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Selective Cleavage and Modification of the Intersubunit Disulfide Bonds of Bovine Dopamine β-Monooxygenase: Conversion of Tetramer to Active Dimer

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Bovine dopamine β-monooxygenase is a tetramer consisting of two disulfide-linked dimers. To examine the role(s) of the intersubunit disulfide bonds in the protein structure and activity, the enzyme was treated with DTT at pH 7.5 and 25°C under nondenaturing conditions. A 15-min incubation with 0.5 mM DTT selectively cleaved half of the intersubunit disulfide bonds. The cleavage did not affect the activity or tetrameric structure of the enzyme. Upon chemical modification of the reduced cysteine residues with 0.1 M iodoacetamide (IAA) for 60 min, half of the tetramer was converted to a dimeric species. The resulting dimeric and tetrameric species exhibited similar kinetic properties, and the V_{max} values were decreased by 30% compared to that of the native enzyme. Upon treatment with IAA alone, no dimer species was detected but the enzyme lost 30% of the original activity. Cys514 and Cys516 were selectively modified by the treatment with DTT and IAA. From these results, we concluded that: (i) chemical modification of the intermolecular disulfide bonds strongly destabilizes the intersubunit interaction; (ii) breakage of the intersubunit interaction does not affect the activity. The reduction mechanism of the intersubunit disulfide bonds and the roles of the intersubunit interactions are discussed.

Key words: chemical modification, disulfide bonds, dithiothreitol, dopamine β-monooxygenase, intersubunit interaction.

Dopamine β-monooxygenase (EC 1.14.17.1, DβM) catalyzes the conversion of dopamine to noradrenaline within catecholamine-secreting vesicles of adrenal medullary cells and adrenergic neurons (for reviews, see Refs. *1* and *2*). The bovine enzyme is a tetramer and exists as both soluble and membrane-bound forms (*3, 4*). The subunit exhibits heterogeneity in the N-terminal portion and in glycosylation due to different post-translational modifications of the protomer encoded by a single mRNA (*5-7*). Each subunit contains six intramolecular disulfide bonds, and, as schematically shown in Fig. 1, subunits A and C, and B and D are cross-linked through two intersubunit disulfide bridges (Cys514-Cys514′ and Cys516-Cys516′) (*8*). DβM can therefore be regarded as a dimer of disulfide-linked AC and BD (*8, 9*). The cysteine residues involved in the intra- and inter-molecular disulfide bonds are strictly conserved among the human, bovine, and mouse enzymes (*10-14*), suggesting an important role of each disulfide bond in the structure and activity of DβM. However, the domain structure and the nature of the interdomain and intersubunit interactions are unknown, since the three-dimensional structure of the enzyme has not been determined.

Each subunit contains an active site which has two distinct copper ions essential for the activity (*15-22*). No cooperativity based on subunit-subunit interactions has been observed in the kinetics or ligand binding. Saxena *et al.* (*23*) showed that the association of AC and BD dimers is pH-dependent, and that AC (or BD) dimer exhibits a lower K_m for tyramine than the tetramer. However, the putative K_m difference between the dimer and tetramer has not been confirmed by others (*2, 24*, and our unpublished results). Thus, it is unclear what roles individual subunit-subunit interactions play in the expression of the enzyme activity and in the structural stabilization of the enzyme. In this study, we successfully perturbed the intersubunit interaction between the subunits A and C (B and D) by a selective modification of the intermolecular disulfide bonds. Under mild reducing conditions, DTT selectively cleaved the intersubunit disulfide bonds, and the chemical modification of the reduced cysteine residues destabilized the tetramer, resulting in dissociation to active dimers. Based on these results, we discuss the role of the intersubunit interactions.

EXPERIMENTAL PROCEDURES

Materials—Tyramine hydrochloride, DTT, TPCK-trypsin, and Con A-Sepharose were purchased from Sigma; disodium fumarate, ascorbic acid, *N*-ethylmaleimide (NEM), iodoacetamide (IAA), 5,5′-dithiobis(2-nitrobenzoic acid), 2-(*N*-morpholino)ethanesulfonic acid (MES), and HEPES were from Nacalai Tesque; *N*-iodoacetyl-*N*′-

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Abbreviations: DβM, dopamine β-monooxygenase; DTT, dithiothreitol; *I*, ionic strength; IAA, iodoacetamide; I-AEDANS, *N*-iodoacetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; MES, 2-(*N*-morpholino)ethanesulfonic acid; NEM, *N*-ethylmaleimide; TPCK, *N*-tosyl-L-phenylalanyl chloromethyl ketone.

