Analysis of Elements in the Substrate Required for Processing by Mitochondrial Processing Peptidase

(Received for publication, May 25, 1995, and in revised form, October 6, 1995)

Tadashi Ogishima, Takuro Niidome, Kunitoshi Shimokata, Sakae Kitada, and Akio Ito

From the Department of Chemistry, Kyushu University, Fukuoka 812, Japan

We have recently demonstrated that synthetic peptides modeled on the extension peptide of malate dehydrogenase can be a good substrate of mitochondrial processing peptidase and that arginine residues present at positions −2 or −3 and distant from the cleavage point were important for recognition by the enzyme (Niidome, T., Kitada, S., Shimokata, K., Ogishima, T., and Ito, A. (1994) J. Biol. Chem. 269, 24719–24722). We further investigated the elements required for substrates of the protease. To analyze the reaction by a more rapid yet quantitative method, we have developed intramolecularly quenched fluorescent substrates. Using the fluorogenic substrates we demonstrated that at least one of the proline and glycine between the distal and proximal arginine residues was also important while other connecting sequences were dispensable. In addition, the protease showed considerable preference for aromatic and, to a lesser extent, hydrophobic amino acids in the P$_2$-position. These results together with the previous data suggest that the proximal and distal arginine residues, proline and glycine between them, and P$_2$ amino acid could be critical determinants for the specific cleavage of the substrates by the protease.

Most mitochondrial proteins are synthesized on cytoplasmic ribosomes and transported into their correct mitochondrial component. The majority of them carry N-terminal extension peptides that target the protein molecules to the organelle. Mitochondrial processing peptidase (MPP) is localized in the mitochondrial matrix and is responsible for proteolytic cleavage of the extension peptides after or during the transportation (1–3). The enzyme is a metalloprotease and forms a heterodimer consisting of structurally related α- and β-subunits (4–11). We have recently demonstrated that the β-subunit is a catalytic one (12). MPP acts exclusively on the precursor forms of mitochondrial proteins, whose extension peptides are heterogeneous in sequence (13–15). The absence of apparent sequence homologies raises a question about how MPP specifically recognizes the extension peptides and cleaves them at a single site. Attempts have been made to solve the question, mainly by use of in vitro translated and radiolabeled mutants of mitochondrial precursors. By detecting mature forms from the in vitro translated precursors on SDS-polyacrylamide gel electrophoresis followed by fluorography, the processing activity has so far been evaluated. Those studies pointed out the importance of basic amino acids near the C terminus and neutral amino acids in the middle portion of the extension peptides (16–20). The method, however, is time-consuming, and the obtained data are not quantitative. Thus the conventional method is unsuitable for kinetic analysis of the reaction. To overcome the limitation of the assay method, we developed a new method (21), which employed as the substrate synthetic peptides that were modeled on the extension peptide of rat mitochondrial dehydrogenase. This method enabled us to evaluate the importance of arginine residues that are located at the −2 or −3 position and at a position distant from the cleavage point. To further investigate the structural elements in the substrates required for recognition by MPP, we developed new substrates that are based on intramolecular quenching of fluorescence. The fluorescent substrates had a 2-aminoxybenzoic acid fluorophore at the N-terminal end and a 3-nitrotyrosine quencher at a C-terminal portion from the scissile bond in the peptides modeled on the malate dehydrogenase extension peptide (22). They were efficiently cleaved if they contained the distal and proximal arginine residues. The results from kinetic studies using the substrates revealed that arginine residues between the critical arginine residues were also important while other connecting sequences were dispensable. They also indicated that the enzyme exhibited considerable preference for aromatic and, to a lesser extent, hydrophobic amino acids at the P$_2$-position.  

