			(4)
<i>Acinetobacter calcoaceticus</i>	+			(2)
<i>Azomonas agilis</i> **	+			(2)
<i>Azotobacter vinelandii</i> **	+			(2)
<i>Leucothrix mucor</i> **	+			(2)
<i>Paracoccus denitrificans</i> **	+			(2)
<i>Deinococcus radiophilus</i> ††	+			(2)
<i>Stenotrophomonas maltophilia</i>			+	(2)
<i>Xanthomonas campestris</i>			+	(2)
<i>Lysobacter enzymogenes</i>			+	(2)
Gram-positive				
<i>Bacillus subtilis</i> ‡			+	(5)
<i>Streptococcus faecalis</i> ‡			+	(6)
<i>Staphylococcus epidermidis</i> ‡			+	(7)

* +, ATCase activity corresponds with this molecular mass (smaller molecular mass forms have sometimes been observed in addition to those reported here but these account for only a small percentage of the total activity).

† 1, Wild *et al.* (1980); 2, this investigation; 3, Bergh *et al.* (1993); 4, Schurr *et al.* (1995); 5, Brabson & Switzer (1975); 6, Chang *et al.* (1974); 7, M. Shepherdson, unpublished.

‡ Obtained by gel filtration.

§ Obtained by gel filtration and sucrose density gradient centrifugation.

|| Obtained by gel filtration and gradient electrophoresis.

¶ Obtained by gradient electrophoresis and from the derived amino acid sequence of the cloned gene.

** Obtained by gradient gel electrophoresis.

†† Despite staining Gram-positive, Deinococcaceae are thought to be more closely allied to the Gram-negative bacteria (Murray, 1986).

The ATCase from *Leu. mucor*, which proved to be of the class A type, is interesting in that according to Woese *et al.* (1985), this species is peripherally related to the enterics and vibrios, and so may be near the bifurcation point dividing species possessing the class A and class B enzymes.

So far we have not considered the functional importance of the two types of non-catalytic subunit and their genetic origins. The most extensively studied ATCase of the class

B type is from *E. coli*. In this enzyme there is no doubt that the non-catalytic subunit plays an extremely important part in the allosteric regulation of enzyme activity [although this is less clear for other enterobacteria (Foltermann *et al.*, 1981)]. Possibly the need for highly effective regulation was the driving force for the acquisition of this subunit. If so, what was its genetic origin? Did it appear as the result of duplication of the gene for a nucleotide-binding protein serving some other function?

The situation with regard to the function and genetic origin of the larger non-catalytic subunit may turn out to be more comprehensible, at least in broad concept. Schurr *et al.* (1995) have shown that the non-catalytic 44·2 kDa polypeptide chains in *Ps. putida* ATCase are homologous with the next enzyme in the pathway, dihydroorotase, although it does not itself possess dihydroorotase activity. Presumably this polypeptide has arisen as a result of gene duplication. It is not clear what functional role, if any, these six polypeptide chains play. From an evolutionary point of view, the association of the catalytic subunit together with a dihydroorotase homologue may be significant since, in eukaryotic cells, gene fusion has given rise to multifunctional enzymes, e.g. the CAD protein in mammals in which the first three enzyme activities in the biosynthetic pathway are all present on the same polypeptide. This has led Schurr *et al.* (1995) to suggest that class A enzymes may be a vestige of the eukaryotic CAD complex (reviewed by Davidson *et al.*, 1993) or its progenitor (Schurr *et al.*, 1995); the latter may be more probable.

A closer link in the evolutionary relationship between the bacterial and higher eukaryote ATCases may be the yeast *URA2* gene (Souciet *et al.*, 1989). This gene encodes a multifunctional protein which has both carbamoyl phosphate synthetase and ATCase activities. Situated between these two genes is a domain encoding a non-functional dihydroorotase which is homologous with the CAD dihydroorotase, although not with its monofunctional counterparts in bacteria and yeast.

While preparing this manuscript we have seen a preliminary communication reporting similar findings and conclusions (O'Donovan & Shanley, 1995).

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