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Catabolic ornithine carbamoyltransferase of *Pseudomonas aeruginosa*

Changes of allosteric properties resulting from modifications at the C-terminus

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Ornithine carbamoyltransferases (OTCases) catalyse the formation of citrulline and phosphate from ornithine and carbamoylphosphate by a thermodynamically favoured reaction. *In vivo*, catabolic OTCase of *Pseudomonas aeruginosa* promotes the reverse reaction, the phosphorolysis of citrulline. Although the enzyme is assayed *in vitro* in the direction of citrulline synthesis, the enzyme cannot perform this reaction *in vivo* due to poor affinity for carbamoylphosphate and high cooperativity towards this substrate. Furthermore, the dodecameric catabolic OTCase is an allosteric enzyme; the enzyme is stimulated by nucleoside monophosphates and inhibited by polyamines (e.g. spermidine). A previous study showed that a modification of the C-terminus of the catabolic OTCase alters the homotropic cooperativity of the enzyme. We have now investigated the importance of the C-terminus for homotropic and heterotropic cooperativity by site-directed mutagenesis. Deletion of the C-terminal Ile335 residue strongly reduced cooperativity for carbamoylphosphate and sensitivity to spermidine. These properties were essentially restored when the two C-terminal amino acids (Asp334 and Ile335) were removed by deletion. However, in this variant enzyme, AMP failed to abolish carbamoylphosphate cooperativity completely, whereas the wild-type enzyme was rendered virtually non-cooperative by AMP. An extension of catabolic ornithine carbamoyltransferase by 15 amino acid residues interfered with both homotropic and heterotropic interactions and lowered the maximal velocity. All variant enzymes had the same dodecameric structure as the wild type and differed only slightly in affinity for the second substrate ornithine. A structural model of the dodecamer, at 0.3-nm resolution, suggests that the C-terminus could be involved in trimer/trimer interaction. We propose that modifications at the C-terminus alter the trimer/trimer interface and, in addition, removes the salt bridge His5-Ile335 within a monomer. These changes profoundly and indirectly modify the allosteric transition and consequently the interactions of the dodecamer with carbamoylphosphate and effectors.

Pseudomonas aeruginosa has two ornithine carbamoyltransferases (OTCase). The anabolic enzyme is encoded by the *argF* gene, participates in arginine synthesis and catalyses the formation of citrulline and phosphate (P_i) from ornithine and carbamoylphosphate (Stalon et al., 1967; Haas et al., 1979). This enzyme is a trimer consisting of identical subunits of M_r 34000 (Itoh et al., 1988). The catabolic OTCase is the second enzyme of the arginine deiminase pathway, which is encoded by the *arcDABC* operon and allows ATP synthesis in the absence of respiration (Mercenier et al., 1980; Vander Wauven et al., 1984; Lüthi et al., 1990). The catabolic OTCase has 12 identical subunits of M_r 38000 (Baur et al., 1987; Marcq et al., 1991). *In vivo*, this enzyme promotes the cleavage of citrulline, the reverse of the biosynthetic reaction. The thermodynamic equilibrium of the OTCase reaction strongly favours citrulline synthesis. Therefore, *in vitro* assays of catabolic OTCase are generally performed in the anabolic direction (Stalon et al., 1977). *In vivo*, the situation is different. The reaction of the catabolic OTCase is driven by the hydrolysis of carbamoylphosphate, which is catalysed by carbamate kinase, the *arcC* product; the catabolic OTCase does not perform the biosynthetic reaction for two reasons: the enzyme has both a poor affinity for carbamoylphosphate and a high cooperativity towards this substrate (Haas et al., 1979; Baur et al., 1990; Tricot et al., 1993). The catabolic OTCase of *P. aeruginosa* also shows manifestations of an allosteric enzyme, i.e. activation by P_i and PP_i, which may interact with carbamoylphosphate-binding sites, and allosteric control of activity (Tricot et al., 1993). Positive allosteric effectors include AMP and other nucleoside monophosphates (NMP); polyamines (e.g. spermidine) are negative effectors (Tricot et al., 1993). The *P. aeruginosa* catabolic enzyme is the only allosteric OTCase

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Abbreviations. OTCase, ornithine transcarbamoylase (carbamoyltransferase); $[S]_{0.5}^{cp}$, concentration of carbamoylphosphate giving half-maximal velocity.

Enzymes. Catabolic and anabolic ornithine carbamoyltransferases (EC 2.1.3.3).

for which the primary structure is known (Baur et al., 1987) and its three-dimensional structure has been solved to 0.45-nm resolution (Marcq et al., 1991).