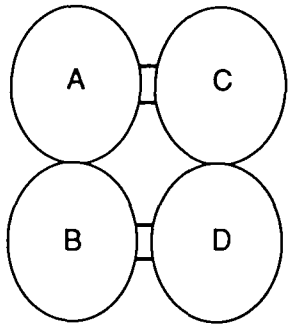

Fig. 1. **Schematic representation of possible tetrameric organizations of dopamine β-monooxygenase.** Four subunits in the tetramer are designated as A, B, C, and D. Two intersubunit disulfide bonds (Cys514-Cys514′ and Cys516-Cys516′) exist at the A-C and B-D dimer interfaces (*8*). For simplicity, possible direct interactions between subunits A and D (or B and C) are not explicitly shown in the models.

(5-sulfo-1-naphthyl)ethylenediamine (I-AEDANS) was provided by Research Organics; DEAE-cellulose (DE-52) was from Whatman; and catalase was obtained as a crystalline suspension (65,000 units/mg) from Boehringer Mannheim. All other chemicals used were of analytical grade.

Enzyme Preparation—Soluble DβM was purified from bovine adrenal medullae according to Ljones *et al.* (*25*) with the following modifications. Catalase (0.1 mg/ml) was included in the homogenization step and the polyethylene glycol fractionation step (*26*). After Con A-Sepharose chromatography the enzyme was further purified by gel chromatography on a TSK-GEL G3000SW column (Tosoh, Tokyo; 7.5×600 mm) using 10 mM MES containing 0.2 M NaCl, pH 6.5. The purified enzyme was concentrated with an Amicon Centriflo CF25 membrane filter to a final concentration of 10-17 mg/ml, and stored at −70°C. The specific activity of the purified enzyme was 15-20 μmol/min/mg under standard assay conditions (see below). The enzyme concentration was determined spectrophotometrically using $E^{1\%}_{280}=12.4$ (*27*). UV-VIS spectra were obtained with a Shimadzu UV-2200 spectrophotometer equipped with a Shimadzu ultra micro cell holder, and 0.1 ml of samples were used for spectroscopic measurements.

Assay of Dopamine β-Monooxygenase Activity—The enzyme activity was assayed at 25°C as the oxygen consumption rate using a Yellow Springs model 53 polarographic oxygen electrode equipped with a Shimadzu C-R4A integrator to amplify the electrode signals, or as the octopamine formation rate using the spectroscopic method described by Wallace *et al.* (*5*) and modified by Ljones *et al.* (*25*). In both assays, the standard assay mixtures (2.7 ml for oxygen assay and 0.25 ml for octopamine assay) contained 10 mM tyramine, 10 mM ascorbate, 0.2 mM fumarate (*24*), 2 μM $CuSO_4$, 0.1 mg/ml catalase, and 50 mM MES buffer (pH 5.1 and ionic strength $I=0.15$). To obtain kinetic parameters, tyramine concentration was varied from 0.1 to 10 mM.

Treatments of Dopamine β-Monooxygenase with DTT and NEM—Enzyme solutions (2 mg/ml) in 50 mM HEPES, pH 7.5, $I=0.15$, were prepared from the enzyme stored at −70°C by gel chromatography on a TSK-GEL G3000SW$_{XL}$ column (Tosoh, 7.8×300 mm) followed by concentration with an Amicon microconcentrator, Microcon 30. To this enzyme solution, an equal volume of 1 mM DTT in the same buffer was added, and the mixture was incubated at 25°C for 0-60 min. The reduction reaction was quenched by the addition of an equal volume of 100 mM NEM (*28, 29*). The samples were immediately subjected to enzyme assay, to SDS-PAGE under nonreducing conditions to determine the extent of the intersubunit disulfide bond cleavage, or to gel chromatography to examine structural changes such as dissociation to dimers or monomers. A high concentration of NEM (50 mM) was used to inhibit possible rearrangement of disulfide bonds during the sample treatment for SDS-PAGE. To determine the free sulfhydryl content of the reduced enzyme, the reduction reaction was acid-quenched by the addition of 1 M HCl, the final pH being around 2 (*29, 30*).

To modify the free sulfhydryl groups of the enzyme pretreated with DTT, an equal volume of 200 mM IAA in the same HEPES buffer containing 4 mM NEM was added to the enzyme-DTT mixtures, and the samples were incubated for 60 min. To instantaneously block all free thiol groups of DTT, NEM (twofold excess over the SH content from DTT) was included, since it took more than 10 min for IAA alone to completely block the SH groups (data not shown).

