Use of a designed fusion protein dissociates allostery properties from the dodecameric state of *Pseudomonas aeruginosa* catabolic ornithine carbamoyltransferase

(Alloster/Assembly/glutathione S-transferase/thermostability)

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ABSTRACT The catabolic ornithine carbamoyltransferase from *Pseudomonas aeruginosa*, an enzyme consisting of 12 identical 38-kDa subunits, displays allostery properties, namely carbamoylphosphate homotropic cooperativity and heterototropic activation by AMP and other nucleoside monophosphates and inhibition by polyamines. To shed light on the effect of the oligomeric organization on the enzyme’s activity and/or allostery behavior, a hybrid ornithine carbamoyltransferase/glutathione S-transferase (OTCase–GST) molecule was constructed by fusing the 3’ end of the *P. aeruginosa arcB* gene (OTCase) to the 5’ end of the cDNA encoding *Musca domestica* GST by using a polyglycine encoding sequence as a linker. The fusion protein was overexpressed in *Escherichia coli* and purified from cell extracts by affinity chromatography, making use of the GST domain. It was found to exist as a trimer and to retain both the homotropic and heterotropic characteristic interactions of the wild-type catabolic OTCase but to a lower extent as compared with the wild-type OTCase. The dodecameric organization of catabolic *P. aeruginosa* OTCase may therefore be related to an enhancement of the substrate cooperativity already present in its trimers (and perhaps also to the thermostability of the enzyme).

Protein oligomerization is a very important process in biology. Since the discovery of oligomeric proteins (1), much effort has been devoted to the study of the assembly and disassembly of polymeric proteins and the effect of this process on their biological function (2, 3).

We are currently using ornithine carbamoyltransferase (OTCase) as a model system to study the role of assembly and disassembly on the biological activity of the enzyme. OTCase catalyzes the conversion of ornithine and carbamoylphosphate (CP) into citrulline and phosphate. The anabolic OTCases from various sources have similar properties. They are generally trimeric molecules, made up of identical 35- to 40-kDa subunits, and display Michaelis–Menten kinetics with both substrates. *Pseudomonas aeruginosa* possesses another OTCase, involved in the arginine deiminase pathway, which supplies energy derived from arginine under anaerobic conditions (4). The catabolic enzyme is a dodecamer, made up of identical 38-kDa subunits organized as four trimers (5–7). In vivo, it catalyzes the phosphorolysis of citrulline, the reverse of the anabolic reaction. Since the thermodynamic equilibrium of the reaction strongly favors citrulline synthesis, in vitro assays are performed in this direction (8). The catabolic OTCase exhibits the characteristics of an allostery enzyme, such as homotropic cooperativity with respect to the substrate, carbamoylphosphate, and heterotropic activation by AMP and other nucleoside monophosphates and inhibition by polyamines (9). Homotropic cooperativity and low affinity for CP prevent the catabolic activity from performing the anabolic reaction in vivo (10, 11). The structure of the catabolic OTCase is known at 3-Å resolution (7).

In the *Escherichia coli* trimeric anabolic OTCase, substitution of Arg-106 by Gly creates an enzyme with reduced cooperativity for both substrates (12). Moreover, the aspartate carbamoyltransferase of *Bacillus subtilis*, a trimeric enzyme structurally related to *P. aeruginosa* anabolic OTCase, normally shows Michaelis–Menten kinetics for aspartate, but can also be transformed by an Arg/Ala substitution at position 107 (equivalent to Arg-106 in *E. coli* OTCase) into an enzyme with cooperative kinetics (13).

To understand the role of the dodecameric structure in both the homotropic and heterotropic cooperativity, we decided to interfere with the assembly process by adjunction of a protein with a different oligomeric organization by fusing two genes. This paper describes the design, using molecular modeling, the construction of the fused gene, and the production and characterization of the OTCase–glutathione S-transferase (GST) protein. Although the fusion protein could have been dimeric (as GST), trimeric (as most OTCases), or dodecameric, it was found to be trimeric and to retain both the homotropic and heterotropic properties of wild-type *P. aeruginosa* catabolic OTCase.

