Mitochondrial processing peptidase (MPP), a metalloendopeptidase consisting of α- and β-subunits, specifically cleaves off the N-terminal presequence of the mitochondrial protein precursor. Structural information of the substrate bound to MPP was obtained using fluorescence resonance energy transfer (FRET) measurement. A series of the peptide substrates, which have distal arginine residues required for effective cleavage at positions −7, −10, −14, and −17 from the cleavage site, were synthesized and covalently labeled with 7-diethylaminocoumarin-3-carboxylic acid at the N termini and \(N,N'\)-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazole-4-yl)ethylenediamine (IANBD) at position +4, as fluorescent donor and acceptor, respectively. When the peptides were bound to MPP, substantially the same distances were obtained between the two probes, irrespective of the length of the intervening sequence between the two probes. When 7-diethylamino-3-(4′-maleimidylphenyl)-4-methyl coumarin was introduced into a single cysteine residue in β-MPP as a donor and IANBD was coupled either at the N terminus or the +4 position of the peptide substrate as an acceptor, intermolecular FRET measurements also demonstrated that distances of the donor-acceptor pair were essentially the same among the peptides with different lengths of intervening sequences. The results indicate that the N-terminal portion and the portion around the cleavage site of the presequence interact with specific sites in the MPP molecule, irrespective of the length of the intervening sequence between the two portions, suggesting the structure of the intervening sequence is flexible when bound to the MPP.

Numerous mitochondrial proteins are translated on cytoplasmic ribosomes as larger precursors. An N-terminal presequence of the mitochondrial protein precursor functions as a targeting signal for their transport to mitochondria (1–3). During import of precursors into mitochondria, the presequences are recognized by multiple proteins (4, 5), such as molecular chaperones, translocases of the mitochondrial outer and inner membranes, and peptidases from inside mitochondria. Despite the identification of various proteins that interact with the mitochondrial precursors, the mechanism of recognition of the presequence by these components has not yet been understood.

The lack of sequence homology of the presequences, even though they are characterized by the positively charged residues and the formation property of amphiphilic α-helices, has inhibited clarification of the recognition mechanism (6).

Mitochondrial processing peptidase (MPP), located in the matrix of the mitochondria, cleaves off most presequences of the imported precursors. MPP consists of two structurally related subunits, α-MPP and β-MPP. Complex formation with the two subunits is essential for both enzymatic activity (7, 8) and substrate binding (9).

Earlier studies indicated that some structural elements of the presequence are required for recognition by MPP. An arginine residue at position −2, the so-called “proximal arginine,” from the cleavage site, which is usually found among most precursor proteins, plays a critical role in cleavage reaction (10–12). Distal basic amino acid residue(s) around position −10 are also important for effective cleavage (10–12). The length between the proximal arginine and the distal basic residues is not so fixed, and 4–10 amino acids are allowed (13). Our more recent studies have shown a requirement for effective cleavage of flexible linker sequences containing proline and glycine between the two basic residues (13, 14), a hydrophobic residue at position +1 (12, 13), and serine or threonine residues at position +2 and/or +3 (12, 15).

Some functional amino acid residues in MPP were determined using mutational analysis; His-101, Glu-104, and His-105 in rat β-MPP, which form a metal binding site, HxxEH, conserved among a pitrilysin metallopeptidase superfamily (16), are the catalytic center of MPP (17, 18). Glu-181 is the third metal-binding residue (19). Glu-174 may participate in the catalytic reaction (18). Glu-124, which is in a characteristic acidic amino acid cluster conserved in β-MPP, may interact with the N-terminal portion of the presequence in the cleavage reaction (18). On the other hand, Glu-390 and Asp-391 in yeast α-MPP interact with the distal arginine residues, which are required especially for cleavage of precursors with a longer presequence (19). Deletion of three residues in the glycine-rich segment characteristic of α-MPP resulted in a drastic reduction in affinity to the substrate (20).²

Findings on functional amino acid residues both in precursor

¹ The abbreviations used are: MPP, mitochondrial processing peptidase; CPM, 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin; DAC, 7-diethylaminocoumarin-3-carboxylic acid; FRET, fluorescence resonance energy transfer; IANBD, \(N,N'\)-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazole-4-yl)ethylenediamine; MDH, malate dehydrogenase; α- and β-MPP, α- and β-subunits, respectively, of the mitochondrial processing peptidase.