EXPERIMENTAL PROCEDURES

Preparation of Peptides—The fluorogenic substrates contained 2-aminoxybenzoic acid (ABz) as a fluorescent group at the N-terminal end and 3-nitrotyrosine (Tyr(NO$_2$)) as a quenching group between the MPP cleavage site and the C-terminal end. We synthesized the peptides manually in solid phase employing Fmoc-(N$_2$-9-fluorenylethoxycarbonyl (Fmoc) strategy (23) by situ coupling method (24–26). The following Fmoc amino acid derivatives were used: Fmoc-(N$_2$-methoxy-2,3,6-trimethylbenzenesulfonyl)Arg, Fmoc-(N$_2$-9-tryptyl)Asn, Fmoc-(N$_2$-9-tryptyl)-Gln, Fmoc-(O-t-buty1)Ser, Fmoc-(O-t-buty1)Thr, and Fmoc-(O-t-buty1)-Tyr. Other Fmoc amino acids including Fmoc-3-nitrotyrosine were used without side-chain protection. The completed peptides were deprotected and cleaved from the resin with trifluoroacetic acid in situ in correspondence with the binding site. The fluorogenic substrates that had various amino acid residues at P$_2$ were synthesized by a fragment condensation method. Briefly, a series of

*This work was supported in part by Grant-in-aid for Scientific Research on Priority Areas No. 04259101 from the Ministry of Education, Science, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡Present address: Dept. of Applied Chemistry, Nagasaki University, Nagasaki 852, Japan.

§To whom correspondence and reprint requests should be addressed: Dept. of Chemistry, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka, 812-811 Japan. Tel.: 81-92-641-1101 (ext. 4265); Fax: 81-92-632-2734; E-mail: a.itoscc@mbox.nc.kyushu-u.ac.jp.

1The abbreviations used are: MPP, mitochondrial processing peptidase; ABz, 2-aminoxybenzoic acid; Tyr(NO$_2$), 3-nitrotyrosine; Fmoc, N$_2$-9-fluorenylethoxycarbonyl; HPLC, high performance liquid chromatography.

2In accordance with Schechter and Berger (39), the enzyme binding sites are denoted S$_1$, S$_2$, ..., S$_n$, and S$_1'$, S$_2'$, ..., S$_n'$ away from the scissile bond toward the N and the C terminus, respectively. Amino acid residues in the substrates are referred to as P$_1$, P$_2$, ..., P$_n$, and P$_1'$, P$_2'$, ..., P$_n'$ in correspondence with the binding site.
met-Leu-Ser-Ala-Leu-Leu-arg-pro-val-Gly-Ala-Ala-Leu-Arg-arg
ser-Phe-Ser-Thr-Ser-Ala-Gln-Asn-Asn-_mature portion

Fig. 1. Amino acid sequence of rat malate dehydrogenase precursor. The arrows indicate the first and second cleavage sites by MPP and mitochondrial intermediate peptidase (MIP), respectively.

XSTY(NO₂)AQNN peptides were synthesized on p-alkoxybenzyl-alcohol-polystyrene resin as a C-terminal half of the substrates. Here, X was F, Y, L, V, A, G, S, or R. Fmoc-Gly was attached to 2-chlorotriylchloride resin (28) as the P₂ amino acid, and the N-terminal counterpart was synthesized on the resin. After completion, the side-chain protected peptide was cleaved from the resin with acetic acid/trifluoroethanol/dichloromethane (1:2:7) as previously described (29). The side-chain protected peptide was coupled with the XSTY(NO₂)AQNN-p-alkoxybenzyl-alcohol-polystyrene resin. The condensed peptides were deprotected and cleaved from the resin as described above. Peptides for inhibition experiment were synthesized as peptide amides on trialkoxy-diphenylmethyl-alcohol-polystyrene resin (30).

Analytical Methods—Fluorescence was measured at 25°C on a Hitachi F-2000 fluorescence spectrophotometer. Excitation was at 315 nm, and emission was measured at 420 nm. The peptide concentrations were determined from the final fluorescence (F₄₅₀) after cleavage of the peptides with trypsin or chymotrypsin using ABz-Gly-NH₂ as a standard. The standard was synthesized as described elsewhere (22). Concentrations of peptides were determined by the ninhydrin method (31) after the peptides were hydrolyzed in 2.5 n NaOH at 100°C for 90 min.

Enzyme Assay—The reaction mixture contained 30 nM substrate and 0.05–0.1 pmol of purified MPP in 1 ml of 20 mM HEPES-NaOH buffer (pH 7.6). The reaction was started by addition of the enzyme. Increase of the fluorescence intensity was monitored, and the initial velocities, which had been corrected for the initial fluorescence (F₀₄₅₀) of the substrates, were obtained. The kₘₐₓ/Kₘₐₜ values were determined from the equation, v = (kₗₓ/Kₗₓ)[E][S], where [E] and [S] are enzyme and substrate concentrations, respectively. This equation is valid when the substrate concentrations are much lower than Kₗₓ.

