Converting Catabolic Ornithine Carbamoyltransferase to an Anabolic Enzyme*

(Received for publication, May 3, 1990)

Heinz Baur, Catherine Tricot, Victor Stalon, and Dieter Haas

From the Mikrobiologisches Institut, Eidgenössische Technische Hochschule, CH-8092 Zürich, Switzerland and the Laboratoire de Microbiologie, Université Libre de Bruxelles, B-1070 Bruxelles, Belgium

Pseudomonas aeruginosa has an anabolic and a catabolic ornithine carbamoyltransferase (OTCase). In vitro, these homologous enzymes catalyze the same reaction (ornithine + carbamoyl phosphate (CP) \( \rightarrow \text{citrulline + Pi} \)), yet in vivo they function unidirectionally owing to specific kinetic properties. The catabolic OTCase cannot promote the anabolic reaction (citrulline formation) in vivo because of a sigmoidal CP saturation curve and a high CP concentration for half-maximal velocity. The structural basis for this kinetic specialization was examined. The catabolic OTCase lost most of its homotropic cooperativity and gained anabolic activity when an amino acid residue near the CP binding site, Glu-106, was replaced by alanine or glycine. In the anabolic OTCase of Escherichia coli the glutamic acid residue corresponding to Glu-106 was exchanged by a leucine, and a few crystals of N-methyl-N′-nitro-N-nitrosoguanidine were placed in the center of the plate. Arginine-prototrophic colonies growing around the mutagen were isolated as described (18). Four PA0532 derivatives were retained for further study: PA06238 (arcB6238(Su)), PA06240 (arcB6240(Su)), PA06241 (arcB6241(Su)), and PA06254 (arcB6254(Su)). These strains have a second, 14728

Osmotic carbamoyltransferases (OTCases,² EC 2.1.3.3) catalyze the reaction: L-ornithine + carbamoyl phosphate \( \rightarrow \) L-citrulline + P₄. Anabolic OTCases promote citrulline formation from ornithine as part of arginine biosynthesis (1). A number of procaryotes including Pseudomonas aeruginosa have second, 14728

*This work was supported by Schweizerische Nationalfonds Project 3.585-0.87, Fund for Joint Basic Research Grant 2.9003.88, European Communities Research Contract BAP-0345-B, and an “Action de Recherche Concertée” of the Belgian Government. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. ²To whom correspondence should be addressed. Tel.: 01 256 33 15; Fax: 01 709 96 13

The abbreviations used are: OTCase, ornithine transcarbamylase (carbamoyltransferase); ATCase, aspartate transcarbamylase (carbamoyltransferase); CP, carbamoyl phosphate; kb, kilobase.
catabolic OTCase which participates in the anaerobic degradation of arginine via citrulnine to ornithine (1–3). OTCases are structurally related to aspartate carbamoyltransferase (ATCase; EC 2.1.3.2) of Escherichia coli, an allosteric enzyme par excellence (4–7). Because of these sequence identities, the residues involved in carbamoyl phosphate (CP) binding and the secondary structure of OTCases can be predicted on the basis of the known ATCase structure (4–11). The catalytic subunit of ATCase (the pyrB gene product) forms an active trimer, which displays Michaelis-Menten kinetics. In vivo, two catalytic ATCase trimers are complexed with three regulatory dimers, and this holoenzyme has allosteric properties and a sigmoidal aspartate saturation curve (4, 5). The anabolic OTCases of E. coli (the argF and arg products) and P. aeruginosa (the argF product) are trimers resembling the ATCase catalytic trimer (4, 6, 8, 12, 13). The substrate saturation curves of the E. coli anabolic OTCase and of the catalytic ATCase trimer are changed from hyperbolic to sigmoidal when a conserved residue, Arg-106, involved in CP binding (4), is converted to a nonpolar amino acid residue by site-directed mutagenesis (14, 15).² We are interested in the reciprocal situation. How can we strip the catabolic OTCase of its cooperativity? The catabolic OTCase of P. aeruginosa (the arcB product) is a trimeric enzyme aggregated to a complex form, i.e., a nonamer or dodecamer (16). The CP saturation curve is strongly sigmoidal and a high CP concentration is needed for half-maximal velocity, \( [\text{CP}]_{1/2} = 17 \text{ to } 18 \). Therefore, the wild-type enzyme cannot perform the anabolic reaction in vivo, but mutant enzymes have been obtained which can (17, 18). This study characterizes the sequences and kinetics of such mutant enzymes. Then, an in vivo recombination method is described which allows the isolation of arcB-argF gene fusions. This technique, which should be widely applicable, has led to the identification of further residues involved in homotropic cooperativity of catabolic OTCase.