SH Content Determination—The acid-quenched enzyme preparation (55 μg) was desalted by gel filtration on a Sephadex G-25 column (1×5 cm) equilibrated with 20 mM HCl, then concentrated to about 50 μl with Microcon 30, and lyophilized with a Savant Speed Vac concentrator. The preparation was dissolved in 0.2 ml of 100 mM sodium phosphate, pH 7.2, containing 6 M guanidine-HCl and 10 mM EDTA, and titrated with 5,5′-dithiobis(2-nitrobenzoic acid). A molar extinction coefficient for 2-nitro-5-thiobenzoate of $1.37\times10^4\ M^{-1}\cdot cm^{-1}$ at 412 nm was used (*31*).

Polyacrylamide Gel Electrophoresis—SDS-PAGE was performed on a 7.5% gel according to the method of Laemmli (*32*). To determine the extent of reduction of the intersubunit disulfides, the NEM-quenched samples were incubated with 2% SDS in 62.5 mM Tris-HCl, pH 6.8, at 70-80°C for 3 min, and then subjected to SDS-PAGE under nonreducing conditions. Proteins were stained with Coomassie Brilliant Blue R-250. Densitometry was carried out with a Shimadzu CS-910 densitometer equipped with a Shimadzu C-R4A integrator in a dual-wavelength mode; sample and reference wavelengths were 550 and 500 nm, respectively.

Molecular Weight Determination—The molecular weights of native DβM and enzymes treated with DTT and IAA were determined at 25°C by low-angle laser light scattering measurement combined with gel chromatography (*33*). A TSK-GEL G4000SW$_{XL}$ column (7.8×300 mm), a UV-8011 uv detector, an LS-8000 low-angle laser light scattering photometer, all from Tosoh, and a Shimadzu RID-6A differential refractometer were used. The detectors were connected in tandem in this order. The buffer was 50 mM MES, pH 5.75, $I=0.15$, and the flow rate was 0.5 ml/min. The molecular weight standards used were albumin monomer (bovine serum, $M_r=66{,}300$), catechol 2,3-dioxygenase (*Pseudomonas putida*, $M_r=140{,}000$), and glutamate dehydrogenase (yeast, $M_r=297{,}000$); the former two proteins were purified by using reported methods (*34, 35*), and the third was from Oriental Yeast, Tokyo.

Mapping of Cysteine-Containing Peptides—Native and modified enzyme preparations (10 μg each) were lyophilized and then reduced at 37°C with 5 mM DTT in 7 M guanidine-HCl, 0.2 M Tris-HCl, pH 8.7, and 5 mM EDTA. After 20 min, an equal volume of 20 mM I-AEDANS was added to label free thiols. After 4 h of incubation at 37°C under a nitrogen atmosphere, 2-mercaptoethanol (2 μl) was added to the mixture, and it was dialyzed against 1 liter of 50 mM sodium phosphate buffer, pH 7.5, at 4°C overnight. After dialysis, an equal volume of 8 M urea was added, and then each sample was digested with 1 μg of TPCK-trypsin at 25°C for 48 h. Peptide fragments were separated by reverse-phase HPLC on a Cosmosil 5C18 column (4.6×250 mm, Nacalai Tesque) at 25°C. Fluorescence at 480 nm was monitored with excitation at 340 nm by means of a Shimadzu RF-535 fluorescence detector. To assign cysteine-containing peptide fragments, the native enzyme (1.5 mg) was labeled with I-AEDANS and digested by the same method. The main peptide peaks were collected, and the NH_2-terminal amino acid sequence of each peptide fragment was determined using a protein sequencer, Applied Biosystems Model 470A, with an on-line PTH-amino acid analyzer, Model 120A.

RESULTS

Cleavage of Intersubunit Disulfide Bonds—DβM was incubated with 0.5 mM DTT in 50 mM HEPES buffer, pH 7.5, and the reaction was NEM-quenched at various times. The quenched samples were analyzed by SDS-PAGE under nonreducing conditions (Fig. 2A). Native DβM gave only a single component with an apparent molecular mass of ~160 kDa (0 min in Fig. 2A), this band being the disulfide-linked dimer (S-S dimer). With increase in the reaction time, the S-S dimer content decreased, and concomitantly three monomer bands, one major (70 kDa) and two additional bands (74 and 66 kDa), appeared and their contents increased. The results indicate that the intermolecular disulfide bonds were cleaved by DTT. After 15-min incubation, half of the intersubunit disulfide bonds were reduced (Fig. 2C). The cleavage rate of the intermolecular disulfide bonds by DTT increased almost linearly with an increase of DTT concentration from 0.5 to 10 mM (data not shown). However, substantial reduction of the intramolecular disulfide bonds was also observed.

To examine whether the DTT treatment caused a conformational change of DβM, we analyzed samples by gel chromatography at various incubation times. As shown in Fig. 2B, after 15-min incubation with 0.5 mM DTT, DβM eluted as essentially a single peak at the same elution volume as native DβM, although trace amounts of species with larger and smaller Stokes radii than that of native DβM were detected. These species increased substantially when the enzyme was incubated with DTT for a longer time (~60 min) or with a higher concentration of DTT (data not shown). The enzyme activity did not change during the 15-min incubation (Fig. 2C).

