Articles

In Vitro and in Vivo Activities of T4 Endonuclease V Mutants Altered in the C-Terminal Aromatic Region†

Mitsuyoshi Ishida, Yuriko Kanamori, Naoko Hori, Tetsuya Inaoka, and Eiko Ohtsuka*
Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan
Received September 5, 1989; Revised Manuscript Received December 1, 1989

ABSTRACT: Genes encoding mutants of the thymine photodimer repair enzyme from bacteriophage T4 (T4 endonuclease V) having an amino acid substitution (T127M, W128A, W128S, Y129A, K130L, Y131A, Y132A) were constructed by use of a previously obtained synthetic gene and expressed in *Escherichia coli* under the control of the *E. coli* tryptophan promoter. An in vitro assay of partially fractionated mutant proteins for glycosylase activity was performed with chemically synthesized substrates containing a thymine photodimer. T127M and K130L showed almost the same activity as the wild-type protein. Although W128S, Y131A, and Y132A were slightly active, W128A and Y129A lost activity. The results indicated that the aromatic amino acids around position 130 may be important for the glycosylase activity. Mutant T127M was purified, and the K_m value was found to be of the same order as that of the wild type (10^{-8} M). In vivo activities for all mutants were characterized with UV-sensitive *E. coli*. The results showed that substitution of Thr-127 with Met or Lys-130 with Leu did not have an effect on the survival of the bacteria but substitution of aromatic amino acids (128–132) had various effects on survival.

T4 endonuclease V (T4 Endo V)[1] encoded by the T4 *den*V gene (Yasuda & Sekiguchi, 1970; Sancar & Sancar, 1988) consists of 138 amino acids and serves as a repair enzyme for thymine photodimer lesion of double-stranded DNA. The enzyme has pyrimidine dimer–DNA glycosylase (PD-glycosylase), which cleaves the *N*-glycoside linkage of the 5′-side thymine of a thymine dimer, and apurinic/apyrimidinic-nicking (AP-nicking) activities (Nakabeppu & Sekiguchi, 1981). Recent NMR studies suggested that the second step involved a β-elimination reaction (Monoharan et al., 1988; Kim & Linn, 1988). The gene of T4 phage *den*V has been cloned (Lloyd & Hanawalt, 1981; Valerie et al., 1984; Radany et al., 1984), and the sequence of the 138 amino acids of this endonuclease was deduced from the base sequence (Valerie et al., 1984). The cloned gene has been expressed in *Escherichia coli*, and site-directed mutagenesis of the gene has been reported (Recinos & Lloyd, 1988; Stump & Lloyd, 1988). In contrast to these approaches, we have previously synthesized a gene for this endonuclease by ligation of oligonucleotide fragments; this gene was expressed efficiently in *E. coli* under the control of the *trp* promoter (Inaoka et al., 1989). This made it possible to obtain a large quantity of the enzyme for kinetic studies using synthetic substrates containing a thymine photodimer. The synthetic gene had been designed to facilitate mutagenesis at the carboxy-terminal region, which has a cluster of aromatic amino acids near lysine-130. The ε-amino group of Lys-130 has been assumed to act as a base for β-elimination of the 3′-phosphate at the apyrimidinic site from the previously reported nicking activity of simple tripeptides such as Lys-Trp-Lys and Lys-Tyr-Lys (Behmoaras et al., 1981a,b; Pierre & Laval, 1981). However, the result reported by Recinos and Lloyd (1988) suggests that Lys-130 is associated with pyrimidine dimer specific binding but not with the cleavage reaction. The importance of Tyr-129 and -131 in pyrimidine dimer specific binding of the enzyme has also been reported (Stump & Lloyd, 1988). In this paper we describe construction and expression of genes encoding mutants with single amino acid substitutions at positions 127–132. The PD-glycosylase activity of the mutants was assayed in vitro with a defined oligodeoxyribonucleotide duplex containing a thymine photodimer. Although the present results also indicate the importance of the aromatic amino acids around Lys-130 for activity, substitution of Trp-128 with Ser resulted in an active mutant, in contrast to the substitution with Ala which gave a completely inactive enzyme.

