J. Biochem. 125, 109-114 (1999)

Effects of C-Terminal Deletion on the Activity and Thermostability of Orotate Phosphoribosyltransferase from *Thermus thermophilus*

Hiroshi Hamana and Takao Shinozawa[1]

Department of Biological and Chemical Engineering, Faculty of Engineering, Gunma University, Kiryu, Gunma 376-8515

Received July 15, 1998; accepted September 30, 1998

To investigate the role of the C-terminal region on the activity and thermostability of orotate phosphoribosyltransferase (OPRTase, EC 2.4.2.10) from *Thermus thermophilus*, four C-terminal amino acid-deleted OPRTases (1, 2, 3, and 5 residues deleted) were constructed. The activities of all the mutant OPRTases were lower than that of wild-type OPRTase at all temperatures investigated (50-80°C). V- and EV-OPRTase, mutants with Val and Glu-Val deletions, respectively, showed 63 to 75% of the activity of wild-type OPRTase at the temperatures investigated. EEV- and PLEEV-OPRTase, with Glu-Glu-Val and Pro-Leu-Glu-Glu-Val deletions, respectively, had activities of 22 to 35% of the wild-type. The K_m values for orotate of all mutant OPRTases were more than 4-fold higher than that of the wild-type (25 μM). On the other hand, the K_m for PRPP of the wild-type was 34 μM, and there were no significant differences between the wild-type and mutant OPRTases. The k_{cat} values of the V- and EV-OPRTases were similar to that of the wild-type, but those of the EEV- and PLEEV-OPRTases were less than 50% that of the wild-type. The optimum temperature of all mutant OPRTases, 70°C, was 10°C lower than that of the wild-type. The remaining activities of wild-type and V-OPRTase after incubation at 90°C for 20 min were 70 and 60% of the non-treated OPRTase activity, respectively. Although the remaining activity of EV-OPRTase was only 14% of the non-treated OPRTase activity, the addition of 200 mM KCl during heat treatment increased it to 70%. Circular dichroism spectroscopy revealed that V- and EV-OPRTase denature more easily than the wild-type OPRTase. The results suggest that the C-terminal valine and glutamic acid residues are important for the activity and thermostability of *T. thermophilus* OPRTase.

Key words: carboxyterminal deletion, mutant enzyme, orotate phosphoribosyltransferase, protein thermostability, *Thermus thermophilus*.

An understanding of the thermostability of thermophilic bacterial proteins can lead to improvements in the stability of enzymes for industry and medicine. Additionally, knowledge obtained from studies of thermostable proteins can provide insight into the general mechanism of protein folding and stabilization. There are two main approaches to finding factors involved in protein thermostability. One is to compare of amino acid composition, hydrogen bonds, salt bridges, tertiary structure, *etc.* between thermophilic proteins and their mesophilic counterparts (*1-4*). The other is to introduce mutations into the protein and investigate their effects on the thermostability (*5-7*).

Orotate phosphoribosyltransferase (OPRTase, EC 2.4.2.10) plays an essential role in the *de novo* biosynthesis of pyrimidine nucleotides. It catalyzes the formation of the nucleotide orotidine monophosphate from α-D-5-phosphoribosyl-1-pyrophosphate (PRPP) and orotic acid. Homologous enzymes have been identified in bacteria (*8-10*), fungi (*11-13*), insects (*14*), plants (*15*), and mammals (*16, 17*). OPRTases from bacteria and fungi have M_rs between 20,000 and 24,000. The working form of bacterial OPRTase is a dimer comprising two identical subunits that has been reported to operate by a random sequential kinetic mechanism (*18*). The counterpart of OPRTase in higher organisms, such as insects, plants, and mammals, is uridine-5'-monophosphate synthase (UMP synthase). UMP synthases have M_rs of about 50,000 and are bifunctional proteins comprising an OPRTase domain and an orotidylate decarboxylase domain (*19*).

OPRTase from *Thermus thermophilus* HB27 has been overexpressed in *Escherichia coli* and characterized. This OPRTase is a dimer consisting of two identical subunits of M_r 20,130. It shows high thermostability with an optimum temperature at 75°C and is stable for 20 min at 85°C (*20*).