MATERIALS AND METHODS

Molecular Design of the Fusion Protein. All carbamoyltransferases form functional trimers. However, the catabolic OTCase from *P. aeruginosa* has a higher symmetry: a 23-point group (7). The oligomeric molecule consists of four trimers, organized like in a tetrahedron (Fig. 1). In the 23 symmetry, there are three two-fold and four three-fold axes. The fusion protein was generated by gene fusion between the catabolic *P. aeruginosa* OTCase and the *Musca domestica* GST, the latter enzyme being selected for four reasons. (i) Native GST forms very stable homodimers. (ii) The crystal structures of homologous proteins are known (for a review, see ref. 14). (iii) The enzyme assays are very easy to perform. (iv) The fusion protein can be purified by a single-step glutathione affinity chromatography.

Abbreviations: OTCase, ornithine transcarbamylase(carbamoyltransferase); GST, glutathione S-transferase; OTCase-GST, fusion protein; [S] coli, concentration of carbamoylphosphate giving half-maximum velocity; CP, carbamoylphosphate. 

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The GSTs (EC 2.5.1.18) are a group of isoenzymes that play an important role in the detoxification and metabolism of many xenobiotic and endobiotic compounds. Cytosolic GSTs are dimeric enzymes that catalyze the reaction between the tripeptide glutathione and a wide variety of electrophilic alkylation agents. These proteins are encoded by at least four distinct gene classes: \(a\), \(\mu\), \(\pi\), and \(\sigma\). To model \textit{M. domestica} GST, we first aligned the sequence of this protein with published amino acid sequences for classes \(a\) (human A1-1), \(\mu\) (rat 3–3), and \(\pi\) (pig) (see Figure 1 in ref. 15), using the program HCA-PLOT (16). The length of each region of secondary structure in the parent protein, in the present case, rat 3–3 (class \(\mu\), was retained (17). For the purpose of the present work, a fly GST model was constructed by replacing the side chains of the residues involved in secondary structures in rat GST by their equivalents in the fly sequence and by modeling loop regions. This model was then improved by energy minimization (18). For the graphical visualization we used the program TURBO-FRODO (19).

To design the fusion protein, we constructed a dodecameric protein with 23-point group symmetry. The monomer was duplicated to form six GST dimers. The two-fold axis of each dimer was superimposed on either side of each of the three two-fold axes of the OTCase dodecameric molecule. Each dimer was translated and rotated along this axis to yield a compact oligomer with a 23-point group symmetry. On the basis of this crude model, we decided to fuse the OTCase C terminus to the GST N terminus by using a 10-glycine residue linker. The order chosen for the fusion protein was OTCase–linker–GST since the glutathione binding site is near the C terminus of GST (17). However, in practice, such a designed protein may not be expressed in the cell or expressed with another oligomeric organization. A clear advantage of our design is the possibility of following, by enzymatic assays, the relationship between enzyme activity (GST, OTCase, or both) and protein oligomerization, for the dimer, trimer, or dodecamer, respectively.

**Bacterial Strains and Plasmids.** \textit{E. coli} XL1-Blue MRF’ [\(\Delta(mcrA)\) 183, \(\Delta(mcrCB-hsdSMR-mry)\) 173, supE44, thi-1, recA1, gyrA96, endA1, lac, l-F', proAB, lacF' \(\Delta M15\), \(\text{Tn10} (\text{Terf})\) ] and JM110 [\(\text{proL, thr, leu, thi-1, lacY, galK, galT, ara, tonA, tsx, dam, dcm, supE44, \Delta(lac, proAB), F' traD36, proAB, lac\) \(\Delta M15\)] were used for cloning. OTCase–GST was expressed in \textit{E. coli} BL21(DE3) [\(\text{hsd S gal (\lambda Ic 857 ind 1 Sam 7 min 5 lacUV5-T7 gene 1)}\)]; this strain contains a chromosomal copy of the T7 RNA polymerase gene under lacUV5 control. The plasmids used in this work were pCR-Script SK(+) (Stratagene) and pET5a (Novagen).

**Construction of the OTCase–GST Protein.** The OTCase-encoding \(arcB\) gene and the cDNA encoding GST were modified by PCR at the 5' and 3' ends. The primers used for the construction were designed as follows. Primer A (5'-CATATGGCCTTCAAACATGCAC-3') incorporates a \textit{NdeI} restriction site at the 5' end of \(arcB\) gene. Primer B (5'-ATCGATGCGAGGTTCGAGACGAG-3') introduces a \textit{ClaI} restriction site before the TAA stop codon \(arcB\) gene. This modification replaces the last 2 amino acid residues of OTCase: Asp-334/Ile-335.