A truncated gene lacking 11 amino acids from the C-terminus has also been prepared, and the expressed product was detected with monoclonal antibodies raised against the wild-type enzyme.

The in vivo activity of the mutants was tested by measurement of survival abilities of a UV light sensitive *E. coli* strain transformed with these genes, and the results were compared with the in vitro activity. CD spectra for two mutants were measured and compared with that of the wild-type enzyme.

Materials and Methods

Oligodeoxyribonucleotides. Oligodeoxyribonucleotides used for construction of mutant genes were prepared by a DNA synthesizer, and those containing a thymine photodimer were synthesized as described previously (Inaoka et al., 1989).

Construction of Mutant Plasmids. A truncated mutant plasmid (127-truncated) was prepared from pEndV by re-

†This work was supported in part by research grants from the Ministry of Education, Science and Culture of Japan.

[1] Abbreviations: T4 Endo V, T4 endonuclease V; PD-glycosylase, pyrimidine dimer–DNA glycosylase; AP, apurinic/apyrimidinic; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PEME, phosphate/ethylene glycol/2-mercaptoethanol/EDTA; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; $\overline{TT}$, thymine photodimer; T127M, Thr-127 is replaced by Met; C127-truncated, C-terminal-truncated mutant with 127 amino acids.

0006-2960/90/0429-3817$02.50/0

placement of four DNA fragments of the C-terminus in the *Hin*dIII-*Cla*I fragment of the synthetic gene with two oligonucleotides (dGTCCTACTTGATAG and dTCGACTATCAAG). Other mutants containing an amino acid substitution were constructed with this plasmid (pEVC127) by replacement of the restriction fragment prepared by ligation of appropriate DNA fragments. Codons used for Ala (128, 129, and 132), Ala (131), Ser (128), and Leu (130) were GCU, GCA, UCU, and CUG, respectively. These plasmids were used to transform *E. coli* HB101. The base sequences of mutated genes were analyzed by the dideoxy method (Sanger et al., 1977) using synthetic DNA fragments as primers.

Expression of Genes. *E. coli* HB101 harboring mutated plasmids were precultured in L-broth containing ampicillin and transferred to M9CA medium containing ampicillin as described for the expression of the wild-type gene. 3-Indolylacrylic acid was added (40 μg/mL) at a turbidity of 0.05–0.1. Other methods for characterization of gene products were as described previously (Inaoka et al., 1989). The product was isolated by the procedure of Nakabeppu et al. (1982) unless otherwise specified. Fractions I and V refer to the lysate and purified fractions, respectively.

Renaturation of Mutant Proteins. The precipitate from sonicated mixtures was dissolved in 8 M urea and PME buffer (10 mM potassium phosphate, pH 6.5, 10 mM 2-mercaptoethanol, 2 mM EDTA). The solution was dialyzed against 4 M urea and 2 M urea containing PEME buffer (10 mM potassium phosphate, pH 6.5, 10 mM 2-mercaptoethanol, 2 mM EDTA, 10% ethyleneglycol) and finally against buffer alone. Precipitates were removed by centrifugation at 4000*g* for 30 min, and the supernatant (fraction RU) was used as the renatured fraction.

Assay for Activities. The in vitro assay for PD-glycosylase and AP-nicking activities was performed as described previously with a 5′-labeled 14-mer containing a thymine photodimer, d(CGAAGG$\overline{TT}$GGAAGC), in the presence of the complementary strand (Inaoka et al., 1989). Lineweaver–Burk plots with $\overline{TT}$ 14-mer duplex for the wild-type and mutant enzymes were recorded after treatment with alkali using 0.5 ng of enzyme in 100 μL at 30 °C. The radioactivity of the cleaved oligonucleotide having a 5′-^{32}P was counted at 5, 10, 15, and 20 min.