To determine the free SH content of DβM as a function of the incubation time with DTT, the reaction was acid-quenched at various times. Native DβM contained no free thiols. The SH content increased with incubation time, and was 1.1 mol/mol subunit after 15 min (Fig. 2C). Since the cleavage of half of the intermolecular disulfide bonds (Fig. 2, A and C) resulted in 1 mol SH/mol subunit, it was concluded that the intersubunit disulfide bonds were selectively reduced by 15-min incubation with 0.5 mM DTT (Fig. 2C). Thus, in the following study, we treated DβM with 0.5 mM DTT for up to 15 min to selectively cleave the intersubunit disulfide bonds.

Modification of Reduced Intersubunit Disulfides—Following the incubation with 0.5 mM DTT for various times (0–15 min), DβM was treated with 0.1 M IAA containing 2 mM NEM for 60 min. The free SH groups of unreacted DTT were immediately blocked with a twofold excess of NEM (data not shown). After 60-min incubation with IAA, the samples were subjected to gel chromatography (Fig. 3A). Depending on the preincubation time with DTT, a novel species of DβM with a small Stokes radius compared to that of native enzyme was formed. For simplicity, we designate this species as E2 and the one with the same Stokes radius as native DβM as E1. As shown in Fig. 3B, the time course of E2 formation was almost linearly correlated with that of the intersubunit disulfide reduction (the decrease of the S-S dimer content). The result indicates that the structure of DβM changed due to the modification by IAA of the reduced cysteine residues

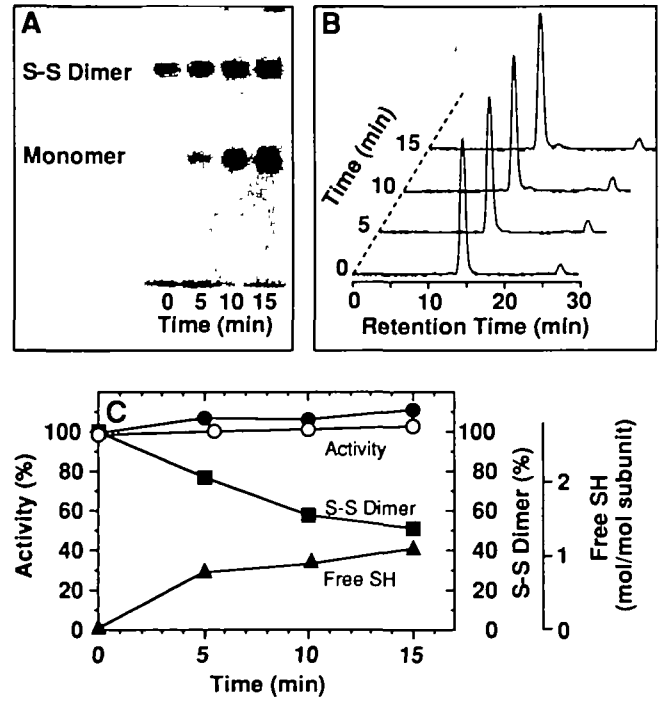

Fig. 2. **Reductive cleavage of intersubunit disulfide bonds by DTT under native conditions.** Dopamine β-monooxygenase (1.0 mg/ml) was incubated with 0.5 mM DTT at 25°C for 0–15 min in 50 mM HEPES, pH 7.5, I=0.15. The reaction was stopped by the addition of an equal volume of NEM (100 mM in the same buffer). A: The reduced enzyme (0.5 μg) was subjected to SDS-PAGE (7.5% separating gel) under nonreducing conditions. B: The reduced enzyme (4 μg) was subjected to gel filtration on a TSK-GEL G3000SW$_{XL}$ column (7.8×300 mm) equilibrated with the same buffer. The flow rate was 0.4 ml/min and the absorbance at 280 nm was monitored. C: The activity (●), the S-S dimer content (■), and the free SH content (▲) were determined at various incubation times with DTT. The time course of the activity in the absence of DTT (○) was also determined. In the case of the SH content assay, the reduction reaction was acid-quenched.

responsible for the intersubunit disulfide bonds.

