J. Biochem. 129, 155–161 (2001)

Electrostatic Recognition of Matrix Targeting Signal by Mitochondrial Processing Peptidase[1]

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Received October 23, 2000; accepted November 6, 2000

Mitochondrial processing peptidase (MPP), a metalloendopeptidase consisting of α and β subunits, specifically recognizes a large variety of mitochondrial precursor proteins and cleaves off the N-terminal basic matrix targeting signals (MTS). Basic residues in MTS and acidic sites in MPP are required for effective processing. To elucidate whether the enzyme recognizes the MTS through electrostatic interaction, we investigated the effects of various salts on MPP activity. Decreases in the activity depended on the ionic strength and increases in the Michaelis constant value correlated clearly with the ionic strength, indicating a lower affinity of the enzyme for the substrate. Direct determination of the affinity between MPP and a MTS peptide using surface plasmon resonance showed a decrease in the association rate with high salt and that dissociation constant values were decreased. The effect of salt on the processing activity towards a variety of precursors was confirmed using five precursors with different sequences and lengths of MTS. Thus, we propose that electrostatic interactions are indispensable for the association between various MTS and MPP.

Key words: ionic strength, matrix targeting signal, mitochondrial processing peptidase, substrate recognition, surface plasmon resonance.

Most nuclear encoded mitochondrial matrix proteins are synthesized on cytoplasmic ribosomes as larger precursors with N-terminal basic extension peptides to target into the matrix (*1–3*). During or after the import of precursors, the matrix targeting signals (MTS) are immediately removed in the matrix by a metalloendopeptidase, general mitochondrial processing peptidase (MPP; EC 3.4.24.64) (*4*, *5*). MPP has been purified from mitochondria of *Neurospora crassa* (*6*), yeast *Saccharomyces cerevisiae* (*7*), rat liver (*8*, *9*), and some species of plants (*10*, *11*). The enzymes, except for those of plants, are soluble proteins composed of α and β subunits in the mitochondria. Yeast and rat MPPs form stable heterodimers and their activity is sensitive to metal chelators (*7*, *8*). The subunits show significant sequence homology to a family of endopeptidases, the pitrilysin family (*12*, *13*), that includes *Escherichia coli* pitrilysin, the insulin-degrading enzymes from mammals and insects. Despite the metalloendopeptidase, they lack the thermolysin-like zinc-binding motif, HExxH, but all members, except α-MPPs, have the inverted HxxEH motif that is involved in enzyme activity. Therefore, β-MPP is the catalytic subunit (*14*, *15*). The α- and β-subunits of MPP are also homologous to core proteins, core II and core I, respectively, of mitochondrial ubiquinol–cytochrome *c* oxidoreductase (bc_1 complex), a component of the respiratory chain (*16*). In potato and spinach mitochondria, two subunits are identical to the core proteins and are integrated into the bc_1 complex (*10*, *11*).

MPP specifically recognizes a wide variety of mitochondrial precursor proteins and cleaves off the MTS at single sites (*8*). Contrary to the strict substrate specificity of the enzyme, the amino acid sequences of MTS are vary widely in length, but show weak similarity (*1*). Recent kinetic experiments using synthetic MTS peptides revealed the structural elements required for specific recognition as a substrate. MPP requires distal and proximal basic amino acid residues in the MTS peptide from the cleavage site for effective processing (*17*, *18*) and prefers a hydrophobic residue at the P_1'-position (*19*). Selective cross-linking of a precursor protein to α-MPP suggests that this subunit may function in substrate recognition (*20*). Indeed, mutagenesis studies indicate that acidic amino acid sites in α-MPP participate in the recognition of distal basic sites, especially in long MTS (*21*). An environment-sensitive fluorescence probe attached to the peptide was also used to quantify binding of the peptide to MPP, and complex formation of the subunits may perhaps be involved in the high affinity interaction to the substrate (*22*). Thus, MPP seems to recognize several elements in MTS as a result of cooperative functions of subunits at multiple sites (*5*).

Previous characterizations of MPP purified from rat and fungus mitochondria revealed that MPP activity is highly sensitive to salt (*6*, *8*). In the presence of NaCl or KCl, the processing activity toward the precursor protein is reduced. To better understand the recognition mechanisms of MTS,

[1] This work was supported in part by Grants-in-aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (to A.I., T.O., and S.K.) and for Core Research for Evolutional Science and Technology in Japan (to A.I.).

