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Fluorescence Quenching of Aminopeptidase-I from Pronase

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Aminopeptidase-I from pronase contains five tryptophan residues, determined by modification with *N*-bromosuccinimide and MCD measurments. The fluorescence of the enzyme is quenched by acrylamide, a neutral quencher, but not by iodide, an anionic quencher. The quenching constant for acrylamide is about one tenth that of acetyltryptophan in aqueous solution because of the steric effect of the large protein molecule. The inaccessibility of iodide to the tryptophans is probably due to the anionic environment of the fluorophores created by the large content of Asp and Glu in the enzyme. Aminopeptidase-I is quenched by Cu(II) and Co(II) through the formation of a nonfluorescent ground-state complex. The results indicate that there are three accessible tryptophans for Cu(II) and two for Co(II) suggesting that some tryptophans are buried inside the enzyme and are thus inaccessible to the quencher. In the presence of a competitive inhibitor, the enzyme contains one fewer accessible tryptophan for Cu(II) indicating that this tryptophan is located in the active center. The CD spectrum of the enzyme in the far UV region is unaltered by the presence of Ca(II), whereas that in the aromatic region is enhanced significantly. The perturbation of the CD spectrum probably results from the local conformational change induced by Ca(II) or from the rigidity of the tryptophan being enhanced by the bound Ca(II).

INTRODUCTION

There are three major classes of proteases: endopeptidases (e.g. thermolysin), carboxypeptidases, and aminopeptidases. The structural bases and mechanisms of action of carboxypeptidase A[1] and thermolysin[2] have already been well doccumented. In contrast, the mechanism of action of aminopeptidase has not been fully elucidated and great efforts have been attempted to collect further structural and catalytic informations for this class of enzyme in order to delineate its catalytic mechanism and structure-function relationships.

Pronase E (*streptomyces grisens* neutral protease, EC 3.4. 24.4), which contains several exo- and endopeptidases, hydrolyzes practically all peptide linkages in proteins and peptides. We purified to homogeneity two stable, monomeric aminopeptidases with similar molar masses 32000 from pronase.[3] These two enzymes are capable of cleaving the *N*-terminal peptide bond of the substrate without the assistance of metal ions and exhibit a strong preference for a large, hydrophobic *N*-terminal amino acid side chain (e.g., Leu or Phe). The presence of a calcium ion tends to enhance their thermal stabilities, substrate affinities and turnover rates.[3] Chemical modificaion of the enzymes[4] revealed that a readily modified histidine residue with pK_a 6.9 is essential for enzyme catalysis, whereas another histidine residue with pK_a 7.7 serves as a Ca(II)-binding ligand and the bound Ca(II) is likely to be involved in substrate binding and turnover. Although some general properties of these enzymes are known, their mechanisms of action (i.e. how the calcium ion, the histidines or other amino-acid residues are involved in enzyme catalysis along the reaction pathway) are unclear. If the enzyme contains an intrisic chromophore, especially that located at or near the active center, to serve as a sensitive probe, more structural and catalytic informations is easily collected to aid understanding the reaction mechanism.

Intrinsic protein fluorescence is extensively used as a powerful tool to measure binding (of substrate, inhibitor, or other cofactor), monitor conformational changes, or follow a reaction. The fluorescence of most proteins is dominated by the tryptophans; these residues are highly sensitive to solvent polarity. As a result, the emission spectra of Trp residues can reflect the polarity of their environment. The sensitivity of Trp fluorescence to quenchers allows determination of the accessibility of Trp residues in proteins by quenching measurements. In this work, we determined the number of Trp residues in pronase aminopeptidase by chemical modification with *N*-bromosuccinimide (NBS) and by magnetic circular dichroism (MCD) measurments. We investigated quenching of Trp fluorescence of the enzyme by quenchers such as acrylamide, iodide, and metal

Dedicated to Professor Fa-Ching Chen (陳發清) on the occasion of his eightieth birthday.

