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Structural and Functional Roles of Cysteine Residues of *Bacillus polymyxa* β-Amylase

Nobuyuki Uozumi, Tsukasa Matsuda, Norihiro Tsukagoshi,* and Shigezo Udaka

Department of Food Science and Technology, Faculty of Agriculture, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan

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ABSTRACT: *Bacillus polymyxa* β-amylase contains three cysteine residues at positions 83, 91, and 323, which can react with sulfhydryl reagents. To determine the role of cysteine residues in the catalytic reaction, cysteine residues were mutated to construct four mutant enzymes, C83S, C91V, C323S, and C-free. Wild-type and mutant forms of the enzyme were expressed in, and purified to homogeneity from, *Bacillus subtilis*. A disulfide bond between Cys^{83} and Cys^{91} was identified by isolation of tryptic peptides bearing a fluorescent label, IAEDANS, from wild-type and C91V enzymes followed by amino acid sequencing. Therefore, only Cys^{323} contains a free SH group. Replacement of cysteine residues with serine or valine residues resulted in a significant decrease in the k_{cat}/K_m value of the enzyme. C323S, containing no free SH group, however, retained a high specific activity, approximately 20% of the wild-type enzyme. None of the cysteine residues participate directly in the catalytic reaction.

The enzyme β-amylase (α-1,4-glucan maltohydrolase, EC 3.2.1.2) catalyzes the liberation of β-anomeric maltose from the nonreducing ends of α-1,4-glucan and is present in certain bacteria (Marshall, 1974; Murao et al., 1979; Shinke et al., 1974; Takasaki, 1976; Hyum & Zeikus, 1985) as well as in higher plants (Bernfeld, 1955; Kreis et al., 1987). Five genes encoding β-amylase have been cloned and sequenced from both prokaryotes and eukaryotes (Kawazu et al., 1987; Rhodes et al., 1987; Kitamoto et al., 1988; Toda et al., 1988; Kreis et al., 1987; Mikami et al., 1988). Three highly conserved sequences are recognized among them and are suggested to comprise the active site (Mikami et al., 1988). β-Amylases characterized to date are sensitive to various sulfhydryl-modifying reagents and are considered to contain an SH group essential for the activity (Murao et al., 1979; Hyum & Zeikus, 1985; Gertler & Birk, 1966; Spradlin & Thomas, 1970; Higashihara & Okada, 1974; Uehara & Mannen, 1979). The exact role of cysteinyl residues in the enzymatic action, however, remains uncertain, since the derivatization of the SH groups with alkylating reagents might inhibit the catalytic action only by steric hindrance at the active site (Mikami et al., 1980).

* To whom correspondence should be addressed.

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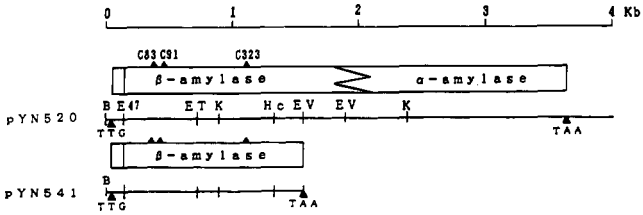

FIGURE 1: Diagrammatic presentation of the β-amylase gene from *B. polymyxa*. The upper half represents the correlation of the β- and α-amylase coding regions on plasmid pYN520, while the lower half shows the amylase gene on plasmid pYN541 used in this study. Closed triangles indicate the positions of cysteine residues. The start codon (TTG) and stop codon (TAA) are also indicated. The abbreviations for restriction enzyme sites are B, *Bam*HI; E47, *Eco*47III; ET, *Eco*T22I; EV, *Eco*RV; Hc, *Hin*cII; and K, *Kpn*I.

Bacillus polymyxa produces multiform β-amylases with approximate molecular masses of 70, 56, and 42 kDa and a 48-kDa α-amylase (Kawazu et al., 1987). The amylase gene contains in-phase β- and α-amylase coding sequences in the 5′ and 3′ regions, respectively. A precursor protein, a 130-kDa amylase, has both β- and α-amylase activities and is proteolytically cleaved to produce multiform β-amylases and a 48-kDa α-amylase after secretion (Uozumi et al., 1989). The three major β-amylases contain three cysteine residues and have essentially the same enzymatic properties (Kawazu et al., 1987).