Inhibitory Effect of Synthetic Peptides on the Reaction with Fluorogenic Substrates—The processing reaction was analyzed in a total volume of 1 ml. The substrate was 30 pmol of ABz-LARPVGAALRRSFSTY(NO₂)AQNN, and inhibitors were the peptides at various concentrations that consisted of partial sequences of the malate dehydrogenase extension peptide.

Analysis of Reaction Products—The reaction mixture was performed with 0.5 pmol of the substrate and 0.2 pmol of purified MPP in 0.2 ml of 20 mM HEPES-NaOH buffer (pH 7.6) for 30 min at 25°C. The reaction was terminated by addition of 0.2 ml of acetonitrile and 10 µl of 10% trifluoroacetic acid. After centrifugation at 10,000 x g for 5 min, the supernatant was applied to a Cosmosil 5C18-AR column (10 x 250 mm; Nacalai Tesque, Kyoto, Japan) equilibrated with 1% acetonitrile containing 0.1% trifluoroacetic acid. The products were separated by a linear gradient of acetonitrile (1–51%, 30 min) and collected. The separated products were analyzed by amino acid analysis.

Materials—MPP was purified from bovine or rat liver mitochondria according to the method of Ou et al. (6). Solvents for peptide synthesis were purchased from Kanto Chemical Co. Ltd. (Tokyo). 2-Chlorotriylchloride resin was purchased from Novabiochem (Lauffelfingen, Switzerland). Other reagents for peptide synthesis were from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). Fmoc-Tyr(NO₂) was prepared by coupling of Tyr(NO₂) (Jensen Chimica, Geel, Belgium) with Fmoc-N-succinimidyl carbonate as previously described (32), and the di-Fmoc derivative was removed on Wako gel C-100 (Wako Pure Chemical Industries, Osaka, Japan).

RESULTS AND DISCUSSION

Design of Fluorogenic Substrates—Fig. 1 shows the sequence of the extension peptide of rat malate dehydrogenase. Consisting of 24 amino acids, the peptide has an MPP cleavage site between Ser and Phe and a mitochondrial intermediate peptidase cleavage site between Asn and Ala (14, 33). The fluorescent donor ABz and acceptor Tyr(NO₂) were introduced at the N-terminal end and at a position C-terminal to the scissile bond, respectively, in the extension peptide. We first synthesized a derivative of the full-length malate dehydrogenase extension peptide, where Tyr(NO₂) was introduced at the 20th position (numbering from the original N terminus in pre-malate dehydrogenase), and ABz was connected to Met⁴. Fluorescence increased upon addition of MPP, and the increase continued linearly, at least for the first 2–3 min. Addition of EDTA-Na at a final concentration of 0.1–10 mM inhibited the increase of fluorescence (Fig. 2) as had been observed in processing of both in vitro synthesized precursors (6) and nonfluorogenic synthetic peptides (21). The inhibition was not rapid, indicating that the metal binds tightly at the active pocket of MPP.

Although the fluorogenic peptide of full-length was efficiently cleaved, it showed initially about 50% of the fluorescence of the fully cleaved form. A substrate with such a high initial fluorescence has a limited increase of fluorescence, which lowers the sensitivity of the detection. Since long range resonance energy transfer is considered to operate in this quenching (22), the efficiency should improve as distance between the donor and acceptor is reduced. Thus, we synthesized a series of N-terminally truncated substrates to obtain well quenched and yet efficiently cleaved substrates. Initial fluorescence intensity of the peptides decreased, i.e. the quenching efficiencies improved as the distance between the chromophores was reduced (Table I). MPP cleaved the peptides that started from Ser⁴ and Leu⁶ as efficiently as that from Met⁴. It also cleaved the peptides that started from Arg⁹ a little slower. The cleavage rates dropped dramatically for the peptides that lacked the sequences from Met⁴ to Arg⁹. Here, we could confirm the importance of the distal arginine for substrate recognition as we have previously shown by using the nonfluorogenic substrates (21).

