Structural and Functional Roles of the Cysteine Residues of *Bacillus stearothermophilus* Farnesyl Diphosphate Synthase†

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ABSTRACT: *p*-(Chloromercuri)benzoic acid inhibited the catalytic activity of *Bacillus stearothermophilus* farnesyl diphosphate synthase (FPP synthase), which contains only two cysteine residues at positions 73 and 289. In order to explore the role of the cysteine residues, either or both of them were replaced with phenylalanine or serine. Five mutant enzymes, C73F, C73S, C289F, C289S, and C73S-C289S, were overproduced in *Escherichia coli* and purified to homogeneity. All of them were active as farnesyl diphosphate synthase, showing specific activities comparable to that of the wild-type enzyme. These results indicate that neither of the cysteines is essential for catalytic function. The C73F mutant, however, was very sensitive to heat treatment, while C73S was as highly stable as the wild type. The K_m value of C289F for isopentenyl diphosphate is 10 times that of the wild type. The wild-type enzyme was converted into an oxidized form which was separable from the native enzyme on ion-exchange chromatography, and this conversion was accelerated by cupric ions. Similar conversion has previously been reported by several reseachers, who found the occurrence of two forms of pig liver FPP synthase and who attributed this phenomenon to the oxidoreduction of sulfhydryl and disulfide groups. However, even the C73S-C289S mutant, which has no cysteine residues, was also converted into an oxidized form as in the case of the wild-type enzyme. These results provide evidence that residues other than cysteine are involved in the conversion of this enzyme into the oxidized form.

Prenyltransferases are the enzymes that catalyze isoprenoid chain elongation by sequential condensation of isopentenyl diphosphate (IPP[1]) with allylic diphosphates to produce prenyl diphosphates (Poulter & Rilling, 1982). These enzymes are extremely interesting from a mechanistic viewpoint in that they catalyze the repetition of stereospecific condensation of IPP with allylic diphosphates to give prenyl diphosphates with chain lengths depending on the specificities of the individual enzymes (Ito et al., 1987; Ohnuma et al., 1991).

Farnesyl diphosphate synthase (EC 2.5.1.10), one of this class of enzymes, occupies a central branch point of isoprenoid biosynthetic pathways. It catalyzes the condensations of IPP with dimethylallyl diphosphate (DMAPP) and with geranyl diphosphate (GPP) to produce (*all-E*)-farnesyl diphosphate (FPP) as the ultimate reaction product (Poulter & Rilling, 1982). Recently, cDNAs or genomic clones encoding FPP synthase have been isolated from various organisms, and their nucleotide sequences have been determined and compared (Clarke et al., 1987; Anderson et al., 1989; Wilkin et al., 1990; Fujisaki et al., 1990; Koyama et al., 1993).

Meanwhile, it has been suggested that cysteine residues play important roles in the catalytic function or the substrate binding of several prenyltransferases (Holloway & Popják, 1967; Barnard & Popják, 1981; Yoshida et al., 1989). Moreover, there have been several reports on the multiple forms of FPP synthase from porcine liver (Koyama et al., 1977; Yeh & Rilling, 1977; Barnard et al., 1978) and of FPP/geranylgeranyl diphosphate synthase from *Methanobacterium thermoautotrophicum* (Chen & Poulter, 1993), all of which are attributed to oxidation–reduction behavior of cysteine residues of the enzymes.

We have shown that FPP synthase from *Bacillus stearothermophilus* is thermostable and unique in that it possesses only two cysteine residues in contrast to the other enzymes, which have four to six cysteines (Koyama et al., 1993). It is therefore advantageous to study the role of cysteine residues using this synthase. This paper describes the effect of mutation at the two cysteine residues in this thermophilic FPP synthase.

MATERIALS AND METHODS

Materials. [1-^{14}C]IPP (1.95 GBq/mol) was purchased from Amersham Corp. Nonlabeled IPP, DMAPP, and GPP were the same preparations as used in the previous work (Koyama et al., 1993). Restriction enzymes and other DNA-modifying enzymes were purchased from Takara Shuzo Co., Ltd., and Toyobo Co., Ltd., unless otherwise stated.