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Abbreviations: Ad, adrenodoxin; α-MPP, α subunit of mitochondrial processing peptidase; β-MPP, β subunit of mitochondrial processing peptidase; MDH, malate dehydrogenase; MPP, mitochondrial processing peptidase; MTS, matrix targeting signal; SPR, surface plasmon resonance.

we analyzed the effects of salt on MPP activity and on substrate binding. We found that MPP is highly sensitive to salt with dramatic decreases in association to the MTS peptide. The electrostatic recognition mechanisms of MTS by MPP are discussed on the basis of analogy with the structures of core proteins in the bc_1 complex.

MATERIALS AND METHODS

Expression and Purification of MPP—Native MPP was partially purified from bovine liver mitochondria as described (*8*). To obtain recombinant MPP, we wanted to purify recombinant rat MPP from *E. coli* cells. However, most rat α-MPP is produced as inclusion bodies or insoluble aggregates in the cell, in contrast to the β-MPP subunit that is mostly in a soluble form, and the amount of the active dimer was insufficient for purification. We then used yeast α-MPP, most of which is expressed in a soluble monomeric form, as the partner to mutant rat β-MPP. Yeast α-MPP and rat β-MPP tagged with a hexahistidine at the C-terminus were individually expressed in *E. coli* strain BL21 (DE3). The recombinant subunits were purified on a Ni^{2+}-chelating Sepharose column (Amersham Pharmacia Biotech; His trap) to a purity exceeded 95%. We then mixed equal molar amounts of yeast α- and rat β-MPPs to reconstitute the enzyme complex *in vitro*. The recombinant MPP used in the experiments consists of two subunits from different organisms, and exhibits processing activity to various precursor proteins translated *in vitro*. The kinetic parameters for a synthetic oligopeptide substrate were essentially the same as those of purified bovine liver MPP (*23*).

Assays of MPP Activity—Kinetic parameters were determined using the MDH 1-21 peptide, which is based on the N-terminal sequence of rat malate dehydrogenase precursor as a substrate. The synthetic peptide was incubated with MPP in 400 μl of 10 mM HEPES-KOH buffer, pH 7.4, containing 0.1% Tween 20 at 25°C. The reaction was stopped by adding 0.05% trifluoroacetic acid. The processing products were analyzed by reversed phase HPLC as described (*17*). K_m and k_{cat} values for MPPs were determined from Lineweaver-Burk plots. Enzyme activity was also determined in a continuous fluorometric assay as described by Ogishima *et al.* (*19*) using aminobenzoyl-LARPVGAAL-RRS-FSTY(NO_2)AQNN peptide as the substrate. The recombinant MPP was incubated with the fluorogenic peptide in 10 mM HEPES-KOH buffer, pH 7.4, containing 0.01% Tween 20 at 25°C. The reaction was monitored at 420 nm (excitation at 315 nm).

Cleavage of Precursor Proteins by MPP—Radio-labeled precursor proteins were synthesized in a rabbit reticulocyte lysate containing [^{35}S]methionine. The *in vitro* translated proteins and recombinant MPP were incubated for 30 min at 30°C. The reaction was stopped by adding sample buffer for SDS-PAGE and boiling. The processing product was separated on SDS-PAGE and visualized using an Imaging Analyzer (Fuji Film; Bas1000). The processing efficiency of MPP was determined by quantifying the band density of the cleaved protein for the total protein using Image Gauge 3.0 (Fuji Photo Film, and Koshin Graphic Systems).

Binding Analysis of MTS to MPP Using Surface Plasmon Resonance (SPR)—Binding between MPP and the MDH1-21 peptide was analyzed by surface plasmon resonance measurements, SPR670 (Nippon Laser Electronics). The basic principles have been reviewed previously (*24*) and the measurements were made essentially according to demands of the customer. In brief, 60 μl of purified inactive yeast MPP complex, an alanine mutant of β-MPP at the Glu active center, was immobilized onto dithiobutyrate-coated sensor chips using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl and *N*-hydroxysuccinimide at a flow rate of 15 μl/min at 25°C. After blocking the activated carboxyl groups on the sensor chip two times with 0.5 M ethanolamine-HCl (pH 8.0), the chips were washed in 10 mM HEPES-KOH buffer, pH 7.4, containing 0.2 mM *n*-dodecyl-maltoside (Running buffer) until the baseline resonance stabilized. No MPP protein was loaded onto the control chip and the chip was blocked with 0.5 M ethanolamine-HCl (pH 8.0) to prove the specificity of binding. For kinetic measurements, MDH1-21 was injected over a range of concentrations between 0.25 μM and 50 μM. The increase in the degree of resonance (R) depended on the amount of peptide bound to MPP. We then determined the maximum values of the degree of resonance (R_{max}). The association and dissociation rates, k_{ass} and k_{diss}, were determined by calculating the dR/dT plot for the initial linear portion of the association phase and introducing the R_{max} values into the equation as follows, $dR/dT = k_{ass}[A]R_{max} - (k_{ass}[A] + k_{diss})R$, in which T and [A] are reaction time and concentration of MDH1-21, respectively. A plot of dR/dT *versus* R yields a linear function with a slope of $k_{ass}[A] + k_{diss}$ and an ordinate intercept of $k_{ass}[A]R_{max}$. The values of k_{ass} and k_{diss} were calculated based on the plot, and then introduced into the equation $K_d = k_{diss}/k_{ass}$ to determine the K_d value.