To determine whether the *B. polymyxa* β-amylase contains an essential active cysteine residue, we employed site-directed mutagenesis to replace three cysteine residues by serine or valine residues utilizing the DNA fragment encoding the 42-kDa β-amylase. The mutant enzymes were purified and characterized. The *B. polymyxa* β-amylase contained a disulfide linkage. None of the cysteine residues appear to be essential for catalytic activity.

Materials and Methods

Bacterial Strains, Media, and Transformation. L broth (Miller, 1972) and antibiotic medium 3 (Difco) were used to grow *Escherichia coli* JM103 (Maniatis et al., 1982) and *Bacillus subtilis* 1A289 (*amy*E *sac*A321 *aro*I906 *met*B5; *Bacillus* Genetic Stock Center, The Ohio State University, Columbus), respectively. When required, ampicillin and kanamycin were added at 50 and 10 μg/mL, respectively. The transformation of *E. coli* and *B. subtilis* was carried out as described previously (Kawazu et al., 1987; Uozumi et al., 1989). β-Amylase-producing transformants were identified by staining the plates with a 1.7 mM I_2–KI solution (Tsukagoshi et al., 1984).

Plasmids. pYN520 containing in-phase β- and α-amylase coding sequences of the *B. polymyxa* amylase gene was described previously (Uozumi et al., 1989). pYN4941 was constructed as follows: a 2.4-kb *Bam*HI–*Kpn*I fragment containlng the β-amylase coding region was isolated from pYN520 and inserted between the *Bam*HI and *Kpn*I sites on pUC118 (Figure 1). A universal translation terminator, 5′-GCTTAATTAATTAAGC-3′ (Pharmacia Fine Chemicals, Piscataway, NJ), was inserted into the *Eco*RV site after removal of the 0.3-kb *Eco*RV fragment on the resultant plasmid. pYN541 was constructed as follows: a 2.1-kb *Bam*HI–*Eco*RI fragment was isolated from pYN4941 by use of the *Eco*RI site located 12 bp downstream of the 3′ end of the insert and inserted between the *Bam*HI and *Eco*RI sites on pUB110, followed by the transformation of *B. subtilis* 1A289 to kanamycin resistance.

Site-Directed Mutagenesis of the β-Amylase Gene. The 0.6-kb *Eco*47III–*Eco*T22I and 0.4-kb *Kpn*I–*Hin*cII fragments on pYN4941 containing regions I and III conserved among β-amylases, respectively, were inserted between the corresponding sites on M13mp19. Single-stranded M13mp19 DNA containing the insert was subjected to mutagenesis with the aid of the oligonucleotide-directed in vitro mutagenesis system (Amersham Corp.). To confirm that only the desired mutations occurred during the manipulations, the gene from each was sequenced for its entirety by the dideoxy chain-termination method (Sanger et al., 1977). The *Eco*47III–*Eco*T22I or *Kpn*I–*Hin*cII fragment with the confirmed mutations was inserted between the corresponding sites on pYN4941 and used for the transformation of *E. coli* JM103 to ampicillin resistance. To analyze the gene products, the *Bam*HI–*Eco*RI fragment containing the mutated gene was subcloned onto pUB110 as described above and expressed in *B. subtilis*. Mutagenic deoxyoligonucleotides 5′-CAACGCATAAGAGTGGAGG-3′, 5′-GTAGGAGATGACGTCAACATCCC-3′, and 5′-GACATTTACTAGCCTGGAG-3′ were synthesized at the Center for Gene Research of Nagoya University and were used to obtain pYN541-C83S, pYN541-C91V, and pYN541-C323S, respectively. pYN541-C-free was constructed as follows: the *Eco*47III–*Eco*T22I fragment on pYN541-C83S containing region I was further mutated at codon 91 to produce double mutations, C83S-C91V, followed by insertion in the corresponding region on pYN541-C323S.