The in vivo assay was performed by transfection of mutant plasmids into the UV-sensitive *E. coli* strain AB1886 (*uvr*A6), which was provided by Drs. Sekiguchi and Nakabeppu. *E. coli* strain AB1157 (*uvr*A$^+$) was used as the resistant control. Transformed *E. coli* were analyzed with restriction enzymes after plasmids had been isolated by the rapid-boiling method (Holmes & Quigley, 1981). *E. coli* (pEndV/AB1886) and the other strain were cultured and finally diluted in M9CA (+Amp) medium and then subjected to UV irradiation on agar plates by a germicidal lamp (10 W) from a distance of 1 m. The incident dose rate was measured at 254 nm by a photometer (UVX type, UVP Co.). Bacteria were cultured at 37 °C overnight in the dark and handled under lights above 500 nm with a yellow lamp (Toshiba FLR40 S-Y-Nu). *E. coli* were diluted to 500–1000 colonies/plate, and survival rates were obtained from an average of three experiments.

Preparation of Monoclonal Antibodies for T4 Endo V. The wild-type T4 Endo V obtained previously (100 μg) was used for each BALB/C mouse as the antigen, and monoclonal antibodies were prepared according to the method reported (Gelfre et al., 1977). Mice were immunized at 2-week intervals. Three days after the final injection, spleen cells were

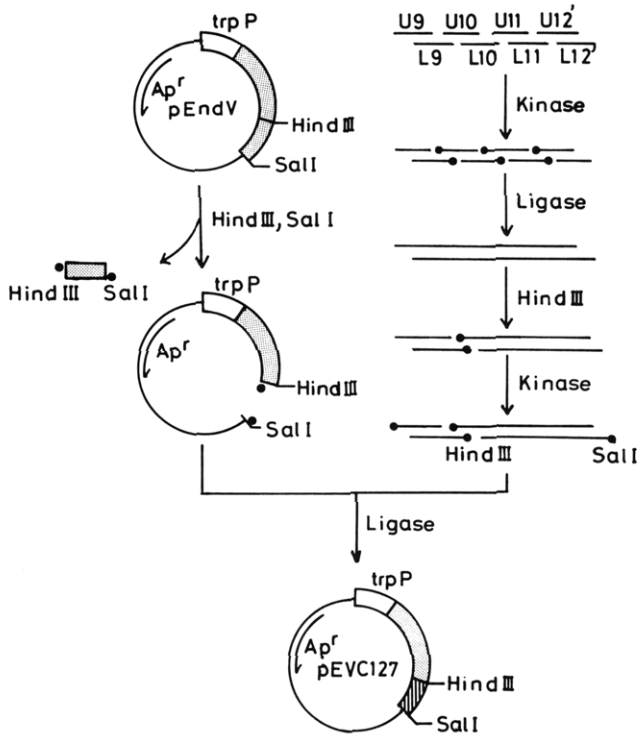

FIGURE 1: Construction of an expression vector containing the gene for the truncated enzyme (127-truncated).

fused with mouse myeloma cells (P3-X63-Ag-635) and hybridomas selected in hypoxanthine–aminopterin–thymidine medium. Specific antibodies were screened by the enzyme-linked immunosorbent assay (ELISA) (Nakane & Deguchi, 1982). The hybridoma producing specific antibodies were cloned by the limiting dilution method. Anti-mouse IgG+IgM conjugated with horseradish peroxidase (HRP) and *o*-phenylenediamine were employed for the assay. Subclasses of antibodies were assayed by a kit obtained from Zyme Co. Out of seven clones, 6-3D and 57-2H were found to be suitable for testing. Monoclonal antibodies were prepared from the ascitic fluid by inoculation of pristen-primed mice with the hybridomas and by precipitation with ammonium sulfate followed by gel filtration.

Western blotting was performed on a nitrocellulose filter at 37 °C for 1 h and detected by HRP-conjugated anti-mouse IgG+IgM.

RESULTS

Construction of Plasmids Containing Genes for T4 Endonuclease V Mutants. It was planned to alter each amino acid in a cluster of aromatic acids around lysine-130. A truncated mutant with a deletion of 11 amino acids from the C-terminus and a mutant at position 127 (Thr to Met) were also prepared by modification of the gene. A plasmid containing the truncated mutant was constructed by use of restriction sites for *Hin*dIII and *Sal*I as shown in Figure 1. Two oligonucleotides containing termination codons were prepared and joined to the six previously obtained fragments to yield a cassette, which then was linked to a large fragment from plasmid pEndV containing the gene for the wild-type enzyme.