We also synthesized peptides that had the Tyr(NO₂) acceptor at the 17th (P₁*) or 18th (P₂*) position. The peptide that had the acceptor at the P₁* position (peptide 8 in Table I) had a drastically reduced cleavage efficiency compared to the peptide that had the acceptor at the 20th (peptide 1 in Table I). Introduction of Tyr(NO₂) into the P₂* position significantly elevated the cleavage efficiency when compared with the peptide having the acceptor at the P₁* position but not when compared with the peptide having the acceptor at the 20th position (compare peptides 9 and 3 in Table I). Since the substrates that connected ABz to Met⁴, Ser⁶, and Leu⁶ were cleaved at nearly the same rate, we hereafter used ABz-LARPVGAALRRSFSTY(NO₂)-AQNN as a typical substrate designating it as ABzL5Y*20.

Identification of the Cleavage Site in the Peptides That Reacted with MPP—The reaction products from the substrates that connected ABz to Met⁴, Leu⁶, and Arg⁹ were separated by HPLC. Fig. 3 shows that each chromatogram gave only three components when monitored at 215 nm. The peptide eluted latest corresponded to the substrate. The earliest eluted fractions had an identical retention time about 22.0 min. The first and third fractions showed an absorbance at 280 nm. They
Effects of the length of peptides and position of Tyr(NO₂) on self-quenching and cleavage efficiencies of the substrates

Derivatives of the extension peptide of rat malate dehydrogenase that had N-terminal deletions and Tyr(NO₂) at various positions were synthesized. ABz was attached to the N terminus. Tyr(NO₂) was introduced at P₄, P₅, or P₆. Fₐₜ and Fₙₙ were fluorescence intensities measured for the substrates and for the final products obtained by hydrolysis with trypsin or chymotrypsin, respectively. The kₐₜ/Kₘ values were determined by the reaction with purified MPP using steady-state enzyme kinetics. See “Experimental Procedures” for details.

| Substrate* | kₐₜ/Kₘ | % |
|------------|--------|---|
| ABz-MLSALARPVGAALRRS | FSTY(NO₂)AQNN | (1) | 53 | 2.1 |
| ABz-SALAIRPVGAALRRS | FSTY(NO₂)AQNN | (2) | 47 | 1.9 |
| ABz-ARPVGAALRRS | FSTY(NO₂)AQNN | (3) | 30 | 2.2 |
| ABz-RPVGAALRRS | FSTY(NO₂)AQNN | (4) | 24 | 1.5 |
| ABz-VGAALRRS | FSTY(NO₂)AQNN | (5) | 47 | 0.12 |
| ABz-LRRA | FSTY(NO₂)AQNN | (6) | 17 | 0.12 |
| ABz-RRS | FSTY(NO₂)AQNN | (7) | 12 | <0.05 |
| ABz-MLSPAIRPVGAALRRS | Y(NO₂)STSAQNN | (8) | 52 | 0.06 |
| ABz-LARPVGAALRRS | FY(NO₂)TSAQNN | (9) | 27 | 0.73 |

* The arrow (↑) indicates the putative scissile bond.

Fig. 3. Identification of the reaction products by HPLC. The substrates were reacted with MPP, and the reaction products as well as the substrates were applied to a reversed-phase HPLC. The substrates were: A, ABz-MLSALARPV- GAALRRSFSTY(NO₂)AQNN; B, ABz-LA- RPVGAALRRSFSTY(NO₂)AQNN; and C, ABz-RPVGAALRRSFSTY(NO₂)AQNN. The peak fractions were collected and analyzed for amino acid composition. See “Experimental Procedures” for details.

TABLE I

| Substrate* | kₐₜ/Kₘ | % |
|------------|--------|---|
| ABz-MLSPAIRPVGAALRRS | FSTY(NO₂)AQNN | (1) | 53 | 2.1 |
| ABz-SALAIRPVGAALRRS | FSTY(NO₂)AQNN | (2) | 47 | 1.9 |
| ABz-ARPVGAALRRS | FSTY(NO₂)AQNN | (3) | 30 | 2.2 |
| ABz-RPVGAALRRS | FSTY(NO₂)AQNN | (4) | 24 | 1.5 |
| ABz-VGAALRRS | FSTY(NO₂)AQNN | (5) | 47 | 0.12 |
| ABz-LRRA | FSTY(NO₂)AQNN | (6) | 17 | 0.12 |
| ABz-RRS | FSTY(NO₂)AQNN | (7) | 12 | <0.05 |
| ABz-MLSPAIRPVGAALRRS | Y(NO₂)STSAQNN | (8) | 52 | 0.06 |
| ABz-LARPVGAALRRS | FY(NO₂)TSAQNN | (9) | 27 | 0.73 |