² In our previous works, the residues of MPP were numbered from the N terminus of the mature protein reported in the data base. In this studies, we numbered the residues according to the full-length MPP precursors. For instance, His-101, Glu-104, and His-105 in rat β-MPP were represented as His-56, Glu-59, and His-60, respectively, in our previous papers.

Katsuhiko Kojima, Sakae Kitada, Tadashi Ogishima, and Akio Ito†
From the Department of Chemistry, Faculty of Science, Kyushu University, Fukuoka 812-8581, Japan

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proteins and MPP required for the processing reaction, especially for precursor recognition by MPP, suggest that the two subunits of MPP cooperatively form the substrate binding pocket and that they have several substrate binding sites to cope with different structural elements in the extension peptide. To elucidate the recognition mechanism that makes feasible strict substrate specificity for MPP, it is necessary to determine the structure of the precursor bound to the enzyme.

In the present study, fluorescence resonance energy transfer (FRET) experiments provide the first evidence that the distal arginine and the portion around the cleavage site of the precursor are located at specific sites in the MPP molecule, irrespective of the position of the distal arginine. An induced-fit mechanism of substrate recognition of MPP seems likely.

**Experimental Procedures**

**Preparation of fluorescence-labeled peptides**—The fluorescent dyes, 7-diethylaminocoumarin-3-carboxylic acid (DAC), N,N’-dimethyl-N-(iodoacetyl)-N’-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (IANBD), and 7-diethylamino-3-(4’-maleimidylphenyl)-4-methylcoumarin (CPM) were purchased from Molecular Probes, Inc. (Eugene, OR). Peptide synthesis and DAC labeling were done as described (9). IANBD amide labeling with the cysteine residue of peptides for intramolecular FRET experiments was done essentially the same as DAC labeling. Peptide authenticity was identified by MALDI-TOF mass spectrometry (Voyager, PerSeptive Biosystems). The concentrations of the fluorescence peptides were calculated from the molar extinction coefficient of 45,000 (m⁻¹ cm⁻¹) for DAC or 23,500 (m⁻¹ cm⁻¹) for IANBD amide.

**Preparation of fluorescence-labeled MPP**—A hexahistidine-tagged yeast α-MPP and yeast α/E73QHis complex were purified as described (9). Purification and fluorescent labeling of a hexahistidine-tagged yeast β-E73QHis were done as follows. The supernatant from the BL21(DE3) strain carrying pET-βE73QHis was loaded on a 5-ml Hi-trap chelating column (Amersham Pharmacia Biotech) equilibrated with buffer A (20 mM Hepes-KOH, pH 7.4, containing 500 mM NaCl). The column was washed with 50 ml of buffer A containing 50 mM imidazole. The β-MPP was eluted with buffer A containing 200 mM imidazole. To the fractions containing β-MPP, 20 mM Hepes-KOH, pH 7.4, containing 500 mM NaCl. The column was washed with 50 ml of the same buffer, and then the protein was eluted with 30 ml of the same buffer. CPM-labeled MPP was then applied onto 2 ml of Q-Sepharose FF (Amersham Pharmacia Biotech) equilibrated with buffer A (20 mM Hepes-KOH, pH 7.4, containing 100 mM NaCl). The purity of CPM-labeled β-MPP was confirmed by SDS-polyacrylamide gel electrophoresis followed by CPM staining. The labeling efficiency was calculated from a molar extinction coefficient of 33,000 (m⁻¹ cm⁻¹) for CPM. The labeling procedure resulted in the incorporation of 0.11 μmol of CPM/mol of β-MPP.

**Fluorescence measurements**—Fluorescence measurements were performed using a Hitachi F-4500 fluorescence spectrophotometer equipped with automatic fluorescence polarization system. In the intramolecular FRET measurement of the double-labeled peptide, excitation of DAC was measured at 390 nm and the emission intensity at 480 nm. MPP was then added to the IANBD-labeled peptides in the presence of various concentrations of MPP. The excitation and emission intensity at 390 nm and the emission intensity at 480 nm were measured. The dissociation constant, $K_d$, was determined as follows: $F = (F_{max} - [Enz]) / K_d + [Enz]$, where $F$ and $F_{max}$ are the measured and maximal fluorescence intensity of the peptides, respectively, and [Enz] represents the concentrations of the enzyme. A plot of [Enz]/F versus [Enz] yields a linear function with a slope of $1/F$ and an ordinate intercept of $K_d$.