Purification of β-Amylases. B. subtilis 1A289 cells with plasmids containing wild-type and mutant β-amylase genes were grown for 40 h at 28 °C in antibiotic medium 3 supplemented with 0.5% soluble starch and 10 μg/mL kanamycin. β-Amylases were purified as described previously (Kawazu et al., 1987) with some modifications. The nonbinding fractions containing β-amylase on a DEAE-cellulose column were pooled, dialyzed against 10 mM acetate buffer (pH 6.0), and applied to a column of CM-cellulose equilibrated with the same buffer. The nonbinding fractions containing the enzyme activity were pooled and dialyzed extensively against 100 mM phosphate buffer (pH 7.0) containing 25% ammonium sulfate, followed by fractionation on a phenyl-Sepharose CL-4B column previously equilibrated with the same buffer. The enzyme was eluted at a flow rate of 18 mL/h from the column with 10 mM phosphate buffer (pH 7.0). The β-amylase-containing fractions were pooled and dialyzed against 50 mM phosphate buffer (pH 7.0).

Protein Derivatization. The general conditions for protein labeling and isolation of resultant derivatives were as described previously (Sanger et al., 1977; Gorman et al., 1987). Wild-type and mutant C91V β-amylases (400 μg of each) were labeled at 25 °C for 2 h with 4.5 mM *N*-(iodoacetyl)-*N*′-(8-sulfo-1-naphthyl)ethylenediamine (IAEDANS)[1] in 75 mM Tris-HCl buffer (pH 8.0) containing 6 M guanidine hydrochloride and 4.5 mM EDTA. The derivatized proteins were protected from light to avoid photodecomposition of the fluorophore. This also applied to the analytical procedures described below. The reactions were stopped by adjustment of the pH of the reaction mixture to 5 with acetic acid, followed by extensive dialysis against 100 mM Tris-HCl buffer (pH 8.0), and then dialysis against the same buffer containing 1 mM $CaCl_2$.

Protein Cleavage and Peptide Separation. Protein derivatives were digested by the addition of aliquots of 1% (w/v)

