Evolutionary Relationships among Bacterial Carbamoyltransferases

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(Received 7 February 1989; revised 17 May 1989; accepted 6 June 1989)

An immunological approach was used for the study of ornithine carbamoyltransferase (OTCase) evolution in bacteria. Antisera were prepared against the anabolic and catabolic OTCases of *Pseudomonas aeruginosa* and *Aeromonas formicans* as well as against OTCase and putrescine carbamoyltransferases from *Streptococcus faecalis*; these antisera were then tested against the unpurified OTCases, either anabolic or catabolic, of 34 bacterial strains. Extensive cross-reactions were observed between the antisera to catabolic OTCases from *P. aeruginosa*, *A. formicans* and *S. faecalis* and the catabolic enzymes from other species or genera. These antisera cross-reacted also with the anabolic OTCases of strains of the Enterobacteriaceae but not with the anabolic OTCases of the same species or of other species or genera. The cross-reaction measured between the antisera against *P. aeruginosa* anabolic OTCase and the anabolic OTCases of other *Pseudomonas* were largely in agreement with the phylogenic subdivision of *Pseudomonas* proposed by N. J. Palleroni. The correlation was also significantly higher with the anabolic enzyme of an archaeobacterium, *Methanobacterium thermoaceticum*, than with the catabolic or anabolic OTCases from other genera in the eubacterial line. The antiserum raised against *A. formicans* anabolic OTCase was quite specific for its antigen and appeared to be raised against the heaviest of the various oligomeric structures of the enzyme.

INTRODUCTION

Anabolic ornithine carbamoyltransferase (OTCase; EC 2.1.3.3) functions in the biosynthesis of arginine and catalyses the formation of citrulline and phosphate from ornithine and carbamoylphosphate. The anabolic OTCase from a variety of organisms occurs as a trimeric molecule with a subunit molecular mass ranging from 35 to 40 kDa. This trimeric structure also exists in other enzymes involved in carbamoylphosphate metabolism, namely in aspartate carbamoyltransferase (ATCase; EC 2.1.3.2) and in putrescine carbamoyltransferase (PTCase; EC 2.1.3.6) (Vickers, 1981; Cunin *et al.*, 1986).

Catabolic OTCase is part of the catabolic arginine deiminase pathway and catalyses the formation of carbamoylphosphate and ornithine from citrulline and phosphate. Catabolic OTCases are usually large proteins composed of three, six, eight, nine or more identical subunits (Legrain *et al.*, 1977; Marshall & Cohen, 1972; Baur *et al.*, 1987).

Arginine prototrophic organisms that possess the arginine deiminase pathway have two OTCases, one anabolic and the other catabolic. In *Pseudomonas putida* and *Pseudomonas aeruginosa*, both enzymes function unidirectionally. The *Pseudomonas* catabolic enzymes display intense allosteric behaviour which probably results from their large oligomeric structure,

Abbreviations: ATCase, aspartate carbamoyltransferase; OTCase, ornithine carbamoyltransferase; PTCase, putrescine carbamoyltransferase.

octameric in *P. putida* OTCase, nonameric or dodecameric in the *P. aeruginosa* enzyme (Baur *et al.*, 1987). In this context it is not clear whether trimeric and multimeric OTCases are similar proteins resulting from convergence, or homologous proteins resulting from the differentiation and specialization of an ancestral carbamoyltransferase.

To answer this question we have determined the amino acid sequence of the two *P. aeruginosa* OTCases (Baur *et al.*, 1987; Itoh *et al.*, 1988) and compared them with the known primary structure of other bacterial OTCases and ATCases. All these enzymes bear substantial sequence similarities which indicate a close evolutionary relationship.

More information concerning the structural similarities between the carbamoyltransferases was obtained by exploring the immunological relationships of the enzymes from various bacteria. Previous immunological studies (Falmagne *et al.*, 1985) have shown that the catabolic OTCase from *P. putida* shares common antigenic determinants with other catabolic OTCases and also with the anabolic OTCase from Enterobacteriaceae. Using specific antisera directed against the *P. putida* catabolic enzyme or the *E. coli* anabolic OTCase we have observed no heterologous cross-reactions with either the *P. putida* anabolic OTCase or the anabolic OTCases from representatives of other bacterial genera (Falmagne *et al.*, 1985). In the present work we have used antisera against the anabolic and catabolic enzymes from *P. aeruginosa* and *Aeromonas formicans* as well as against OTCase and PTCase from *Streptococcus faecalis*. These enzymes were selected for three reasons. Firstly, the primary structure of both the anabolic and catabolic OTCases from *P. aeruginosa* PAO has recently been determined; secondly, the enzymes from *Aeromonas* have been shown to exist in various active aggregates (Legrain *et al.*, 1977) which could have different immunological properties; thirdly, PTCase displays OTCase activity and, for this reason, was included in the immunochemical comparison with the OTCases. Finally, whenever possible, NH₂-terminal sequences were determined for purified enzymes in order to complete their structural comparison.

METHODS

Strains. The strains used and their origins are listed in Table 1. *Methanobacterium thermoautotrophicum* (DMS 2133) was obtained from T. Leisinger, Zürich, Switzerland.

Media and growth conditions. Conditions for bacterial growth and cell-free extract preparation have been described previously (Falmagne *et al.*, 1985). Lactic bacteria were grown in the presence of 20 mM-glucose, yeast extract and tryptone, as described by Wargnies *et al.* (1979). Arginine (50 mM) was used as an added energy source for the production of the catabolic OTCase, whereas 25 mM-agsmatine was added for the production of PTCase.