As shown in Fig. 3C, the enzyme activity gradually decreased during the incubation with IAA. A similar extent of inactivation was also observed when the enzyme was treated with IAA alone (Fig. 3C). Thus, this inactivation was not due to modification of the disulfides. To determine the kinetic properties of E1 and E2, we isolated both DβM species by gel chromatography and further purified them by rechromatography. From 190 μg of native DβM we obtained 50 and 30 μg of pure E1 and E2, respectively (see the inset of Fig. 4A). Using these E1 and E2 preparations we examined the kinetics by two assay methods. Both E1 and E2 showed a hyperbolic dependence of the initial velocity on tyramine concentration. The results are summarized in Table I. The K_m values of E1 and E2 were similar to that of native DβM. E1 and E2 exhibited similar V_{max} values, but the values were about 70% of that of native DβM. The decrease in V_{max} values corresponds to the net decrease in activity shown in Fig. 3C, suggesting that the modification of the intermolecular disulfide bonds did not affect the activity, but modification by IAA of amino acid residues not involved the disulfide bonds caused the inactivation of the enzyme.

Characterization of E2—The molecular weight of E2 was determined to be 1.3×10^5 by low-angle laser light scattering photometry coupled with high-performance gel chromatography (*33, 35*), whereas those of native DβM and E1 were found to be 2.6×10^5 (Fig. 4A). Since the subunit molecular weight estimated from cDNA is 6.5×10^4 (*11-13*), we can conclude that E2 is a dimer and E1 is a

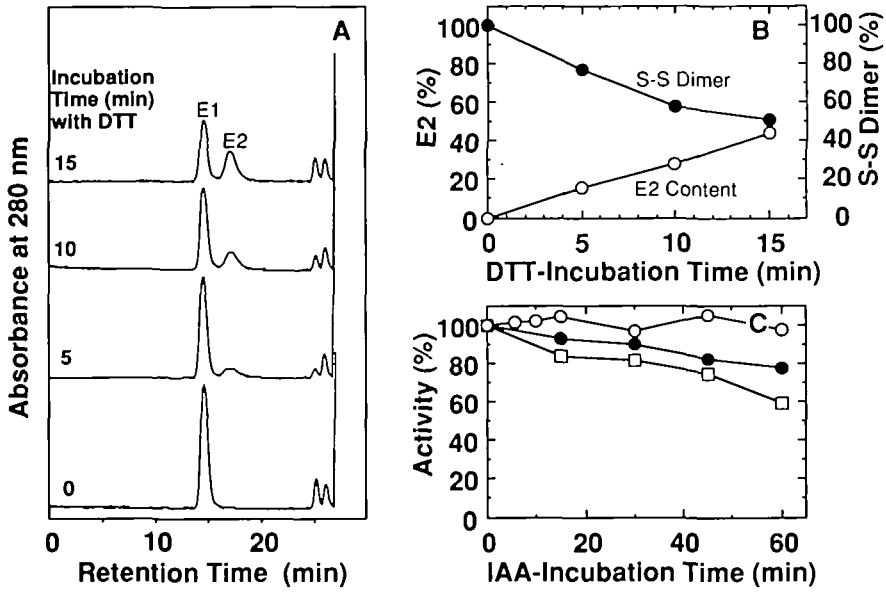

Fig. 3. **Formation of a novel species of dopamine β-monooxygenase by iodoacetamide treatment under native conditions of the DTT-reduced enzyme.** The native enzyme (1.0 mg/ml) was first incubated with 0.5 mM DTT in 50 mM HEPES (pH 7.5, $I=0.15$) at 25°C for 0-15 min and then with 100 mM IAA and 2 mM NEM in the same buffer for 0-60 min. A: The enzyme samples pretreated with DTT for 0-15 min were incubated with IAA for 60 min, and then each (4 μg) was analyzed by gel filtration on a TSK-GEL G3000SW$_{XL}$ column as described in the legend to Fig. 2. The faster- and slower-eluted species are designated by E1 and E2. B: The contents of E2 and S-S dimer were determined at various preincubation times with DTT. IAA treatment was done for 60 min. Open and filled circles show E2 and S-S dimer contents, respectively. C: The activity of the enzymes pretreated with DTT for 0 min (●) and 15 min (□) was determined at various incubation times with IAA. The time course of the activity of native enzyme (○) in the absence of DTT and IAA is also shown.