From the decrease in fluorescence of donor DAC or CPM, induced in the presence of acceptor IANBD, the energy transfer efficiency $E$ was calculated from

$$E = 1 - Q_	ext{obs}/Q_0$$

where $Q_0$ stands for the unquenched quantum yield of the donor and $Q_{obs}$ is the quantum yield in the presence of the acceptor. Quantum yield was substituted for emission maximum intensity $F$. From the energy transfer efficiency results, the distance between donor and acceptor was calculated according to the Förster theory (21),

$$R = R_0(E^{-1} - 1)^{1/6}$$

where $R$ is the calculated distance and $R_0$ is the distance at which 50% energy transfer would occur between the donor-acceptor pair; it is given in angstroms, as shown in Equation 3.

**Table I**

Amino acid sequences of the fluorescence-labeled peptides for intramolecular FRET measurements

| Peptides | Position of the distal Arg | Sequences |
|----------|--------------------------|-----------|
| MDH-AAAL | -7 | (α-DAC) LARPVGAS - PSTC (S-IANBD) AQNN |
| MDH-14A  | -10 | (α-DAC) LARPVGAS - PSTC (S-IANBD) AQNN |
| MDH-AdAR | -14 | (α-DAC) LARGPGLGGGAVATRS FSTC (S-IANBD) AQNN |
| MDH-AdRA | -17 | (α-DAC) LAGSGAAPGLGGGAVATRS FSTC (S-IANBD) AQNN |

**Figure 1**

Spectral change of the emission of DAC- and DAC/IANBD-labeled peptides with the addition of MPP. The emission spectra of DAC-labeled (A) and DAC/IANBD-labeled (B) MDH14A (0.5 μM, respectively) were taken in the presence of various concentrations (0–3 μM) of yeast MPP (α/E73Q). The excitation wavelength of DAC was 390 nm. Note the differences in the range of Fluorescence. The spectra changed from the spectrum 1 to the spectrum 2 with the addition of MPP; a.u., arbitrary units.
$R_0 = 9790(J^2Qd^2)^{1/6}$  \hspace{1cm} (Eq. 3)

where $J$, the overlap integral, is the degree of spectral overlap of donor emission $F_D(\lambda)$ and acceptor absorbance $e_A(\lambda)$, as defined by Equation 4.

$$J = \int F_D(\lambda)e_A(\lambda)\lambda^2d\lambda$$

$\kappa^2$ is assumed to be 2/3. The refractive index of the solvent, $n$, is used at a value of 1.4. $Q_D$, the quantum yield for the donor, was given as $Q_D = Q_0\times(F_0\timesA_0)(F_\lambda\timesA_\lambda)$  \hspace{1cm} (Eq. 5)

where $Q_D$ is the quantum yield for the reference dye, $F_0$ and $F_\lambda$ are the fluorescence intensities for the donor and reference dye, respectively, and $A_0$ and $A_\lambda$ are the fluorescence intensity for the donor and reference dye, respectively. Fluorescein was used as the reference dye and was assumed to have a quantum yield of 0.92 in 0.1 N NaOH.

Although $\kappa^2$ was taken as 2/3 for the calculation of distances, the maximum and minimum values of $\kappa^2$ were estimated according to the method of Dale et al. (21).

$$\kappa_{\text{max}}^2 = (2/3)\times(1 + d_0 + d_\lambda + 3d_\delta d_\lambda)$$

$$\kappa_{\text{min}}^2 = (2/3)\times(1 - (d_0 + d_\lambda)/2)$$

where $d_0 = (\text{rD}0.452; d_\lambda = (\text{rD}0.452$; $r_D$ and $r_\lambda$ are the limiting anisotropies of the donor and acceptor, respectively. Using these values for the orientation factor, the maximum and minimum distances between probes were calculated and were regarded as the probable error limits of the distance (R-limits).