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MATERIALS AND METHODS

Materials

Pronase E, all metal salts, acrylamide, NaOH, HCl, NaCl and KI were purchased from E. Merck (Darmstadt, Germany). All buffers (Tris, Hepes), urea, NBS, dialysis tubing, acetic acid and L-leucine hydroxamate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Buffers and substrates were prepared in water purified to a specific resistance 18 MΩ/cm by a Milli-Q system (Millipore) and further rendered free of metals with Chelex-100 resin (Bio-Rad) and dithizone extraction as described;[5] dialysis tubing was treated as recommended.[6] The aminopeptidases from pronase were purified as described previously.[3]

Spectral measurements

Absorption and fluorescence spectra were measured on a Hitachi U-3210 spectrophotometer (Hitachi, Japan) and an SLM 8000C spectrofluorimeter (SLM Instruments, USA), respectively. The CD and MCD spectra were conducted on a JASCO-720 spectropolarimeter (Japan Spectroscopic Co.).

Chemical modification of the enzyme with NBS

Modification of Trp residues with NBS was performed as described.[7] Aliquots of freshly prepared NBS solutions were added sequentially at 25 °C to aminopeptidase (2 mL, 22 μM) in a quartz cuvette in acetate buffer (0.1 M, pH 4.0) which also contained urea (10 M) to unfold the protein to make all Trp residues accessible to the modification reagent. The absorption spectra of the enzyme were measured for each addition of NBS (the solution was stirred for 15 min for each addition of NBS to assure the completion of the reaction) and the number of modified Trp residues was estimated from variation of absorbance at 280 nm.

MCD measurement of the enzyme

Trp is the only naturally occurring amino acid that gives a positive MCD band (at 290-294 nm). Furthermore, this band being completely separate from overlapping contributions by other bands provides a convenient means to determine the Trp content in proteins. The MCD spectrum of aminopepitdase (21 μM) in Tris buffer (1 mM, pH 8.), NaCl (0.02 M) was measured using a 5 mm quartz cell in a magnetic field 0.8 T; the number of Trp residues was estimated by the MCD intensity at 293 nm using a molar absorptivity per tesla 2.8 $M^{-1}cm^{-1}T^{-1}$.[8]

Quenching of the Trp fluorescence of the enzyme

Quenching mechanisms of two types are considered here. The dynamic quenching is due to deactivation of the excited state fluorophore through collision with a quencher; static quenching resulted from the formation of a ground state complex between the fluorophore and the quencher. Quenching of both types is described by the Stern-Volmer equation:[9]

$$F_o/F = 1 + K_{sv}[Q] \quad (1)$$

in which F_o and F are the fluorescent intensities of the fluorophore in the absence and presence of quencher, respectively; [Q] is the concentration of the quencher; K_{sv} the Stern-Volmer constant, is equal to the formation constant of fluorophore and quencher in static quenching and to $\tau_o k_q$ in dynamic quencing; k_q is the bimolecular collisional quenching constant and τ_o is the lifetime of the fluorophore in the absence of quencher.

Quenching of two populations of fluorophores, one of which is inaccessible to quencher, can be analyzed according to a modified form of the Stern-Volmer equation:[10]

$$F_o/\Delta F = 1/f_a + 1/(f_a K[Q]) \quad (2)$$

in which $\Delta F = F_o - F$, f_a is the fraction of fluorophore that is accessible to quencher and K is the Stern-Volmer quenching constant of the accessible fraction. Therefore, measurements of fluorescent intensities at varied quencher concentration allow the determination of the quenching constant and the number of accessible Trp residues in proteins using linear plots of Eqs. (1) and (2).

RESULTS AND DISCUSSION

As the two aminopeptidases from pronase are similar,[3] only aminopeptidase-I (AP-I) was investigated. Modification of *N*-acetyltryptophamide (NATA), a model compound of Trp residue in protein, with NBS in acetate (0.1 M, pH 4.0), urea (10 M), NaCl (0.02 M) at 25 °C gave a molar absorptivity $(4.14 \pm 0.03) \times 10^3$ $M^{-1}cm^{-1}$ for the absorbance change at 280 nm, in excellent agreement with the literature value 4.20×10^3 $M^{-1}cm^{-1}$.[11] Two isosbestic points at 264 and 298 nm were observed during reaction of NATA with NBS. Modification of AP-I (20 μM) with successive addition of NBS is illustrated in Fig. 1. The number of Trp residues was estimated to be 4.92 ± 0.07. Two sets of isosbestic points were observed during the modification (Fig. 1). The first set

(at 267 and 298 nm) accounts for the modification of two Trp residues and the second set (at 261 and 298 nm) for the remaining three Trp residues. The results indicate that, even in the unfolded enzyme, two populations of Trp residues with distinct environments, hence distinct absorption spectra, were created by nearby amino-acid residues surrounding the tryptophans.