The GST cDNA was modified by introducing a SalI restriction site at the 5' end by using primer C (5'-GTCGACATGATTTTACATGCCGACGAG-3') introduces a \textit{ClaI} restriction site before the TAA stop codon of the GST cDNA.

The PCR conditions used for the modification of \(arcB\) gene and the GST cDNA were as follows: 95°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min for 30 cycles. Each PCR mixture (50 pmol total volume) contained 2 ng template DNA, 50 pmol primers, 0.2 mmol 2'-deoxynucleotide 5'-triphosphates, and 1 unit Vent DNA polymerase (New England Biolabs). The resulting two DNA fragments were purified by gel electrophoresis on a 1% agarose gel by using GeneClean kits (Bio 101), and the isolated fragments cloned into the pCR-Script SK(+) plasmid. The plasmids containing the modified \(arcB\) gene or the modified GST cDNA were digested, respectively, with the restriction enzymes \textit{NdeI}/\textit{ClaI} or \textit{SalI}/\textit{BglII}.

The synthetic linker encoding the polyglycine sequence was obtained by annealing two oligonucleotides as follows:

\[
\begin{align*}
5'\text{CGATGCGCAGTTGCGCGCCGGCGAGCGACGAG-3'} & \text{ at } \text{SalI} \\
3'\text{TACCGCAGACACCCACACCCGCGCCACCGCGCAGG-5'} & \text{ at } \text{NdeI}
\end{align*}
\]

The \textit{NdeI–ClaI} fragment, the \textit{ClaI–SalI} linker, and the \textit{SalI–BglII} fragment were ligated simultaneously into the expression plasmid pET5a, previously treated with \textit{NdeI} and \textit{BamHI} (Fig. 2). The resulting expression plasmid, carrying the new gene encoding the fusion protein OTCase–GST, was named pNM1.

The constructs were checked by sequencing the double-stranded DNA, using the dideoxy-chain termination method (20).

**Protein Purification.** \textit{E. coli} strain BL21(DE3) carrying the pNM1 plasmid was routinely grown at 37°C on Luria–Bertani...
Fig. 2. Construction of the expression plasmid pNM1. The blunt-ended PCR products, designed as described in Materials and Methods, were cloned into SrfI-digested pCR-Script SK(+) giving, respectively, pCR-arcB and pCR-gst. The fragments NdeI/Clal, BamHI, and the synthetic linker ClaI/SalI were ligated simultaneously in pET5α which has been digested by NdeI and BamHI, finally giving pNM1.

broth, supplemented with 100 μg of ampicillin per ml. Cultures were incubated until the cell density reaches 10^9/cell/ml, then expression of the OTCase–GST protein was induced by the addition of 400 μM isopropyl β-D-thiogalactoside and culture continued for a further 3 h. The cells were harvested by centrifugation (10,000 x g, 10 minutes at 4°C) and washed with PBS buffer (150 mM NaCl/16 mM Na₂HPO₄/4 mM NaH₂PO₄, pH 7.3). Cell extracts were prepared by suspending the cells in PBS buffer and disrupting them by sonication with a Rayton sonic oscillator at 10 kHz. Cell debris was removed by centrifugation at 25,000 x g for 30 min.

The cell extract was loaded onto a glutathione–Sepharose 4B affinity column (Pharmacia) (1 x 10 cm), previously equilibrated with PBS buffer. The column was washed with the buffer until the optical density at 280 nm reached the baseline, then the fusion protein was eluted with 50 mM Tris-HCl (pH 8), containing 10 mM glutathione.

Criteria of Purity. The purity of the enzyme was assessed by analytical gel electrophoresis under native and denatured conditions as described (5). Mass spectrum was obtained on an API III+ triple-quadrupole mass spectrometer (Perkin–Elmer Sciex) equipped with a nebulizer-assisted electrospray source (ionspray) operating at atmospheric pressure. A 5-kV voltage was applied to the electrospray needle. The mass spectrometer was scanned from m/z 1100 to 2000, with steps of 1 m/z unit with a 2-ms dwell time. An average of 10 scans were obtained in the flow injection analyses mode. The molecular species produced a series of multiply charged protonated molecular ions. The reconstructed molecular mass profile was determined with a data system by using a deconvolution algorithm

(Perkin–Elmer Sciex). Horse heart myoglobin (16951.5 Da) was used for calibration. Molecular mass is given as isotope-average values. In flow injection analyses, the sample dissolved in ammonium acetate buffer (pH 7) was introduced by means of a Harvard 22 syringe pump at a flow rate of 5 μl/min on a Valco C6W injector equipped with a 1 μl internal loop. The solvent used for infusion consisted of methanol/water/acetic acid (25/74/1; vol/vol), and 30 pmol of protein was used to obtain the spectrum.