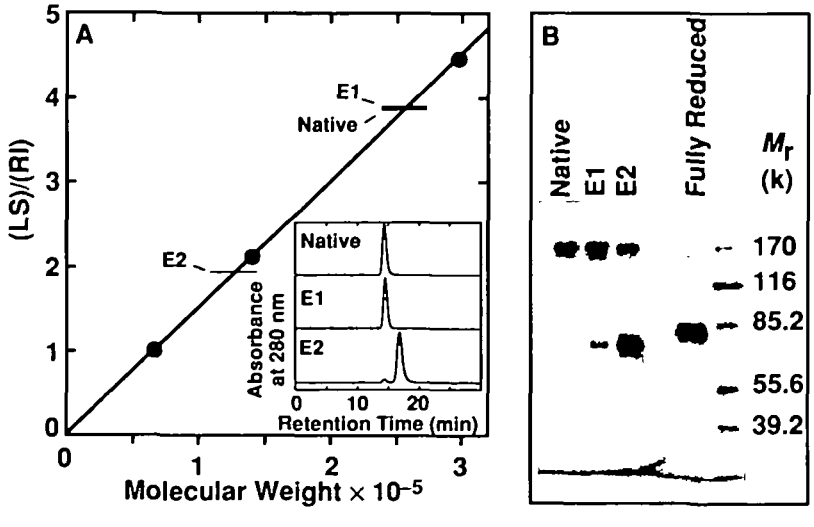

Fig. 4. **Characterization of a novel species of dopamine β-monooxygenase produced by treatment with DTT and NEM under native conditions in terms of S-S dimer content and molecular weight.** A: E1 and E2, 10 μg each, were each subjected to gel filtration on a column of TSK-GEL G4000SW$_{XL}$ (7.8×300 mm) at 25°C, and the components in the eluates were detected by a UV monitor, a low-angle laser light scattering photometer, and a differential refractometer (RI monitor) in this order. The elution buffer was 50 mM MES, pH 5.72, $I=0.15$, and the flow rate was 0.5 ml/min. Bovine serum albumin monomer (66,300), catechol 2,3-dioxygenase (140,000), and yeast glutamate dehydrogenase (297,000) were used as standards. The ratio of the output of the light scattering photometer (LS) to that of the RI monitor (RI) was plotted against molecular weight. The inset shows the elution profiles of each enzyme species detected by the UV monitor. B: Native enzyme, E1 and E2 (0.5 μg each) were analyzed by SDS-PAGE (7.5% gel) under nonreducing conditions. For comparison, native enzyme and marker proteins (kits from Boehringer Mannheim) were fully reduced according to Laemmli (*32*) and subjected to SDS-PAGE.

TABLE I. **Comparison of kinetic parameters for native dopamine β-monooxygenase, E1, and E2.** Native DβM (0.19 mg) was incubated with 0.5 mM DTT for 15 min, and then treated with 100 mM IAA containing 2 mM NEM for 60 min (see the legend to Fig. 3). The E1 and E2 species were isolated by preparative gel filtration on a TSK-GEL G3000SW$_{XL}$ column as described in the legend to Fig. 2, and purified by rechromatography on the same column. The values of K_m and V_{max} were obtained from *v versus v/s* plots, where *v* is the initial velocity and *s* is tyramine concentration. Dioxygen concentration in air-saturated buffer of 50 mM MES, pH 5.1, was determined to be 290 μM at 25°C by an oxygen electrode (*35*).

Enzyme species	O_2 consumption		Octopamine formation	
	K_m (mM)	V_{max} (μmol/min/mg)	K_m (mM)	V_{max} (μmol/min/mg)
Native enzyme	0.51	17.7	0.77	18.5
E1	0.58	12.9	0.64	13.6
E2	0.46	11.4	0.78	14.2

tetramer. Next, the disulfide-linked dimer contents of E1 and E2 were determined by SDS-PAGE under nonreducing conditions (Fig. 4B). E2 gave mainly the monomer bands, and E1 gave the disulfide-linked dimer band as a dominant component. The results show that E2 is an AB (or AD) dimer consisting of two subunits associated through noncovalent interaction. E2 was stable for at least several days at 4°C in terms of both activity and dimeric structure.

Identification of Cysteine Residues Modified—To assign the IAA-modified cysteine residues of E1 and E2, all disulfides of the native enzyme, E1 and E2 were reduced with DTT under denaturing conditions, and then all free thiols were labeled with I-AEDANS. After TPCK-trypsin digestion, cysteine-containing peptides from each enzyme sample were separated by reverse-phase HPLC (Fig. 5). Of all cysteine-containing peptides from native DβM, a single peptide indicated by an arrowhead in Fig. 5 was absent in the peptide map of E2, and only weakly detected in that of E1, whereas the other peptides were detected in essentially the same amount among native DβM, E1, and E2. The NH_2-terminal sequence of the peptide was determined to be FNSEEC*TC*P, where C* is an AEDANS-labeled cysteine residue and it could not be detected as a peak upon usual PTH-amino acid analysis by a protein sequencer. Judging from the primary structure of DβM (*11-13*), the C*s were Cys 514 and Cys 516. That is, these cysteines participate in the intersubunit disulfide bridges, and can be selectively reduced by DTT and modified by IAA.