Site-Directed Mutagenesis. Site-directed mutagenesis was conducted as described in a previous paper (Koyama et al., 1994), and a series of mutated plasmids, pMu102 (C73F), pMu122 (C73S), pMu93 (C289F), and pMu119 (C289S), were obtained. The nucleotide sequences of the mutagenic oligonucleotides employed for introducing the mutations are shown in Figure 1. Introduction of the mutation was confirmed by sequencing the whole nucleotide sequence using the dideoxy chain-termination method (Sanger et al., 1977) with a DNA sequencer (Applied Biosystems, Model 373A).

Construction of Expression Vector Systems for FPP Synthase Mutants. The *Nco*I/*Hin*dIII fragments of the four

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[1] Abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, (*all-E*)-farnesyl diphosphate; bp, base pair(s); IPTG, isopropyl β-D-thiogalactopyranoside; PCMB, *p*-(chloromercuri)benzoic acid; EDTA, ethylenediamine-tetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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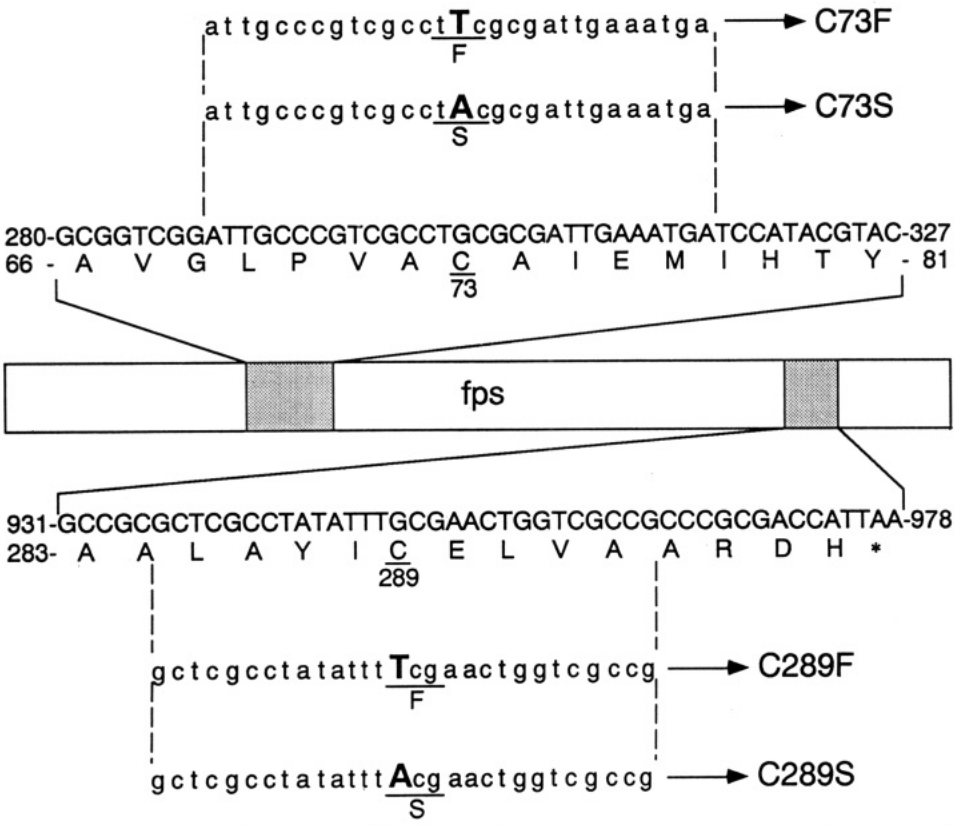