RESULTS

Effect of Various Ions on MPP Activity—When the processing activity of highly purified recombinant MPP from *E. coli* was measured using a fluorogenic peptide substrate in the presence of various concentrations of NaCl, the activity decreased to less than 5%, even in the presence of 100 mM NaCl (Fig. 1A). The sensitivity of the recombinant enzyme activity was almost the same as that of native MPP (Fig. 1A) and similar to previous data obtained using precursor proteins as substrates instead of the peptide (*6*). Thus, the salt sensitive decrease in the activity is a property intrinsic to MPP.

We examined the effect of various ions on the MPP activity to determine whether the enzyme is sensitive to salting out effects. These effects are well represented by the following Hofmeister's series: $Cl^- > Br^- > I^-$, and $NH_4^+ > K^+ > Na^+$ (*25*). Therefore, we changed the cations or anions in this series and examined the effects of concentrations on the enzyme activity. At various concentrations of monovalent ions, there was no correlation between the activity and the series (Fig. 2, B and C). Thus, the decrease in enzyme activity is not due to salting out effects, but rather depends on salt concentration. We then examined the effects of divalent cations as divalent ions have a higher ionic strength than monovalent ions at the same concentration. The MPP activity was more sensitive to divalent cations than to monovalent cations (Fig. 2D). Even 10 mM magnesium or calcium chloride led to a marked reduction in the activity to less than 20% of that in case of no salt. Magnesium ion had slightly stronger effect on the activity than calcium ion. The