Enzyme assays. The anabolic OTCases were routinely assayed in 50 mM-EDTA/NaOH buffer, pH 8.5, containing 10 mM each of carbamoylphosphate and ornithine. The anabolic OTCase of *Aeromonas* was assayed in the presence of 50 mM-ornithine. The catabolic OTCases from *Pseudomonas* were assayed as described by Falmagne *et al.* (1985); the catabolic OTCase and PTCase from lactic bacteria were assayed as in Wargnies *et al.* (1979); the anabolic and catabolic OTCases from *Bacillus licheniformis* were assayed as described by Broman *et al.* (1978). The OTCase from *Methanobacterium thermoautotrophicum* was assayed according to Meile & Leisinger (1984).

The detailed preparation of the purified transcarbamylases used in this study is described in Baur *et al.* (1987) for the *P. aeruginosa* PAO catabolic OTCase, in Itoh *et al.* (1988) for the *P. aeruginosa* anabolic OTCase, in Wargnies *et al.* (1979) for the OTCase and PTCase from *S. faecalis*, in Falmagne *et al.* (1985) for the *E. coli* *argF* and *argI* products and in Legrain *et al.* (1977) for the catabolic OTCase from *Bacillus* and *Aeromonas*. The catabolic OTCase of *P. putida* was purified according to Halleux *et al.* (1972). The catalytic subunit of *E. coli* K12 ATCase was a gift from G. Hervé (Gif-sur-Yvette, France).

Purification of the anabolic OTCase from *A. formicans*. *A. formicans* NCIB 9232 was grown aerobically at 30 °C on minimal medium 154 (Stalon *et al.*, 1972) supplemented with glucose and ammonia (Stalon & Mercenier, 1984) in a 15 l fermenter. Cells were collected by centrifugation at the end of the exponential phase (8×10^8 cells ml⁻¹) and sonicated (Legrain *et al.*, 1977) in the presence of 200 ml EDTA/NaOH buffer (25 mM, pH 6.8) containing 100 mM each of potassium phosphate and ornithine, and 3 mM-2-mercaptoethanol. After centrifugation at 20000 g for 20 min the crude extract was heated at 60 °C for 15 min. The coagulated solids were discarded by centrifugation. The supernatant was fractionated by ammonium sulphate precipitation and the 30–60% saturation fraction was collected. This fraction was dialysed against 25 mM-EDTA/NaOH buffer, pH 6.8, and applied to a DEAE-Sephacrose CL6B column (5 × 40 cm) equilibrated with the same buffer. OTCase was eluted with a linear gradient of potassium phosphate (50–400 mM; 800 ml), concentrated by ultrafiltration with a Diaflow PM10 membrane

Table 1. *Bacterial strains used in this investigation*

Species	Strain or source	Species	Strain or source
<i>Pseudomonas aeruginosa</i>	PAO	<i>Proteus vulgaris</i>	IRC 9623
<i>Pseudomonas putida</i>	IRC 204	<i>Escherichia coli</i> C 600 OTC ⁻ Δ(pMC20)	This laboratory
<i>Pseudomonas putida</i>	ATCC 12633	<i>Escherichia coli</i> C 600 OTC ⁻ Δ(pA11)	This laboratory
<i>Pseudomonas fluorescens</i>	ATCC 13525	<i>Aeromonas formicans</i>	NCIB 9232
<i>Pseudomonas aureofaciens</i>	ATCC 13985	<i>Streptococcus faecalis</i>	ATCC 11700
<i>Pseudomonas mendocina</i>	NCIB 10541	<i>Streptococcus faecalis</i>	IRC
<i>Pseudomonas cepacia</i>	NCTC 10743	<i>Streptococcus faecalis</i>	ATCC 14508
<i>Pseudomonas caryophylli</i>	ATCC 25418	<i>Streptococcus faecium</i>	ATCC 9790
<i>Pseudomonas syringae</i>	ATCC 19310	<i>Lactobacillus brevis</i>	IP 7135
<i>Pseudomonas stutzeri</i>	ATCC 11607	<i>Lactobacillus fermentum</i>	ATCC 9338
<i>Pseudomonas pseudoalcaligenes</i>	ATCC 17440	<i>Streptococcus lactis</i>	NCIB 8586
<i>Pseudomonas indigofera</i>	ATCC 19706	<i>Staphylococcus aureus</i> Cowan	ATCC12598
<i>Pseudomonas maltophilia</i>	ATCC 13637	<i>Bacillus licheniformis</i>	ATCC 14580
<i>Pseudomonas acidovorans</i>	ATCC 17439	<i>Bacillus subtilis</i>	Marburg C4
<i>Pseudomonas testosteroni</i>	ATCC 11996	<i>Corynebacterium glutamicum</i>	ATCC 21659
<i>Salmonella typhi</i>	ATCC 6539	<i>Brevibacterium fluvum</i>	ATCC 21493
<i>Klebsiella aerogenes</i>	MK53*	<i>Agrobacterium tumefaciens</i>	R10

* Received from B. Magasanik. Massachusetts Institute of Technology, Boston, MA, USA.