[1] Abbreviations: pCMB, *p*-chloromercuribenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoate); α-EPG, 2,3-epoxypropyl α-D-glucopyranoside; IAEDANS, *N*-(iodoacetyl)-*N*′-(8-sulfo-1-naphthyl)ethylenediamine, NEM, *N*-ethylmaleimide.

```
                                    I                                         II                     III
                              83        91                                                          323
                              ▲         ▲                                                            ▲
B.polymyxa             77 IISTHKCGGNVGDDCNIPLPSW 98  159 GPSGELRYPSYYP 172 319 LTFTCLEMSDS 329
                          * *** *******  *** ***         *********** *             ******* **
C.thermosulfurogenes   77 IMSTHACGGNVGDTVNIPIPSW 98  159 GPSGELRYPSYNP 172 320 MTFTCLEMDDS 330
                          *** * ******* ****** *         ** ******** *               ****** **
Soybean                89 IMSFHQCGGNVGDIVNIPIPQW 110 182 GPAGELRYPSY-P 193 339 LNFTCLEMRDS 349
                          ************* ********         ***** ***** *              **** ****
Barley                 87 IMSFHQCGGNVGDAVNIPIPQW 108 180 GPAGEMRYPSY-P 191 337 INFTCAEMRDL 347
                          *********** *** ******         * *** ***** *              **** ****
Sweet Potato           90 IMSFHQCGGNVADAVFIPIPQW 111 184 GAAGELRYPSY-P 195 341 LNFTCLEMRDS 351

CONSERVED                 I-S-H-CGGNV-D---IP-P-W         G--GE-RYPSY-P              --FTC-EM-D-
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FIGURE 2: Conserved amino acid sequences in three homologous regions (I, II, and III) of β-amylases. Identical residues are denoted by asterisks between the sequences. The amino acids are numbered from the N terminus of each mature enzyme. Closed triangles indicate positions of cysteine residues of the *B. polymyxa* β-amylase. Amino acid sequences of the following amylases were aligned to maximize the homologies: *B. polymyxa* (Kawazu et al., 1987; Rhodes et al., 1987; Uozumi et al., 1989), *C. thermosulfurogenes* (Kitamoto et al., 1988), soybean (Mikami et al., 1988), barley (Kreis et al., 1987), and sweet potato (Toda et al., 1988). Conserved amino acids among five β-amylases are shown at the bottom.

trypsin at a ratio of β-amylase to trypsin by weight of 50 to 1. After incubation at 37 °C for 16 h, digestion was terminated by freezing the reaction mixture and subsequent lyophilization. The resultant peptides were dissolved in 0.1% CF_3COOH in H_2O and separated by HPLC (JASCO, Tokyo, Japan) on a reversed-phase column (Biofine RPC-PO, 4.6 × 150 mm; JASCO). Chromatography was performed at a flow rate of 1 mL/min with a linear gradient of increasing percentages of CH_3CN relative to H_2O while a constant concentration of 0.1% (v/v) CF_3COOH was maintained. Programs for rates and extents of increase in CH_3CN were optimized for each particular separation. Absorbance of the eluates was monitored at 220 nm in addition to fluorescence emission monitored at 540 nm due to excitation at 340 nm. Peaks of fluorescence were collected manually and lyophilized. If necessary, samples were digested further with trypsin, and peptides bearing fluorescence were isolated as described above.

Other Analytical Procedures. The NH_2-terminal amino acid sequences were determined with a gas-phase sequence analyzer (ABI 477A-120A protein sequencer). The circular dichroism spectrum of each protein was measured at room temperature with a JASCO J-500C spectropolarimeter. The β-amylase activity was determined at 25 °C with soluble starch as a substrate (Kawazu et al., 1987). Protein was determined with bovine serum albumin as the standard (Lowry et al., 1951). The purity of protein was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli, 1970).