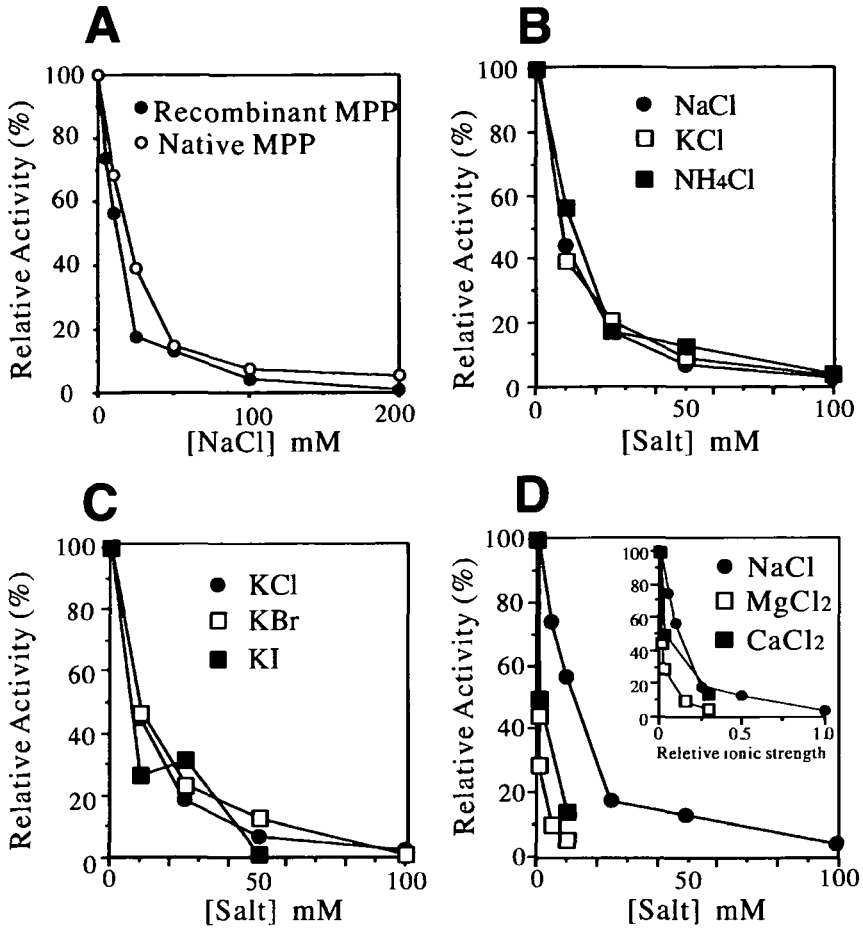

Fig. 1. **Effect of various ions on MPP activity.** The activity of MPP was measured using a fluorogenic peptide as substrate in HEPES buffer containing various concentration and types of ions at 25°C. The reaction was monitored at 420 nm (excitation at 315 nm). The activity is given as the percentage of the activity in the absence of salt. A: Effect of salt on the activity of native and recombinant MPP. Native MPP was partially purified from bovine liver mitochondria. The hexahistidine-tagged α- and β-MPP subunits were individually expressed in *E. coli* and purified using a Ni^{2+}-chelating Sepharose column, and active MPP was reconstituted *in vitro* by mixing of the two subunits. B: The decrease in MPP activity depends on the concentration of the monovalent cation. NH_4Cl (filled squares), KCl (open squares), NaCl (filled circles). C: The decrease in MPP activity depends on the concentration of the monovalent anions. KCl (filled circles), KBr (open squares), KI (filled squares). D: The decrease in MPP activity depends on the concentration of the divalent cation and the relative ionic strength. Relative MPP activities in the presence of various divalent cations, Na^+ (filled circles), Mg^{2+} (open squares), or Ca^{2+} (filled squares) are indicated. The insert represents the effects of relative ionic strength on MPP activity at the concentration of 100 mM NaCl used as a standard.

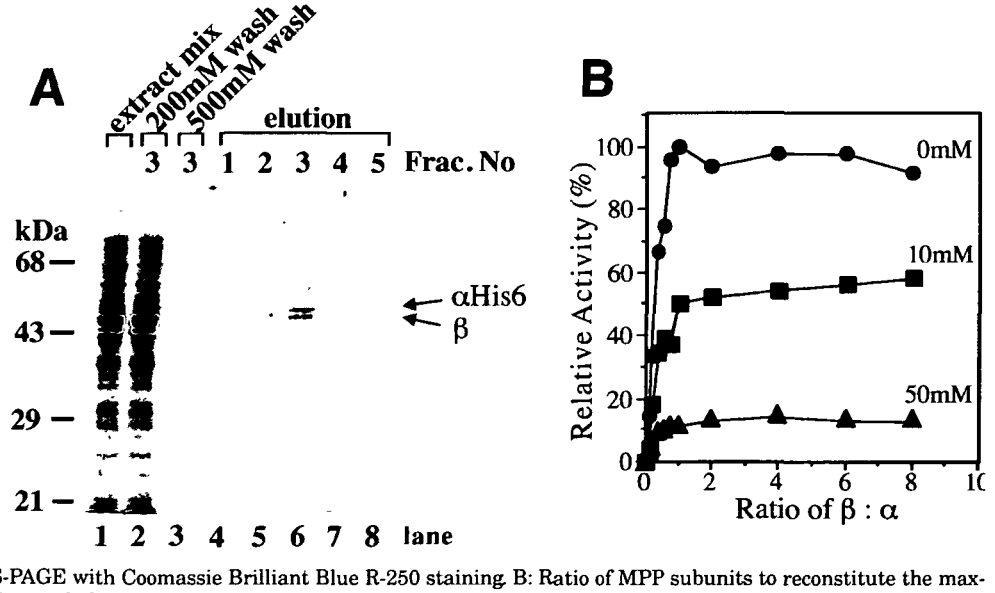

Fig. 2. **Interaction of MPP subunits in salt solution.** A: Co-elution of MPP subunits after washing the Ni^{2+}-chelating column under high salt conditions. Interactions between the α- and β-MPPs were detected using a Ni^{2+}-chelating column. Purified yeast hexahistidine-tagged α-MPP (αHis6) was mixed with bacterial extract expressing non-tagged β-MPP, and the mixture (extract mix) was applied onto a Ni^{2+}-chelating Sepharose column (bed volume: 1 ml). After washing the column with HEPES-KOH buffer (pH 7.5) containing 200 and 500 mM NaCl, the proteins were eluted with buffer containing 500 mM imidazole. The eluted proteins (fraction volume: 1 ml) were analyzed by SDS-PAGE with Coomassie Brilliant Blue R-250 staining. B: Ratio of MPP subunits to reconstitute the maximum activity in salt solution. Highly purified subunits were mixed to reconstitute the active enzyme. The β-MPP was added to the α-MPP in salt solution, 0 mM (filled circles), 10 mM (filled squares), or 50 mM (filled triangles) NaCl, and the reconstituted activity was measured using a fluorogenic peptide as a substrate in HEPES buffer containing varying concentrations of NaCl at 25°C. The relative activity is given as the percentage of the activity at an equimolar ratio of subunits in the absence of NaCl.

effects of the relative ionic strengths of monovalent and divalent ions on the activity are shown in the inset in Fig. 1. Compared with salt concentration, the decrease in activity showed a much closer dependency on ionic strength

between monovalent and divalent ions, which means that ionic strength is mainly involved in the salt sensitivity of the enzyme.