The tertiary structures of the OPRTases in the meso-

[1] To whom correspondence should be addressed. Phone: +81-277-30-1431, Fax: +81-277-30-1431, E-mail: shinozawa@bce.gunma-u.ac.jp

Abbreviations: DMSO, dimethyl sulfoxide; EEV-OPRTase, OPRTase with Glu, Glu, and Val deleted at C-terminus; EV-OPRTase, OPRTase with Glu and Val deleted at C-terminus; IPTG, isopropyl-β-D-thiogalactopyranoside; OPRTase, orotate phosphoribosyltransferase; PLEEV-OPRTase, OPRTase with Pro, Leu, Glu, Glu, and Val deleted at C-terminus; PRPP, α-D-5-phosphoribosyl-1-pyrophosphate; *pyrE* gene, the gene of OPRTase; UMP synthase, uridine-5'-monophosphate synthase; V-OPRTase, OPRTase with Val deleted at C-terminus.

philic bacteria *Salmonella typhimurium* (*21, 22*) and *Escherichia coli* (*23*) have been determined. To compare the tertiary structures of *T. thermophilus* and mesophilic bacterial OPRTases, *T. thermophilus* OPRTase has been crystallized (*24*). However the tertiary structure of the enzyme has not been determined by the molecular replacement method. Because the amino acid sequence of *T. thermophilus* OPRTase is 28 residues shorter than those of the *E. coli* and *S. typhimurium* proteins, and the amino acid sequence of *T. thermophilus* OPRTase shows less than 30% identity to the other OPRTases, structural analysis by heavy atom isomorphous replacement is now underway.

To find factors involved in the thermostability of *T. thermophilus* OPRTase, a protein engineering approach was attempted. Based on the crystal structures of *S. typhimurium* and *E. coli* OPRTases, it appears that the N-terminal and C-terminal regions are not involved in substrate binding and dimer formation (*21-23*). To confirm this point in *T. thermophilus* OPRTase, we attempted to construct an N-terminal deletion mutant and overexpress it in *E. coli*. However, protein expression was not observed. On the other hand, the five C-terminal amino acid-deleted OPRTase was constructed and showed unexpected decreases in activity and optimum temperature. To investigate the role of the C-terminal region, a series of C-terminal-deleted OPRTases was constructed and their thermostabilities and activities were investigated. The results show the importance of the C-terminal amino acids in both activity and thermostability.

MATERIALS AND METHODS

Materials—AmpliTaq® DNA polymerase was a product of Perkin Elmer (Foster City, CA). *Xba*I and *Hin*dIII were obtained from New England Biolabs (Beverly, MA). The DNA Ligation Kit ver.1 was from Takara (Shiga). Thermo Sequenase™ was a product of Amersham Life Science (Buckinghamshire, England). Blue Sepharose CL-6B was purchased from Pharmacia Biotech (Uppsala, Sweden). High Q (anion exchange resin) was a product of Bio-Rad (Hercules, CA). TSKgel Toyopearl HW-50F was obtained from Tosoh (Tokyo). PRPP and sodium orotate were purchased from Sigma (St. Louis, MO). DMSO, Ampicillin, and IPTG were from Wako Pure Chemicals (Osaka). *E. coli* strain XL1-Blue was a laboratory stock.

Construction of C-Terminal Deletion Enzymes—A plasmid pTD-tac(N)/*pyr E*(PCR) has been constructed for the overexpression of *T. thermophilus* OPRTase in *E. coli* (*20*). C-terminal deleted OPRTases were constructed by PCR using this plasmid and primers containing a stop codon and a *Hin*dIII restriction site (Fig. 1). PCR amplification was performed with AmpliTaq DNA polymerase in the presence of 10% DMSO on an Air Thermo-Cycler Model 1605 (Idaho Technology). The conditions for PCR were an initial denaturation step at 98°C for 15 s, followed by 40 cycles of 98°C for 10 s, 50°C for 10 s, 70°C for 20 s, and a final extension step at 70°C for 1 min. The PCR product was digested with *Xba*I and *Hin*dIII, then ligated into the plasmid pTD-tac(N)/*pyr E*(PCR) previously digested with *Xba*I and *Hin*dIII, using the DNA Ligation Kit. The plasmids obtained were named pTD-tac(N)/C-del *pyr E*(PCR). To confirm the deletion of the C-terminal coding sequence and the absence of any other mutations, DNA sequencing was performed with Thermo Sequenase and a DSQ-1000 DNA Sequencer (Shimadzu). Plasmids containing the mutant OPRTase genes were transformed into *E. coli* XL1-Blue.