FIGURE 1: Schematic presentation of wild-type and mutated FPP synthase gene sequences around Cys-73 (upper) and Cys-289 (lower). The nucleotide sequences of the FPP synthase of *B. stearothermophilus* for residues 280–327 (upper) and 931–978 (lower) are shown along with the corresponding amino acid sequences. The sequences of mutagenic oligonucleotides are aligned above/below the wild-type sequence corresponding to the FPP synthase gene (fps). Nucleotides identical to those in the wild-type enzyme are indicated by lowercase letters, and altered nucleotides are indicated by capital letters. All mutant genes have been sequenced to verify point mutations. C73S-C289S is constructed by combining the mutated gene fragments corresponding to C73S and C289S.

kinds of pMu plasmids, pMu102, pMu122, pMu93, and pMu119, were ligated into the *Nco*I/*Hin*dIII site of pTrc99A (Pharmacia) to construct a series of recombinant plasmids, pEX140 (C73F), pEX145 (C289F), pEX162 (C73S), and pEX153 (C289S), respectively.

The construction of pEX165, which is for the expression of C73S-C289S, was carried out by ligation of the *Nco*I/*Nru*I fragment (668 bp) of pMu122 and the *Nru*I/*Hin*dIII fragment (229 bp) of pMu119 with the *Nco*I/*Hin*dIII site of pTrc99A.

Overproduction and Purification of FPP Synthase Mutants. The procedures employed for the overproduction and purification of FPP synthase mutants were essentially similar to those for the wild-type enzyme previously reported by us (Koyama et al., 1993). *Escherichia coli* JM105 was transformed with each of the expression plasmids, pEX140, pEX145, pEX162, pEX153, and pEX165. An overnight culture of each of the *E. coli* JM105 transformants harboring the pEX plasmids in L-B medium (Sambrook et al., 1989) containing 50 μg/mL of ampicillin was inoculated into a 250-fold volume of M9YG medium (Koyama et al., 1993) with 50 μg/mL ampicillin. The *E. coli* cells were cultured at 30 °C to an OD_{600} of 0.6–0.8, and then IPTG was added to a final concentration of 1 mM. The incubation was continued for an additional 3 h at 30 °C.

The mutated enzymes were purified essentially according to reported procedures (Koyama et al., 1993) including heat treatment followed by two chromatographic steps, except that the heat treatment was omitted for the purification procedure of C73F (see Results).

FPP Synthase Assay. The enzyme activity was measured by determination of the amount of [1-^{14}C]IPP incorporated into butanol-extractable polyprenyl diphosphates. In a standard experiment, the incubation mixture contained, in a final volume of 1.0 mL, 50 mM Tris-HCl buffer, pH 8.5, 50 mM $MgCl_2$, 50 mM NH_4Cl, 5 mM KF, 50 mM 2-mercaptoethanol, 25 μM DMAPP or GPP, 25 μM [1-^{14}C]IPP (sp. act., 37 GBq/mol), and a suitable amount of enzyme. After incubation at 55 °C for 15 min, the reaction products were immediately extracted with 1-butanol. The butanol layer was washed with water, and an aliquot of the extract was counted for radioactivity.

Product Analysis. After enzymatic reaction, the radioactive prenyl diphosphate produced in the reaction mixture was hydrolyzed to the corresponding alcohol with potato acid phosphatase (Fujii et al., 1982).

The hydrolysate was extracted with pentane and analyzed by reversed-phase thin-layer chromatography with LKC-18 (Whatman) in a solvent system of acetone/water (7:1). The positions of authentic standards were visualized with iodine vapor, and the distribution of radioactivity was detected.

SDS-PAGE Analysis. The enzyme samples were electrophoresed on a 14% polyacrylamide gel containing 0.1% SDS essentially according to the standard methods (Laemmli, 1970) and stained with Coomassie Brilliant Blue. Preparation of the enzyme samples for PAGE was carried out in two ways. (a) Reductive conditions: The sample solution contained 62.5 mM Tris-HCl buffer, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue. The solution was heated on a boiling water bath for 3 min. (b) Non-reductive conditions: A solution containing the same constituents as described above except that 2-mercaptoethanol was omitted was applied to gels without heating. Electrophoresis was carried out with a current of 3 mA per lane until the bromophenol blue marker nearly reached the bottom of the gel.