Effect of Salt on the MPP Complex and Structure of the MTS Peptide—Protein–protein interactions are often reduced in high ionic strength solutions. To determine if the decrease in enzyme activity is due to dissociation of the subunits under salt conditions, the complex bound to a Ni^{2+}-chelating column *via* C-terminal polyhistidine-tag of α-MPP was exposed to 200 and 500 mM NaCl. When the salt was washed from the column, we detected only a small amount of β-MPP dissociated from the complex (Fig. 2A, lane 3), and practically the same amount of the two subunits was recovered using imidazole (Fig. 2A, lane 6). Therefore, tight binding between the subunits probably exists under salt conditions. Molecular titration experiments using *in vitro* MPP reconstituted from the subunits revealed that equal moles of the two subunits are adequate to obtain maximum activity, even in the presence of 50 mM NaCl (Fig. 2B). The active dimer formation did not change in relation to ionic strength. Thus, the salt sensitivity of the enzyme is apparently not the result of subunit dissociation.

To determine whether salt-dependent decreases in MPP activity are due to conformational changes in the substrate, we next examined the secondary structure of the MTS peptide of rat MDH in 0 and 100 mM NaCl using a CD spectrophotometer. Under our assay conditions, the peptide formed a random coil structure in both low and high salt solution (data not shown). We observed no drastic change in peptide conformation. Thus salt has practically no effect on substrate structure, and a change in the substrate structure by salt can not explain the salt sensitive activity of MPP.

Increase in the Michaelis Constant of the Enzyme with Ionic Strength—We determined the kinetic parameters of MPP using the MDH 1-21 peptide as substrate. Table I summarizes the effects of salt concentration on the kinetic parameters of MPP activity. The K_m value is sensitive to salt, with the value increasing more than 100-fold at 100 mM NaCl over the level in the absence of salt or $MgCl_2$; even at 1 mM NaCl, the K_m value was increased about 10 times. On the other hand, the turnover number, k_{cat}, is slightly decreased by salt and this results in a dramatic reduction of cleavage efficiency. As shown Fig. 3, plots of K_m values for relative ionic strength indicate that an increase in the K_m value correlates well with ionic strength. Thus, an increase in ionic strength likely leads to a change in the affinity of the peptide for MPP, and results in a drastic reduction of MPP activity.

Lowering the Affinity Is the Result of Decreases in the Association Rate (k_{ass}) between MPP and the MTS Peptide—We examined interactions of the MTS peptide with yeast

TABLE I. **Effect of various salts on kinetics parameters of MPP.** The kinetic parameters of MPP were measured using the MDH 1-21 peptide, as described in "MATERIALS AND METHODS" and calculated from Lineweaver-Burk plots.

	Salts (mM)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\cdot\mu M^{-1}$)
	0	0.074	84	1,200
NaCl				
	10	0.87	83	95
	50	2.4	33	14
	100	8.2	22	2.7
$MgCl_2$				
	1	0.61	93	140
	10	2.1	32	15

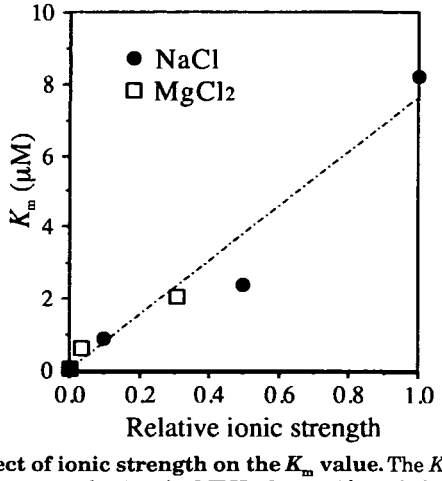

Fig. 3. **Effect of ionic strength on the K_m value.** The K_m values of MPP were measured using the MDH1-21 peptide and plotted for relative ionic strength compared to a concentration of 100 mM NaCl, used as a standard. The K_m values in the presence of NaCl and $MgCl_2$ are indicated by filled circles and open squares, respectively.