(Amicon), and dialysed against 25 mM-EDTA/NaOH buffer, pH 8.0, supplemented with 50 mM-ornithine and 3 mM-2-mercaptoethanol. A G-200 Sephadex column (2.5 × 40 cm) equilibrated with the same buffer was loaded with the OTCase fraction. The fractions containing the highest activities were pooled, concentrated and dialysed against 25 mM-EDTA/NaOH buffer, pH 6.0, containing 50 mM-potassium phosphate and 100 mM-KCl and then rechromatographed on a G-200 column (2.5 × 40 cm) equilibrated with the same buffer. The fractions containing the highest activities were again pooled, concentrated and dialysed against 25 mM-potassium phosphate buffer, pH 7.0, and stored at -20 °C.

Thermostability. Tris/HCl buffer (50 mM; 1.9 ml; pH 8.0) containing the desired effector was heated at 60 °C for 5 min, then 0.1 ml of the enzyme (200 units) was added to the test-tube and rapidly mixed. At intervals, 50 µl samples of the solution were removed and rapidly chilled in a test-tube containing the reaction mixture (minus carbamoylphosphate) for the OTCase assay. After 3 min the reaction was started at 30 °C by the addition of carbamoylphosphate.

Molecular sieving: determination of the molecular mass of the *Aeromonas* anabolic OTCase. A 2.5 × 40 cm column was packed full with swollen Sephadex G-200. To assure constant bed-height and equilibration with eluent, the column was washed for 36 h with eluting buffer containing 3 mM-2-mercaptoethanol in each case. A flow rate of 12 ml h⁻¹ was maintained during the elution process. Complete elution of the OTCase activity was achieved within 8 h. Before each run, the native enzyme (2000 units) was dialysed extensively for 24 h against 3 × 1 l changes of buffer (see Results).

Electrophoresis. SDS-PAGE and the cross-linking of the subunits of the native enzyme were done as described previously (Legrain & Stalon, 1976).

Preparation and analysis of antisera. Purified carbamoyltransferases were used to immunize rabbits. Material for injections was emulsified in an equal volume of Freund's complete adjuvant (total volume 2.0 ml). For each protein, two animals received first multiple intradermal and one intramuscular injections. Three subsequent injections of enzyme were administered at 3-week-intervals. The total amount of enzyme given to each rabbit was 0.5 mg. Each animal was bled on several occasions to test for antisera. Antisera were titrated against the homologous antigen. Those having the highest titre were pooled and stored at -80 °C. Antisera used for precipitation tests were heated for 30 min at 56 °C and coagulated materials were discarded by centrifugation.

Quantitative immunoprecipitation. Quantitative measurements of cross-reactivity were made by incubating various dilutions of antiserum ranging from 10 nl to 100 µl of antiserum with a fixed amount of enzyme activity (10 units). A given sample of antiserum was assayed in duplicate or triplicate. The immunoprecipitated antibodies linked to staphylococcal protein A were removed by centrifugation as described by Falmagne *et al.* (1985). Tubes with non-immune serum were always included to correct for nonspecific precipitation. With each antiserum, it was checked that similar levels of antiserum were needed to precipitate the specific purified antigen and a cell-free extract preparation. With the exception of *A. formicans* anabolic OTCase (see Results), less than 10% variation was observed with purified or crude cell extract preparation. In each case, a check was made to ensure that no inhibition of enzyme activity occurred in the range of antibodies used in this study, even when the highest amount

of antiserum was incubated with its antigen. The titre is defined as the antiserum concentration at which 75% of the antigen activity was precipitated. The index of dissimilarity (ID) refers to the ratio of antiserum concentration needed to precipitate 75% of the heterologous and homologous activities. Preparation of antisera, amino acid analysis and NH_2 -terminal amino acid sequences are described in detail in a previous publication (Falmagne *et al.*, 1985).

RESULTS

Anabolic OTCase of A. formicans

Purification. *A. formicans* anabolic OTCase was purified from a crude extract of the wild-type strain which had a specific activity of 110 units h^{-1} (mg protein) $^{-1}$. After 1600-fold purification, as described under Methods, 5 mg of an electrophoretically 95% homogeneous preparation, specific activity 175000 units h^{-1} (mg protein) $^{-1}$, was obtained with a yield of 15%. SDS-PAGE gave a single band corresponding to a subunit of molecular mass of 36 kDa. The results of cross-linking experiments followed by SDS-PAGE revealed five major bands; when the migration of these bands was plotted versus the molecular mass, the covalently cross-linked species produced by amidination appeared to be multiples of the 36 kDa protomer. The five protein species corresponded respectively to the monomer, the dimer, the trimer, the hexamer and the nonamer of the subunit. Increasing the amidination time increased the amount of the higher molecular mass species and decreased that of the low molecular mass species. This indicates that the higher molecular mass species may well result from an association of trimeric species. With an extended amidination time a minor additional band of higher molecular mass than the nonamer could be observed, suggesting a small amount of intermolecular cross-linking.

Molecular sieving: molecular mass determination. The molecular mass of the native enzyme was estimated by molecular sieving in the presence of several eluents. Representative results are given in Fig. 1. Depending on the conditions used, two molecular forms of the enzyme were obtained. Under acidic conditions (pH 6.0) in the presence of either 25 mM-citrate/NaOH, 25 mM-EDTA/NaOH or 25 mM-potassium phosphate, the prominent activity eluted at a molecular mass of 360 kDa, corresponding probably to a nonameric species. In contrast, basic pH (8.5) increased the amount of a trimeric species with a molecular mass of 120 kDa. Dissociation into trimers could also be promoted by ornithine and phosphate, or by ethylene glycol and other non-aqueous solvents. Enzyme dissociation was prevented by salt (100 mM-KCl). In all cases, the molecular mass of the enzyme was unaffected by the absence or presence of thiol agents (10 mM-2-mercaptoethanol), thus indicating that the bonds between the subunit are entirely noncovalent. The same pattern of enzyme distribution was observed with either crude or purified preparations. The variation of molecular mass of the native enzyme with KCl, ethylene glycol and pH suggests that hydrophobic bonds are involved in the quaternary structure of this enzyme.