**RESULTS**

**Distance between the N-terminal End and the C-terminal Portion of the Peptide Substrates Bound to MPP**—Sequence data on the presequence of mitochondrial protein precursors show that position of the distal basic acid is not so fixed among the extension peptides and is located −7 to −17 from the cleavage site. To elucidate the structure of the substrate peptide and MPP. In attempting this elucidation, we measured the distance between the distal arginine and the residue around the cleavage site, using intramolecular FRET. We synthesized a series of peptides with different lengths of intervening sequence between the proximal and distal arginines, labeling them at the N-terminal end with fluorescent donor DAC and at position +4 cysteine with the acceptor IANBD amide (Table I). These sites were two and five amino acid residues away from the distal and proximal arginines, respectively, to avoid interference by probes in the interaction between the arginines and other recognition elements of the peptide and MPP. In MDH-14A, arginine at position −3 (position 14 from the N-terminal end) was replaced with alanine in the original sequence of rat MDH, as it is not necessary for the residue at this position to be arginine (10). This peptide has a distal arginine at position −10 and is used as a standard peptide in the present work. In MDH-ΔAAL, the alkylating amino acid residues between proximal and distal arginines were deleted so that the distal arginine was at position −7. This peptide was found to have the minimum length for effective cleavage by the MPP (13). MDH-AdAR and MDH-AdRA have the distal arginine residue at position −14 and −17, respectively. In these peptides, the linker sequence in the presequence of the bovine adrenodoxin precursor was introduced between the proximal and distal arginines instead of the original one of the MDH presequence.

The emission maximum of DAC is at 470 nm (Fig. 1). The absorption spectrum of IANBD, which gives the peak at 498 nm, has an excellent overlap with the emission spectrum of DAC (data not shown). The spectral overlap, $J$, between these spectra is calculated to be $9.31 \times 10^{-14} \text{M}^{-1} \text{cm}^{-1} \text{nm}^{-4}$. The spectral characteristics of all the peptides were essentially the same. For the intermolecular FRET measurements, fixed concentrations (0.5 μM) of the DAC or DAC/IANBD-labeled peptides were added to various concentrations (usually 0–3 μM) of the purified α/βE73Q, in which β-MPP is an inactive mutant with glutamine substituting for the glutamate residue of the active center (18) (Fig. 2). As demonstrated in our previous study (9), the fluorescence of DAC introduced to the peptide substrate bound to MPP increases with environmental change around the dye (Fig. 1). Titration of the DAC-labeled peptides gave the dissociation constant, $K_D$, of peptides for binding to MPP (Fig. 2). All of the peptides bound to MPP with a high affinity to the same extent.

Introduction of IANBD into the peptides led to a drastic suppression of increase in DAC fluorescence through FRET in all of the peptides studied (Fig. 2). In the titration of DAC- or DAC/IANBD-labeled peptides with MPP, the fitting curves showed a biphasic nature (Fig. 2). Because fluorescence anisotropy change of the peptides was saturated at a stoichiometric amount of the enzyme (data not shown), the biphasic fluorescence change in the titration experiments might be due to increased scattering by increasing the concentration of the enzyme. For calculation of FRET efficiency, $E$, the fluorescence intensities, $F_D$ and $F_{\lambda\lambda}$ for DAC- and DAC/IANBD-labeled peptides, respectively, were taken as extrapolating the second phase curve to the ordinate. The calculated FRET efficiencies of all the peptides showed a range of 80–85% (Table II). The quantum yield, $Q$, of the fluorescence donor DAC showed a gradual decrease with the increasing length of the peptides; this also resulted in a decrease in the distance at which 50% FRET occurred between the donor-acceptor pair, $R_0$, suggest-
ing that the mobility of the N-terminal portion of the peptides bound to MPP increases with length of the presequence. Taken together with the parameters described above, the distances of the donor-acceptor pair, R, were substantially the same among all of the peptides measured and were calculated to be ~28 Å (Table II); this indicates that the distance between the N-terminal end of the peptides and cysteine residue at position +4 is fixed, irrespective of the length of the intervening sequence between the proximal and distal arginines. Our findings suggest that the distal basic amino acid interacts with a specific site, probably the acidic residue(s), in the enzyme.