TABLE II

Deletion and replacement of the intervening sequences between the proximal and distal arginine residues

Cleavage efficiencies of the fluorogenic peptides that lacked the intervening sequences between the distal and proximal arginine residues and that had replacement of proline or proline and glycine were measured. See “Experimental Procedures” for details.

| Substrate* | kₐₜ/Kₘ | % |
|------------|--------|---|
| ABz-ARPVGAALRRS | FSTY(NO₂)AQNN | (1) | 53 | 2.1 |
| ABz-LARPVGAALRRS | FSTY(NO₂)AQNN | (2) | 47 | 1.9 |
| ABz-VGAALRRS | FSTY(NO₂)AQNN | (3) | 24 | 1.5 |
| ABz-LRRA | FSTY(NO₂)AQNN | (4) | 47 | 0.12 |
| ABz-RS | FSTY(NO₂)AQNN | (5) | 17 | 0.12 |
| ABz-Aarro | FSTY(NO₂)AQNN | (6) | 12 | <0.05 |
| ABz-LARPVGAALRRS | FY(NO₂)TSAQNN | (7) | 52 | 0.06 |
| ABz-LARPVGAALRRS | FY(NO₂)TSAQNN | (8) | 27 | 0.73 |

* The arrow (↑) indicates the putative scissile bond.

Gly did not significantly alter the cleavage efficiency of the peptide. However, replacement of Pro⁶ with Ala reduced the cleavage efficiency. In a separate experiment using the synthetic peptide/HPLC assay system, we have also obtained a similar result that MPP cleaved MSLALARVAALRRSFSTSA less efficiently than MSLALARPVGAALRRSFSTSA with Kₘ values of 6.0 μM (versus 0.7 μM) without

should contain Tyr(NO₂) since it has an absorption maximum at 276 nm at pH 2.0. Amino acid analysis of the fractions revealed that the peptide with retention time of 22.0 was FSTY(NO₂)AQNN and that the peptides of the second peak were the N-terminal counterpart. Other fragments than those peptides did not appear after a prolonged incubation with MPP (data not shown). These results demonstrate that the substrates were cleaved at the correct site by MPP.

Deletion and Replacement of the Intervening Sequences between the Proximal and Distal Arginine Residues—The arginine residue that is located at the −2 or −3 position from the scissile bond in the extension peptide of malate dehydrogenase together with the basic amino acid residue at a position distant therefrom is important for recognition by MPP as previously shown (21). We then analyzed the role of the intervening sequences between the proximal and distal arginine residues (Table II). Cleavage efficiencies of the substrates that lacked Ala¹¹, Ala¹³-Ala¹₂, and Ala¹¹-Ala¹₂-Leu¹³ were essentially the same as ABzLSY*20. The efficiency dramatically reduced, however, when deletion extended to Arg¹⁴. This seemed to be discrepant with our previous result that alanine was replaceable for Arg¹⁴ (21). Although we are unable to clearly explain the discrepancy right now this arginine could serve only as a spacer to give an appropriate distance between the proximal (Arg¹³) and distal (Arg¹) arginine residues. Replacement of Pro⁶ with
**Substrate Recognition by Mitochondrial Processing Peptidase**

Inhibitory Effects of Synthetic Peptides That Have Partial Sequences of the Malate Dehydrogenase Extension Peptide.

The reaction toward ABzL5Y*20 was measured in the presence of various concentrations of the synthetic peptides. The remaining activity was determined and is given as percentage of the activity in the absence of added peptide. See "Experimental Procedures" for details.

Significant change of V_{max} values (5.0 versus 8.4 pmol/min).\(^3\) These results indicate that the sequence from Ala\(^{13}\) to Leu\(^{13}\) was dispensable while at least one of the \(\alpha\)-helix-breaking amino acid residues (Pro\(^9\) and Gly\(^{10}\)) was important.