RESULTS

Construction and Expression of the Mutant β-Amylase Genes. The amino acid sequences deduced from various β-amylase genes were optimally aligned to the *B. polymyxa* sequence. The alignment revealed three highly conserved regions (Figure 2). The *B. polymyxa* β-amylase contained one additional cysteine residue in region I, which was replaced with a valine residue in the other enzymes. Taking into consideration the sequence similarity, we constructed four mutant genes by means of oligonucleotide-directed mutagenesis. The cysteine codons at 83 and 323 were replaced by a serine codon to construct C83S and C323S, respectively. C91V, with the cysteine codon at 91 replaced by a valine codon, contained the sequence most homologous to the other enzymes. In C-free, three cysteine codons were replaced by serine codons at codons 83 and 323 and by a valine codon at codon 91. The mutant enzymes were expressed in *B. subtilis* and purified by the same procedure used for the wild-type (Wt) enzyme. The proteins obtained were homogeneous as judged by SDS–polyacrylamide gel electrophoresis and had approximate molecular masses of 42 kDa (Figure 3).

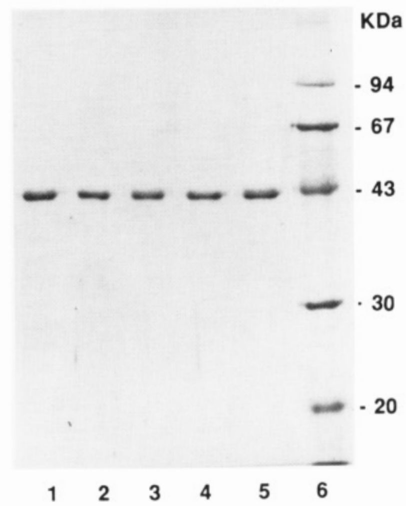

FIGURE 3: SDS–polyacrylamide gel electrophoretic profiles of the purified β-amylases. The β-amylases were purified from *B. subtilis* carrying pNY541 (lane 1), pNY541-C83S (lane 2), pNY541-C91V (lane 3), pNY541-C323S (lane 4), or pNY541-C-free (lane 5). A 0.4-μg sample of each protein was analyzed. Molecular weight marker proteins (phosphorylase *b*, 94 000; bovine serum albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; soybean trypsin inhibitor, 20 100; α-lactoalbumin, 14 400) were simultaneously electrophoresed (lane 6). The gel was stained with Coomassie brilliant blue R-250.

Identification of Disulfide Bond. To assess whether two cysteine residues at 83 and 91 in the *B. polymyxa* β-amylase form a disulfide bond, both Wt and C91V enzymes were derivatized with a fluorescent label, IAEDANS, under denaturating conditions. Tryptic digestion of fluorescent derivatives followed by high-performance liquid chromatograhy yielded two and three major peptides bearing the fluorescent label from Wt and C91V proteins, respectively (Figure 4). Among those five peptides, peptide C91V-3 was unique and should be generated as the consequence of mutation. Wt-1 and -2 were eluted from a Biofine RPC-PO column at exactly the same retention time as that of C91V-1 and -2, respectively. Upon further digestion of C91V-2 with trypsin, a major peptide, C91V-2′, bearing the fluorescent label was eluted at nearly the same position as those of Wt-1 and C91V-1. All the major fluorescent peptides, in which cysteine residues should be derivatized with IAEDANS, were subjected to amino acid sequencing. The NH_2-terminal amino acid sequences of those peptides are shown along with the deduced amino acid sequence (Figure 5). The unique fluorescent peptide, C91V-3, contained an NH_2-terminal amino acid sequence exactly the same as the deduced sequence from residues 2 (Gly^{84}) to 13 (Leu^{95}) except for residue 9 (Cys^{91}), which was replaced with a valine residue as expected from the muta-

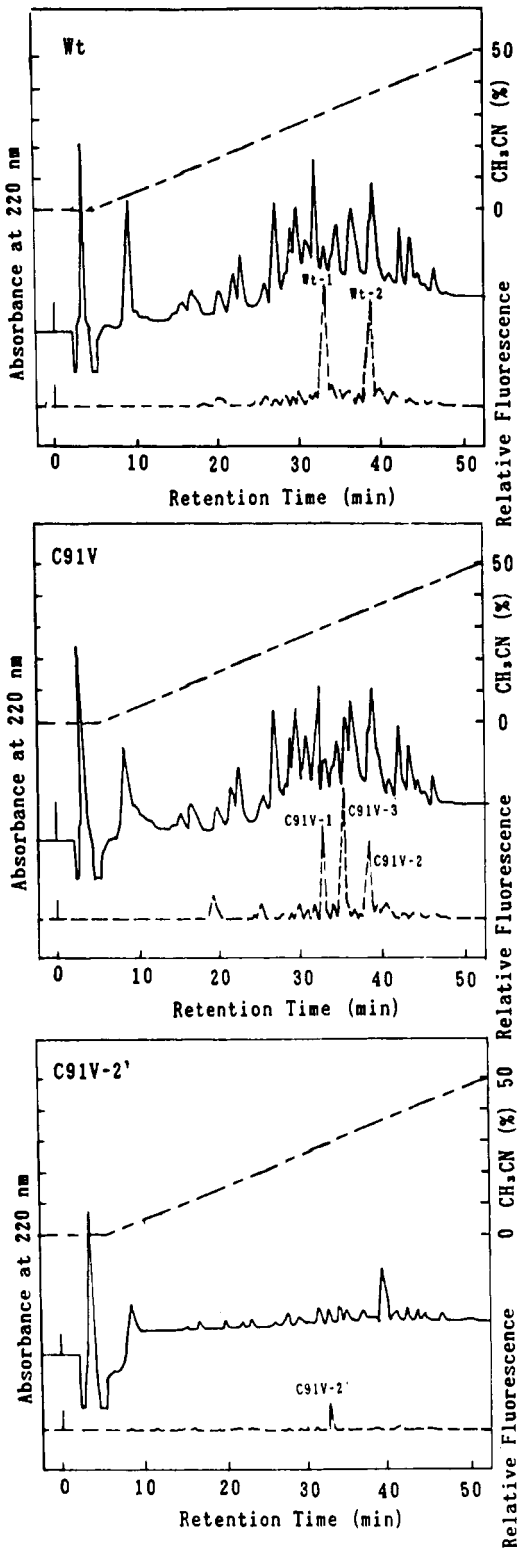

FIGURE 4: Separation of fluorescent peptides from the tryptic digests of Wt and C91V derivatized with IAEDANS. The tryptic digests were fractionated on a reversed-phase HPLC column. See the text for details.