Purification of OPRTase—Wild-type and all mutant OPRTases were purified by the same method, and all column steps were carried out at room temperature. Two liters of 2×YT containing 50 μg/ml ampicillin and 50 ml of an overnight culture of transformed *E. coli* XL1-Blue was incubated at 37°C for 5-6 h with shaking. Overexpression of OPRTase was induced by adding 1 mM IPTG, and incubation was continued at 37°C for 18 h without shaking. The harvested cells were suspended in 50 ml of Buffer A (20 mM Tris-HCl, pH 8.0) and disrupted by 30 cycles of 10 s bursts of sonication with 10 s intervals between each burst at 4°C using an Ultrasonic Disruptor (Model UR-200P, Tomy Seiko, Tokyo). The cell lysate was then centrifuged at 10,000×*g* for 30 min at 4°C. The supernatant was applied to a Blue Sepharose CL-6B column (1 cmφ×7 cm) equilibrated with Buffer A. Proteins were eluted with a linear gradient of 0-0.5 M KCl in Buffer A at a flow rate of 0.5 ml/min, and 2-ml fractions were collected. Fractions containing OPRTase were identified by SDS-PAGE and pooled. The pooled fractions were diluted 3-fold with Buffer A and applied to a High Q column (1 cmφ×2 cm) equilibrated with Buffer A. Proteins were eluted with a linear gradient of 0-0.5 M KCl in Buffer A at a flow rate of 1 ml/min, and 1-ml fractions were collected. OPRTase-containing fractions were pooled, and solid ammonium sulfate was added to 40% saturation on ice. After centrifugation at 10,000×*g* for 15 min at 4°C, the pellet was suspended in 1 ml Buffer A with 0.1 M KCl. Insoluble matter was removed by centrifugation and the

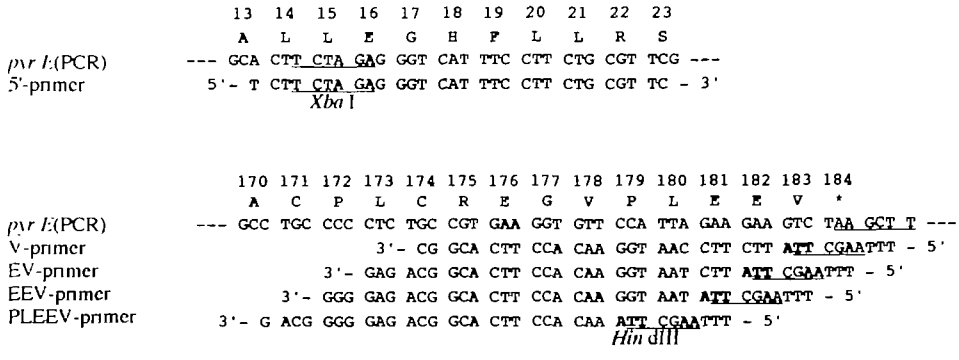

Fig. 1. **PCR primers used for the construction of mutant OPRTases.** The partial amino acid and nucleotide sequences of wild-type OPRTase are labeled *pyr E*(PCR). Numbers above the amino acids indicate position from the N-terminus.

supernatant was applied to a TSKgel Toyopearl HW-50F column (1.5 cmϕ × 45 cm) equilibrated with Buffer A containing 0.1 M KCl. Proteins were eluted with the same buffer at a flow rate of 0.125 ml/min, and 1-ml fractions were collected. OPRTase fractions were pooled and dialyzed overnight against Buffer A. The concentration of purified OPRTase was determined by the micro biuret method (*25*) using BSA as a standard. The purified protein was stored at 4°C.

SDS-PAGE—Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 15% polyacrylamide resolving gel and 5% polyacrylamide stacking gel was carried out for the analysis of protein composition as described by Laemmli (*26*). The solubilizing solution for the proteins contained 7% SDS, 70 mM DTT, 20 mM EDTA, pH 7.0, 5% glycerol, and 0.05% bromophenol blue. The electrophoresed proteins were stained with Coomassie Brilliant Blue R-250. Scanning of the stained proteins in the gel was carried out with a color image scanner JX-250 (Sharp). The purity of each OPRTase was calculated with a Macintosh computer using the NIH image program (developed at the U.S. National Institutes of Health and available on the Internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, Virginia, part number PB95-500195GEI).

Assay of OPRTase Activity—OPRTase activity was measured spectrophotometrically at 50-80°C. The assay solution (1 ml) contained 200 μM PRPP, 300 μM sodium orotate, 2 mM $MgCl_2$, 150 mM KCl, 20 mM Tris-HCl, pH 8.0 (adjusted at 50-80°C), and 2 μg of protein. The decrease in orotate was monitored at 295 nm in a spectrophotometer (Hitachi U-2000) with a thermoelectric cell holder as previously described (*20*). The activity was calculated from the change in absorbance of orotate during the first 5 s. One unit of enzyme activity was defined as the amount of OPRTase required to convert 1 μmol of orotate to OMP per minute.