Thermostability of the enzyme. When purified enzyme was incubated in 50 mM-Tris/HCl buffer, pH 8.8, at 60 °C, the half-life of the enzyme was about 10 min. However, when 10% (v/v) ethylene glycol or 10 mM-ornithine was added to the buffer, the enzyme was more rapidly inactivated (half-life 2–3 min). The presence of 100 mM-KCl prolonged the half-life to about 2 h.

These results may indicate that the different conformational states of the enzyme have different thermostabilities and suggest that the most dissociated active form of the enzyme is the most labile. This is strengthened by the fact that the molecular mass species of 120 kDa obtained after gel filtration was completely destroyed within 1 min exposure to 60 °C, while the molecular mass species of 360 kDa had a half-life of about 15 min under the same conditions.

Specificity of the antiserum to OTCases of A. formicans and P. aeruginosa

Confirming our previous results, antisera raised against the catabolic OTCase of either *A. formicans* or *P. aeruginosa* cross-reacted with the other catabolic OTCases (Table 2). The corresponding enzymes of Gram-positive bacteria showed lower activity with the two antisera. In addition, cross-reactivity was also observed with some anabolic enzymes of Enterobacteriaceae. However, strong differences in the cross-reactivities of the two antisera were observed

Fig. 1. Distribution of *A. formicans* anabolic OTCase activity after gel filtration on a Sephadex G200 column (2.5×40 cm). V_e , elution volume; V_0 , void volume. Elution buffers used were: A, potassium phosphate (50 mM, pH 6.0); B, potassium phosphate (50 mM, pH 8.0) supplemented with 50 mM-ornithine; C, EDTA/NaOH (20 mM, pH 8.5) supplemented with 50 mM-ornithine; D, EDTA/NaOH (20 mM, pH 8.5) supplemented with 50 mM-ornithine and 25% (v/v) ethylene glycol.

with *argF* and *argI* products of *E. coli* K12 (Table 2). These isoenzymes show an overall identity of 86% at the amino acid level (Van Vliet *et al.*, 1984), while they present a 57.1% and 55.7% identity respectively with the *P. aeruginosa* catabolic OTCase (Baur *et al.*, 1987). This strong discrepancy between the cross-reactivity of the two *E. coli* isoenzymes with the *P. aeruginosa* or *A. formicans* catabolic OTCase (Table 2) could conceivably be due to only a few key antigenic sites on the enzyme molecules. Consequently, any alteration in those sites could greatly exaggerate the dissimilarity established by immunological tests. A simple method for predicting antigenic protein determinants from amino acid sequence was described by Hopp & Woods (1985). It involves the calculation of local hydrophilicity values using the six amino acids surrounding each position in the sequence, and plotting hydrophilicity against position. Hopp and Woods have shown, for different proteins studied previously, that the major hydrophilicity peaks correspond to antigenic determinants. The rules of Hopp and Woods, when applied to the amino acid sequences of *P. aeruginosa* catabolic OTCases and of the *E. coli* anabolic isoenzymes, showed that a hydrophilicity peak present in the profile of *P. aeruginosa* OTCase and of *E. coli* *argF* isoenzyme is absent in the *E. coli* *argI* OTCase. This hydrophilicity peak is located at residues 201–210 with a magnitude of 1.3 in the catabolic OTCase and 1.2 in the *argF* isoenzyme, whereas the same region appears to be fairly hydrophobic in the *argI* sequence. However, the prediction rules of Hopp and Woods do not take into account the fact that hydrophobic and aromatic residues are often implicated in epitopes. On the other hand, regions corresponding to the amino- or carboxy-terminus often elicit antibody formation (Westhof *et al.*, 1984). In this context, it is noteworthy that both the catabolic OTCase and the *E. coli* *argF* OTCase bear

Table 2. *Indices of dissimilarity for OTCases with the catabolic OTCases from P. aeruginosa and A. formicans as reference points*

Values are representative of two experiments; variations are less than 5%. The titre (nl antiserum required to precipitate 75% of 10 units of antigen activity) was 21 nl and 43 nl for the catabolic OTCases from *A. formicans* and *P. aeruginosa* respectively. The numbers in parentheses indicate the true dissimilarity index which is defined as the ratio of antiserum concentration needed to precipitate 1 pmol of heterologous and homologous antigen. Previously published data for the turnover number of catabolic OTCases for *P. putida* (Halleux *et al.*, 1972), *P. aeruginosa* (Baur *et al.*, 1987), *Streptococcus faecalis* (Wargnies *et al.*, 1979) were used. In the calculation, values for *E. coli* K12 OTCase isoenzymes were similar, as previously found for *E. coli* W (Legrain & Stalon, 1976).