Enzyme Assays. OTCase activity was determined by quantifying citrulline production by the method of Prescott and Jones (21). The standard assay mixture consisted of 2.0 ml of 150 mM imidazole/HCl (pH 6.8), 10 mM L-ornithine and varying amounts of carbamoylphosphate (and effectors, where appropriate) (22). The reaction was started by the addition of carbamoylphosphate, allowed to continue for 10 min at 37°C, then stopped by the addition of 1.0 ml of antipyrene diacetylmonoxime, used for the colorimetric assay of citrulline. The tubes were placed in a boiling water bath for 20 min, and the absorbance was measured at 466 nm. Assays were performed in duplicate and the data are the average of at least two independent determinations. Specific activities are expressed as mmol citrulline formed/h/mg protein. The steady-state kinetic data were analyzed by Lineweaver–Burk, Hanes, and Eadie plots to determine the maximal velocity. Cooperativity was analyzed by standard graphical methods and the Hill coefficient (nH) was determined by the Hill equation as described (9).

GST catalyzes the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) to glutathione to form a CDNB-glutathione product with a strong molar absorptivity at 340 nm (ε = 9.6 mM⁻¹·cm⁻¹). The standard assay mixture consisted of 1 mM CDNB, 5 mM glutathione and 100 mM Tris-HCl (pH 8.5). Optical density changes were recorded at 340 nm (23).

Heat Inactivation Assays. The OTCase–GST and native OTCase were incubated at 60°C in 0.02 M potassium phosphate buffer (pH 6.8). Samples were withdrawn after various periods of time and the residual activities were determined in presence of 150 mM imidazole (pH 6.8), 10 mM L-ornithine, 10 mM phosphate, and 20 mM carbamoylphosphate at 37°C.

Molecular Weight of the Native Enzyme. The molecular weight of the fusion protein was determined by three different methods: (i) analytical centrifugation, (ii) gel filtration on a Sephacryl column, and (iii) native gel electrophoresis.

The ultracentrifugation experiments were performed at 10°C in 50 mM Tris-HCl (pH 8.0) in a Beckman XL-A analytical ultracentrifuge, equipped with a UV scanning system, using a four holes An-60 Ti rotor with double-sector centerpieces of 1.2 cm path-length. The S value was determined at 30,000 rpm from the position of the point of inflection. The equilibrium sedimentation data obtained after 22 h at 10,000 rpm were analyzed by using the Optima XL-A data analysis software, version 2.0, from Beckman, using a value for the partial specific volume for OTCase–GST of 0.73 cm³/g, determined from its amino acid composition (24).

Gel filtration studies were carried out as described (25) using a column (80 x 2.6 cm) of Pharmacia Sephacryl S300, equilibrated and eluted with 150 mM imidazole-HCl (pH 6.8) without effector, or with either 10 mM AMP or 10 mM spermidine. OTCase–GST (200 μg) was loaded onto the column and the elution profile followed by the absorbance at 280 nm. A standard curve of partition coefficient, Kp = (ve - vo)/(ve + vo), versus molecular mass was prepared using blue dextran 2000 and proteins of 25, 43, 158, 232, 440, and 669 kDa.

Electrophoresis was performed in native conditions in Tris-Bicine (pH 8.6) (Phast system; Pharmacia) as described (26).

RESULTS AND DISCUSSION

Production of OTCase–GST. After designing monomeric OTCase–GST by molecular modeling, the fusion gene was
constructed (Fig. 2) (see Materials and Methods). The fusion protein was then overexpressed in E. coli and purified to homogeneity by affinity chromatography on glutathione-Sepharose. Analysis of purity by SDS/polyacylamide gels showed a single band with an electrophoretic mobility corresponding to an apparent molecular weight of \( \approx 64 \text{ kDa} \). Mass spectrometry analysis gave a value of 62,698 ± 6 Da, including one adduct of glutathione molecule consistent with that predicted from the DNA sequence (62,396 Da). The yield was 14 mg per liter of culture medium.