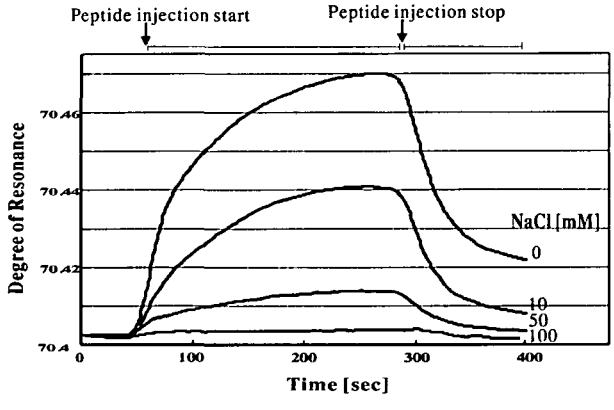

Fig. 4. **Effect of salt on the interaction between MPP and the MDH peptide as detected by SPR.** Purified inactive mutant MPP complex (6 μg) was loaded and immobilized onto the sensor at a flow rate of 15 μl/min at 25°C. After blocking the activated carboxyl groups on the sensor chip with ethanolamine-HCl, the chips were equilibrated in running buffer containing 0 to 100 mM NaCl. MDH1-21 peptide (20 μM) was then infused over the surface of the chip and the degree of resonance depends on the interaction between MPP and the peptide. The start and stop times of peptide injection are indicated by arrows.

TABLE II. **Effect of salt on the association and dissociation rates between MPP and the MTS peptide.** The association and dissociation rates of the peptide to MPP were measured by real-time, direct observations of the protein–peptide interaction using the SPR technique. The association and dissociation rates (k_{ass} and k_{diss}) were determined by calculating the d*R*/d*T* plot for the initial linear portion of the association phase.

NaCl (mM)	k_{ass} (1/Ms)	k_{diss} (1/s)	K_d (M)
0	3.5×10^3	1.4×10^{-3}	0.38×10^{-6}
10	1.4×10^3	2.3×10^{-3}	1.7×10^{-6}
50	0.47×10^3	1.9×10^{-3}	4.0×10^{-6}