Enzyme source	Enzyme function	Index of dissimilarity	
		Anti- <i>A. formicans</i> OTCase	Anti- <i>P. aeruginosa</i> OTCase
<i>Aeromonas formicans</i> NCIB 9232	C	1	1.6 (10.4)
<i>Pseudomonas aeruginosa</i> PAO	C	192 (42)	1
<i>Pseudomonas putida</i> IRC 204	C	941 (128)	3.3 (2.3)
<i>Pseudomonas putida</i> ATCC 12633	C	NT	3.4
<i>Pseudomonas fluorescens</i> ATCC 13525	C	1322	6.0
<i>Pseudomonas mendocina</i> NCIB 10541	C	340	1.2
<i>Proteus vulgaris</i> IRC 9623	A	394	3.5
<i>Escherichia coli</i> K12 <i>argF</i> product	A	71 (25)	3.7 (8.2)
<i>Escherichia coli</i> K12 <i>argI</i> product	A	780 (279)	NC
<i>Klebsiella aerogenes</i> MK53	A	617	NT
<i>Salmonella typhi</i> ATCC 6539	A	NC	3.7
<i>Bacillus licheniformis</i> ATCC 14580	C	+	64
<i>Streptococcus faecalis</i> ATCC 11700	C	+	181 (801)

Abbreviations: NC, no cross-reaction; NT, not tested; A, anabolic OTCase; C, catabolic OTCase; +, positive cross-reaction at the highest level of antiserum concentration (100 µl).

anionic residues in the carboxy-terminal region while a lysine is present at the same position in the *E. coli argI* product. Finally, antibodies could be directed against topographic determinants involving residues far apart in the amino acid sequence, but brought together on the surface of the native molecule. The residues where the *E. coli argI* product differs from both the *argF* product and the catabolic OTCase of *Pseudomonas* are Gln41, Gly122, the tripeptide Leu-Ala-Asp at position 139–141, the lysine residues at positions 190, 224 and 242, Ala200, Glu207 and Glu242. Anionic residues appear at position 196–197 in both the catabolic enzyme from *Pseudomonas* PAO and *E. coli argF* isoenzyme, whereas two alanines are present in the corresponding *argI* sequence. The precise delineation of antigenic domains will require a complete structure determination by X-ray diffraction analysis, and this is under way for the *P. aeruginosa* catabolic OTCase.

Antiserum to *A. formicans* and to *P. aeruginosa* catabolic OTCases do not cross-react with the anabolic enzymes of other bacteria including their own genera. The hydrophilicity profile of the *P. aeruginosa* anabolic enzyme also showed peaks of hydrophilicity quite different from those of either the catabolic enzyme of the same organism or of the *E. coli argF* and *argI* anabolic isoenzymes.

Specificity of the antiserum to A. formicans anabolic OTCase

Quite different levels of antisera were needed to precipitate the crude and the purified anabolic OTCase. However, when the preparations were dialysed in the presence of various effectors, the same levels of antisera were required to precipitate both enzymes. The enzyme dialysed in 50 mM-potassium phosphate, pH 7.0, with 100 mM-KCl required the lowest level of antiserum, 20 nl, while in the presence of 10% ethylene glycol five times more antiserum was required to precipitate the enzyme dialysed against the same buffer adjusted to pH 8.0. In addition, when ornithine was included in the dialysis buffer, only partial precipitation (60%)

Table 3. Indices of dissimilarity for anabolic OTCases with *P. aeruginosa* anabolic OTCase as a reference point

Values are representative of two experiments; variations are less than 5%. The titre (nl of antibody required to precipitate 75% of 10 units of *P. aeruginosa* anabolic OTCase) was 36 nl.

Enzyme source	Taxonomic classification (according to Palleroni <i>et al.</i> , 1974)	Index of dissimilarity
<i>Pseudomonas aeruginosa</i> PAO	I	1.0
<i>Pseudomonas stutzeri</i> ATCC 11607	I	3.4
<i>Pseudomonas fluorescens</i> ATCC 13525	I	3.5
<i>Pseudomonas mendocina</i> NCIB 10541	I	5.1
<i>Pseudomonas syringae</i> ATCC 19310	I	6.2
<i>Pseudomonas pseudoalcaligenes</i> ATCC 17440	I	6.4
<i>Pseudomonas putida</i> IRC 204	I	7.1
<i>Pseudomonas putida</i> ATCC 12633	I	7.2
<i>Pseudomonas aureofaciens</i> ATCC 13985	I	14.7
<i>Pseudomonas caryophilli</i> ATCC 25418	II	+ (> 500)
<i>Pseudomonas cepacia</i> NCTC 10743	II	+ (> 500)
<i>Pseudomonas acidovorans</i> IP 60-78	III	+ (> 500)
<i>Pseudomonas testosteroni</i> ATCC 11996	III	NC
<i>Pseudomonas maltophilia</i> ATCC 13607	V	NC
<i>Pseudomonas indigofera</i> ATCC 19706	U	416.0
<i>Methanobacterium thermoaceticum</i>		34.7
<i>Agrobacterium tumefaciens</i> R10		+ (> 500)

Abbreviations: NC, no cross-reaction; U, unclassified; +, positive cross-reactivity at the highest level of antiserum (100 µl).

was observed, even with the highest level of antisera (10 µl). These observations led to the conclusions: (i) that antibodies are directed against determinants which undergo conformational changes upon the addition of ligands; (ii) that some antibodies are able to combine with only one conformational state. Since a different quaternary structure is reached in the presence of either KCl or ethylene glycol, two possibilities must be considered to explain the fact that antibodies react more effectively in the presence of KCl, which favours the nonameric structure of the enzyme: (i) some antigenic determinants are formed by the assembly of the trimeric structures into the nonameric form; (ii) some antigenic determinants have a different conformation in the nonameric and in the trimeric states.