Mutants of the catabolic enzyme have been obtained that perform the anabolic function *in vivo* (Haas et al., 1979; Baur et al., 1990). A single amino acid replacement, alanine or glycine for glutamate at position 105, causes a strong reduction of cooperativity for carbamoylphosphate, a markedly lower $[S]_{0.5}^{CP}$ value (concentration of carbamoylphosphate giving half-maximal velocity) and an improvement of specific activity (Baur et al., 1990). In previous studies, the amino acid residues of catabolic OTCase were numbered from 1 (methionine) to 336 (isoleucine). Since the N-terminal methionine residue is removed post-transcriptionally (Baur et al., 1987), we now assign the residues of the mature polypeptide from 1 (alanine) to 335 (isoleucine). By an *in vivo* gene fusion technique, the nine C-terminal amino acids of catabolic OTCase have been replaced by the homologous eight amino acids of anabolic OTCase from *Escherichia coli*. This dodecameric hybrid enzyme also has a reduced homotropic cooperativity for carbamoylphosphate (Baur et al., 1990), indicating that the C-terminus influences carbamoylphosphate binding.

Since these results suggest that the C-terminal region of the polypeptide chain has an important role in modulating the cooperative effects, we have engineered proteins in which the C-terminus is modified by deletion or elongation in order to investigate the resulting homotropic and heterotropic properties of the mutant catabolic OTCases. Preliminary crystallographic data at 0.3-nm resolution (unpublished results) obtained for the mutant enzyme in which glutamate at position 105 is replaced by glycine, allow us to interpret the kinetic data in terms of interactions of C-terminal residues with N-terminal residues in catabolic OTCases.

MATERIALS AND METHODS

Bacteria, plasmids and growth conditions

E. coli strain ED8767 (*met hsdS supE supF recA56*) (Borck et al., 1976), JM105 (*thi rpsL endA sbcB15 hsdR4 Δ(lac proAB)*, F′ *traD36 proAB*$^+$ *lacI*^{q}Z *Δ*M15) (Yanisch-Perron et al., 1985), CJ236 (*ung-1 dut-1 thi-1 relA1*, pCJ105) (Kunkel et al., 1987) and MV1190 (*thi Δ(lac proAB) supE Δ(srl-recA)306*::Tn*10*, F′ *traD36 proAB*$^+$ *lacI*^{q}Z *Δ*M15) (Bio-Rad) were used for cloning and mutagenesis. *P. aeruginosa* strains PTO6091 (*arcD*::Tn*5−751 recA102 strA*) and PTO6095 (*arcB*::Tn*5−751 recA102 strA*) (Lüthi et al., 1986, 1990) served to express OTCases encoded by recombinant plasmids. Plasmids pME179 (Baur et al., 1989), pME183, pME190 (Lüthi et al., 1986) and pME3606 (Baur et al., 1990) have been described in previous studies; newly constructed plasmids are shown in Fig. 1. The composition of growth media and selective antibiotic concentrations were as described previously (Vander Wauven et al., 1984; Jeenes et al., 1986; Lüthi et al., 1986).

Recombinant DNA methods

Plasmid DNA was isolated from *E. coli* host strains (Del Sal et al., 1988), digested with restriction endonucleases or *Bal*31 nuclease, ligated and transferred into *E. coli* by transformation according to standard protocols (Sambrook et al., 1989). Oligonucleotide-directed mutagenesis (Kunkel et al., 1987) of the 3′ end of the *arcB* gene (Fig. 1) was performed after subcloning appropriate *arcB* fragments into M13mp18, using the Muta-Gene *in vitro* mutagenesis kit (Bio-Rad). The mutated *arcB* gene was then re-integrated into the *arc* operon in plasmid pME183, producing pME3610 and pME3612 (Fig. 1). The presence of the introduced mutations in these plasmids was checked by DNA sequencing performed with denatured double-stranded DNA (Del Sal et al., 1988) and Sequenase (US Biochemical Corporation) by the dideoxynucleotide chain-termination method (Sambrook et al., 1989).