The MCD spectrum of AP-I (21 μM) in Tris buffer (1 mM, pH 8.0) at 25 °C is shown in Fig. 2. The spectrum exhibits a maximum at 293 nm and a minimum at 272 nm similar to those observed for other Trp-containing proteins.[12] The number of Trp residues was estimated to be 4.9 from the MCD intensity at 293 nm using a $\Delta\varepsilon_M$ = 2.8 $M^{-1}cm^{-1}T^{-1}$. Therefore, the enzyme contains five Trp residues.

AP-I exhibits an absorption maximum at 280 nm and a fluorescence maxima (λ_{ex} = 280 nm) at 330 nm. The fluorescence maximum is blue-shifted compared to that of tryptophan in aqueous soluton suggesting that the average microenvironments of the tryptophans in the enzyme are moderately hydrophobic.[13] The fluorescence intensity (λ_{ex} = 295 nm, λ_{em} = 330 nm) of AP-I was be quenched by acrylamide, a neutral quencher, but not by I^-, an anionic quencher. The Stern-Volmer plots (Eq. (1)) for the quenching of AP-I (1 μM) by the two quenchers in Hepes buffer (10

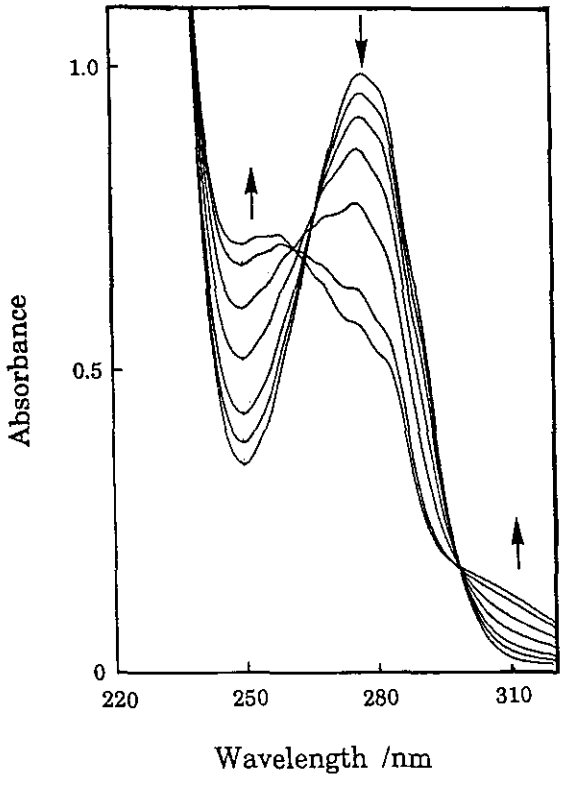

Fig. 1. Absorption spectra for the modification of aminopepitdase-I (22 μM) with N-bromosuccinimide in acetate-buffer (0.1 M, pH 4.0) and urea (10 M) at 25 °C. The arrows indicate the directions for the alterations of the absorption spectra. [NBS] = 0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30 mM. The modification resulted in decreased absorbance at 280 nm and increased absorbance at 250 nm.

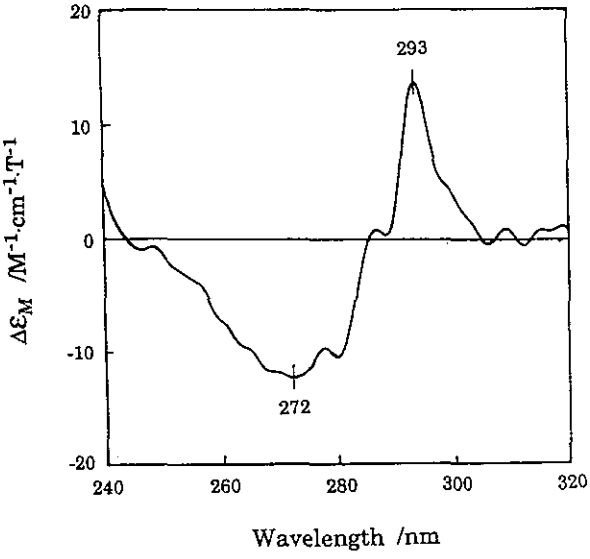

Fig. 2. MCD spectrum of aminopeptidase-I (21 μM) in the presence of a magnetic field (0.8 T) in Tris-buffer (1 mM, pH 8.0), NaCl (0.02 M), at 25 °C using a quartz cuvette (5 mm). The spectrum exhibits a positive peak at 293 nm and a negative peak at 272 nm.