genized nucleotide sequence. Furthermore, the amino acid sequence chemically determined suggests that the NH_2-terminal amino acid of the peptide should be Cys^{83}, since the NH_2-terminal amino acid was not assigned most probably as the consequence of modification with IAEDANS. The other four fluorescent peptides, Wt-1, Wt-2, C91V-1, and C91V-2', appeared to contain essentially the same peptide region. The NH_2-terminal amino acid sequences of peptides Wt-1 and C91V-1 starting from Ile^{280} suggest that both peptides could include a cysteine residue reactive with IAEDANS at position 323, since they beared the fluorescent label. Therefore, the differences in the number and the position of modified cysteine residues betwen Wt and C91V proteins demonstrate the presence of a disulfide bond between Cys^{83} and Cys^{91} in the wild-type enzyme. This further indicates that Cys^{323} contains a free SH group.

Table I: Kinetic Parameters of Various β-Amylases with Soluble Starch as a Substrate[a]

	k_m (mg/mL)	k_{cat} (s^{-1})	k_{cat}/k_m (mL/mg s^{-1})
Wt	0.071 ± 0.014	128 ± 2	1820
C83S	0.460 ± 0.007	14.5 ± 0.1	31.5
C91V	0.099 ± 0.021	9.5 ± 0.1	96.6
C323S	0.118 ± 0.012	40.5 ± 4.0	343
C-free	0.549 ± 0.005	16.2 ± 0.2	29.5

[a] Initial reaction rates were determined at 25 °C as described in Materials and Methods. Kinetic data were fitted to the Michaelis-Menten relationship to evaluate K_m and V_{max}. Turnover number (k_{cat}) was calculated on the basis of the amount of enzyme used.

Table II: Effects of Various Sulfhydryl Group Reagents on the Activity of β-Amylases[a]

	pCMB	NEM		DTNB	
	40 μM	2 mM	10 mM	1 mM	5 mM
Wt	<1	112	95	94	95
C83S	<1	90	98	103	100
C91V	<1	25	<1	46	53
C323S	115	86	97	100	100
C-free	103	92	97	102	99

[a] Enzyme (200 nM) was incubated at 4 °C for 60 min in 50 mM phosphate buffer (pH 7.0) with various sulfhydyl group reagents at the concentrations indicated, followed by measurement of the activity at 25 °C with soluble starch as a substrate.

Kinetic Parameters of Wild-Type and Mutant Enzymes. Kinetic constants K_m and k_{cat} were determined from initial rate measurements for hydrolysis of soluble starch (Table I). The mutants C91V and C323S had K_m values for this substrate comparable with that of Wt, while the mutants C83S and C-free exhibited a marked increase in the K_m, approximately 7- and 8-fold increases, respectively. For all the mutants, the k_{cat}/K_m ratio, which reflects the specific activity for soluble starch of the enzyme, was reduced drastically compared to that for Wt: approximately 5-fold for C323S, 20-fold for C91V, and 60-fold for both C83S and C-free.

Inactivation of Wild-Type and Mutant Enzymes by SH Reagents. To determine whether substitution of cysteine residues has any effect on inactivation, the mutant enzymes as well as the Wt enzyme were subjected to modification of SH reagents such as pCMB, NEM, and DTNB and then assayed for enzyme activity (Table II). Reaction of pCMB with enzymes resulted in the complete inactivation of Wt, C83S, and C91V. Both C323S and C-free retained full activity under the same condition. Of the mutant enzymes, only C91V was inactivated by all three SH reagents, although DNTB modification caused only a partial inactivation of its activity. Assuming that a loss of the enzyme activity is due to the chemical modification of cysteine residues, all these data demonstrate that the cysteine residues are not essential for enzyme activity.

Discussion

β-Amylases from diverse origins have been shown to be sensitive to inactivation by sulfhydryl reagents (Murao et al., 1979; Hyum & Zeikus, 1985; Gertler & Birk, 1966; Spradlin

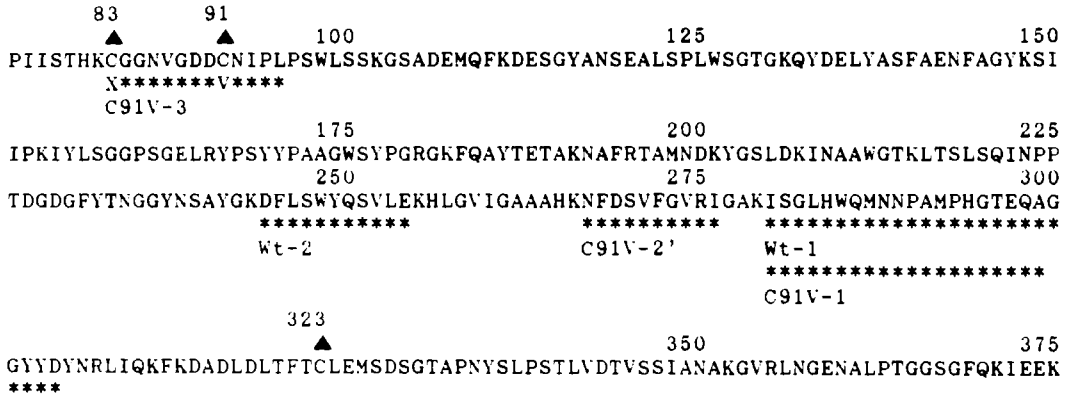

FIGURE 5: Alignments of amino acid sequences of tryptic peptides bearing fluorescence and the deduced sequence of β-amylase. Wt-1, Wt-2, C91V-1, C91V-2′, and C91V-3 were isolated as fluorescent peptides from the tryptic digests. Numbers indicate amino acid positions from the amino terminus. Asterisks denote amino acids identical with the deduced amino acid. X indicates an amino acid not assigned.