Enzyme assay

OTCase activity was determined by quantifying citrulline by the method of Prescott and Jones (1969). The standard assay mixture consisted of 2.0 ml containing 150 mM imidazole/HCl, pH 6.7, 10 mM L-ornithine and varying amounts of carbamoylphosphate (and effectors when appropriate), as previously described (Baur et al., 1990). The carbamoylphosphate solution was prepared just before use. The reaction was started by addition of carbamoylphosphate, allowed to continue for 10 min at 37 °C and stopped by addition of a 1.0 ml antipyrine diacetylmonoxime used for the colorimetric assay of citrulline. The assay tubes were placed in a water bath at 100 °C for 20 min and the absorbance was measured at 466 nm. Citrulline standards contained all the assay components. This is essential since purine nucleotides interfere with the colorimetric assay. Blank controls contained all the reactants except for the enzyme. Chemical carbamoylation of ornithine and spermidine at high carbamoylphosphate concentrations has some chromogenic effect. All solutions of effectors were adjusted to the desired pH and the final pH of the reaction mixture was verified. Assays were performed in duplicate and the data are averages of at least two independent determinations. The standard deviations were 5−10%. Specific activities are expressed in mmol citrulline formed · h^{-1} · mg protein^{-1}. The steady-state kinetic data were analyzed in terms of Lineweaver-Burk (1934), Hanes (1932) and Eadie (1942) plots to derive maximal velocities (V_{max}) and the carbamoylphosphate concentrations required for half-maximal velocity ($[S]_{0.5}^{CP}$). Cooperativity was analyzed by standard graphical methods and the Hill coefficient (n_H) was determined by the Hill equation (1910).

Enzyme purification

Wild-type and mutant OTCases were purified as previously described (Baur et al., 1987; Marcq et al., 1991) after *in vivo* overproduction in strains PTO6091 or PTO6095 carrying one of the recombinant plasmids listed in Table 1. Cells containing the recombinant plasmids were grown in a medium containing, in 1 l 10 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl, 0.7 g K_2HPO_4, 0.3 g, KH_2PO_4, 4.2 g glutamate, 5.2 g arginine and 300 mg carbenicillin. Cells were grown semi-anaerobically to induce the OTCase as previously described (Baur et al., 1987). Enzymes were greater than 95% pure as judged by Phast gel electrophoresis in denatured or native conditions. Protein concentrations of the pure wild-type enzymes were measured at 280 nm using an absorption coefficient of 3.6×10^5 M^{-1} · cm^{-1}. Protein concentrations of the mutant enzymes were determined by the Lowry procedure or according to the A_{280}/A_{260} values (Kalckar, 1947).

Molecular masses were estimated by gel filtration on a Sephacryl HR300 (2.6 cm×60 cm) column equilibrated in

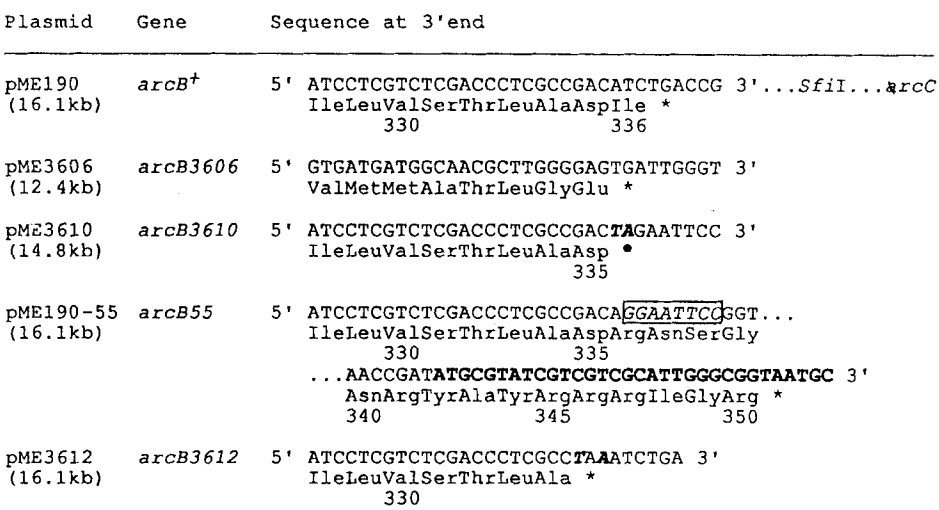

Fig. 1. Recombinant plasmids possessing an *arcB* gene modified at the 3′ end. Construction of pME190 (Lüthi et al., 1986) and pME3606 (Baur et al., 1990) has been reported. The deletion plasmids pME3610 and pME3612 were derived from pME190-55 and pME190, respectively, by site-directed mutagenesis (exchanged nucleotides are in bold-face italics). The construction of pME190-55 is described in the text; the *Eco*RI linker is boxed and nucleotides shown in bold-face type are derived from *arcC*.

20 mM potassium phosphate, pH 7.5, and calibrated with the following markers: thyroglobulin (M_r 670000), ferritin (M_r 440000), catalase (M_r 232000), aldolase (M_r 158000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000) and Blue dextran 2000 as a void-volume indicator. Blue dextran was determined by measuring the absorbance at 600 nm and markers were located by determining the absorbance at 280 nm. Purified variant OTCases were located by measuring the absorbance at 280 nm and by enzyme assay.