EXPERIMENTAL PROCEDURES

Mutant Isolation and Transductional Mapping—P. aeruginosa PA0532 (argF2, his-35) was plated on minimal medium supplemented with histidine, and a few crystals of N-methyl-N′-nitro-N-nitrosoguanidine were placed in the center of the plate. Arginine-prototrophic colonies growing around the mutagen were isolated as described (18). Four PA0532 derivatives were retained for further study: PA06238 (arcB6238(Su)), PA06240 (arcB6240(Su)), PA06241 (arcB6241(Su)), and PA06254 (arcB6254(Su)). These strains and a mutant previously obtained, PA06245 (arcF2, pur-106, arcB(Su); Ref. 18), were analyzed for the presence of the original argF mutation by cotransduction with argG and for transductional linkage between his-30 and arcB(Su), by the procedures of Haas et al. (18) using the generalized transducing phage G101.

In Vivo Cloning of arcB/Su Alleles—P. aeruginosa strains PA06238, PA06240, PA06241, and PA06254 were transformed with pME183, which carries the wild-type 5-kb arcDABC operon on the mobilizable broad host range vector pRK240 (19, 20). By homologous recombination, an exchange (double cross-over) could take place between the chromosomal arcB(Su) allele and the plasmid-borne arcB∗ gene. To isolate such pME183arcB(Su) derivatives, we introduced pUB307 (a conjugative plasmid capable of pRK240 mobilization)
tion; Ref. 21) into the arcB(Su) strain and did a plate-mating with the recombination-deficient argF recipient PTO6009 (argF10, leu 10, recA102; Ref. 21), with selection for arginine prototrophy and the carbenicillin resistance (Cb') marker of PME183. Cb' argF transconjugants arose at \( \sim 10^{-3} \) / donor and carried an arcB(Su) plasmid (designated PME183-38, PME183-40, PME183-41, or PME183-54, respectively). These plasmids were isolated and checked for their ability to restore arginine prototrophy in strain PTO6009.

Plasmid Manipulation and Oligonucleotide-directed Mutagenesis DNA was extracted and manipulated by standard techniques (21-23). Chromosomal DNA from strain PAO524 (arcB(Su)) was digested with HindIII and Smal. Fragments of \( \sim 5 \) kb were cloned into the Pseudomonas vector pME290 (Ref. 22) cut with HindIII and Smal; the resulting recombinant plasmid pME335 (Cb', 11.8 kb) carried the arcB(Su) allele within the arcDABC operon on a 5.3-kb HindIII-Smal fragment (19) and rendered argF mutants of P. aeruginosa arginine-independent. The arcB(Su) alleles carried by pME335, pME183-38, pME183-40, pME183-41, and pME183-54 were suctioned on appropriate restriction fragments into M13mp18/ polyacrylamide gel electrophoresis. Protein concentrations were determined by the Lowry procedure and according to A280/A260 values carried the arcB(Su) allele within the arcDABC operon on a 5.3-kb HindIII-Smal fragment (19) and rendered argF mutants of P. aeruginosa arginine-independent. The arcB(Su) alleles carried by pME335, pME183-38, pME183-40, pME183-41, and pME183-54 were suctioned on appropriate restriction fragments into M13mp18/ polyacrylamide gel electrophoresis. Protein concentrations were determined by the Lowry procedure and according to A280/A260 values.
still functioned in the catabolic direction.

The Gly-106 and Ala-106 enzymes were purified and their kinetic properties compared to those of the wild-type catabolic enzyme. Both modified OTCases had a strongly reduced cooperativity for CP (as expressed by the Hill coefficient $n_H$), a markedly lower $[S]_{0.5}$ value, and an improved specific activity (Table I). Thus, they were much more efficient in citrulline synthesis than was the wild-type enzyme (Table I), and this is consistent with the arginine prototrophy of the arcB(Su) strains. Other effects of the arcB(Su) mutations were a twofold increase in the apparent $K_m$ for ornithine (at 10 mM CP) and a shift of the pH optimum by ~1 pH unit toward alkaline values (Table I). With respect to the pH optimum, the modified catabolic OTCase resembled the anabolic E. coli OTCase (Table I). The main conclusion is that Gly-106 in the catabolic enzyme is essential for CP cooperativity. Glu-106 is near Arg-108, which corresponds to an essential arginine residue involved in CP binding in ATCase and anabolic OTCase (4, 14, 15).