Determination of Kinetic Constants—The kinetic constants of OPRTase were determined at 70°C. The kinetic constants for orotate were measured in 1-ml assay solutions containing 20, 40, 80, 150, or 300 μM orotate, 200 μM PRPP, 2 mM $MgCl_2$, 150 mM KCl, 20 mM Tris-HCl, pH 8.0, and 2 μg of protein. The kinetic constants for PRPP were measured under the same conditions except for the substrate concentrations (300 μM orotate and 10, 20, 50, 100, or 200 μM PRPP).

CD Spectroscopy—The ellipticities of the wild-type, V-, and EV-OPRTases were measured at 226 nm with a temperature gradient on a Jasco J-720W spectropolarimeter. Protein concentration was set to 300 μg/ml in 20 mM Tris-HCl, pH 8.0. Spectra were recorded in a 0.1 cm cuvette with a 1 nm slit width. The temperature of the sample was raised at a constant rate of 1°C per 6 min in the range from 80 to 100°C.

RESULTS AND DISCUSSION

Construction of Mutant OPRTase—The *T. thermophilus* OPRTase gene has a high G+C content (about 70%). In a previous study involving the overexpression of this gene in *E. coli*, the N-terminal amino acid coding sequence (Asp 2-Leu 19) was replaced by an A+T-rich sequence with major codons for *E. coli* (*20*). We attempted to construct an N-terminal-deleted OPRTase by removing this region (Asp 2-Leu 12), but no overexpression of the protein was detected. This suggested that the N-terminal coding sequence is important for expression. On the other hand, C-terminal deletions did not affect overexpression in *E. coli*. The nucleotide sequences of the C-terminal-deleted OPRTases were analysed, and the deletion of the C-terminal coding sequence and the absence of any other mutations were confirmed.

Purification of OPRTase—The wild-type and all C-terminal-deleted OPRTases were purified as described in "MATERIALS AND METHODS." All C-terminal-deleted OPRTases showed chromatographic behavior similar to wild-type OPRTase in Blue Sepharose CL-6B, High-Q, and TSKgel Toyopearl HW-50F. This suggests that the properties of the mutant OPRTases at room temperature do not differ so much from those of wild-type OPRTase in terms of affinity for the Blue Sepharose CL-6B ligand, pI, or dimer form. The purity of each OPRTase was analyzed by SDS-PAGE (Fig. 2) and calculated with an NIH image program. Each OPRTase was at least 92% pure. In each case, about 3-4 mg of OPRTase was obtained from 2 liters of *E. coli* culture.

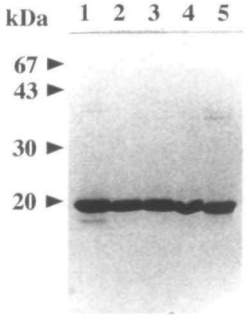

Fig. 2. **SDS-PAGE analysis of purified OPRTases.** The purity of each OPRTase was analyzed by electrophoresis in a 15% polyacrylamide gel. Lanes 1-5: 10 μg each of wild-type, V-, EV-, EEV-, and PLEEV-OPRTase, respectively.

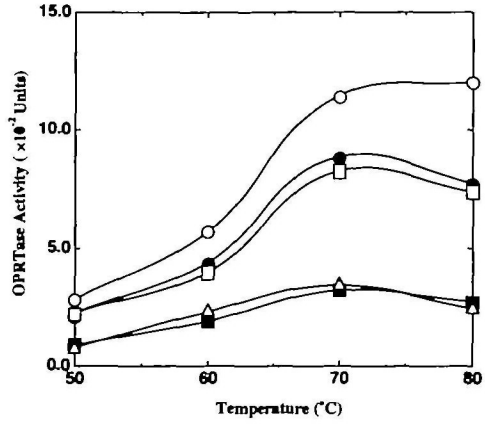

Fig. 3. **Temperature-dependency of the OPRTase activity of wild-type and mutant OPRTases.** Enzyme activity was measured as described in "MATERIALS AND METHODS." Wild-type (○), V-(●), EV-(□), EEV-(■), and PLEEV-OPRTase (△).

OPRTase Activities at Various Temperatures—OPRTase activity was measured at 50-80°C (Fig. 3). The activity of each mutant OPRTase was lower than that of the wild-type at the investigated temperatures. V- and EV-OPRTases showed 63 to 75% of the activity of wild-type OPRTase at 50-80°C, while the EEV- and PLEEV-OPRTases showed much lower activities of 22 to 35%. The maximum activity was observed at 80°C for the wild-type OPRTase but at 70°C for all mutant OPRTases. This decrease in the optimum temperature suggests that the mutant OPRTases have lower thermostabilities than the wild-type OPRTase.