Inhibitory Effect of Synthetic Peptides That Have Partial Sequences of the Malate Dehydrogenase Extension Peptide—We measured the processing reaction toward the fluorogenic substrate (ABzL5Y*20) in the presence of synthetic peptides to elucidate the structural or sequential elements responsible to the specific recognition by MPP. We first synthesized six kinds of peptides with five consecutive sequences of the malate dehydrogenase extension peptide (LARPV-NH\(_2\), PVGAA-NH\(_2\), AALRR-NH\(_2\), RRSFS-NH\(_2\), FSTSA-NH\(_2\), SAQNN-NH\(_2\)) that corresponded to the amino acid sequences from Leu\(^9\) to Asn\(^{14}\). As a control, a synthetic peptide corresponding to the N-terminal 13 amino acid residues of mature malate dehydrogenase did not inhibit the reaction even at 0.1 mM (data not shown). None of the six peptides had any effect on the cleavage of ABzL5Y*20 even at 0.1 mM (data not shown except FSTSA-NH\(_2\)). We then synthesized longer peptides that elongated N-terminally from FSTSA-NH\(_2\) and examined their inhibitory potency (Fig. 4). The peptides connected with Ser and Arg-Ser inhibited the reaction by 10 and 20%, respectively, at 2 \(\mu\)M. Inhibition was enhanced when Arg-Arg-Ser was connected to FSTSA-NH\(_2\). Further elongation of RRSFSTSA-NH\(_2\) with Ala gave only slight enhancement of the inhibitory effect. The peptide connected with Ala, Ala-Ala, and Ala-Ala-Ala-Ala-Ala gave the processing reaction to essentially the same extent. Introduction of arginine to AAAARRSFSTSA-NH\(_2\) at a position that corresponded to the distal position of the substrate, i.e. ARAAARRSFSTSA-NH\(_2\), greatly elevated the inhibitory potency of the peptide. Further inhibition was observed (50% inhibition at 20 \(\mu\)M) by introduction of a PVG sequence between the proximal proximal and distal arginine residues (ARPVGRRSFSTSA-NH\(_2\)). Among the peptides above, at least ARAAARRSFSTSA-NH\(_2\) and ARPVGRRSFSTSA-NH\(_2\) could act as substrates of MPP (data not shown).

Recent NMR studies (34, 35) have indicated the role of proline and glycine in mitochondrial extension peptides. They break a continuous \(\alpha\)-helix from the middle portion of the extension peptides to the cleavage point making the precursors competent for processing. Our result also supported importance of the proline and glycine residues in the extension peptide. The result of the present inhibition experiment indicates that binding ability of synthetic peptides to MPP improves if the \(\alpha\)-helix-breaking amino acids are present between the provisional proximal and distal arginine residues. Tight binding of the peptides having a PVG sequence together with the finding that replacement of the proline and glycine with alanine lead to significant decrease of cleavage efficiency of the peptide (Table II) supports importance of these amino acids.

Preference for Amino Acids at the \(\text{P}_1\)’-position—The resistance to cleavage of the peptide having Tyr(NO\(_2\)) at \(\text{P}_1\)’ (Table I) suggested the limited acceptance of \(\text{P}_1\)’ residues in the substrate by MPP. To confirm this, we synthesized a series of peptides that had varied amino acids at the \(\text{P}_1\)’-position for synthetic convenience, the \(\text{P}_1\) amino acid was replaced by Gly for Ser in the peptides. This conversion resulted in about half reduction of the cleavage efficiency. Fig. 5 shows the cleavage efficiencies of the peptides having altered \(\text{P}_1\)’ amino acids. Alteration of Phe to Tyr reduced the efficiency to about 50%, and that to Leu and Ala further reduced the efficiencies to about 10%. Cleavage efficiencies of the other peptides were below the detection limit. These results demonstrate that MPP exhibits marked preference for aromatic amino acids and, to a lesser extent, hydrophobic amino acids at the \(\text{P}_1\)’-position. Such amino acid residues are abundant at \(\text{P}_1\)’ at least for the mitochondrial precursors that receive two-step cleavage (14). Although this position is considered to be one of the recognition sites for the second processing by mitochondrial intermediate peptidase (36, 37), Arretz et al. (19) have recently reported importance of \(\text{P}_1\)’ for Neurospora MPP. They mutated phenylalanine at the \(\text{P}_1\)’ to lysine in a chimeric precursor of cytochrome b\(_{5}\) and found that the mutated precursor did not undergo processing by purified MPP. We are not sure right now if preference for aromatic and hydrophobic amino acids at the \(\text{P}_1\)’-position could also be applicable to the substrates that undergo one-step cleavage by MPP. At least for the precursors receiving two-step cleavage, the \(\text{P}_1\)’ amino acid together with the proximal, distal, and flexible amino acids could determine the substrate specificity and cleavage position of MPP.