RESULTS

Construction and general properties of mutant catabolic OTCases

In a previous study we have shown that modification of the C-terminus of catabolic OTCase can alter the enzyme homotropic cooperativity for carbamoylphosphate (Baur et al., 1990). Moreover, our current structural model (see Discussion) predicts that C-terminal amino acids form important contacts with N-terminal residues within a monomer and on the interface between trimers. In an engineered hybrid enzyme specified by the *arcB3606* gene (Baur et al., 1990), the nine C-terminal amino acid residues of catabolic OTCase were exchanged for the corresponding eight residues from the anabolic OTCase of *E. coli* (Fig. 1). The altered kinetic properties of this hybrid enzyme may be due to the deletion of the last residue (Ile335) of catabolic OTCase and/or to the amino acid changes in the replaced segment. In order to clarify which sequence differences have an impact on reaction kinetics, we constructed new mutant enzymes. The codons for the C-terminal isoleucine and Asp-Ile residues of the catabolic OTCase were specifically deleted by site-directed mutagenesis, resulting in the *arcB3610* and *arcB3612* genes, respectively (Fig. 1). In a third mutant enzyme, the C-terminus was modified by an extension; a peptide of 15 amino acids (including six arginine residues) was fused to Asp334. This construct (*arcB55*, Fig. 1) was obtained fortuitously during an analysis of the *arcB-arcC* intergenic region (Baur et al., 1989). There is a unique *Sfi*I site downstream of the *arcB* gene and upstream of the *arcC* gene. The $arcB^+C^+$ plasmid pME179 was opened with *Sfi*I, subjected to limited digestion with nuclease *Bal*31, and ligated in the presence of an *Eco*RI linker, as previously described (Baur et al., 1989). In the resulting *arcB55* gene, *arcC* nucleotides were fused, out-of-frame, to the 3′ end of *arcB* (without the last codon), creating a novel, elongated catabolic OTCase (Fig. 1).

Re-insertion of each of the mutated *arcB* genes into the *arc* operon carried by a broad-host-range plasmid allowed high-level expression of the variant OTCases in *P. aeruginosa*; induction was obtained by semi-anaerobic conditions (Lüthi et al., 1990). The modified catabolic OTCases specified by the *arcB3606*, *arcB3610*, *arcB3612* and *arcB55* genes as well as the wild-type catabolic OTCase were purified to near homogeneity. All proteins had similar subunits of approximately M_r 38000 (as determined by gel electrophoresis under denaturing conditions) and similar relative molecular masses of approximately M_r 420000 (as judged by gel filtration and gel electrophoresis under non-denaturing conditions). These data suggest that the modified OTCases have the same dodecameric structure as the wild-type enzyme (Baur et al., 1987; Marcq et al., 1991).

The pH optimum of the wild-type enzyme critically depends on the carbamoylphosphate concentration (Fig. 2). Increasing pH, at any carbamoylphosphate concentration, enhances the degree of sigmoidicity and reduces the affinity of the enzyme for carbamoylphosphate (Tricot et al., 1993). As a result, there is a large decrease of enzyme activity at pH 7.2–7.4 at 10 mM carbamoylphosphate. Although a higher carbamoylphosphate concentration (50 mM) can overcome this problem to some extent (Fig. 2), it is generally difficult to measure enzyme activity above pH 7.4 due to a low affinity for carbamoylphosphate and a high homotropic cooperativity. The pH optima of the genetically modified OTCases were determined at 4 mM carbamoylphosphate and, at this substrate concentration, ranged from pH 6.0–7.0. Consequently, we decided to perform OTCase assays at an intermediate pH value to provide comparable experimental conditions for all enzymes in this study and to avoid very high carbamoylphosphate concentrations which would have led to solubility problems. The pH value previously used for the characterization of the wild-type enzyme (pH 7.25; Baur

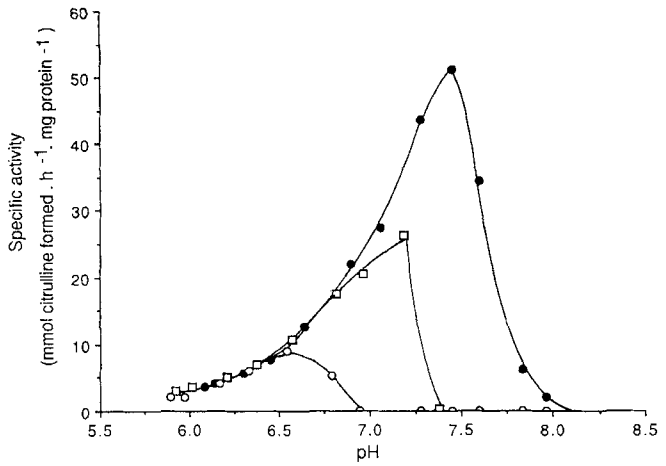

Fig. 2. pH dependence of the activity determined for the wild-type catabolic OTCase in the presence of 2, 10 or 50 mM carbamoylphosphate. The reactions were performed in 150 mM imidazole/HCl in the presence of 10 mM L-ornithine. Typically, the standard deviations were approximately 5% and 5–10% in the presence of 50 mM carbamoylphosphate. The carbamoylphosphate concentrations are: ○, 2; □, 10; ●, 50 mM.