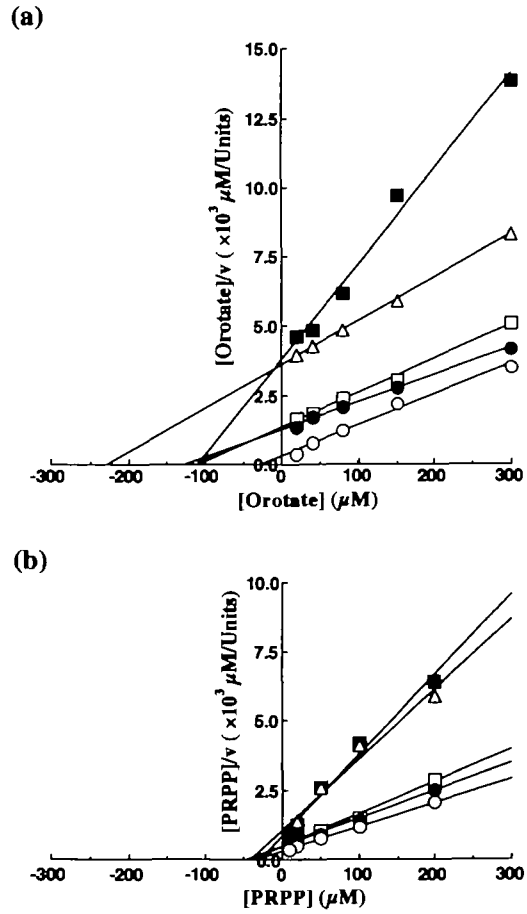

Fig. 4. **Kinetic analysis of wild-type and mutant OPRTases.** (a) [S]/v-[S] plot of initial velocity and orotate concentration at 200 μM PRPP. (b) [S]/v-[S] plot of initial velocity and PRPP concentration at 300 μM orotate. Initial velocity was measured as described in "MATERIALS AND METHODS." Wild-type (○), V-(●), EV-(□), EEV-(■), and PLEEV-OPRTase (△).

TABLE I. **Kinetic constants for Orotate and PRPP at 70°C.**

OPRTase	Substrate					
	Orotate			PRPP		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)
Wild-type	25	16.6	6.64×10^5	34	16.6	4.88×10^5
V-	124	16.3	1.31×10^5	44	16.3	3.70×10^5
EV-	107	13.3	1.24×10^5	39	13.3	3.41×10^5
EEV-	108	5.0	0.46×10^5	28	5.0	1.79×10^5
PLEEV-	227	8.0	0.35×10^5	41	8.0	1.95×10^5

Kinetic Constants of OPRTase for Orotate and PRPP—To investigate why these OPRTases show lower activities, the kinetic constants were determined. The initial velocity of each OPRTase assay was measured at 70°C and used to calculate the kinetic constants (Fig. 4, Table I). The K_m values of all mutant OPRTases for orotate were more than 4-fold higher than that of the wild-type (25 μM). On the other hand, the K_m for PRPP of the wild-type OPRTase was 34 μM, and there were no significant differences between the wild-type and mutant OPRTases. In contrast, the k_{cat} values of V- and EV-OPRTases for both orotate and PRPP were similar to that of wild-type OPRTase, while those of EEV- and PLEEV-OPRTases were less than 50% of the wild-type. These results suggest that Val and Glu-Val at the C-terminus are important for orotate binding to

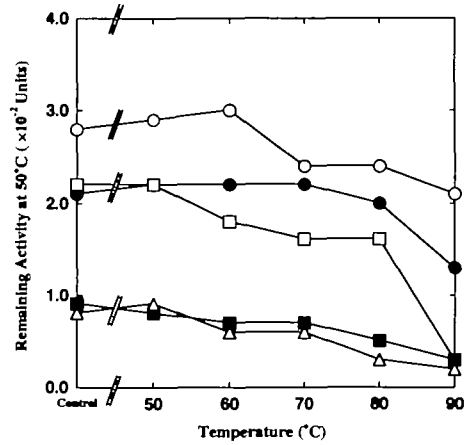

Fig. 5. **Thermostability of wild-type and mutant OPRTases at various temperatures.** Solutions of 100 μg/ml OPRTase were incubated at the indicated temperatures for 20 min in 20 mM Tris-HCl, pH 8.0, and enzyme activity was measured at 50°C. Remaining activities of wild-type (○), V-(●), EV-(□), EEV-(■), and PLEEV-OPRTase (△) are indicated.