Location of Putative Sites in MPP Interacting with the N-terminal and C-terminal Portions of the Substrate Peptide—

The similarity of the calculated distances between donor-acceptor pairs on the substrates might be because of compensation for changes in distance by changes in orientation of the fluorescent dyes. To eliminate this possibility, we next carried intermolecular FRET measurements between a fluorescence donor in the β-MPP and the acceptor in the substrate peptides bound to MPP. This measurement could also estimate the location in the MPP of the N-terminal end of the peptide substrate and the amino acid residue at +4 position from the cleavage site. We first modified a single cysteine residue, Cys-252, in yeast β-MPP (βE73Q) by CPM, as the fluorescent donor. The histidine-tagged βE73Q partially purified by nickel-chelating column chromatography was reacted with CPM (Fig. 3, lane 1). When CPM-labeled βE73Q was purified further with Q-Sepharose chromatography (Fig. 3, lane 3), a single band was obtained by both Coomassie Blue staining and UV transillumination. To confirm the specificity of the labeling to the cysteine residue, the labeling with CPM was done with a mutant enzyme, βE73Q/C252S, in which the single cysteine residue was substituted to serine. No visible band in UV transillumination was obtained with both Coomassie Blue-stained gel (left panel) and a UV-transilluminated Coomassie Blue-stained gel (right panel) following nickel-chelating column chromatography in βE73Q/C252S (lane 2) and CPM. Then, each sample was finally purified by Q-Sepharose chromatography. Lanes 3 and 4 represent the final eluate of βE73Q (6 μg) and βE73Q/C252S (6 μg), respectively. The purification and CPM labeling are described in detail under “Experimental Procedures.”

![fig3.png](image)

**FIG. 3.** Specific modification of Cys-252 in β-MPP by CPM. A Coomassie Blue-stained gel (left panel) and a UV-transilluminated gel (right panel) SDS-polyacrylamide gel (8%) after electrophoresis are shown. βE73Q (lane 1) and βE73Q/C252S (lane 2) following nickel chelating column chromatography were reacted with CPM. Then, each sample was finally purified by Q-Sepharose chromatography. Lanes 3 and 4 represent the final eluate of βE73Q (6 μg) and βE73Q/C252S (6 μg), respectively. The purification and CPM labeling are described in detail under “Experimental Procedures.”

A series of peptides labeled with IANBD amide at the N-terminal end or at position +4 was synthesized (Table III). For labeling with IANBD at the N-terminal end of the peptides, a cysteine residue, instead of leucine, was introduced at the N-terminus. The α-amino group of the N-terminal end of the peptides was acetylated to avoid the effect of the positive charge of the α-amino group in the peptides. In intermolecular FRET measurements, titration of the fixed concentration (0.5 μM) of CPM-labeled αE73Q was done with various concentrations (typically 0–1.5 μM) of the IANBD-labeled peptides. The emission spectrum of CPM, which has a fluorescence maximum at 475 nm, is similar to that of DAC, and overlaps the excitation spectrum of IANBD amide (λ = 9.68 × 10⁻¹⁴ m⁻¹ cm⁻¹ nm⁻¹). The quantum yield of CPM was extremely high (0.90 ± 0.02) relative to that of DAC. The addition of the acceptor-labeled peptides led to a decrease in the fluorescence of CPM and a small increase in that of IANBD, which has a peak at 545 nm (Fig. 4). Because FRET can be detected when donor and acceptor molecules are in close proximity (typically 10–100 Å) and fluorescence quenching by collision between the donor-acceptor pair was negligible, under the conditions of this measurement (data not shown), the observed decrease in fluorescence indicates binding of the IANBD-labeled peptide to the CPM-labeled MPP molecule. The absorption spectra of IANBD did not change with binding to MPP. From the spectral parameters obtained, the Förster distance, R₀, was calculated to be 48.6 ± 0.3 Å. The titration curves were shown in Fig. 5 as a plot of 1 − (F_D/A/F_D) versus [Peptide], where [Peptide] is the concentration of the IANBD-labeled peptides. The Kₐ values calculated from the nonlinear least-squares fit of the data increased slightly relative to those of DAC-labeled peptides. In both cases of the peptides labeled at the N-terminal end and at position +4, the FRET efficiency, E, which is calculated as the limitation value of 1 − (F_D/A/F_D), showed essentially the same value among the peptides that have distal arginine at different positions (Table IV). Distances between CPM in β-MPP and IANBD at position +4 of the peptides were calculated to be in the range of 44 to 46 Å, whereas those between CPM in β-MPP and IANBD at the N-terminus of the peptides were in the range of 43 to 45 Å, irrespective of the position of the distal arginine residue. The finding showing that the distance between the cysteine residue in β-MPP and the N terminus of the peptides is substantially the same among the peptides with various lengths of intervening sequences between the proximal and distal arginines confirmed that the distal basic amino acid residue interacts with the specific site of the enzyme if the distal basic residue is at least within −7 to −17 of the cleavage site.