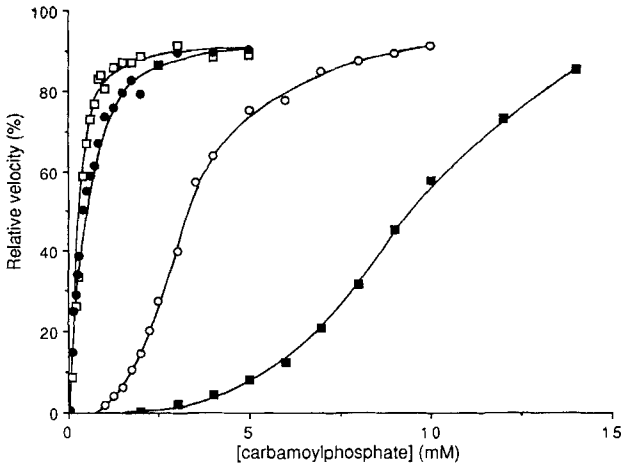

Fig. 3. Carbamoylphosphate saturation curves in the wild-type catabolic OTCase with and without effectors. The reactions were performed in 150 mM imidazole/HCl, pH 6.7, in the presence of 10 mM L-ornithine. ○, without effector; ●, in the presence of 10 mM P_i; □, in the presence of 10 mM AMP; ■, in the presence of 20 mM spermidine. The maximum velocity calculated was set equal to 100%. In general, the standard deviations were approximately 5% and 5–10% in the presence of AMP.

Table 1. Effect of nucleotides on the activity of the wild-type catabolic OTCase. The assays were performed at pH 6.7 as described in the Materials and Methods section with 1 mM carbamoylphosphate in the presence of 10 mM effector. The results are the means of values from at least two separate experiments. The activity of OTCase in the presence of P_i was set at 100%.

Effector	Relative activity
	%
None	2 ± 0.5
P_i	100
AMP	97 ± 7
GMP	26 ± 3
CMP	81 ± 4
UMP	14 ± 1

et al., 1990) was inadequate for some modified OTCases because of poor affinity for carbamoylphosphate at this pH.

Determination of effector responses at pH 6.7

Wild-type catabolic OTCase of *P. aeruginosa* is activated by P_i, presumably via interaction with the carbamoylphosphate-binding sites. Furthermore, the enzyme is allosterically regulated by activators (NMP) and inhibitors (spermidine and other polyamines; Tricot et al., 1993). Since the allosteric behaviour may depend on the assay pH, the response to allosteric effectors was reassesed at pH 6.7. NMP effectors were tested at a relatively low carbamoylphosphate concentration (1 mM) in order to maximize potential effects. AMP and CMP were the strongest allosteric activators and were almost as effective as P_i, whereas GMP and UMP had smaller effects (Table 1). Polyamines were tested as inhibitors at 3 mM carbamoylphosphate, i.e. at a concentrations near the $[S]_{0.5}^{CP}$ value (Table 2). Spermidine (10 mM) and putrescine (10 mM) reduced the activity of wild-type catabolic OTCase to 7% and 55%, respectively, relative to a non-inhibited control. The effects of the most important modulators (P_i, AMP and spermidine) on the carbamoylphosphate saturation curves are illustrated for the wild-type enzyme (Fig. 3). The responses of each mutant enzyme are compared to those of the wild-type enzyme (Table 2) and a summary of the activation and inhibition data is presented in Table 3.