Table 1: Effect of PCMB on the Wild-Type and C73S-C289S Activities[a]

PCMB concn (μM)	% enzyme activity[b]	
	wild type	C73S-C289S
0	100	100
50	63.5	104.3
100	51.3	90.5
150	17.6	89.4

[a] Each enzyme (80 nmol) was incubated with the indicated concentrations of PCMB in 1.0 mL of 0.33 M acetate buffer, pH 5.6, at 37 °C for 60 min. Then the enzymatic activity of 50 μL of the resulting solution was assayed according to the procedure described in Materials and Methods except that 2-mercaptoethanol was omitted in the incubation mixture. Each value represents the mean of three determinations. [b] The activity in the absence of PCMB was taken as a standard value of 100%.

RESULTS

Effect of SH Reagent on FPP Synthase Activity. It has been shown that several prenyltransferases such as FPP synthases from pig liver (Holloway & Popják, 1967) and from human liver (Barnard & Popják, 1981) and hexaprenyl diphosphate synthase from *Micrococcus luteus* B-P 26 (Yoshida et al., 1989) are inactivated by some SH reagents. Table 1 shows the effect of *p*-(chloromercuri)benzoic acid (PCMB) on the activity of *B. stearothermophilus* FPP synthase (wild type). At high concentration ranges, the thiol-specific reagent inactivates the thermophilic enzyme.

Production of FPP Synthase Mutants. In order to explore the role of the cysteine residues, oligonucleotide-mediated site-directed mutagenesis studies were undertaken with this thermostable FPP synthase. Figure 1 shows the sequences around Cys-73 and Cys-289 of the wild-type enzyme and its mutated enzymes along with the sequences of mutagenic oligonucleotides.

The cysteine residue at position 73, which is fully conserved in region B of FPP synthases (Koyama et al., 1993), was changed to phenylalanine or serine to produce C73F or C73S, respectively. The other cysteine residue at position 289 was also replaced with phenylalanine or serine to produce C289F or C289S, respectively. A mutant enzyme (C73S-C289S) which has replacements of both the 73-Cys and the 289-Cys residue with serine was also made by connecting mutated gene fragments derived from C73S and C289S.

Overexpression and subsequent purification of the mutant enzymes were carried out as previously described (Koyama et al., 1993, 1994).

Heat Stability of FPP Synthase Mutants. We found that C73F lost most of its enzymatic activity after heat treatment at 55 °C for 60 min. However, as shown in Figure 2, only C73F is sensitive to heat treatment, while the other mutants, C73S, C289F, C289S, and C73S-C289S, are as stable as the wild-type enzyme. The thermostability curves of the wild-type and C73F enzymes indicate that the replacement of Cys-73 with phenylalanine resulted in decreases of more than 15 °C in the half-activity temperature, at which 50% of the enzyme activity is lost in 20 min (data not shown).

Kinetic Analysis of FPP Synthase Mutants. As described above, all of the five mutant enzymes showed prenyltransferase activity, indicating that neither of the cysteine residues at positions 73 and 289 is essential for catalytic function. Table 2 shows kinetic parameters of the purified mutants along with those of the wild-type enzyme. The mutant enzymes having replacements with serine, C73S, C289S, and C73S-C289S showed specific activities comparable to that of the wild-type enzyme.[2] However, the specific activities of the mutants having phenylalanine in place of cysteine, C73F and C289F,

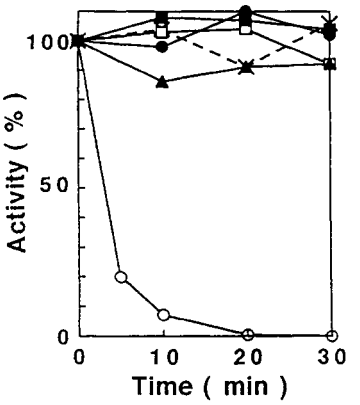

FIGURE 2: Heat stability of the FPP synthase mutants. Each of the mutant enzyme (5 μg/mL) was heated at 55 °C for an appropriate period, and then the remaining enzymatic activity was assayed as described in Materials and Methods., ●, wild type; ○, C73F; □, C73S; ×, C289F; ■, C289S; ▲, C73S-C289S. Each point represents the mean of three determinations.