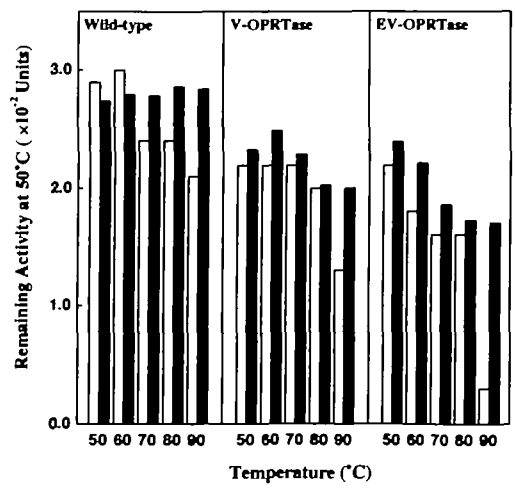

Fig. 6. **Effect of KCl on the thermostability of wild-type and mutant OPRTases at various temperatures.** OPRTases were incubated at various temperatures for 20 min in the absence ( ) or presence (▨) of 200 mM KCl, and the remaining activity was measured at 50°C.

OPRTase and enzyme activity, respectively, and the deletion of a few amino acids from the C-terminus caused structural changes in the active site.

Thermostability of OPRTase—The effects of C-terminal deletions on thermostability were investigated. After incubation at 50-90°C for 20 min, OPRTase activity was measured at 50°C (Fig. 5). The remaining activities of wild-type and V-OPRTase after incubation at 90°C were 70 and 60% of those of the respective non-heated OPRTases. In the case of EV-OPRTase, the amount of activity remaining relative to that of the non-heated EV-OPRTase was 70% after incubation at 80°C, but only 14% after incubation at 90°C. EEV- and PLEEV-OPRTases had about 30% activity relative to wild-type OPRTase and the remaining activity decreased gradually with increasing incubation temperature. As above, the deletion of Glu and Val from the C-terminus greatly reduced thermostability during 20 min of incubation at 90°C. Glu and Val have been reported to play important roles in the stabilization of some proteins through electrostatic interactions (salt bridges, hydrogen bonds) (*27-30*) and hydrophobic interactions (*31, 32*), respectively. Similarly, these amino acid residues at the C-terminus probably contribute to the stabilization of *T. thermophilus* OPRTase.

An earlier study (*20*) revealed that KCl enhances the activity of *T. thermophilus* OPRTase, and thus the effect of KCl on the thermostability of the wild-type, V-, and EV-OPRTases was investigated (Fig. 6). Increases in the stability were observed in all three OPRTases at high temperatures. The effect on EV-OPRTase was particularly large. The remaining activity after incubation at 90°C increased from 14% (absence of KCl) to 70% (presence of KCl) of that of the non-heated EV-OPRTase. These results indicate that the deletion of C-terminal amino acids probably led to the decrease in the hydrophobic effect involved in protein folding.

Thermal Denaturation—Protein unfolding of the wild-type, V-, and EV-OPRTases was monitored by the changes in alpha-helical ellipticity at 226 nm (Fig. 7). At temperatures from 80°C to 90°C, there were no changes in the ellipticities of these OPRTases (data are not shown). In the case of wild-type OPRTase, the ellipticity increased slowly in the range from 92 to 100°C, and the ellipticity at 100°C was about −5 mdeg. In the case of EV-OPRTase, a rapid increase in ellipticity started at 92°C and reached −1.0 mdeg at 95°C. The ellipticity of V-OPRTase increased slowly in the range from 92 to 96°C, then increased rapidly from 96°C, reaching about −1.0 mdeg at 100°C. This result also suggests that Glu and Val at the C-terminus are involved in protein folding at high temperatures.

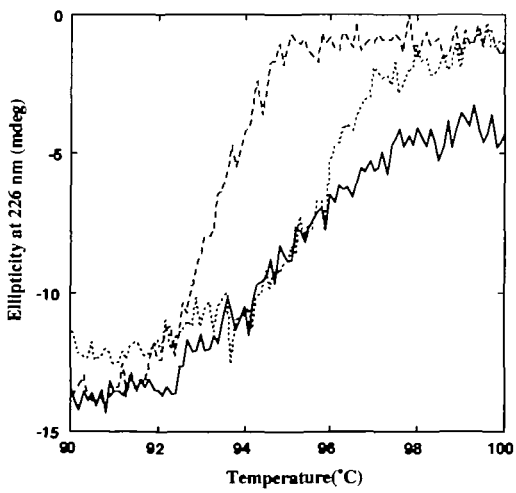

Fig. 7. **Thermal denaturation as monitored by CD spectroscopy.** Ellipticities at 226 nm of three enzyme types (wild-type ——, V- ····, and EV-OPRTase ---) were measured during the increase in temperature from 90°C to 100°C at a rate of 1°C per 6 min.