DISCUSSION

In the present study we examined what role(s) the intermolecular disulfide bonds of DβM play in the activity and structural integrity of DβM. The selective cleavage of the intermolecular disulfide bonds by DTT alone did not change the activity or tetrameric structure of DβM. However, when the reduced cysteine residues, which were confirmed to be Cys 514 and Cys 516, were chemically modified by IAA, DβM dissociated to an active dimeric species. The dimer was thought to be stabilized by a noncovalent subunit-subunit interaction at the A-B (or A-D) interface. The dimer exhibited similar kinetic properties to the tetramer. The results clearly show that the intermolecular disulfide bonds are not essential for intact conformation of the active site of DβM. Considering the strict conservation

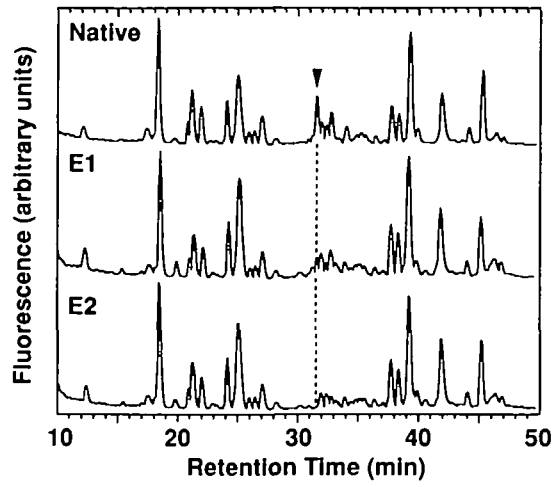

Fig. 5. **Identification of cysteine residues responsible for intermolecular disulfide bonds.** Native enzyme, E1 and E2, 10 μg each, were fully reduced, and the free thiols were labeled with I-AEDANS as described in "EXPERIMENTAL PROCEDURES." Tryptic peptide fragments containing AEDANS-labeled cysteine residue(s) were separated at 25°C by reverse-phase HPLC on a column of Cosmosil 5C18 (4.6×250 mm) and detected on the basis of the fluorescence due to AEDANS (excitation at 340 nm, emission at 480 nm). Elution was carried out with a linear H_2O-acetonitrile gradient (0.575% per min and the initial concentration of 14%) containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min.

of the cysteine residues responsible for the intermolecular disulfide bonds among species (*10-14*), the intermolecular disulfide bonds and the cysteine residues required for the disulfide bonds may play an important role in the protein folding process of DβM *in vivo*.

The reduction kinetics of the intermolecular disulfide bonds of DβM is relatively complex, because there are many possible intermediates of the reduced DβM due to the presence of two intermolecular disulfide bonds close together (see Fig. 6). DβM can be regarded as a dimer consisting of two identical disulfide-linked dimers, AC and BD, and no strong cooperativity between AC and BD has been found. Thus, we presume that the intersubunit disulfide bonds of the AC and BD units react independently and identically with DTT. Therefore, to analyze the results, it is enough to consider the reaction of the intersubunit disulfide bonds of the AC dimer with DTT. After incubation with DTT, the AC dimer may contain five possible states of the intersubunit disulfide bridges (Fig. 6): both intersubunit disulfide bonds are intact (State 1); one of the two intersubunit disulfide bonds is cleaved (State 2); one intersubunit disulfide bond exists between Cys514 and Cys516′, and the other two cysteine residues are in reduced form (State 3); Cys514 and Cys516 of one subunit form an intrasubunit disulfide bond and those of the other subunit are in reduced form (State 4); both intersubunit disulfide bonds are cleaved (State 5). The SH content of the dimer is expressed by the following equation:

$$\text{SH (mol/mol of dimer)} = 2P_2 + 2P_3 + 2P_4 + 4P_5 \qquad (1)$$

where P_i is the probability of observing the AC dimer in the state i, $i=2$-5. The amount of S-S dimer which can be determined by SDS-PAGE under nonreducing conditions (Fig. 2) is given by:

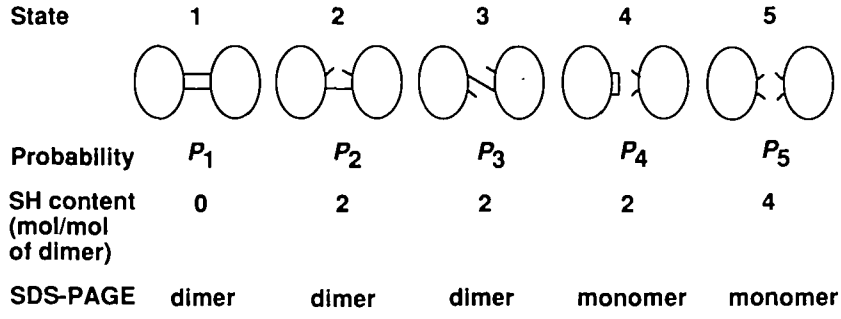

Fig. 6. **Possible states of the AC (or BD) dimer after incubation with DTT.** Intersubunit disulfide bonds are depicted by lines connecting the two subunits, and free SH groups of reduced cysteine residues by short lines. The long line in State 3 shows the intersubunit disulfide bond between Cys514 and Cys516′. In State 4, Cys514 and Cys516 of the left subunit form an intrasubunit disulfide bond.