Table 2: Kinetic Parameters of the Wild-Type and Mutant Enzymes[a]

enzyme	sp act.[c]	GPP		IPP[b]	
		K_m[d]	V_{max}[c]	K_m[d]	V_{max}[c]
45 °C[e]					
wild type	2610	5.8	4270	4.7	3410
C73F	1300	4.9	2650	4.8	2020
55 °C					
wild type	4690	8.4	7330	13.0	7200
C289F	350	7.5	2630	130	2760
C73S	4300	7.3	9810	11.6	7850
C289S	3940	6.4	9330	14.3	9170
C73S-C289S	3970	5.6	7810	13.3	7540

[a] Each value represents the mean of two determinations. [b] For the reaction with GPP. [c] Nanomoles of [1-^{14}C]IPP converted to FPP/min/(mg of enzyme). [d] μM. [e] As C73F lost its activity at 55 °C, kinetic data at 45 °C were obtained.

were approximately one-half and one-thirteenth of that of the wild-type enzyme, as assayed at 45 and 55 °C, respectively.

Although the K_m values of the mutant enzymes for GPP are all similar to that of the wild-type enzyme, the K_m value for IPP of C289F is 10 times that of the wild-type enzyme.[2]

Effect of pH. All of the mutant enzymes showed pH activity profiles similar to that of the wild-type enzyme (data not shown).

Reaction Product. All of the mutant enzymes catalyzed the synthesis of FPP exclusively (data not shown).

Two Forms of FPP Synthase. During the purification of C73S-C289S the enzymatic activity was found in two well-resolved fractions from a Mono Q chromatography column, one being eluted by 80 mM NaCl and the other by 100 mM NaCl (Figure 3A). Careful inspection revealed that the wild-type enzyme was also eluted similarly in two fractions on Mono Q chromatography especially when the wild-type enzyme was applied on the Mono Q column after being kept at 4 °C for several weeks (Figure 3B). Similar observations have been reported for pig liver FPP synthase (Koyama et al., 1977; Yeh & Rilling, 1977; Barnard et al., 1978) and geranylgeranyl diphosphate synthase of *M. thermoautotrophicum* (Chen & Poulter, 1993). These authors have attributed these phenomena to the oxidation–reduction of sulfhydryl groups of the enzymes. However, the present study indicates

[2] The kinetic parameters of the wild-type enzyme are different from those reported earlier (Koyama et al., 1994), because the previously estimated concentrations of the enzyme protein and of the allylic substrate, GPP, were found to be incorrect.