Some cross-reactivity was observed with the *A. formicans* catabolic OTCase, which also exists as various oligomeric species. The ID value for the catabolic OTCase relative to the anabolic enzyme was 100. However, no cross-reaction could be detected, either with the anabolic OTCases from various arginine prototrophic organisms, or with catabolic OTCases from other genera used in this study.

Specificity of the antiserum to *P. aeruginosa* anabolic OTCase

Specific antisera gave heterologous cross-reactions of various intensities with the anabolic OTCases from Pseudomonadaceae (Table 3). The anabolic OTCases from the *Pseudomonas* of group I according to the classification of Palleroni *et al.* (1974) showed heterologous cross-reactions of considerable strength with antiserum to *P. aeruginosa* anabolic OTCase (Table 3). Weak cross-reactions were observed with the anabolic enzyme from *Pseudomonas* of group II (*P. caryophilli* and *P. cepacia*). With the two strains of group III used in this study, one, *P. acidovorans* showed a weak cross-reaction while the other, *P. testosteroni*, showed no cross-reactivity. *P. maltophilia*, a strain of group V, did not show any reaction at all. Outside the genus *Pseudomonas*, only the anabolic enzyme of *Agrobacterium* cross-reacted with the antiserum to anabolic OTCase while, outside the eubacterial kingdom, cross-reactivity was also observed with *Methanobacterium thermoautotrophicum*. Neither the anabolic enzymes of Enterobacteriaceae, *Aeromonas*, *Bacillus licheniformis* and *Arthrobacter*, nor the catabolic OTCases from *Pseudomonas*, *Aeromonas*, *Bacillus licheniformis* and *Streptococcus faecalis* showed any cross-reaction with the antiserum.

Table 4. *Indices of dissimilarity for OTCases with Streptococcus faecalis catabolic OTCase as a reference point*

Values are the means of a single experiment done in duplicate. The titre (nl antiserum required to precipitate 75% of 10 units of *S. faecalis* catabolic OTCase) was 41 nl.

Enzyme source	Enzyme function	Index of dissimilarity
<i>Streptococcus faecalis</i> ATCC 11700	C	1.0
<i>Streptococcus faecalis</i> IRC	C	1.1
<i>Streptococcus faecalis</i> ATCC 14508	C	1.0
<i>Streptococcus faecium</i> ATCC 9790	C	3.1
<i>Streptococcus lactis</i> NCIB 8586	C	3.4
<i>Lactobacillus brevis</i> IP 7135	C	2.2
<i>Lactobacillus fermentum</i> ATCC 9338	C	8.0
<i>Staphylococcus aureus</i> Cowan ATCC 12595	C	18.4
<i>Bacillus licheniformis</i> ATCC 14580	C	105.0
<i>Aeromonas formicans</i> NCIB 9232	C	178.0
<i>Pseudomonas putida</i>	C	+
<i>Pseudomonas aeruginosa</i> PAO	C	NC
<i>Escherichia coli</i> K12 <i>argF</i> product	A	+
<i>Escherichia coli</i> K12 <i>argI</i> product	A	NC

Abbreviations: A, anabolic OTCase; C, catabolic OTCase; +, positive cross-reaction at the highest level of antiserum concentration; NC, no cross-reaction.

Specificity of antiserum to PTCase

Antibodies raised against *S. faecalis* ATCC 11700 PTCase (titre 57 nl) cross-reacted only with the homologous PTCase from the other *S. faecalis* species and with a PTCase found in a *Lactobacillus brevis* isolate. The index of dissimilarity in the case of *S. faecalis* IRC, *S. faecalis* ATCC 14508 and *Lactobacillus brevis* IP 7135 were 1.4, 1.5 and 2.1 respectively. No PTCase was produced by other lactic bacteria, or by any other bacterium used in this study. None of the five antisera to OTCases used in this study showed any cross-reactivity with these PTCases.

Specificity of the antiserum to S. faecalis catabolic OTCase

The OTCases of faecal and other streptococci, and of *Lactobacillus* and *Staphylococcus* show a high degree of resemblance to each other (Table 4). These species form a super cluster within the so-called clostridia subbranch of Gram-positive bacteria. That these organisms are distantly related to the genus *Bacillus* (Stackebrandt & Teuber, 1988) is confirmed by the weaker reactivity of the *Bacillus licheniformis* catabolic OTCase with the antiserum to *S. faecalis* OTCase. Outside the genus, cross-reactions were also observed with the catabolic enzyme of *A. formicans*. At the highest level of antiserum used (100 µl), an extremely weak cross-reaction was produced with the *P. putida* catabolic OTCase or *E. coli argF* product. However, no cross-reaction could be detected with the *P. aeruginosa* catabolic enzyme or with the anabolic enzymes of either Enterobacteriaceae, Pseudomonadaceae, *Bacillus* or *Aeromonas*.

NH₂-terminal sequences

The alignment of the NH₂-terminal sequences of several carbamoyltransferases is shown in Fig. 2. Outside bacterial genera the comparison of the OTCases from yeast, *Aspergillus* and man

Fig. 2. Comparison of the NH₂-terminal amino acid sequences of carbamoyltransferases. The sequences are aligned on *E. coli* catalytic ATCase (*pyrB* product) (Hoover *et al.*, 1983) according to their best scoring using the algorithm of Pearson & Lipman (1988). +, Residue positively charged; −, residue negatively charged; ●, hydrophobic residue, ○, polar residue. The *argI* and *argF* sequences of *E. coli* K12 were obtained from Bencini *et al.* (1983) and Van Vliet *et al.* (1984). The ATCase sequence of *B. subtilis* is from Lerner & Switzer (1986). The eukaryotic carbamoyltransferase sequences (*Saccharomyces*, *Aspergillus* and human) are from Huygen *et al.* (1987). The *arcB* sequences of *P. putida* and *A. formicans* are from Falmagne *et al.* (1985), and the *arcB* and *argF* products of *P. aeruginosa* from Baur *et al.* (1987) and Itoh *et al.* (1988). The amino sequences of *S. faecalis* catabolic OTCase and PTCase and *A. formicans* anabolic OTCase were determined in this study.