Recognition Mechanism of the Substrates by MPP—In the present study, we determined several critical residues or positions in the extension peptide of malate dehydrogenase. We also found that none of the peptides having five consecutive residues of the malate dehydrogenase extension peptide sequence inhibited the processing activity though they had the important sequences. This implies either that the substrate-binding sites of the protease are multiple and dependent each other or that the distal and proximal arginine residues are spatially close by the aid of the proline and/or glycine and form a specific structure in combination with the \(\text{P}_1\)’ amino acid to present in the enzyme pocket the scissile bond to an active

---

3 T. Niidome, K. Shimokata, S. Kitada, T. Ogishima, and A. Ito, unpublished data.
water on the metal. We suppose that such structure should be a type of induced fitting since the substrate peptides lack a secondary structure in aqueous (without phospholipids or detergents) environment as previously reported on other model extension peptides.

Implications for the Development of Real-time Measurements of MPP Activity—Our recent establishment of the MPP assay system that consists of synthetic peptides and HPLC enabled us to study the recognition mechanism of MPP kinetically. Such a study has so far virtually been impracticable in the conventional assay system with in vitro synthesized precursor proteins. The synthetic peptide and HPLC system is, however, time-consuming and insensitive. Although kinetic parameters obtainable with the new method described in this paper are limited essentially to $k_{cat}/K_{m}$, this substrate allows an easy, rapid, sensitive, and accurate measurement of the reaction.

The following observations demonstrate that cleavage of the fluorogenic substrates by MPP is specific. (i) The cleavage point was always Ser-Phe as shown in Fig. 3 (Gly-Tyr for P$_1$-fluorogenic substrates by MPP is specific. (i) The cleavage point limited essentially to obtainable with the new method described in this paper).

Acknowledgments—We gratefully acknowledge Prof. S. Iwanaga and Dr. S. Kawabata for performance of amino acid analysis.