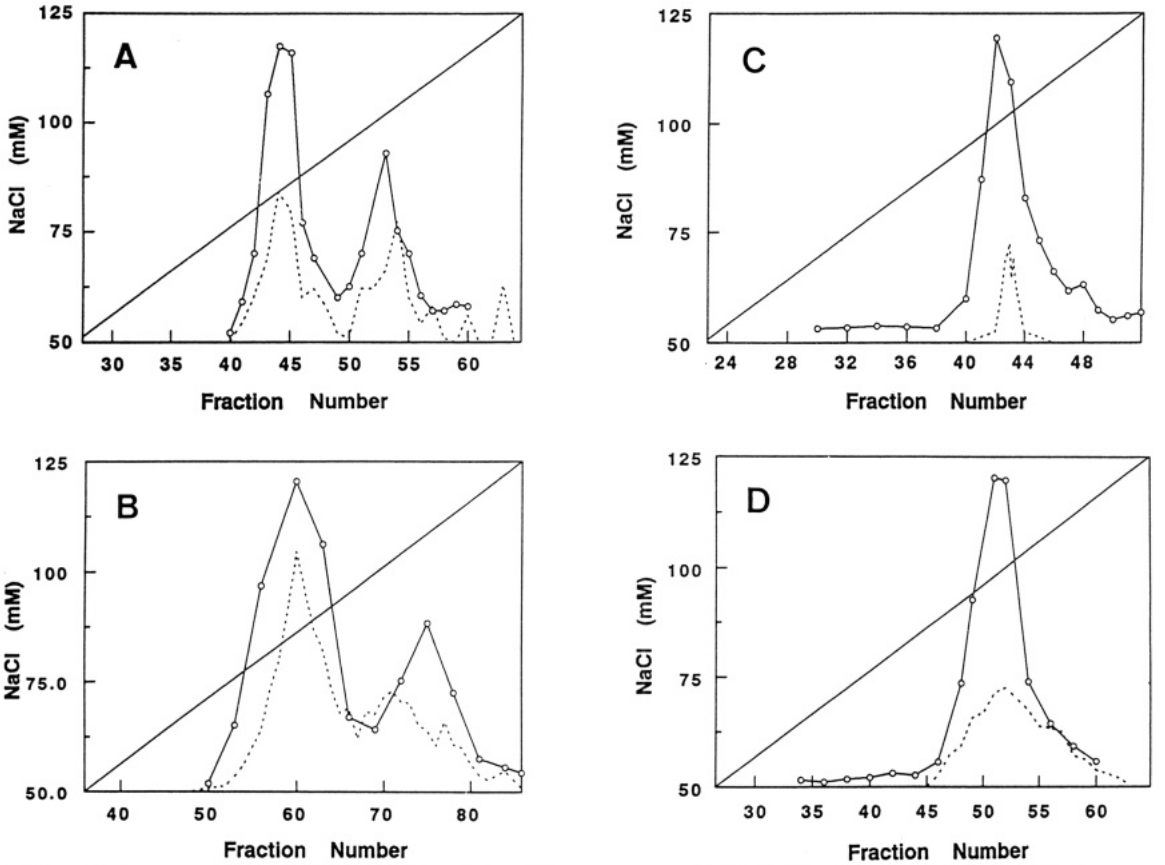

FIGURE 3: Mono Q chromatography of C73S-C289S and the wild-type enzyme. Buffer I, 25 mM Tris-HCl buffer, pH 7.7, containing 10 mM 2-mercaptoethanol and 1 mM EDTA; Buffer II, 0.5 M NaCl in buffer I; buffer III, 25 mM Tris-HCl buffer, pH 7.7; Elution: A 480-mL linear gradient from 0 to 125 mM NaCl at 1.0 mL/min was used: (A) C73S-C289S; (B) wild-type enzyme after it was kept at 4 °C for several weeks; C, C73S-C289S after dialysis against 100 μM $CuSO_4$ in buffer III; (D) wild-type enzyme after dialysis against 100 μM $CuSO_4$ in buffer III. (- - -) Absorbance at 280 nm; (-O-) FPP synthase activity; (—) NaCl gradient.