	1	5	10	15	20	25	30	35	40																																								
<i>Escherichia coli pyrB</i>	A	N	P	L	Y	Q	K	H	I	I	S	I	N	D	L	S	R	R	D	D	L	N	L	V	L	A	T	A	A	K	L	K	A	N	P	Q	P	E	L	L	K	H	K						
						+																																											
<i>Escherichia coli argI</i>	S	G	F	Y	H	K	H	F	L	K	L	L	D	F	T	P	A	E	L	N	S	L	L	Q	L	A	A	K	L	K	A	D	K	K	S	G	K	E	E	A	K								
						+																																											
<i>Escherichia coli argF</i>	S	D	L	Y	K	K	H	F	L	K	L	L	D	F	T	P	A	Q	F	T	S	L	L	T	L	A	A	Q	L	K	A	D	K	K	N	G	K	E	V	Q	K								
						+																																											
<i>Bacillus subtilis pyrB</i>	K	H	L	T	M	S	E	L	S	T	E	E	I	K	D	L	L	Q	T	A	Q	E	L	K	S	G	K	T	D	N	Q	L	T	G	K														
						+																																											
<i>Saccharomyces cerevisiae</i>	S	T	T	A	A	S	T	P	S	L	R	H	L	I	S	I	K	D	L	S	D	E	E	F	R	I	L	V	Q	R	A	Q	H	F	K	N	V	F	K	A	N	K	T	N	D	F			
<i>ARG3</i>						+																																											
<i>Aspergillus niger</i>	. . . P	P	A	S	P	F	A	P	R	H	F	L	S	I	A	D	L	S	P	S	E	F	A	T	L	V	R	N	A	S	S	H	K	R	A	I	K	S	G	M	P	Q	N	L					
						+																																											
<i>Streptococcus faecalis arcB</i>	N	S	V	F	Q	G	R	S	L	L	A	E	K	N	F	T	R	A	E	L	E	Y	L	V	D	F	S	I	H	L	K	E	P																
						+																																											
<i>Pseudomonas putida arcB</i>	A	F	N	I	H	N	R	N	L	L	S	L	E	H	H	T	N	R	E	L	R	Y	L	D	L	T	R	D	L	K	K	A	K																
						+																																											
<i>Pseudomonas aeruginosa argF</i>	S	V	R	H	F	L	S	F	N	D	Y	S	P	E	E	L	I	G	L	I	R	R	G	S	E	L	K	D	L	R	N	G	V	L															
						+																																											
<i>Aeromonas formicans arcB</i>	A	F	N	L	R	N	R	N	F	L	K	L	D	F	T	P	R	E	I	Q	Y	M	I	D	L	A	I	D	L	K																			
						+																																											
<i>Pseudomonas aeruginosa arcB</i>	A	F	N	M	H	N	R	N	L	L	S	L	M	H	H	S	T	R	E	L	R	Y	L	D	L	S	R	D	L	K	R	A	K	Y	T	G	T	E	Q	Q	H								
						+																																											
<i>Human OTCase</i>	. . . Q	N	K	V	Q	L	K	G	R	D	L	L	T	L	K	N	F	T	G	E	E	I	K	Y	M	L	W	L	S	A	D	L	K	F	R	I	K	Q	K	G	E	Y	L	P					
						+																																											
<i>Streptococcus faecalis aguB</i>	K	R	D	Y	V	T	T	E	T	Y	T	K	E	E	M	H	Y	L	V	D	L	S	L	K	I	K	E	A	I																				
						+																																											
<i>Aeromonas formicans argF</i>	K	H	L	L	K	D	S	D	L	N	K	S	H	I	E	X	L																																
						+

Fig. 3. Stereo view of the backbone folding observed in the crystalline state for *E. coli* catalytic ATCase. Modelling of *E. coli* ATCase was done using the coordinate of Monaco *et al.* (1978) and records in the protein data bank (Bernstein *et al.*, 1977). Amino acids which appear to be conserved in the eleven sequences shown in Fig. 2 are depicted by the same symbols as in that Figure.

are included (Huygen *et al.*, 1987). In this study, three new NH₂-terminal sequences were determined, those of *S. faecalis* OTCase and PTCase and of *A. formicans* anabolic OTCase. The alignment was performed by using the optimal algorithm of Goad & Kanehisa (1982) as improved by Pearson & Lipman (1988). Carbamoyltransferases are aligned according to the best scoring relative to the catalytic subunit of *E. coli* K12 ATCase, for which the crystallographic structure is known at 2.6 Å (0.26 nm) resolution (Kim *et al.*, 1987). The amino acids in positions 7, 9, 10, 16, 19, 20, 23, 24, 30 and 31 are most conserved. In the three-dimensional structure of ATCase (Fig. 3), Leu20, Val23 and Leu30 define a hydrophobic face on helix 1, which is in contact with helix 5 and 9. The side-chain of the conserved polar residues Lys7 and Lys31 point outside the protein while Ser16, which is located at the beginning of helix 1, might play a role in initiating the folding of this first helix. These observations suggest that the tertiary structure observed for ATCase in the NH₂-terminal region may well be maintained in the spatial arrangement of the other carbamoyltransferases.