In this study, we analyzed the contribution of C-terminal amino acids to the activity and thermostability of *T. thermophilus* OPRTase by constructing OPRTase mutants with amino acid deletions. In conclusion, Val and Glu-Val are important for orotate binding and for activity and thermostability, respectively. The determination of the tertiary structure will help to clarify the role of these amino acids in the activity and thermostability of the enzyme. X-ray crystallography of wild-type OPRTase is now in progress.

We are grateful to Dr. R. Katakai and Mr. T. Ooyama for cooperation with CD spectroscopy.

REFERENCES

1. Argos, P., Rossman, M.G., Grau, U.M., Zuber, H., Frank, G., and Tratschin, J.D. (1979) Thermal stability and protein structure. *Biochemistry* **18**, 5698-5703
2. Vogt, G. and Argos, P. (1997) Protein thermal stability: hydrogen bonds or internal packing? *Fold. Des.* **2**, S40-S46
3. Schäfer, T., Bönisch, H., Kardinahl, S., Schmidt, C., and Schäfer, G. (1996) Three extremely thermostable proteins from *Sulfolobus* and a reappraisal of the 'traffic rules.' *Biol. Chem.* **377**, 505-512
4. Querol, E., Perez, P.J., and Mozo, V.A. (1996) Analysis of protein conformational characteristics related to thermostability. *Protein Eng.* **9**, 265-271
5. Akanuma, S., Qu, C., Yamagishi, A., Tanaka, N., and Oshima, T. (1997) Effect of polar side chains at position 172 on thermal stability of 3-isopropylmalate dehydrogenase from *Thermus thermophilus*. *FEBS Lett.* **410**, 141-144
6. Nicholson, H., Tronrud, D.E., Becktel, W.J., and Matthews, B.W. (1992) Analysis of the effectiveness of proline substitutions and glycine replacements in increasing the stability of phage T4 lysozyme. *Biopolymers* **32**, 1431-1441
7. Ishikawa, K., Kimura, S., Kanaya, S., Morikawa, K., and Nakamura, H. (1993) Structural study of mutants of *Escherichia coli* ribonuclease HI with enhanced thermostability. *Protein Eng.* **6**, 85-91
8. MacGregor, C.H., Arora, S.K., Hager, P.W., Dail, M.B., and Phibbs, P.J. (1996) The nucleotide sequence of the *Pseudomonas aeruginosa* pyrE-crc-rph region and the purification of the crc gene product. *J. Bacteriol.* **178**, 5627-5635
9. Ghim, S.Y., Nielsen, P., and Neuhard, J. (1994) Molecular characterization of pyrimidine biosynthesis genes from the thermophile *Bacillus caldolyticus*. *Microbiology* **140**, 479-491
10. Quinn, C.L., Stephenson, B.T., and Switzer, R.L. (1991) Functional organization and nucleotide sequence of the *Bacillus subtilis* pyrimidine biosynthetic operon. *J. Biol. Chem.* **266**, 9113-9127
11. Berges, T., Perrot, M., and Barreau, C. (1990) Nucleotide sequences of the *Trichoderma reesei* ura3 (OMPdecase) and ura5 (OPRTase) genes. *Nucleic Acids Res.* **18**, 7183
12. Edman, J.C. and Kwon-Chung, K.J. (1990) Isolation of the URA5 gene from *Cryptococcus neoformans* var. *neoformans* and its use as a selective marker for transformation. *Mol. Cell. Biol.* **10**, 4538-4544
13. Sánchez, M., Prado, M., Iglesias, F.J., and Domínguez, A. (1995)