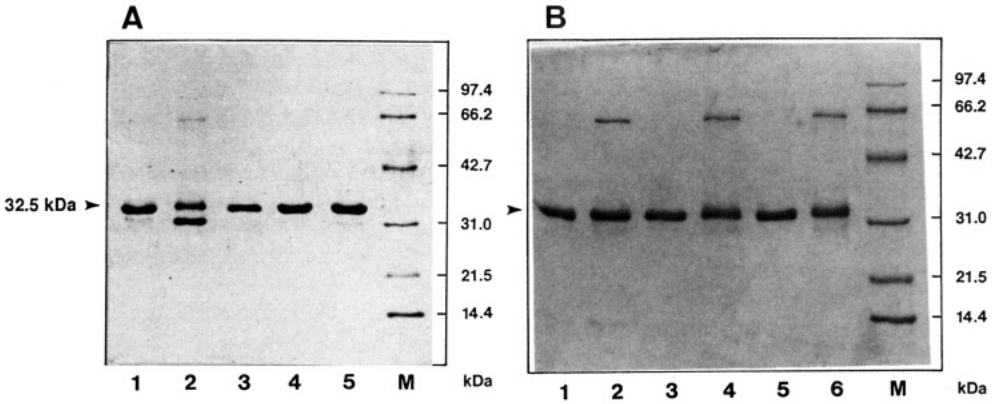

FIGURE 4: SDS–PAGE analyses of FPP synthase and its mutants treated under oxidative or reductive conditions. (A) Lane 1, wild-type enzyme after ordinary purification procedures; lane 2, wild-type enzyme dialyzed against 100 μM $CuSO_4$ in buffer III described in the caption to Figure 3; lane 3, wild-type enzyme subjected to Cu^{2+} treatment followed by dialysis against 10 mM 2-mercaptoethanol in buffer III; lane 4, C73S-C289S after ordinary purification; lane 5, C73S-C289S dialyzed against 100 μM $CuSO_4$ in buffer III. (B) Lane 1, C73S; lane 2, C73S dialyzed against 100 μM $CuSO_4$ in buffer III; lane 3, C289F; lane 4, C289F dialyzed against 100 μM $CuSO_4$ in buffer III; lane 5, C289S; lane 6, C289S dialyzed against 100 μM $CuSO_4$ in buffer III; M, molecular mass markers. The protein samples of lanes 2 and 5 in panel A and lanes 2, 4, and 6 in panel B were prepared under the nonreductive conditions described in Materials and Methods. Other samples were prepared under reductive conditions.

that even C73S-C289S, which has no cysteine residues, is also resolved into two fractions. Therefore, the chromatographic resolution of FPP synthase should not necessarily be understood as a sole problem of cysteine residues.

When wild-type and C73S-C289S enzymes that had been dialyzed against 100 μM $CuSO_4$ in 25 mM Tris-HCl buffer, pH 7.7, were chromatographed, both were eluted by 100 mM NaCl (Figure 3D). Therefore it seems that the fractions eluted by 100 mM NaCl in Mono Q chromatography are related to oxidation which is stimulated by Cu^{2+}, regardless of whether SH groups are present or not. The Cu^{2+}-treated enzyme gave a band at 31 kDa in addition to the band at 32.5 kDa (Figure 4A, lane 2). The 31-kDa band disappeared on electrophoresis under reductive conditions (Figure 4A, lane 3). Therefore the 31-kDa band represents a Cu^{2+}-mediated oxidation product with an intrasubunit S–S bond, while the 32.5-kDa band seems to correspond to an oxidized form without involvement of sulfhydryl oxidation. C73S-C289S gave a single band at 32.5 kDa regardless of Cu^{2+} treatment (Figure 4A, lanes 4 and 5), but Cu^{2+}-treated C73S-C289S was eluted as a single peak by 100 mM NaCl (Figure 3C). From a comparison of the SDS–PAGE and Mono Q chromatography analyses of the wild-

type enzyme, it is likely that about half of the 100 mM NaCl eluate corresponds to the 31-kDa band and that the rest of it corresponds to the 32.5-kDa band. Native PAGE analysis of the wild-type enzyme or C73S-C289S, either of which gives two activity peaks on Mono Q chromatography, gave only a single band (data not shown).