Inorganic phosphate is an activator of wild-type catabolic OTCase (17, 18). Thus, in the presence of 10 mM P, the homotropic cooperativity of this enzyme disappeared ($n_H = 1.0$), the $[S]_{0.5}$ value was 1.9 mM and the specific activity of the pure enzyme was 27.1 mmol h$^{-1}$ (mg protein)$^{-1}$ at pH 7.25. Interestingly, the mutant OTCases had lower $[S]_{0.5}$ values and higher specific activities (Table I) than had the P$_F$-activated wild-type catabolic OTCase.

The catabolic OTCase of P. aeruginosa has more amino acid sequence identity with the anabolic OTCase of E. coli than with the anabolic enzyme of P. aeruginosa (13). We have therefore focused on the first two enzymes. Glu-106 of the catabolic OTCase corresponds to a glutamine residue in the argF enzyme of E. coli. Does a Gly-106 → Glu replacement confer CP cooperativity on the anabolic OTCase? This was tested by site-directed mutagenesis of the E. coli argF gene cloned into a pBR322 derivative (pME3604; Fig. 1). However, the engineered argF (Glu-106 → Glu) enzyme, in extracts from E. coli, retained Michaelis-Menten kinetics and, in fact, had a lower apparent $K_m$ value for CP (0.06 mM, at pH 8.5) than had the wild-type argF enzyme ($K_m = 0.36$ mM, at pH 8.5). Thus, in the context of the anabolic OTCase of E. coli, Glu-106 did not produce a sigmoidal CP saturation curve, and the cooperativity of the catabolic OTCase must be due to particular sequence features in addition to Glu-106.

To identify such sequences we constructed arcB-argF fusions in vivo (Fig. 2a). A truncated arcB$^{36}$ gene (with a 0.05-kb deletion in the distal part) and a truncated arcF gene (with a 0.12-kb deletion in the proximal part) were inserted into the multi-copy, broad host range plasmid vector pKT240 in the same orientation. These shortened genes were separated by 1.8 kb (Fig. 2a) and neither specified an active OTCase. The E. coli host used (SC1800; argI90 A(proAB lac argF)) was recombination-proficient and OTCase-negative. Selection for arginine prototrophy, i.e. for OTCase function, resulted in the recovery of arcB-argF fusions (Fig. 2a) at frequencies of 10$^{-6}$ to 10$^{-7}$. Recombination occurred between arcB and ‘argF’ in three different 30-base pair regions having ~80% nucleotide sequence identities (data not shown), the overall arcB/argF identity being 63% (16). One recombinant, pME3606, was characterized in detail; it specified a modified catabolic OTCase with 8 C-terminal amino acids originating from the anabolic OTCase (Fig. 2b). This hybrid enzyme functioned in the anabolic direction in both E. coli and P. aeruginosa, owing to lowered $[S]_{0.5}$ and $n_H$ values (Table I).

\textsuperscript{3}Glu-106 is at position 104 in anabolic OTCase of E. coli.
Thus, the C terminus of catabolic OTCase is important for cooperativity.

All arcB(Su) enzymes including the one specified by pME3606 had the same molecular weight as wild-type catabolic OTCase (~420,000), as judged by Sepharose 4B gel filtration and Phast gel electrophoresis under non-denaturing conditions. We have no evidence for dissociation of these enzymes into catalytically active, low molecular weight forms (e.g. trimers) without cooperative properties. However, we cannot exclude this type of dissociation during enzyme assays when protein concentrations were ~100 times lower than during gel filtration.

Protein engineering experiments involving in vitro constructions may give rise to some inactive enzymes. The in vivo fusion strategy described here (Fig. 2a) has the advantage of producing active enzymes, does not require perfect sequence identity and should be applicable to other studies on directed enzyme evolution.

Acknowledgments—We thank A. Boukhzar for help with kinetic experiments, E. Jäggi and H. Paul for secretarial assistance, and G. Hervé and O. Dideberg for discussion.