The newly constructed plasmids (pEVC127) were used to transform *E. coli*, and the structure of the truncated gene was analyzed by restriction analysis as well as base sequencing. pEVC127 was convenient for the construction of plasmids containing other mutant genes. Genes that coded mutants were obtained by replacement of U12 and L12 of the *Hin*dIII–*Sal*I fragment of the wild-type synthetic gene and joined

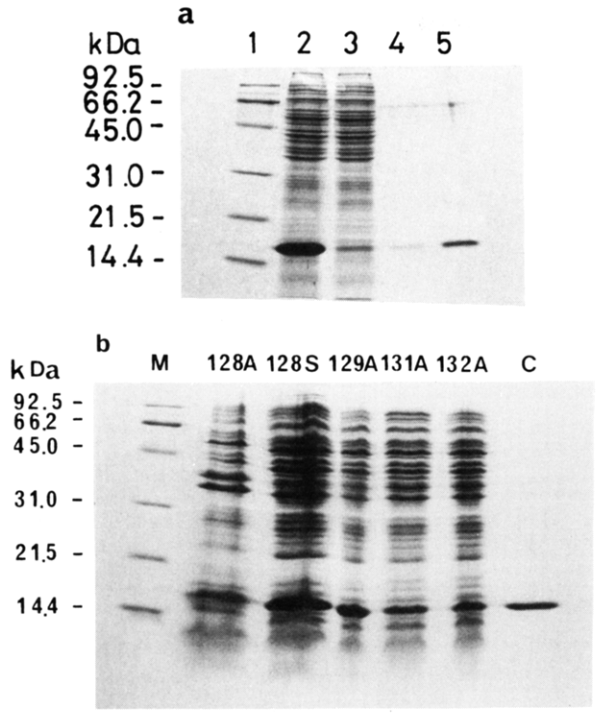

FIGURE 2: Analyses of the products from the gene for mutants T127M (a) and others (b) on 15% SDS–PAGE. (a) 1, markers; 2, *E. coli* whole cell proteins; 3, fraction I, 4, fraction III, 5, fraction V. (b) M, markers; C, a purified sample of the wild-type enzyme.

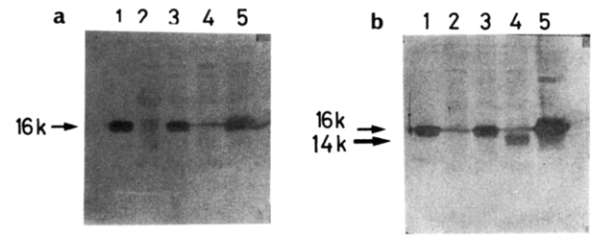

FIGURE 3: Analysis of the truncated mutant (127-truncated) by blotting with monoclonal antibodies: 6-3D (a) and 57-2H (b). (1) Purified wile-type enzyme; (2) proteins from *E. coli* harboring plasmids containing a human growth hormone gene; (3) wild-type enzyme (fraction I); (4) 127-truncate (fraction I); (5) K130L (fraction I).

to the *Hin*dIII–*Sal*I site of pEVC127. These mutant plasmids could easily be screened by restriction analyses using the *Nsi*I site at the C-terminus. The base sequences for these mutants were confirmed.

Expression of Mutant Genes. *E. coli* harboring the mutant plasmid were cultured, and the gene was induced under the control of the *trp* promoter by addition of 3-indolylacrylic acid. In contrast to the wild type protein which accumulated to a maximum level after 2 h, a mutant protein (K130L) reached a maximum amount after 24 h. The amount was estimated by PAGE and found to be 28% of the total protein. Another mutant protein (T127M) was analyzed similarly as shown in Figure 2a and estimated to be 20% of the total protein. Figure 2b shows whole-cell proteins of mutants (W128A, W128S, Y129A, Y131A, Y132A) after 8 h of induction with 3-indolylacrylic acid. It was planned to express the truncated mutant gene by the same procedure. However, the product was not overproduced and was difficult to detect as a distinct band. By use of a partially purified fraction, a 14K protein was characterized on a nitrocellulose membrane as shown in Figure 3b by blotting with a monoclonal antibody (57-2H) which was raised with the wild-type T4 endoV. The truncated protein did not seem to be recognized by another antibody (6-3D) as shown in Figure 3a. It is likely that these monoclonal antibodies recognize different parts of the enzyme.