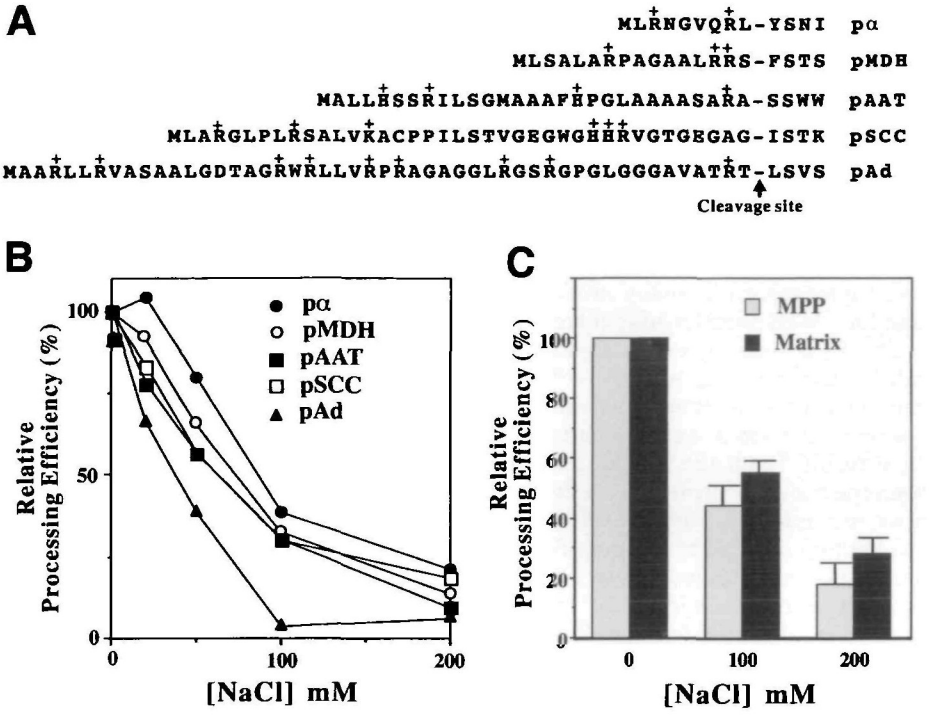

Fig. 5. **Salt dependent decrease in processing efficiency toward precursor proteins.** A: Amino acid sequences of the N-terminal regions of the precursors. (+) indicates positive charged residues in the MTS regions. The arrow indicates the MPP cleavage site. B: Effect of salt on the processing of precursor proteins by MPP. Radiolabeled precursors and recombinant MPP were incubated in reaction buffer containing NaCl for 30 min at 30°C. The processing product was separated on SDS-PAGE and visualized with an Imaging Analyzer. The processing efficiency of MPP was determined by quantifying the band density of the cleaved protein relative to total protein, and the relative activity is given as a percentage of activity in the absence of NaCl. pα, precursor of yeast α-MPP; pMDH, precursor of mouse malate dehydrogenase; pAAT, precursor of mouse aspartate aminotransferase; pSCC, precursor of bovine cytochrome P-450 (scc); pAd, precursor of bovine adrenodoxin. C: Comparison of the salt sensitivity of MPP activities between the purified enzyme and matrix fraction. The radiolabeled precursor protein (MDH) was incubated with recombinant MPP (50 pg) or yeast mitochondrial matrix fraction (50 ng) in reaction buffer containing NaCl for 15 min at 30°C.

MPP, the catalytic glutamate of which was mutated to alanine, by SPR analysis. This system makes it possible to determine the rates of association (k_{ass}) and dissociation (k_{diss}) between the enzyme and the peptide in real time and directly without a proteolytic reaction. As expected, the binding of the peptide to MPP immobilized on the sensor chip was observed in low salt buffer, while there was little binding in high salt buffer (Fig. 4). This binding is specific, as the MPP did not efficiently receive peptides with alanine substitutions at the distal or proximal arginine, even under low salt conditions (data not shown). Table II shows the k_{ass}, k_{diss}, and K_d values at various salt concentrations calculated as described in "MATERIALS AND METHODS." The k_{ass} values decreased to less than one-tenth as the NaCl concentration was reduced from 0 to 50 mM, whereas the k_{diss} values remained essentially the same. This analysis reveals that a decrease in the k_{ass} value in high salt leads to a reduction in the K_d value between MPP and MTS. Thus, ionic strength inhibits the accessibility of the MTS peptide to MPP and weakens the affinity between the enzyme and substrate.

Effect of Salt on the Processing Activity toward Precursors—Until now, we used the MDH peptide to investigate the salt sensitivity of MPP. We next analyzed the processing efficiency toward five mitochondrial precursor proteins with different MTS sequences containing 9 (in the case of pα) to 50 (in the case of pAd) amino acids (Fig. 5A). The processing efficiency of all precursors decreased at higher concentrations of NaCl, although the sensitivities differed among the precursors (Fig. 5B). Thus, salt sensitive decreases in activity seem to be shared by various MTS.

The maximum suppression of MPP activity by salt is obtained within the physiological salt concentration range *in vitro*. However *in vivo*, all of the mitochondrial precursor proteins are converted into the mature form. Therefore, we compared the salt sensitivities of the activities between the MPP and mitochondrial matrix fraction (Fig. 5C). At the physiological salt concentration range of 100 to 200 mM NaCl, no substantial differences in sensitivity were observed between the two preparations, indicting that MPP activity in the matrix fraction is also sensitive to salt. Thus, we prefer an interpretation that salt sensitive activity is an intrinsic property of MPP rather than being due to differences in the microenvironment surrounding the enzyme or to differences between the conformations *in vitro* and *in vivo*.

DISCUSSION

The evidence we obtained shows that the affinity between MPP and the MTS peptide is highly sensitive to ionic strength and that MPP associates with MTS in precursor proteins through electrostatic interactions. The K_m value of the enzyme is increased under high salt conditions and the increases correlate with the ionic strength of the assay buffer. Quantitative measurements of protein–peptide interactions using SPR indicated that the reduced affinity is the result of decreases in the association rates in between MPP and the MTS peptide.

In the present work, we observed significant differences in salt sensitivity among the precursors. The processing efficiency for pAd is greatly decreased by salt, while that for pα is not so sensitive. The arginine on the N-terminal side of the MTS of pα is not involved in processing by MPP (*21*). Thus, the actual N-terminal basic net charge required for recognition by MPP is much lower than that in pAd; MTS has nine basic sites in its fifty amino acids. The

strength of the electrostatic interaction with MPP may depend on the degree of positive net charge in MTS. This assumption is consistent with our recent data that the synthetic MTS peptide of bovine pAd has much lower K_m and K_d values than the MTS peptide of rat MDH (Yamasaki *et al.*, unpublished results). The pAd has many basic residues in its long MTS, whereas the MTS region of MDH contains three basic residues in a short N-terminal stretch (Fig. 5A). The basic sites in a long MTS could contribute to the stabilization of the enzyme-substrate complex through electrostatic interactions at many acidic sites on MPP, meaning that long MTS peptides have a high affinity for MPP.