REFERENCES

1. Cunin, R., Glansdorff, N., Piérard, A., and Stalon, V. (1986) Microbiol. Rev. 50, 314–352
2. Stalon, V., Ramos, F., Piérard, A., and Wiame, J. M. (1961) Biochim. Biophys. Acta 139, 91–97
3. Vander Wauven, C., Piérard, A., Kley-Raymann, M., and Haas, D. (1984) J. Bacteriol. 160, 928–934
4. Kantrowitz, E. R., and Lipscomb, W. N. (1988) Science 241, 669–674
5. Hervé, G. (1989) in Allotrophic Enzymes (Hervé, G., ed) pp. 61–79, CRC Press, Boca Raton, FL
6. Van Vliet, F., Cunin, R., Jacobs, A., Piétte, J., Gigot, D., Lamurelwe, M., Piérard, A., and Glansdorff, N. (1984) Nucleic Acids Res. 12, 6277–6289
7. Haas, D., Baur, J., Tricot, C., Galimand, M., and Stalon, V. (1989) in Recent Advances in Microbial Ecology (Hattori, T., Ishida, Y., Maruyama, Y., Morita, R. Y. and Uchida, A., eds) pp. 617–621, Japan Scientific Societies Press, Tokyo
8. Houghton, J. E., Bencini, D. A., O’Donovan, G. A., and Wild, J. R. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 4864–4868
9. Houghton, J. E., O’Donovan, G. A., and Wild, J. R. (1989) Nature 338, 172–174
10. Tricot, C., De Coen, J.-L., Momin, P., Falmane, P., and Stalon, V. (1989) J. Gen. Microbiol. 135, 2453–2464
11. Kuo, L. C., Miller, A. W., Lee, S., and Kozuma, C. (1988) Biochemistry 27, 8823–8832
12. Legrain, C., Halieux, P., Stalon, V., and Glansdorff, N. (1972) Eur. J. Biochem. 27, 93–109
13. Itoh, Y., Soldati, L., Stalon, V., Falmane, P., Terawaki, Y., Leisinger, T., and Haas, D. (1988) J. Bacteriol. 170, 2725–2734
14. Kuo, L. C., Zambidis, I., and Caron, C. (1980) Science 210, 522–524
15. Stebbins, J. W., Xu, W., and Kantrowitz, E. R. (1989) Biochemistry 28, 2529–2550
16. Baur, H., Stalon, V., Falmane, P., Lüthi, E., and Haas, D. (1987) Eur. J. Biochem. 166, 111–117
17. Stalon, V., Ramos, F., Piérard, A., and Wiame, J.-M. (1972) Eur. J. Biochem. 29, 25–35
18. Haas, D., Evans, R., Mercenier, A., Simon, J.-P., and Stalon, V. (1979) J. Bacteriol. 139, 713–720
19. Lüthi, E., Mercenier, A., and Haas, D. (1986) J. Gen. Microbiol. 132, 2667–2675
20. Lüthi, E., Baur, H., Gamper, M., Brunner, F., Villeval, D., Mercenier, A., and Haas, D. (1990) Gene (Amst.) 87, 37–43
21. Jeenes, D. J., Soldati, L., Watson, J. M., Mercenier, A., Reimmann, C., Leisinger, T., and Haas, D. (1986) Mol. Gen. Genet. 203, 421–429
22. Itoh, Y., and Haas, D. (1985) Gene (Amst.) 36, 27–36
23. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Gene (Amst.) 33, 103–119
25. Crahas, J., Charlier, D., Cunin, R., and Glansdorff, N. (1979) Gene (Amst.) 5, 107–111
26. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382
27. Legrain, C., and Stalon, V. (1976) Eur. J. Biochem. 63, 289–301
28. Klenk, H. M. (1947) J. Biol. Chem. 167, 461–475
29. Preocott, L. M., and Jones, M. E. (1969) Anal. Biochem. 32, 408
30. Forsht, A. (1985) Enzyme Structure and Mechanism, 2nd ed., pp. 106–107, 272–270, W. H. Freeman & Co., New York
31. Hanes, C. S. (1932) Biochem. J. 26, 1406–1421
32. Bagdasarian, M. M., Amann, E., Lurz, R., Rückert, B., and Bagdasarian, M. (1985) Gene (Amst.) 26, 273–282
33. York, M. K., and Stodolek, M. (1981) Mol. Gen. Genet. 181, 230–240