**TABLE II**

| Peptides | Kₐ | Q | R₀ | E | R | R-limits |
|----------|----|---|----|---|---|---------|
|          | μM|    |   |   |   |         |
| MDH-AAAL | 0.20 | 0.217 | 38.1 | 0.850 | 28.5 | 21.3–36.5 |
| MDH-14A  | 0.21 | 0.203 | 37.7 | 0.850 | 28.2 | 21.9–36.1 |
| MDH-AdAR | 0.35 | 0.158 | 36.2 | 0.797 | 28.8 | 22.1–36.9 |
| MDH-AdRA | 0.33 | 0.148 | 35.8 | 0.834 | 27.4 | 21.2–35.9 |

**DISCUSSION**

We found that when mitochondrial protein precursors are bound to MPP, distal basic amino acids in its presequences interact with the specific site in the enzyme if the basic residues are present at positions −7 to −17. This means that the intervening sequence between the proximal arginine and the
distal basic amino acid is flexible so that the distal basic residue can fit into a specific binding site in MPP. Thus, the present study is the first to propose the structure of the presequence bound to MPP.

We generated an energy-minimized model of rat MPP (22) based on the crystal structure of the core proteins in the bovine bc1 complex (23, 24). The two subunits form a ball with a crack leading to the internal cavity. Functional amino acid residues predicted in our previous works (17–19) are arranged around the cavity. In the simulation model (Fig. 6), the distance between Glu-73 in yeast β-MPP (corresponding to Glu-104 in rat β-MPP), which is a catalytic center (17, 18), and Glu-390/Asp-391 in yeast α-MPP (corresponds to Glu-446/Asp-447 in rat α-MPP), which we assumed to be residues interacting with the distal arginine in the presequence (19), was calculated to be about 30 Å. The glycine-rich loop in α-MPP, which is conserved among different organisms and has been shown to be essential for MPP function, is close to the metal binding active center in β-MPP and is located about 30 Å from Glu-390/Asp-391 in α-MPP. These values are close to those (about 28 Å) obtained from FRET measurements between the N-terminal end and the amino acid residue at position +4 of the peptides (Table II). On the other hand, in the simulation model, Cys-252 in yeast MDH-ΔAAAL modified with CPM seems to be located on the surface of the C-terminal domain of β-MPP and is about 50 and 40 Å away from Glu-390/Asp-391 and the glycine-rich segment in α-MPP, respectively. Taking the uncertainty of the predicted location of Cys-252 in the model of β-MPP into consideration, these values are also similar to those obtained from FRET measurement of about 45 Å from Cys-252 to the N-terminal end and the amino acid residue at position +4 of the peptides. The scissile bond of the presequence bound to MPP must be close to the active center in β-MPP. Taken together, these findings lead one to expect that the distal basic residue and the proximal and distal arginine residues are indicated by single and double underlines, respectively. Ac indicates an acetyl group.