When C73S, C289F, and C289S were treated with Cu^{2+}, each mutant enzyme gave a new band at 64 kDa on SDS–PAGE under nonreductive conditions (Figure 4B, lanes 2, 4, and 6). The Cu^{2+}-treated wild-type enzyme also showed a 64-kDa, band but the band was much thinner than the two major bands, 31 and 32.5 kDa (Figure 4A, lane 2). Therefore, the wild-type FPP synthase seems to be susceptible to oxidation when treated with Cu^{2+}, forming an intrasubunit disulfide bond rather than intersubunit bonds. In addition, this enzyme appears to undergo unidentified reactions which do not involve sulfhydryl groups.

DISCUSSION

In order to investigate the significance of the two cysteine residues in the FPP synthase of *B. stearothermophilus*, we prepared five mutants: C73F, C73S, C289F, C289S, and C73S-C289S. All of these mutants showed the FPP synthase activity, indicating that neither of the cysteines is essential for the catalytic function.

As compared with the wild-type enzyme, C289F shows a 10 times larger K_m value for IPP but a similar K_m value for GPP. These facts suggest that the binding affinity for IPP is affected by the substitution of Cys-289 with phenylalanine. It is likely that the C-terminal region (region G) containing Cys-289 is located in a space that affects the binding of IPP. This conclusion coincides with our previous results obtained in site-directed mutagenesis studies focused on the three C-terminal amino acids of this enzyme (Koyama et al., 1994).

The replacement of Cys-73 with phenylalanine resulted in a marked decrease in the heat stability of the enzyme, while the replacement of the same cysteine with serine had no such effect. It is reasonable to assume that the conformational change in C73F is so marked that an intersubunit disulfide bond is not formed even in the presence of Cu^{2+}. Though region B corresponds to domain I, which is proposed as the binding site for the homoallylic substrate, IPP (Ashby et al., 1990), the replacement of Cys-73 in this region with phenylalanine or serine did not affect the K_m value for IPP. Thus, Cys-73, which is completely conserved in region B throughout the FPP synthases of various organisms, is not important for the binding of IPP, though it seems to be involved in the stabilization of the enzyme. The inhibition of the wild-type enzyme by relatively high concentrations of PCMB seems to be attributable to a similar conformational change. Probably, the SH reagent inactivates the enzyme as a consequence of the introduction of bulky groups rather than through loss of functional sulfhydryl groups. In fact, PCMB is inhibitory toward the wild-type enzyme but not toward C73S-C289S (Table 1).

The tendency for the FPP synthase to form the disulfide bond implies the proximity of the cysteine residues. This is consistent with the conclusion that region G occupies a position that affects the IPP binding site (Koyama et al, 1994). Region G, which includes Cys-289, seems to be located near region B, the presumed IPP binding site, because the enzyme tends to form the disulfide bond between Cys-73 and Cys-289 spontaneously. The wild-type enzyme does not contain any intersubunit disulfide bonds, because the 64-kDa band was not detected on SDS–PAGE of intact enzyme that had not been treated with 2–mercaptoethanol (data not shown). The SDS–PAGE analysis of the Cu^{2+}-treated wild-type enzyme indicates that the formation of the intrasubunit disulfide bond is predominant over that of the intersubunit disulfide bond (Figure 4A, lane 2).

The FPP synthase or its mutant C73S-C289S displays two forms, which are separable on Mono Q chromatography, one being eluted by 80 mM NaCl and the other by 100 mM NaCl. The latter fraction contains at least two species. A possible explanation is as follows. One corresponds to the band at 31 kDa, which is observed only in the case of the wild-type enzyme and must be attributed to the subunit which contains the intrasubunit disulfide bond formed by oxidation, as reported in the case of the FPP/GGPP synthase of *M. thermoautotrophicum* (Chen & Poulter, 1993). The other is an oxidized form that migrates to the same position (32.5 kDa) in SDS–PAGE as does untreated enzyme. The Cu^{2+}-treated C73S-C289S mutant seems to take such an oxidized form.

It seems that the wild-type FPP synthase of *B. stearothermophilus* is fully reduced and that the oxidized form must be an artifact, because SDS–PAGE of freshly prepared enzyme gave a single 32.5-kDa band even under nonreducing conditions.

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