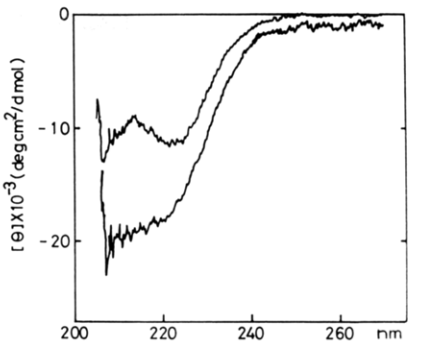

FIGURE 4: CD spectra for wild type (upper curve) and W128S (lower curve). The spectra were measured by a JASCO 500A spectropolarimeter (1-cm light path) with 0.15 mg/mL wild-type and 0.18 mg/mL W128S proteins in PEME buffer. [θ] [(deg cm^2)/dmol] were normalized to mean residue ellipticity.

Table I: Kinetic Data for the PD-glycosylase Activity

	V_{max} [nmol min^{-1} mg^{-1}]	k_{cat} (min^{-1})	K_m (nM)	k_{cat}/K_m ($\times 10^6$ min^{-1} M^{-1})
wild type	68	1.1	15	73
Met-127	31	0.5	15	33

Isolation and CD Spectra for T127M and W128S. The mutant protein T127M was recovered from the supernatant of sonicated cells. All other mutant enzymes could not be recovered in solution in reproducible yield. T127M was purified by essentially the same procedure used for the isolation of the wild-type enzyme (Inaoka et al., 1989). However, the yield of the mutant was 2% of the wild-type enzyme, although the amount in the cell lysate was almost the same as that of the wild type. This may mean that the content of product with the correct conformation becomes smaller by replacement of Thr with Met. In order to compare the effect of amino acid substitution on the conformation of this region, W128S was recovered from the precipitate. Renaturation of the protein was performed by dissolving in 8 M urea and decreasing the concentration of urea by dialysis. Cation-exchange chromatography was not possible due to the nonabsorbable nature of this mutant. The supernatant (fraction RU) was used for further studies. The CD spectra for the wild type and mutant were measured (Figure 4). The CD spectrum for W128S (Figure 4, lower curve) showed some difference from that of the wild-type enzyme. The spectrum for T127M showed a shape similar to that of the wild-type (data not shown) and suggested the main chain of the protein kept a similar conformation.

Kinetic and Binding Properties of T127M. The kinetic properties of the PD-glycosylase activity for purified T127M were compared with those for the wild-type enzyme. Lineweaver–Burk plots were used to deduce the kinetic parameters. The results are listed in Table I. This mutation resulted in a decrease of the V_{max} to half the original value, but the K_m value remained the same. This may mean that the substitution of Thr-127 with Met does not affect the affinity of the enzyme for the 14-mer containing a thymine photodimer. The dissociation constant for T127M was measured and found to be 9.1×10^{-8}. This differs from the value for the wild type (Inaoka et al., 1989) by one order of magnitude.

In Vitro Activities of Other Mutants. Since other mutant enzymes were difficult to dissolve, the PD-glycosylase activities in crude extracts of *E. coli* harboring each mutant gene were compared. These activities were measured in the presence of EDTA and based on the total soluble protein. Since the efficiency and solubility were not equal in each case, exact

Table II: In Vivo Activity vs in Vitro Activity

PD-glycosylase act.	survival activity		
	normal	low	ND[a]
normal	Met-127 Leu-130		
low	Ser-128 Ala-132		Ala-131
ND		Ala-129	Ala-128 C127-truncated

[a] ND, not detected.

comparisons were not made. However, the mutants could be roughly divided into three categories. Mutants T127M and K130L had more than 10% of the PD-glycosylase activity compared to the wild-type enzyme. Without alkaline treatment, T127M and K130L showed almost the same cleavage with increasing amounts of the protein. Three mutants (W128S, Y131A, and Y132A) were found to have low PD-glycosylase activity. Without alkaline treatment these mutants nicked about half of the apyrimidinic sites. W128A, Y129A, and 127-truncated mutants belonged to a group with no activity.