The C-terminal isoleucine residue of catabolic OTCase is essential for homotropic and heterotropic cooperativity

Plasmid pME3610 specifies a mutant OTCase lacking the last amino acid residue Ile335 (Fig. 1). This enzyme, like the hybrid enzyme encoded by *arcB3606* (Fig. 1), showed reduced homotropic cooperativity for carbamoylphosphate (n_H = 1.8–1.9), compared to that of the wild-type catabolic OTCase (n_H = 3.4; Table 2). Moreover, deletion of Ile335 in the *arcB3610*-encoded enzyme led to a reduced affinity for carbamoylphosphate and also to an increase of V_{max} (Fig. 4; Table 2). Thus, the C-terminal isoleucine residue is intimately involved in mediating homotropic cooperativity. The OTCases encoded by the wild-type *arcB*$^+$ and the mutant *arcB3606* and *arcB3610* genes were all inhibited by spermidine and activated by P_i and AMP, but the sensitivities to these effectors differed for the three enzymes (Tables 2 and 3). In particular, the spermidine concentrations required to produce 50% inhibition were higher for the two mutant OTCases than for the wild-type enzymes (Table 3). We conclude that the C-terminal isoleucine residue also contributes to spermidine sensitivity.

A C-terminal extension of catabolic OTCases entails decreased V_{max} values and reduced sensitivity to effectors

The *arcB55* gene codes for an elongated polypeptide of catabolic OTCase which lacks Ile335 (Fig. 1). This enzyme

Table 2. Homotropic and heterotropic cooperativity for carbamoylphosphate in wild-type and variant OTCases. The assays were performed in 150 mM imidazole/HCl, pH 6.7, containing 10 mM L-ornithine and variable carbamoylphosphate concentrations. $[S]_{0.5}^{CP}$, concentration of carbamoylphosphate at one half of the maximum velocity; n_H, Hill coefficient; K_m^{app}, apparent K_m determined at 20 mM carbamoylphosphate. The results are the means of values from at least two independent experiments.

Plasmid (relevant genotype)	Effector	Effector concentration	$[S]_{0.5}^{CP}$	n_H	V_{max}	K_m^{app} for ornithine
		mM			mmol citrulline · h^{-1} · mg protein^{-1}	mM
pME183 (*arcB*$^+$)	none		2.9 ± 0.2 [a]	3.4 ± 0.1 [a]	13.4 ± 0.5 [a]	0.8 ± 0.05
	P_i	5	0.45 ± 0.05	1.0 ± 0.05	12.0 ± 0.5	
	AMP	10	0.3 ± 0.03	1.1 ± 0.05	12.0 ± 1.0	
	spermidine	20	8.6 ± 0.5	4.6 ± 0.5	13.0 ± 1.0	
pME3606 (*arcB3606*)	none		2.0 ± 0.1	1.9 ± 0.1	11.3 ± 0.5	1.1 ± 0.1
	P_i	5	0.35 ± 0.05	1.0 ± 0.05	11.5 ± 0.5	
	AMP	10	0.9 ± 0.1	1.5 ± 0.1	10.2 ± 0.1	
	spermidine	20	4.1 ± 0.2	2.0 ± 0.2	10.2 ± 0.2	
pME3610 (*arcB3610*)	none		10.1 ± 0.5	1.9 ± 0.1	20.5 ± 0.7	1.6 ± 0.1
	P_i	5	2.4 ± 0.15	1.0 ± 0.05	20.5 ± 0.7	
	AMP	10	0.75 ± 0.05	1.3 ± 0.05	19.3 ± 1.2	
	spermidine	20	19.8 ± 1.5	2.3 ± 0.1	18.1 ± 1.5	
pME190-55 (*arcB55*)	none		8.5 ± 0.5	1.8 ± 0.1	0.65 ± 0.05	1.5 ± 0.1
	P_i	5	6.7 ± 0.5	1.7 ± 0.1	0.76 ± 0.05	
	AMP	10	4.0 ± 0.4	1.6 ± 0.1	0.73 ± 0.05	
	spermidine	20	25.0 ± 2	2.4 ± 0.2	0.44 ± 0.04	
pME3612 (*arcB3612*)	none		2.6 ± 0.2	5.5 ± 0.4	13.1 ± 0.5	1.4 ± 0.1
	P_i	5	0.3 ± 0.05	1.7 ± 0.1	14.0 ± 0.5	
	AMP	10	0.4 ± 0.05	2.8 ± 0.2	14.6 ± 0.1	
	spermidine	20	4.9 ± 0.2	5.0 ± 0.4	14.1 ± 0.1	

[a] In previous assays at pH 7.25 (Baur et al., 1990) the following values were obtained: $[S]_{0.5}^{CP} = 8.8$; $n_H = 4.2$; $V_{max} = 21.4$.

Table 3. Activation and inhibition of wild-type and variant OTCases. The assays were performed in 150 mM imidazole/HCl, pH 6.7, containing 10 mM L-ornithine. The carbamoylphosphate concentration was chosen for each variant enzyme at the $[S]_{0.5}^{CP}$ value (see Table 2). $[A]_{0.5}$ is the concentration of a positive effector required to reach half-maximal activation. $[I]_{0.5}$ is the concentration of the allosteric inhibitor spermidine required to reach 50% of the velocity observed in the absence of the effector. The results are the means of values from at least two independent experiments.