**Characterization of the Oligomeric State.** *Ultracentrifugation.* The sedimentation velocity profiles showed a single boundary. From the experimental sedimentation coefficient of 5.7 ± 0.1 S, we can estimate a \( s_{20,w} \) value of the order of 7.5 S, compatible with a homogenous globular protein with a molecular weight of about 150 kDa (27). Equilibrium sedimentation is an absolute method for determining the molar mass of macromolecules in solution. The data, fitted to a model with one single solute, gave a molecular weight of 180 kDa.

**Gel filtration and polyacrylamide gel electrophoresis.** On gel filtration, OTCase–GST eluted as a single symmetrical peak, indicating sample homogeneity. Its partition coefficient compared with those of standard proteins gave an apparent molecular size of 191 kDa (Fig. 3) in the absence of effector or in the presence of AMP or spermidine. The ultracentrifugation and gel filtration experiments show that the OTCase–GST is a trimer of 62-kDa subunits in both 50 mM Tris-HCl buffer (pH 8) and enzyme assay buffer (150 mM imidazole-HCl, pH 6.8).

A molecular mass of \( \approx 190 \text{ kDa} \) was observed for the OTCase–GST enzyme on polyacrylamide gel electrophoresis in native conditions (Fig. 4).

All methods of molecular mass determination indicate that OTCase–GST is a trimer in the absence of effector or in the presence of the positive effector AMP or of the negative effector spermidine. The comparison of the biochemical properties of wild-type dodecameric OTCase and trimeric OTCase–GST should provide information relating the degree of oligomerization with the enzymatic properties of OTCase.

**Thermostability of Wild-Type OTCase and OTCase–GST.** Following incubation of the enzymes for various lengths of time at 60°C in 0.02 M potassium phosphate buffer (pH 6.8), the residual activity was assayed (Fig. 5). Wild-type OTCase was found to be very stable, retaining full activity for at least 2 h, while the OTCase–GST lost 50% of its activity within 10 min.

**Kinetic Properties of the Trimeric OTCase–GST Enzyme.** The maximum enzymatic activities of the fusion enzyme was 50% of that of wild-type GST and 35% of that of wild-type OTCase. Table 1 shows the kinetic and allosteric properties of the fusion enzyme as compared with wild-type dodecameric catabolic OTCase. It will be noted that for wild-type OTCase there is no effect of ornithine concentration between 0.5 to 50 mM on the cooperative carbamoylphosphate saturation curve (9). The apparent \( K_m \) for ornithine determined at saturation of carbamoylphosphate for the OTCase–GST enzyme is 1.4 mM, the same as that determined for the wild-type enzyme (data not shown). The carbamoylphosphate saturation curves of wild-type OTCase and OTCase–GST are shown in Fig. 6, and a summary of the kinetic parameters is given in Table 1. The maximal velocity of the fusion enzyme is decreased by 65% as compared with wild-type OTCase. The cooperativity with respect to carbamoylphosphate is reduced and the concentra-

**Fig. 4.** Polyacrylamide gel electrophoresis of purified OTCase–GST. Electrophoresis in native conditions on a 8–25% polyacrylamide gel. Lanes: 1, wild-type OTCase; 2, OTCase–GST; 3, marker proteins: thyroglobulin (Mr, 669,000), ferritin (Mr, 440,000), catalase (Mr, 232,000), lactate dehydrogenase (Mr, 140,000), bovine serum albumin (Mr, 67,000), ferritin subunit (Mr, 18,000).

**Fig. 5.** Thermostability of the OTCase and OTCase–GST. The enzyme was incubated at 60°C for each time period at a protein concentration of 50 \( \mu \text{g/ml} \) in 0.02 M potassium phosphate buffer (pH 6.8). The remaining activity was assayed in 150 mM imidazole-HCl (pH 6.8), 10 mM L-ornithine, 10 mM phosphate, and 20 mM carbamoylphosphate at 37°C. O, Wild-type OTCase; ● OTCase–GST enzyme.
Table 1. Allosteric properties of the wild-type OTCase and chimeric OTCase–GST