In Vivo Activities of Mutants. Plasmids containing the mutant genes for T4 Endo V were transfected into *E. coli* AB1886, which have a deficient *uvr*A gene. *E. coli* AB1886 are known to be sensitive to UV irradiation and to be complemented in the presence of expressed T4 *den*V. Each strain was subjected to UV irradiation at 254 nm, under the conditions for avoiding reversible reactions by visible lights. After incubation at 37 °C overnight, colonies were counted. Figure 5 shows representative data of the survival experiment for each strain. Four mutants (T127M, K130L, W128S, and Y132A) showed normal survival activity compared to wild type, and Y129A gave low activity. Other mutant plasmids for Y131A, W128A, and 127-truncated showed no effect on the survival of the *uvr*-deficient *E. coli*. Table II summarizes these in vivo experiments together with those from the in vitro assays.

Discussion

The C-terminal region of T4 Endo V has been assumed to be an important site for cleavage of the photodiester linkage at the abasic site of thymine photodimers. The aromatic amino acids around Lys-130 have been postulated to be responsible for the nicking activity on the basis of data from genetic studies (Nakabeppu & Sekiguchi, 1981; Valerie et al., 1984; Seawell et al., 1980) and biochemical experiments with model peptides (Behmoaras et al., 1981a,b; Pierre & Laval, 1981). The present deletion mutant which lacks 11 amino acids from the C-terminus (127-truncated) and a mutant with an amino acid substitution W128A showed complete loss of activities in the PD-glycosylase and survival assays. However, W128S had ca. 3% of the PD-glycosylase, 1.5% of the nicking, and almost normal survival activities with respect to the wild-type enzyme. Nakabeppu et al. (1982) have reported a decreased nicking activity of a crude extract of a revertant W128S. Endonucleolytic systems in *E. coli* may assist in the cleavage of the phosphodiester linkage at the lesion. The other mutant, Y132A, might function by the same mechanism, since this mutant belonged to the same category as shown in Table II.

Substitution of Tyr-129 and -131 with Ala have different results. Y129A showed no PD-glycosylase but low survival activity, and Y131A reacted in the opposite way. Stump and Lloyd (1988) have reported a preparation of mutants Y129N and Y131N and suggested the importance of Tyr-129 and -131 in pyrimidine dimer specific binding and possibly in nicking activity. On the other hand, Lys-130 has been found not to

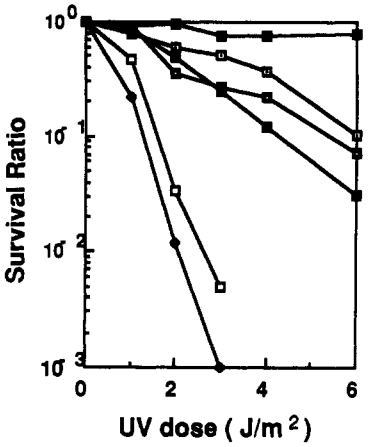

FIGURE 5: In vivo activities of mutants. Plasmids containing a mutant gene were transfected to UV-sensitive *E. coli* AB1886. Plasmid pBR322 was used as the control in *E. coli* AB1886 and in UV-resistant *E. coli* AB1157. (■) pBR322/AB1157; (●) pBR322/AB1886; (thick box) Endo V/AB1886; (⊡) Ser-128/AB1886; (□) Ala-128/AB1886; (box with plus) Met-127/1886.

be essential for dimer-specific binding (Recinos & Lloyd, 1988). Our present results from K130L also showed PD-glycosylase and survival activities were not affected by loss of the α-amino group. Substitution of Thr-127 with Met also retains these activities. Since the CD spectra for this mutant and wild type showed a very similar shape, the main chain of T127M might hold the same conformation as that of the wild type. The CD spectrum for W128S suggested some differences in the main chain structure from that of the wild-type enzyme. The CD spectra suggested a high α-helix content for this enzyme (Chen et al., 1974). It will be interesting to compare these data and those from X-ray crystallography.