Cloning and sequencing of the URA5 gene from the yeast *Yarrowia lipolytica*. *Yeast* **11**, 425-433
14. Eisenberg, M., Gathy, K., Vincent, T., and Rawls, J. (1990) Molecular cloning of the UMP synthase gene rudimentary-like from *Drosophila melanogaster*. *Mol. Gen. Genet.* **222**, 1-8
15. Nasr, F., Bertauche, N., Dufour, M.E., Minet, M., and Lacroute, F. (1994) Heterospecific cloning of *Arabidopsis thaliana* cDNAs by direct complementation of pyrimidine auxotrophic mutants of *Saccharomyces cerevisiae*. I. Cloning and sequence analysis of two cDNAs catalysing the second, fifth and sixth steps of the de novo pyrimidine biosynthesis pathway. *Mol. Gen. Genet.* **244**, 23-32
16. Ohmstede, C.A., Langdon, S.D., Chae, C.B., and Jones, M.E. (1986) Expression and sequence analysis of a cDNA encoding the orotidine-5′-monophosphate decarboxylase domain from Ehrlich ascites uridylate synthase. *J. Biol. Chem.* **261**, 4276-4282
17. Suchi, M., Harada, N., Tsuboi, T., Asai, K., Okajima, K., Wada, Y., and Takagi, Y. (1989) Molecular cloning of human UMP synthase. *Adv. Exp. Med. Biol.* **253**, 511-518
18. Bhatia, M.B., Vinitsky, A., and Grubmeyer, C. (1990) Kinetic mechanism of orotate phosphoribosyltransferase from *Salmonella typhimurium*. *Biochemistry* **29**, 10480-10487
19. McClard, R.W., Black, M.J., Livingstone, L.R., and Jones, M.E. (1980) Isolation and initial characterization of the single polypeptide that synthesizes uridine 5′-monophosphate from orotate in Ehrlich ascites carcinoma. Purification by tandem affinity chromatography of uridine-5′-monophosphate synthase. *Biochemistry* **19**, 4699-4706
20. Bunnak, J., Hamana, H., Ogino, Y., Saheki, T., Yamagishi, A., Oshima, T., Date, T., and Shinozawa, T. (1995) Orotate phosphoribosyltransferase from *Thermus thermophilus*: overexpression in *Escherichia coli*, purification and characterization. *J. Biochem.* **118**, 1261-1267
21. Scapin, G., Grubmeyer, C., and Sacchettini, J.C. (1994) Crystal structure of orotate phosphoribosyltransferase. *Biochemistry* **33**, 1287-1294
22. Scapin, G., Ozturk, D.H., Grubmeyer, C., and Sacchettini, J.C. (1995) The crystal structure of orotate phosphoribosyltransferase complexed with orotate and α-D-5-phosphoribosyl-1-pyrophosphate. *Biochemistry* **34**, 10744-10754
23. Henriksen, A., Aghajari, N., Jensen, K.F., and Gajhede, M. (1996) A flexible loop at the dimer interface is a part of the active site of the adjacent monomer of *Escherichia coli* orotate phosphoribosyltransferase. *Biochemistry* **35**, 3803-3809
24. Hamana, H., Moriyama, H., Shinozawa, T., and Tanaka, N. (1998) Medium temperature, 37°C, provides single crystals of orotate phosphoribosyltransferase from *Thermus thermophilus*. *Acta Crysta. D* (in press)
25. Itzhaki, R.F. and Gill, D.M. (1964) A micro-biuret method for estimating proteins. *Anal. Biochem.* **9**, 401-410
26. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
27. Day, M.W., Hsu, B.T., Joshua, T.L., Park, J.B., Zhou, Z.H., Adams, M.W., and Rees, D.C. (1992) X-ray crystal structures of the oxidized and reduced forms of the rubredoxin from the marine hyperthermophilic archaebacterium *Pyrococcus furiosus*. *Protein Sci.* **1**, 1494-1507
28. He, X.Y., Deng, H., and Yang, S.Y. (1997) Importance of the gamma-carboxyl group of glutamate-462 of the large alpha-subunit for the catalytic function and the stability of the multienzyme complex of fatty acid oxidation from *Escherichia coli*. *Biochemistry* **36**, 261-268
29. Kawamura, S., Tanaka, I., Yamasaki, N., and Kimura, M. (1997) Contribution of a salt bridge to the thermostability of DNA binding protein HU from *Bacillus stearothermophilus* determined by site-directed mutagenesis. *J. Biochem.* **121**, 448-455
30. Yaoi, T., Hayashi, I.Y., and Oshima, T. (1996) Electrostatic interaction between two domains of isocitrate dehydrogenase from *Thermus thermophilus* is important for the catalytic function and protein stability. *FEBS Lett.* **398**, 228-230
31. Kirino, H., Aoki, M., Aoshima, M., Hayashi, Y., Ohba, M., Yamagishi, A., Wakagi, T., and Oshima, T. (1994) Hydrophobic interaction at the subunit interface contributes to the thermostability of 3-isopropylmalate dehydrogenase from an extreme thermophile, *Thermus thermophilus*. *Eur. J. Biochem.* **220**, 275-281
32. Declerck, N., Joyet, P., Trosset, J.Y., Garnier, J., and Gaillardin, C. (1995) Hyperthermostable mutants of *Bacillus licheniformis* alpha-amylase: multiple amino acid replacements and molecular modelling. *Protein Eng.* **8**, 1029-1037