$$\text{S-S dimer (\%)} = 100\ (P_1 + P_2 + P_3) \quad (2)$$

Since each dimer is in one of the five states and the summation of all the individual probabilities of the states is 1,

$$P_1 + P_2 + P_3 + P_4 + P_5 = 1 \quad (3)$$

Besides states 1-5, the following two states are also possible: two intersubunit disulfide bonds, Cys514-Cys516′ and Cys516-Cys514′, exist; and two intrasubunit disulfide bonds, Cys514-Cys516 and Cys514′-Cys516′, exist. However, even if these states are taken into consideration in addition to the above five states, the above approach remains valid.

After 15-min incubation with 0.5 mM DTT, the content of free SH group was 2 mol/mol of dimer (1 mol/mol of subunit), that is, 50% of the intersubunit disulfide bonds were cleaved (Fig. 2C), and the amount of S-S dimer (AC dimer) detectable by SDS-PAGE was 50% (Fig. 2, A and C). Then, using the relations of Eqs. 1 and 2, the following equations are obtained:

$$P_2 + P_3 + P_4 + 2P_5 = 1 \quad (4)$$

$$P_1 + P_2 + P_3 = 0.5 \quad (5)$$

From the relations of Eqs. 3-5, we obtain:

$$P_1 = P_5 \quad (6)$$

$$P_4 = P_2 + P_3 \quad (7)$$

$$P_4 + P_5 = 0.5 \quad (8)$$

If formation of an intrasubunit disulfide bond between Cys514 and Cys516 is difficult owing to steric hindrance (*i.e.*, $P_4 = 0$), then, $P_2 = P_3 = 0$ and $P_1 = P_5 = 0.5$. This means that, when one of the two intersubunit disulfide bonds of the AC dimer is cleaved, the other is rapidly reduced with DTT and thus the singly bridged AC dimer can not exist as a stable species. To examine the reduction mechanism in detail, preparation of DβM lacking one of the two cysteine residues responsible for the intersubunit disulfide bonds by site-directed mutagenesis and comparison of the DTT reduction kinetics of native and engineered DβM may be helpful.

The net 50% reduction of the intersubunit disulfide bonds affected neither the tetrameric structure nor the activity of DβM (Fig. 2). Because about 25% of DβM is expected to consist of two AC (or BD) dimers with completely cleaved intersubunit disulfide bonds ($P_4 + P_5 = 0.5$ and $0.5 \times 0.5 = 0.25$; Fig. 6), the result indicates that the subunit-subunit interaction at the A-C interface is strong even if the intersubunit disulfide bonds are reduced, and that the intersubunit interaction at the A-B (or A-D) interface is not weakened by reduction of the disulfide bonds. However, when the reduced cysteine residues responsible for the intersubunit disulfide bonds were modified with IAA, the tetramer dissociated to a noncovalently associated dimer species (Figs. 3 and 4). All other SH group-modifying reagents tested, NEM, iodoacetate, and I-AEDANS, also induced this dissociation (data not shown). The dimer is active and its kinetic properties are similar to those of native DβM, and no further dissociation to the monomer occurs. Taking the bulkiness and charge of the modifying reagents into account, the obtained dimer is possibly the AB (or CD) dimer. The subunit-subunit interaction at the A-C interface was completely disrupted by the presence of chemically modified cysteine residues at the interfacial region, whereas the intersubunit interaction at the A-B (or A-D) interface was not significantly affected. This is probably because the conformational change of each subunit induced by the modification was local in nature, as would be expected from the fact that the enzyme activity was not significantly affected by the modification (Table I), although further investigation is needed to characterize the nature of the dimer.

Dimeric forms of DβM have been obtained from human plasma and pheochromocytoma (*36-38*). The dimer and tetramer from human plasma were active and mutually noninterconverting (*37*). In the present study, we showed that the subunit-subunit interaction at the A-C interface was disrupted by the chemical modification of the intermolecular disulfide bonds, whereas that at the A-B (or A-D) interface remained intact after the modification. Therefore it is possible that the intersubunit disulfide bonds of the dimer in the blood plasma are chemically modified by physiological compounds. Our results provide a clue to elucidate the formation process of the quaternary structure of DβM, as well as the formation mechanism and the physiological (or pathological) significance of the active dimer *in vivo*.

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