It may be concluded that the C-terminal aromatic cluster is essential for the first step of activity. The fact that substitution of Trp-128 or Tyr-129 with Ala abolished the activity suggests that even a single amino acid plays an important role in this region. The three-dimensional structure that should be obtained from X-ray crystallographic studies (Morikawa et al., 1988) may allow the elucidation of the mode of participation of the aromatic amino acids around position 130.

Acknowledgments

We thank Dr. M. Sekiguchi and Y. Nakabeppu for valuable discussions and providing *E. coli* strains. We also thank Dr. S. N. Farrow for reading the manuscript.

References

Behmoaras, T., Toulme, J. J., & Helene, C. (1981a) *Proc. Natl. Acad. Sci. U.S.A. 78*, 926–930.

Behmoaras, T., Toulme, J. J., & Helene, C. (1981b) *Nature 292*, 858–859.

Chen, Y. H., Yang, J. T., & Chan, K. H. (1974) *Biochemistry 13*, 3350–3359.

Friedberg, E. C., & King, J. J. (1971) *J. Bacteriol. 106*, 500–507.

Gelfre, G., Howe, S. C., Milstein, C., Butcher, G. W., & Howard, J. C. (1977) *Nature 266*, 550–552.

Holmes, D. S., & Quigley, M. (1981) *Anal. Biochem. 114*, 193–197.

Inaoka, T., Ishida, M., & Ohtsuka, E. (1989) *J. Biol. Chem. 264*, 2609–2614.

Kim, J., & Linn, S. (1988) *Nucleic Acids Res. 16*, 1135–1141.

Lloyd, R. S., & Hanawalt, P. C. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2796–2800.

Monoharan, M., Mazamder, A., Ransom, S. C., Gerlt, J. A., & Bolton, P. H. (1988) *J. Am. Chem. Soc. 110*, 2690-2691.
Morikawa, K., Tsujimoto, M., Ikehara, M., Inaoka, T., & Ohtsuka, E. (1988) *J. Mol. Biol. 202*, 683-684.
Nakabeppu, Y., & Sekiguchi, M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2742-2746.
Nakabeppu, Y., Yamashita, K., & Sekiguchi, M. (1982) *J. Biol. Chem. 257*, 2556-2562.
Nakane, M., & Deguchi, T. (1982) *FEBS Lett. 140*, 89-92.
Pierre, J., & Laval, J. (1981) *J. Biol. Chem. 256*, 10217-10220.
Radany, E. H., Naumovski, L., Love, D. L., Gutekunst, K. A., Hall, D. H., & Friedberg, E. C. (1984) *J. Virol. 51*, 846-856.
Recinos, A., III, & Lloyd, R. S. (1988) *Biochemistry 27*, 1832-1838.
Recinos, A., III, Augustine, M. L., & Higgins, K. M. (1986) *J. Bacteriol. 168*, 1014-1018.
Sancar, A., & Sancar, G. B. (1988) *Annu. Rev. Biochem. 57*, 29-67.
Sanger, F., Nicklen, S., & Coulson, A. F. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-6457.
Seawell, P. C., Smith, C. A., & Ganesan, A. K. (1980) *J. Virol. 35*, 790-797.
Stump, D. G., & Lloyd, R. S. (1988) *Biochemistry 27*, 1839-1843.
Valerie, K.; Henderson, E. E., & deRiel, J. K. (1984) *Nucleic Acids Res. 12*, 8085-8096.
Valerie, K., Henderson, E. E., & deRiel, J. K. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4763-4767.
Yasuda, S., & Sekiguchi, M. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1839-1845.