We found that the association rate, not the dissociation rate, of the targeting peptide to MPP decreases at higher salt conditions, as determined in SPR experiments. Thus, electrostatic force is required for to make the peptide accessible to MPP and the peptide does not readily dissociate from the enzyme-substrate complex once the enzyme recognizes and interacts with the MTS peptide at the appropriate binding site. MPP subunits from mammals and fungi are highly homologous to core I and II proteins in the bc_1 complex of mitochondria, and in some species of higher plants the MPP and core complexes are identical. MPP also forms a heterodimer, so the over-all structure of the complex should be similar to that of the core proteins. Thus, the crystal structures of bovine and chicken cytochrome bc_1 complexes (*26–28*) can provide clues as to the recognition mechanisms of MPP. The two core proteins, which are structurally similar, consist of two domains of roughly equal size and an almost identical folding topology. In the dimer, the complex forms a crack leading to a large internal cavity, the wall of which is lined with mostly hydrophilic amino acid residues. Since dimerization of the MPP subunits is essential for high affinity interactions with the MTS peptide, the substrate-binding pocket is provided by both α- and β-MPP (*22*). When MPP recognizes the positive N-terminal MTS, the MTS peptide is likely to be transported electrostatically into the negatively charged cavity formed by both MPP subunits (Fig. 6). The N-terminal basic sites of MTS are mainly bound to the α subunit surface of the cavity, while the cleavage site must be in contact with the reactive site on the β subunit. Since a small compound, such as acylamide, can hardly enter the cavity to quench the fluorescent probe attached to the MTS peptide of MDH (*22*), just after incorporation of the substrate, MPP may close and pack the peptide tightly in the cavity. After that, the MTS is cleaved at the reactive center in β-MPP and the MPP-MTS complex dissociates. It remains unknown why MPP efficiently releases the cleaved MTS peptide and what is the molecular basis for the difference in affinity between uncleaved and cleaved MTS peptides, whose positive net charges are even. Mechanisms related to the productive dissociation of the cleaved MTS peptide from the enzyme-substrate complex remain to be examined.

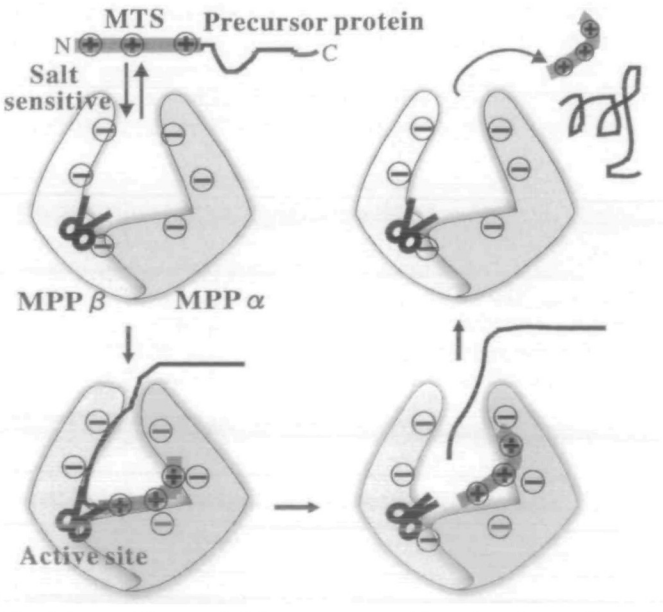

Fig. 6. **A putative model for the recognition of and reaction of MPP with precursor protein.** See "DISCUSSION" for details. + and − symbols represent basic and acidic amino acids, respectively. Bold and narrow lines of precursor proteins indicate the MTS region and the mature portion, respectively. Scissors indicate the active site of the MPP β subunit.

The enzyme activity in the matrix as well as the purified MPP activity are sensitive to salt under isotonic salt concentrations. Under similar conditions *in vivo*, protein precursors are imported into the matrix and processed. We observe little accumulation of precursors in mitochondria *in vivo* or during *in vitro* import experiments into mitochondria. Therefore, MPP should have enough activity to process the precursor proteins under isotonic salt concentration in mitochondria, although it is difficult to estimate only MPP activity *in vivo*. However, the concentrations of ions in mitochondria may vary at different respiratory states. Moreover, mitochondria are essential not only for energy conversion but also for the Ca^{2+} signaling pathway, homeostasis, and cell death (*29–32*). As Ca^{2+} signaling organelles, mitochondria can rapidly take up and slowly release large amounts of Ca^{2+}. Mitochondria can also pump Ca^{2+} out of the cytosol when cellular Ca^{2+} levels are extremely high, usually as a result of cell damage. Under these conditions, MPP would regulate the maturation of newly imported proteins and/or mitochondrial biogenesis by its salt sensitive nature.

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