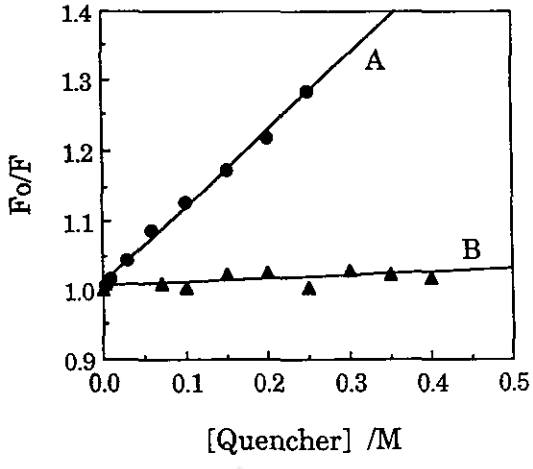

Fig. 3. Stern-Volmer plots for the fluorescence quenching (λ_{ex} = 295 nm, λ_{em} = 330 nm, band pass = 3 nm) of aminopeptidase-I (1 μM) in Hepes-buffer (10 mM, pH 8.0) and NaCl (0.1 M) at 25 °C for (A) acrylamide and (B) KI. The quenching constant is 1.2 M^{-1} for acrylamide.

mM, pH 8.0), NaCl (0.1 M), at 25 °C are shown in Fig. 3. A linear Stern-Volmer plot for acrylamide indicates that all tryptophans in AP-I are accessible to a neutral quencher; the slope gives a quenching constant of 1.2 M^{-1}. This quenching constant is much smaller than that of acetyltryptopan in water (K_{SV} = 28 M^{-1})[14] indicating a poor quenching efficiency probably due to the steric effect from the large protein molecule. The quenching constant is similar to those observed for the tryptophans in proteins (e.g., K_{SV} = 1.0 M^{-1} for chymotrypsin[15]). The fluorescence intensity of AP-I is essentially unaltered by the addition of I^- (Fig. 3B). High contents of Asp and Glu in the enzyme[1] may create an anionic environment around the tryptophans preventing access of iodide due to an electrostatic repulsion.

The fluorescence of AP-I is also quenched by Cu(II) or Co(II) but their Stern-Volmer plots are nonlinear and curved downward (data not shown), indicating the existence of two populations of tryptophans. The modified Stern-Volmer plot (Eq. (2)) for the fluorescence quenching of AP-I by Cu(II) is depicted in Fig. 4A. The values of K and f_a are determined to be 3.8 × 10^5 M^{-1} and 0.64, respectively. The value of K is much larger than that possible for a diffusion-controlled reaction, and therefore the quenching is due to the formation of nonfluorescent complexes between Cu(II) and the tryptophans. A f_a value 0.64 corresponds to 3.2 accessible fluorophores, indicating that about two tryptophans are buried in the interior of the enzyme and are thus inaccessible to the quencher. L-leucine hyroxamate is a strong competitive inhibitor of AP-I (K_i = 0.030 mM at pH 8.0).

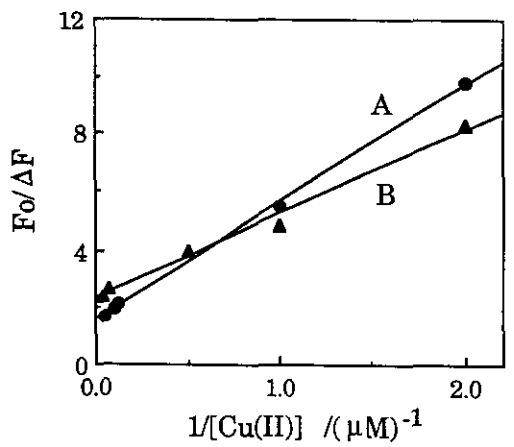

Fig. 4. Modified Stern-Volmer plots for the fluorescence quenching (λ_{ex} = 295 nm, λ_{em} = 330 nm, band pass = 3 nm) of aminopeptidase-I in the absence (A) or presence (B) of L-leucine hydroxamate (1 mM) by Cu(II). Other conditions are as described in Fig. 3.