DISCUSSION

The analogies found among the reaction mechanisms, the amino acid sequences (when available), and the prediction of secondary structures of carbamoyltransferases have suggested that these enzymes belong to a system of acquisitive evolution which has occurred following duplication of a gene encoding an ancestral broad specificity carbamoyltransferase (Legrain *et al.*, 1974, 1977; Hoover *et al.*, 1983; Vickers, 1981; Falmagne *et al.*, 1985; Huygen *et al.*, 1987; Van Vliet *et al.*, 1988).

The complete primary structures of eight carbamoyltransferases are now available: their comparison shows similarity at the amino acid sequence level between *E. coli* ATCase and anabolic as well as catabolic OTCases.

The determination of amino acid sequence and three-dimensional structure of proteins is a cumbersome task. Immunochemical methods could be a shortcut in the detection of structural similarities among proteins. The catabolic enzymes are usually larger proteins composed of six, eight, nine or more identical subunits while the enterobacterial anabolic OTCases generally occur as trimeric molecules. Nevertheless, our study confirms the strong immunological relationships of the catabolic OTCases from various genera with the anabolic enzymes of enterobacteria. Consequently, the variation in quaternary structure between the catabolic OTCases and the anabolic OTCases of enterobacteria reflects the different modes of assembly of a polypeptide whose structure has been relatively conserved.

In contrast, both catabolic OTCase and anabolic OTCase from enterobacteria showed no immunological relationships with the anabolic enzymes from either *Pseudomonas*, *Aeromonas* or *Bacillus* as well as OTCases from other genera. The failure to detect immunological cross-

reactivity among related proteins does not, however, conclusively eliminate the existence of structural similarities between them. This is illustrated by the strong cross-reactivity with the *E. coli argF* product and the weak or nonexistent cross-reactivity with the *E. coli argI* product of the antisera to catabolic OTCase of either *Pseudomonas* or *Aeromonas*. Yet these two *E. coli* isoenzymes bear strong sequence similarities and show an overall identity of 86% at the amino acid sequence level (Van Vliet *et al.*, 1984).

In much the same way, the antigenicity of a protein is also critically dependent on its conformation, as is shown with *A. formicans* anabolic OTCase which exists in various oligomeric forms. The specific antiserum appears to be raised against the higher oligomeric structure and shows less affinity for the trimeric structure.

The use of *P. aeruginosa* anabolic OTCase as an antigen was also justified because this enzyme showed kinetic properties which confer unidirectionality toward citrulline synthesis, by contrast with other catabolic or anabolic OTCases which can work *in vitro* and *in vivo* in both directions of the reaction (Legrain *et al.*, 1972, 1977). The determination of the sequence of this enzyme (Itoh *et al.*, 1988) showed only 45% identity with *E. coli* OTCase isoenzymes or with *P. aeruginosa* catabolic OTCase. No cross-reaction could be observed between the *P. aeruginosa* anabolic OTCase and antisera raised against the *P. aeruginosa* catabolic OTCase and the homologous enzymes of the same or other genera, nor with the antisera raised against the *E. coli* (Falmagne *et al.*, 1985) and *A. formicans* anabolic OTCases.

Conversely, no cross-reaction was observed between the antiserum to *P. aeruginosa* anabolic OTCase and catabolic OTCase as well as anabolic OTCases from the enterobacteria. Strong positive cross-reactions were, nevertheless, observed with antisera to *P. aeruginosa* anabolic OTCase and anabolic OTCase from *Pseudomonas* strains of group I as defined by Palleroni *et al.* (1974). Positive cross-reactions were also obtained with *Pseudomonas* strains of groups II and IV; these groups represent, in fact, entirely separate rRNA homology groups, only interrelated at supragenetic or superfamilial level (Byng *et al.*, 1983). Outside the pseudomonads, positive cross-reaction was observed with the enzyme of *Agrobacterium* and, outside the eubacterial line, with an Archaeobacterium, indicating that structural similarities may be conserved even between *P. aeruginosa* anabolic OTCase and members outside the eubacterial line.

X-ray analysis of *P. aeruginosa* catabolic OTCase is now in progress. Comparison with the results obtained by L. Kuo with the *E. coli* anabolic OTCase will be essential in understanding the molecular components which determine the behaviour of these immunologically related enzymes as well as the allosteric properties of the *P. aeruginosa* catabolic enzyme. On the other hand, as numerous amino acid sequence segments are highly conserved in many parts of these OTCases whose primary structure are known, model-building using computer graphics could be used with a high degree of confidence to quickly derive appropriate models for a number of other carbamoyltransferases from several origins. This work is now under progress in our laboratories.

We thank Françoise Huet, Annick Mercenier, Christiane Legrain, Jean-Paul Simon and Brigitte Wagnies for their participation in some of the experiments, Jean-Marc Godart for his technical assistance and Professor A. Piérard for reading and correcting the manuscript.

This work is supported by grants 2.4521.83 and 2.9003.88 from the Belgian Fund for Joint Basic Research, by a Research Contract EEC no. BAP-0345-B (GDF) and by an 'Action de Recherche Concertée' financed by the Belgian Government. J.-L.D. and V.S. are Research Associates of the National Fund for Scientific Research (Belgium).

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