Plasmid (relevant genotype)	$[A]_{0.5}^{P_i}$	$[A]_{0.5}^{AMP}$	$[I]_{0.5}^{spermidine}$
	mM		
pME183 (*arcB*$^+$)	0.6 ± 0.05	1.5 ± 0.1	3.7 ± 0.3
pME3606 (*arcB3606*)	0.4 ± 0.03	1.0 ± 0.1	16.5 ± 1.5
pME3610 (*arcB3610*)	1.9 ± 0.15	1.9 ± 0.2	11.7 ± 1
pME190-55 (*arcB55*)	4.0 ± 0.2	6.0 ± 0.5	>40
pME3612 (*arcB3612*)	0.32 ± 0.02	1.2 ± 0.1	1.25 ± 1

had low activity and showed a reduced affinity for the effectors P_i and AMP but retained some homotropic cooperativity for carbamoylphosphate (Tables 2 and 3; Fig. 4). Moreover, only partial inhibition by spermidine was observed: 50% inhibition was not reached even at 40 mM spermidine. Thus, elongation of the polypeptide chain can reduce the catalytic efficiency of catabolic OTCases but does not cause inactivation. The salient feature of the elongated mutant enzyme is that the allosteric effectors are less effective compared to the wild-type enzyme.

The C-terminal Asp-Ile residues of catabolic OTCase are dispensable for catalytic and most regulatory properties

The engineered *arcB3612* allele encodes a catabolic OTCase truncated at the C-terminus for Asp334 and Ile335 (Fig. 1). This enzyme was highly active and showed pronounced homotropic and heterotropic cooperativity, which tended to be even higher than in the wild-type enzyme (Tables 2 and 3; Fig. 4). One major difference was apparent; AMP (10 mM) abolished carbamoylphosphate cooperativity in the wild-type catabolic OTCase ($n_H = 1.1$) but failed to do so in the truncated enzyme ($n_H = 2.8$). In summary, the deletion of two C-terminal residues (*arcB3612*) had less pronounced effects on the kinetic properties than the deletion of the C-terminal isoleucine residue alone (*arcB3610*). However, the influence of the C-terminus on the reaction kinetics was also evident in the *arcB3612*-encoded enzyme.

DISCUSSION

Variations in homotropic or heterotropic interactions may occur in bisubstrate enzymes when binding of the second substrate is changed (Pettigrew and Frieden, 1977). However, in the wild-type enzyme, variation of ornithine concentrations from 0.5–50 mM had little effect on carbamoylphosphate cooperativity (Tricot et al., 1993) and in the mutant OTCases the apparent K_m for ornithine showed little variation (Table 1). Therefore, it is unlikely that the major changes observed for the n_H values are a consequence of variations of the affinity for ornithine. All mutant OTCases investigated in this study resembled the wild-type enzyme with respect to their dodecameric structure. Thus, except for the *arcB55*

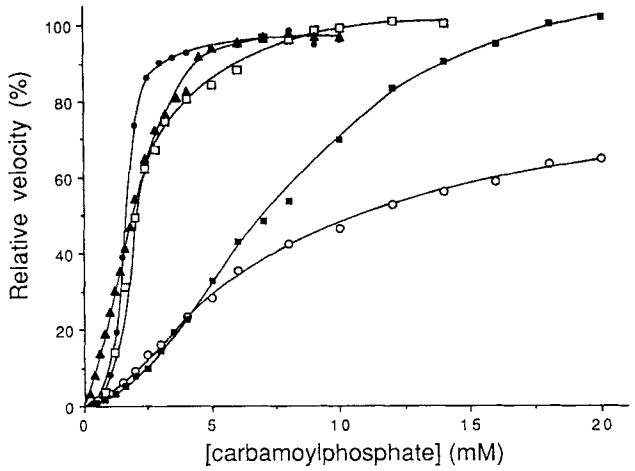

Fig. 4. Carbamoylphosphate saturation curves for wild-type and modified OTCases. The OTCases are specified by wild-type *arcB*$^+$, *arcB3610*, *arcB3612*, *arcB55*, *arcB3606*, or *arcB3612* alleles. The reactions were performed in 150 mM imidazole/HCl, pH 6.7, in the presence of 10 mM L-ornithine. The standard deviations were approximately 5% for wild-type, *arcB3612* and *arcB3606* enzymes and approximately 10% for *arcB3610*-encoded and *arcB55*-encoded OTCases. OTCases are specified by the following: □, wild-type; ○, *arcB3610*; ●, *arcB3612*; ■, *arcB55*; ▲, *arcB3606*.