The presence of L-leucine hyroxamate (1 mM) in AP-I (1 μM) solution assured the occupancy of 97% of the active center of AP-I by the inhibitor. The modified Stern-Volmer plot for the fluorescence quenching of AP-I (1 μM) in Hepes buffer (10 mM, pH 8.0), L-leucine hyroxamate (1 mM), NaCl (0.1 M) at 25 °C is given in Fig. 4B. K and f_a are estimated to be 8.5 × 10^5 M^{-1} and 0.44, respectively. The results indicate that one fewer tryptophan is accessible to the quencher when the active center becomes occupied by the competitive inhibitor. Hence this tryptophan is located in the active center of the enzyme. The modified Stern-Volmer plot for the fluorescence quenching of AP-I by Co(II) gives a quenching constant of 1.9 × 10^2 M^{-1} and 1.9 accessible tryptophans. The results show that the quenching efficiency (in terms of K) of Co(II) is much less than that of Cu(II).

As Ca(II) plays an important role in enzyme catalysis,[3] the Ca(II)-binding site is also located in the active center. The CD spectrum of AP-I in the far UV region (190-240 nm) is unchanged by the presence of Ca(II) indicating that no gross change in secondary structure of the protein occurs. However, the CD spectrum of AP-I in the near W region (240-320 nm) is found to be enhanced by the additon of Ca(II) as illustrated in Fig. 5. The difference spectrum

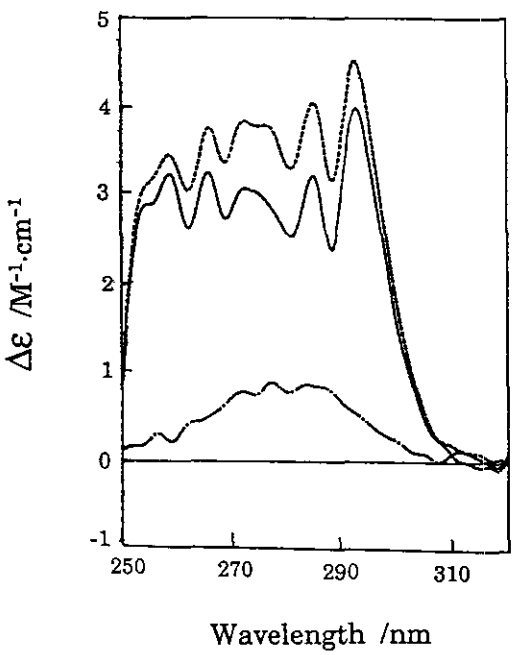

Fig. 5. CD spectra of aminopeptidase-I (22.2 mM) in the absence (solid line) or presence (dashed line) of $CaCl_2$ (5 mM), in Tris-buffer (5 mM, pH 8.0) and NaCl (0.1 M) at 25 °C in a quartz cuvette (10 mm). The difference spectrum (dot-dashed line) exhibits a positive band centered about 280 nm.

shows a positive band ranging from 250 to 310 nm with a maximum around 280 nm. This band is most probably due to the contribution of the tryptophan in the active center resulting from a local conformational alteration induced by presence of Ca(II). Alternatively, the altered CD spectrum may result from enhanced regidity of the Trp residue due to nearby bound Ca(II).

Our results have indicated the presence of Trp in the active center of AP-I. Beacuse the spectral properties of this Trp will likely be changed dramatically upon binding of the substrate or inhibitor, this may serve as a sensitive probe for further catalytic and structural ivestigations. Work already in progress has shown that modification of one Trp residue with *N*-bromosuccinimide causes an inactivation of AP-I indicating the involvement of a Trp residue in enzyme catalysis.

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Key Words

Fluorescence quenching; Pronase aminopeptidase; Modification; MCD; Accessible tryptophans.

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