REFERENCES

1. Mori, M., Miura, S., Tatibana, M., and Cohen, P. P. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 7044-7048
2. McAida, P. C., and Douglas, M. G. (1982) J. Biol. Chem. 257, 3177-3182
3. Controy, J.-G., Fenton, W. A., and Rosenberg, L. E. (1982) Biochem. Biophys. Res. Commun. 105, 1-7
4. Hawlitschek, G., Schneider, H., Schmidt, B., Tropschug, M., Hartl, F.-U., and Neupert, W. (1988) Cell 53, 795-806
5. Yang, M., Jensen, R. E., Yaffe, M. P., Oppliger, W., and Schatz, G. (1988) EMBO J. 7, 3857-3862
6. Ou, W.-J., Ito, A., Okazaki, H., and Omura, T. (1989) EMBO J. 8, 2605-2612
7. Kletter, J., Kalousek, F., Sbaro, M., and Rosenberg, L. E. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 7978-7982
8. Kitada, S., Niidome, T., Nagano, T., Ogishima, T., and Ito, A. (1993) Biochem. Biophys. Res. Commun. 190, 289-293
9. Paces, V., Rosenberg, L. E., Wayne, A. F., and Kalousek, F. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 5335-5338
10. Pollock, R. A., Hartl, F.-U., Cheng, M. Y., Ostermann, J., Horwich, A., and Neupert, W. (1988) EMBO J. 7, 3493-3500
11. Jensen, R. E., and Yaffe, M. P. (1988) EMBO J. 7, 3863-3871
12. Kitada, S., Shimokata, K., Ogishima, T., and Ito, A. (1995) Biochem. (Tokyo) 117, 1148-1150
13. von Heijne, G., Steppuhn, J., and Herrmann, R. G. (1989) Eur. J. Biochem. 180, 533-545
14. Hendrikk, J. P., Hedges, P. E., and Rosenberg, L. E. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 4056-4060
15. A. Tizz, M., Schneider, H., W. Tropschug, U., and Neupert, W. (1991) Biochem. Biophys. Acta 103, 403-417
16. Horwich, A. L., Kalousek, F., Fenton, W. A., Pollock, R. A., and Rosenberg, L. E. (1996) Cell 44, 451-459
17. Kraus, J. P., Novotn' y, J., Kalousek, F., Sbaro, M., and Rosenberg, L. E. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 8905-8909
18. Chu, T. W., Grant, P. M., and Strauss, A. W. (1987) J. Biol. Chem. 262, 15759-15764
19. Arretz, M., Schneider, H., Guiard, B., Brunner, M., and Neupert, W. (1994) J. Biol. Chem. 269, 4959-4967
20. Ou, W.-J., Kumanoto, T., Mihiara, K., Kitada, S., Niidome, T., Ito, A., and Omura, T. (1994) J. Biol. Chem. 269, 24673-24678
21. Niidome, T., Kitada, S., Shimokata, K., Ogishima, T., and Ito, A. (1994) J. Biol. Chem. 269, 24719-24722
22. Médal, M., and Bredamm, K. (1991) Anal. Biochem. 195, 141-147
23. Atherton, E., Christopher, J. L., and Sheppard, R. C. (1981) J. Chem. Soc. Perkin Trans. 1, 538-546
24. Grüssas, A., Jorba, F., Giralt, E., and Pedroso, E. (1989) Int. J. Pept. Protein Res. 33, 386-390
25. Knorr, R., Trzeciak, A., Bannwarth, W., and Gilleissen, D. (1989) Tetrohedron Lett. 30, 1927-1930
26. Knorr, R., Trzeciak, A., Bannwarth, W., and Gilleissen, D. (1990) Pept. Res. 1148-1150
27. Fujii, N., Otaka, A., Sugiyama, N., Hatano, M., and Yajima, H. (1987) Chem. & Pharm. Bull. 35, 3880-3883
28. Barlos, K., Gatos, D., Kutsogianni, S., Papaphotiou, G., Poulos, C., and Tsegou, T. (1991) Int. J. Pept. Protein Res. 38, 562-568
29. Barlos, K., Chatzi, O., Gatos, D., and Stavropoulos, G. (1991) Int. J. Pept. Protein Res. 37, 513-520
30. Richter, H. (1987) Angew. Chem. Int. Ed. 26, 3787-3790
31. Yenen, E. W., and Coking, E. C. (1955) Analyt. Chem. 28, 209-213
32. Fields, C. G., Fields, G. B., Noble, R. L., and Cross, T. A. (1989) Int. J. Pept. Protein Res. 33, 298-303
33. Kalousek, F., Hendrick, J. P., and Rosenberg, L. E. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 7536-7540
34. Thorntor, K., Wang, Y., Weiner, H., and Gorenstein, D. (1993) J. Biol. Chem. 268, 15906-15911
35. Hammen, P. K., Gorenstein, D. G., and Weiner, H. (1994) Biochemistry 33, 8610-8617
36. Isaya, G., Kalousek, F., Wayne, A. F., and Rosenberg, L. E. (1991) J. Chem. Soc. 113, 63-76
37. Isaya, G., Kalousek, F., and Rosenberg, L. E. (1992) J. Biol. Chem. 267, 7904-7910
38. Park, N. G., Aoyagi, S., Lee, T., Kato, S., Jinkawa, W., Ou, W.-J., and Ito, A. (1989) Pept. Res. 2, 178-183
39. Schechter, I., and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 152-172
Analysis of Elements in the Substrate Required for Processing by Mitochondrial Processing Peptidase

Tadashi Ogishima, Takuro Niidome, Kunitoshi Shimokata, Sakae Kitada and Akio Ito

J. Biol. Chem. 1995, 270:30322-30326.
doi: 10.1074/jbc.270.51.30322

Access the most updated version of this article at http://www.jbc.org/content/270/51/30322

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 13 of which can be accessed free at http://www.jbc.org/content/270/51/30322.full.html#ref-list-1