### Table III

| Peptides     | Labeled at position | Sequences                          |
|--------------|---------------------|------------------------------------|
| MDH-ΔAAAL    | +4                  | Ac-LAEPVGABF-FSTC(S-IANBD)AQNN      |
| MDH-14A      | −7                  | Ac-LAEPVGABF-FSTC(S-IANBD)AQNN      |
| MDH-AdAR     | −10                 | Ac-LAEPVGABF-FSTC(S-IANBD)AQNN      |
| MDH-AdRA     | −14                 | Ac-LAEPVGABF-FSTC(S-IANBD)AQNN      |
| Labeled at the N terminus |                 | Ac-LAEPVGABF-FSTC(S-IANBD)AQNN      |
| MDH-ΔAAAL    | −7                  | Ac-C(S-IANBD)ARVPGAR-FSTSAQNN       |
| MDH-14A      | −10                 | Ac-C(S-IANBD)ARVPGAR-FSTSAQNN       |
| MDH-AdAR     | −14                 | Ac-C(S-IANBD)ARVPGAR-FSTSAQNN       |
| MDH-AdRA     | −17                 | Ac-C(S-IANBD)ARVPGAR-FSTSAQNN       |

**Fig. 4.** Spectral change of the emission of CPM-labeled MPP with the addition of IANBD-labeled MDH14A. The emission spectra of CPM-labeled MPP (CPM-βE73Ala) (0.5 μM) were taken in the presence of various concentrations (0–1.5 μM) of IANBD-labeled MDH14A. The excitation wavelength of CPM was 390 nm. The spectra changed from the spectrum 1 to the spectrum 2 with the addition of the peptide. a.u., arbitrary units.

**Fig. 5.** Fluorescence quenching of CPM attached to MPP with the addition of IANBD-labeled peptides. Plots of the fluorescence quenching of CPM attached to MPP, 1 – (Fcp/F0), versus the concentration of the peptides labeled at position +4 (A) or at the N terminus (B). Fcp and F0 represent the fluorescence of CPM in the presence or absence of IANBD-labeled peptides, respectively. CPM-labeled MPP (0.5 μM) was titrated with various concentrations (0–1.5 μM) of IANBD-labeled MDH-ΔAAAL (squares), MDH-14A (diamonds), MDH-AdAR (circles), and MDH-AdRA (triangles). The excitation wavelength of CPM was 390 nm. The fluorescence intensity at 480 nm of CPM-labeled MPP was read. The solid lines are nonlinear least-squares fits of the plots to the equation, 1 – (Fcp/F0) = (E × [L])/(Kd + [L]), where E and [L] represent the calculated maximum of 1 – (Fcp/F0), with regard to the FRET efficiency between the CPM-IANBD pair and the concentration of the IANBD-labeled peptides, respectively. a.u., arbitrary units.
amino acid residue at position +4 of the peptide interact with the acidic residue cluster and the glycine-rich region in α-MPP, respectively, and that the substrate peptide in the cavity of MPP might interact with a nonhelical structure around the cleavage site of the precursor proteins. The formation of an α-helix of the precursor is required apparently for interaction with these components (25–27). The NMR structure of the cytosolic domain of Tom20, a component of the translocase complex in mitochondrial outer membrane, with a synthetic peptide based on the aldehyde dehydrogenase precursor has recently been resolved (28). The peptide that forms an amphiphilic α-helix in a crack of the core structure of Tom20 consists of four helices. The present results indicate that structures required for targeting and processing differ and that a flexible structure is required for the processing, although basic residues in the presequences function as recognition signals for both processes.

Structural convergence between MPP and thermolysin, a Zn²⁺-peptidase with a typical metal-binding motif, HExxH, has been discussed recently (29). Superimposition between the N-terminal domain of core 1 protein of the bc₁ complex and the portion around the active site of thermolysin showed a similar arrangement of secondary structural elements but with different topological connections and in a reverse main chain orientation. This structural architecture is based on four helices, which contain metal ligands and the catalytic glutamate residue, and the neighboring five strands of a β-sheet. A main chain of substrates of thermolysin interacts with the edge of the β-sheet through hydrogen bonds. Like thermolysin, the β-sheet structure around the active center of MPP might interact with a nonhelical structure around the cleavage site of the precursors through hydrogen bonding, to present the scissile bond of the substrate to the active center in β-MPP. Further studies, especially on the crystal structure of MPP, should reveal the precise structure of the presequence bound to MPP and the mechanisms of strict recognition and specific cleavage of precursor proteins by the enzyme.

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Structure of Substrate Peptides Bound to MPP

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