& Thomas, 1970; Higashihara & Okada, 1974; Uehara & Mannen, 1979). To assess the role of cysteine residues in the catalytic activity, we constructed four mutants of the *B. polymyxa* β-amylase and analyzed effects of the mutations on protein structure and on enzymatic properties in combination with protein-modifying reagents.

The *B. polymyxa* β-amylase contains three cysteine residues (Kawazu et al., 1987), two of which, Cys^{83} and Cys^{91}, were shown to form a disulfide bond between them. This, in turn, indicates that Cys^{323} contains a free SH group. No disulfide bonds have been identified in β-amylases from other origins. Mutations to prevent disulfide formation are expected to induce some perturbations in tertiary structure. The mutation of Cys^{83} to Ser^{83} resulted in a gross structural alteration as judged by the CD spectrum, while the mutation of Cys^{91} to Val^{91} had no apparent effect on the structure (data not shown). The enzyme activity of the former mutant at 50 °C was inactivated at almost twice the rate of that of the latter mutant. Val^{91}, a well-conserved residue among β-amylases, might be able to restore, to some extent, the structural perturbations induced by disruption of a disulfide bond, which might result in the simultaneous enhancement of the thermostability (data not shown).

None of the cysteine residues were shown to participate directly in the catalytic reaction based on the enzymatic properties of mutants. This is also the case with soybean β-amylase as described below. The mutant C323S, however, had a rather low specific activity, approximately 20% of that of the wild-type enzyme, suggesting a structural function for Cys^{323}. The thiol hydrogen of Cys^{323} might participate in formation of a hydrogen bond that, if absent, subtly alters the enzyme structure and lowers the specific activity of C323S.

The soybean β-amylase has been well characterized and shown to contain two types of SH groups: highly reactive SH groups (Cys^{82}, Cys^{208}, and Cys^{343}) and essential SH groups (Cys^{95}, Cys^{288}, and Cys^{448}) (Mikami & Morita, 1983; Nomura et al., 1987a). None of the cysteine residues participate either in catalysis or in substrate binding (Mikami et al., 1980). Cys^{95} has, however, been suggested to be located close to the substrate binding site in the active-site region, since modification of Cys^{95} results in the loss of the activity (Nomura et al., 1987b). Furthermore, affinity labeling of the enzyme with α-EPG (2,3-epoxypropyl α-D-glucopyranoside) causes the inactivation of the activity as a consequence of esterification of Glu^{186} with the affinity label (Nitta et al., 1989). Interestingly, these three residues, Cys^{95}, Glu^{186}, and Cys^{343}, in the soybean β-amylase, are included in the highly conserved regions among β-amylases. The *B. polymyxa* β-amylase contains Glu at position 163, equivalent to Glu^{186} in the soybean enzyme. The Glu^{163} residue might participate in the catalytic reaction, since α-EPG caused the inactivation of enzyme activity (Uozumi et al., 1989). In preliminary experiments, the mutant E163Q, in which Glu^{163} was replaced with Gln^{163}, exhibited barely detectable activity for soluble starch, approximately 10^{-4} of the specific activity of the wild-type enzyme. Work in this area is in progress.

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An Investigation of Bovine Serum Amine Oxidase Active Site Stoichiometry: Evidence for an Aminotransferase Mechanism Involving Two Carbonyl Cofactors per Enzyme Dimer†

Susan M. Janes and Judith Pollock Klinman*
Department of Chemistry, University of California, Berkeley, Berkeley, California 94720