<table>
<thead>
<tr>
<th>Effector</th>
<th>mM</th>
<th>[S]_{1/2}^P (mM)</th>
<th>n_H</th>
<th>V_{max} (mmol citrulline h^{-1} x (mg prot)^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>4.0 (0.2)</td>
<td>4.1 (0.2)</td>
<td>13.5 (0.5)</td>
</tr>
<tr>
<td>P_{i}</td>
<td>10</td>
<td>1.2 (0.1)</td>
<td>1.0 (0.05)</td>
<td></td>
</tr>
<tr>
<td>P_{i}</td>
<td>25</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td>10</td>
<td>1.2 (0.1)</td>
<td>2.5 (0.1)</td>
<td></td>
</tr>
<tr>
<td>Spermidine</td>
<td>20</td>
<td>11.0 (0.5)</td>
<td>4.3 (0.2)</td>
<td></td>
</tr>
</tbody>
</table>

The assays were performed in 150 mM imidazole-HCl buffer (pH 6.8) containing 10 mM ornithine at variable CP concentrations. [S]_{1/2}^P, concentration of substrate at half maximum velocity; n_H, Hill index. The results are the mean values from at least two independent experiments. V_{max} is expressed in mmol citrulline formed h^{-1} x (mg prot)^{-1} in the presence of 10 mM l-ornithine and at CP saturation concentration. V_{max} of the enzymes is not changed by the various effectors reported.

Fig. 6. Carbamoylphosphate saturation curves for wild-type OTCase and OTCase–GST. Relative velocities are plotted as a function of the carbamoylphosphate concentration, and were determined in the presence of 10 mM ornithine and 150 mM imidazole-HCl (pH 6.8). V_{max} values are given in Table 1. O, Wild-type OTCase; ●, OTCase–GST enzyme.

AMP, an allosteric effector of the wild-type enzyme, acts primarily by promoting a local conformational change that increases the affinity of the catalytic sites for CP and has a secondary effect on the allosteric transition resulting from the increase in occupancy of the catalytic sites by CP (V.S., unpublished data). A value of 10 mM AMP also reduces the [S]_{1/2}^P value for the OTCase–GST as well as the Hill index, indicating that the heterotropic activation by the nucleoside phosphate is conserved also in the trimeric enzyme form. The allosteric effector AMP was however less effective as an activator since a 5-fold reduction in binding affinity was observed as compared with the wild-type enzyme (Table 2).

Spermidine, an allosteric inhibitor of the wild-type enzyme, also promotes a conformational change that is distinct from those involved in the homotropic cooperativity. When spermidine binds to its regulatory site, it promotes a local conformational change that, in contrast with the effect of AMP, primarily decreases the affinity for CP and secondarily increases the carbamoylphosphate homotropic cooperativity (9).

Like the wild-type dodecameric OTCase, the OTCase–GST is inhibited by spermidine as shown by the increase in the [S]_{1/2}^P value but within experimental error the Hill index does not change, indicating that the spermidine acts primarily on the affinity of the CP site for CP and has no effect on the carbamoylphosphate homotropic cooperativity. The affinity of the OTCase–GST enzyme for spermidine is apparently reduced 2.5-fold by comparison with the wild-type OTCase.

CONCLUSIONS

To elucidate the relationships between the structure and function of the allosteric OTCase we had previously selected variants of the dodecameric OTCase able to carry out the anabolic reaction in vivo (22, 28). In three different mutant catabolic OTCases, a point mutation converting Glu-105 into either Ala, Gly, or Lys largely abolishes carbamoylphosphate cooperativity. Because these mutants remain dodecameric, we engineered hybrid genes which consist of the N-terminal part of E.
coli argF gene encoding a trimeric and nonallosteric enzyme combined with the C-terminal part of the catabolic P. aeruginosa arcB gene (26). This study indicated that the dodecameric structure correlates with carbamoylphosphate cooperativity. Besides, in some trimeric hybrid enzymes that retained CP cooperativity, heterotropic interactions are disconnected from homotropic ones (26).

The results presented here show that it is possible to prepare a trimeric OTCase--GST that retains both the homotropic and heterotropic properties of the wild-type enzyme, clear evidence that allosteric interactions are intrinsic characteristics of the trimer.

As the result of differences in the oligomeric state of OTCase and GST, the monomers associate to yield a functional trimer for the OTCase and one functional dimer and one nonfunctional monomer for the GST. Since the fusion protein retains both homotropic and heterotropic activation and inhibition, the evolutionary advantage for OTCase to pass from a trimeric to a dodecameric structure results in an increase in substrate cooperativity and in thermostability as already noted for hybrid trimeric OTCase (26). It should be noted that another dodecameric OTCase displaying Michaelis–Menten kinetics has been characterized in a hyperthermophilic bacterium, Pyrococcus furiosus.

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