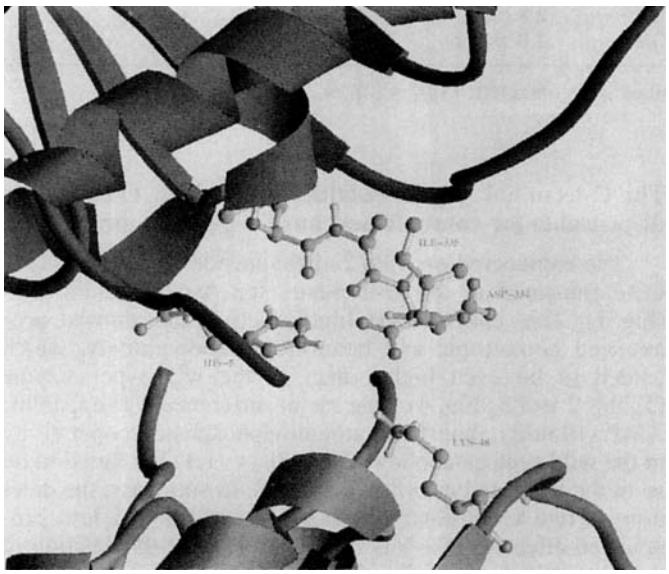

Fig. 5. The interface between two catabolic OTCase trimers. The C-terminal isoleucine residue (Ile335) has two conformations. In the view represented, there is a salt bridge between the C-terminal carboxylate group and His5 on the same monomer. For the other conformation, the second partner of Ile335 is Lys46 from a nearby trimer.

variant enzyme, which has an elongated C-terminus, gross structural changes can also be excluded as the primary reasons for altered allosteric behaviour.

Mutations at the C-terminus had profound effects on the recognition of one substrate (carbamoylphosphate), a substrate analog (P_i) and two allosteric effectors (AMP and spermidine). Carbamoylphosphate binding probably involves at least residues 56–59, 87, 106, 127 and 133 in catabolic OTCase of *P. aeruginosa* (Baur et al., 1987). The properties of artificial hybrids between catabolic and anabolic OTCase suggest that the binding sites for the allosteric effectors are not confined to the C-terminus (unpublished results). Therefore, it seems likely that the mutational changes in the C-terminus of this enzyme do not act directly on binding sites but affect enzyme structure in a more general way.

Sequence alignments between the catabolic OTCase of *P. aeruginosa* and the anabolic OTCases of procaryotic and eucaryotic organisms show that the C-terminal residue Ile335 is not conserved. (Huygen et al., 1987). For instance, the *argF*-encoded, trimeric, anabolic enzymes of *E. coli* and *Neissera gonnorrhoeae* naturally lack an amino acid residue corresponding to Ile335 (Van Vliet et al., 1984; Martin et al., 1990) and terminate with glutamate and aspartate, respectively, which are homologous to Asp334 of the catabolic OTCase. The sequence comparisons, however, do not give any indication as to which amino acid residues in catabolic OTCase might be important for allosteric behaviour and complex quaternary structure. Recently, the X-ray structure of a mutant catabolic OTCase, in which glutamate at position 105 has been replaced by glycine (Baur et al., 1990), has been solved at 0.3-nm resolution (unpublished results). The kinetic properties of this enzyme suggest that it is locked in an active conformation (Baur et al., 1990). The electron-density map of this enzyme reveals two conformations for the C-terminal Ile335 residue, which is 1.23 nm below the surface of the enzyme. In one conformation, the carboxyl group of Ile335 makes a salt bridge with His5 of the same monomer. In the other conformation, a salt bridge with Lys46 on another trimer can be formed (Fig. 5). Any C-terminal deletion or extension can be expected to have profound effects on the interface between the trimers in the dodecamer and hence on the kinetic properties of the enzyme. For instance, deletion of Ile335 or Asp334 and Ile335 will move the new C-terminal carboxylate group away from its native position by 0.35 nm or 0.51 nm, respectively, thereby changing the contacts with positively charged amino acids (such as Arg45 and Lys46) in the trimeric interface. Long-range effects of this type are not uncommon. For instance, amino acid replacements in the C-terminal α-helix of aspartate carbamoyltransferase (catalytic subunit) cause marked changes in the allosteric properties and interactions between the subunits (Peterson and Schachman, 1992).

In conclusion, the preliminary crystallographic analysis of the enzyme with the Glu105→Gly mutation leads us to suggest that alterations of the interface between trimers in the dodecamer are a major reason for the changes in the kinetic properties of the enzyme with alterations at the C-terminus.

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