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ABSTRACT: Recent evidence has shown that the active site cofactor in bovine serum amine oxidase (BSAO) is 2,4,5-trihydroxyphenylalanine or 6-hydroxydopa [Janes et al. (1990) *Science 248*, 981]. However, much ambiguity remains regarding the mechanism of the enzymatic reaction. Conflicting data exist for both the number of functional active sites in the dimeric enzyme and for the oxygen dependence of product release. To resolve these questions, a new method has been developed for the purification of BSAO which leads to the isolation of specific activity ≥0.4 unit/mg of enzyme in 2–3 weeks. This highly active enzyme has been used to quantitate both aldehyde and ammonia release in the reductive half-reaction. Anaerobic incubation of enzyme and substrate resulted in the production of 2 mol of aldehyde/mol of enzyme, indicating the presence of a cofactor at each enzyme subunit. As anticipated for an aminotransferase reaction, no ammonia release was detected under comparable conditions. Active site titration of enzyme samples of varying specific activity with phenylhydrazine extrapolates to 1 mol of inhibitor/mol of enzyme subunit for BSAO of specific activity = 0.48 unit/mg. These findings contrast with numerous, previous reports of only one functional cofactor per enzyme dimer in copper amine oxidases.

Bovine serum amine oxidase (BSAO)[1] catalyzes the oxidative deamination of primary amines using molecular oxygen as a terminal $2e^-$ acceptor:

$$RCH_2NH_3^+ + H_2O + O_2 \rightarrow RCHO + NH_4^+ + H_2O_2 \quad (1)$$

The enzyme is a dimer of 170 kDa, comprised of identical subunits joined by disulfide bonds (Achee et al., 1968). Pure enzyme is peach colored and exhibits a broad absorption band centered around 480 nm. This band is lost when the enzyme is reduced, either with substrate or by inhibitors. Carbonyl reagents such as semicarbazide, phenylhydrazine, and 2,4-dinitrophenylhydrazine are potent inactivators of BSAO (Buffoni, 1966; Yasunobu et al., 1976; Moog et al., 1986). Reductive trapping experiments have shown that the active site prosthetic group is capable of forming a Schiff base with the amine of substrate during the course of catalysis (Hartmann & Klinman, 1987). The enzyme has also been reported to contain two tightly bound coppers per dimeric unit (Yasunobu et al., 1976). Experiments with copper-depleted enzyme indicate that the metal center is necessary for enzyme activity (Suzuki et al., 1986); however, no significant Cu(I) species is formed during catalysis, suggesting that enzyme-bound Cu(II) acts as an electron shuttle in the oxidation of reduced cofactor by molecular oxygen.

Although BSAO has been studied extensively since the 1950s, identification of the carbonyl cofactor has been hindered by its inherent reactivity and the fact that it is covalently linked to enzyme. The inability to isolate and characterize the cofactor has resulted in conflicting hypotheses regarding cofactor structure, which was originally attributed to pyridoxal phosphate (Buffoni, 1966; Buffoni & Cambi, 1990) and, later, to pyrroloquinoline quinone (Lobenstein-Verbeek et al., 1984; Ameyama et al., 1984). Recently, this ambiguity has been resolved with the identification of 6-hydroxydopa (topa) at the active site of BSAO (Janes et al., 1990).

The net reaction catalyzed by serum amine oxidases can be formalized as a series of partial reactions involving, first, proton abstraction and concomitant enzyme reduction, second, hy-

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*Author to whom correspondence should be addressed.

[1] Abbreviations: BSAO, bovine serum amine oxidase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; FPLC, fast protein liquid chromatography; PH, phenylhydrazine; Con-A, concanavalin A; topa, 2,4,5-trihydroxyphenylalanine; NADH, α-nicotinamide adenine dinucleotide, reduced form; ADP, adenosine 5′-diphosphate.

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