Importance of Residues Arg-167 and Gln-231 in both the Allosteric and Catalytic Mechanisms of *Escherichia coli* Aspartate Transcarbamoylase†

Jeffrey W. Stebbins, Yang Zhang, and Evan R. Kantrowitz*
Department of Chemistry, Boston College, Chestnut Hill, Massachusetts 02167
Received November 8, 1989; Revised Manuscript Received December 26, 1989

ABSTRACT: Site-specific mutagenesis has been used to create two mutant versions of aspartate transcarbamoylase. Arg-167 and Gln-231, both previously identified as interacting with the portion of the bisubstrate analogue *N*-(phosphonoacetyl)-L-aspartate (PALA) that corresponds to aspartate [Krause, K. L., Voltz, K. W., & Lipscomb, W. N. (1987) *J. Mol. Biol. 193*, 527-553], were replaced by glutamine and leucine, respectively. The Arg-167 → Gln and the Gln-231 → Leu enzymes show approximately 900-fold and 1500-fold reductions in the maximal observed specific activity, respectively. The aspartate concentration at half the maximal observed specific activity is increased 18-fold for the Gln-231 → Leu enzyme compared to the value for the wild-type enzyme, but is altered little in the case of the Arg-167 → Gln enzyme. The carbamoyl phosphate concentration at half the maximal activity is unchanged by either mutation, suggesting that these mutations result in only local changes to the aspartate binding site. Both mutations eliminate homotropic cooperativity; however, the Gln-231 → Leu enzyme also has altered heterotropic interactions and no longer exhibits substrate inhibition. At relatively low concentrations of aspartate and saturating carbamoyl phosphate, PALA is able to activate the Gln-231 → Leu enzyme, whereas the Arg-167 → Gln enzyme is inhibited at PALA concentrations that normally activate the wild-type enzyme. Equilibrium binding measurements indicate that the Gln-231 → Leu enzyme binds CTP approximately 10-fold more weakly than the wild-type enzyme, even though the mutation is some 70 Å from the regulatory binding site. The binding of carbamoyl phosphate, PALA, and succinate to the Gln-231 → Leu catalytic subunit indicates poor binding for PALA and succinate but normal binding for carbamoyl phosphate. For the Arg-167 → Gln enzyme, there is very little alteration in the binding of these substrates and substrate analogues as compared to the wild-type enzyme. These results indicate that Gln-231 in the wild-type enzyme is critical for the binding of aspartate both in productive and in nonproductive orientations. Gln-231 through its interactions with aspartate and the side chain of Arg-234 is important for both homotropic cooperativity and heterotropic interactions. The function of Gln-231 and Arg-234 in the allosteric and catalytic mechanisms is also discussed.

Escherichia coli aspartate transcarbamoylase (EC 2.1.3.2) catalyzes the committed step of the pyrimidine biosynthesis pathway, the formation of *N*-carbamoyl-L-aspartate from carbamoyl phosphate and L-aspartate. The enzyme exhibits positive cooperativity for both substrates (Bethell et al., 1968; Gerhart & Pardee, 1962), and its activity is feedback-inhibited by CTP and UTP, the end products of the pyrimidine biosynthesis pathway, and is activated by ATP, an end product of the purine biosynthesis pathway (Gerhart & Pardee, 1962; Wild et al., 1989).

The holoenzyme[1] is composed of six catalytic chains (M_r 33 000), which are grouped into two catalytic trimers (i.e., catalytic subunits), and six regulatory chains (M_r 17 000),

†This work was supported by Grants DK1429 and GM26237 from the National Institutes of Health.
* To whom correspondence should be addressed.

[1] Abbreviations: PALA, *N*-(phosphonoacetyl)-L-aspartate; CP, carbamoyl phosphate; T and R states, tight and relaxed states of the enzyme having low and high affinity, respectively, for the substrate; $[S]_{0.5}^{Asp}$, aspartate concentration at half the maximal observed specific activity; $[S]_{0.5}^{CP}$, carbamoyl phosphate concentration at half the maximal observed specific activity; holoenzyme, entire aspartate transcarbamoylase molecule composed of two catalytic subunits and three regulatory subunits.

0006-2960/90/0429-3821$02.50/0