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Studies on the sedimentation behavior of the *Neurospora crassa* plasma membrane H^+-ATPase synthesized in vitro and integrated into homologous microsomal membranes

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RNA transcripts that encoded the *Neurospora crassa* plasma membrane H^+-ATPase (pma^+), a polytopic integral membrane protein, and the pma^+_{344}, a truncated pma^+ with the amino terminal 344 amino acids, were translated in a *N. crassa* in vitro system. The microsomal membranes integrated products were insensitive to extraction by Na_2CO_3 (pH 11.5). The velocity sedimentation behavior of the in vitro synthesized pma^+ were examined under various conditions. The pma^+ migrated on linear sucrose gradients as aggregates which were heterogeneous in size, in the regions of 9–13 S; whereas, these values were reduced when Triton X-100 was presence in the gradients. The formation of these aggregates is interpreted to suggest a mechanism that maintains this polytopic integral membrane protein in a soluble form until it is targeted to the membranes. The sedimentation coefficient of the Triton X-100 solubilized microsomal membranes integrated pma^+ corresponded roughly to a monomer of the pma^+. Furthermore, a comparison of the trypsin cleavage patterns of the in vitro synthesized pma^+ and of the microsomal membranes integrated pma^+ suggest that they have different tertiary, or quaternary, structures. The latter did not give the characteristic trypsin cleavage patterns that have been observed for the native pma^+ in the presence of its ligands MgATP and vanadate (Addison, R. and Scarborough, G.A. (1982) J. Biol. Chem. 257, 10421–10426). This was interpreted to suggest that the microsomal membranes integrated pma^+ cannot interact with its substrates, suggesting that it is catalytically inactive.

Introduction

The *N. crassa* plasma membrane electrogenic, proton-translocating ATPase (pma^+) generates a transmembrane proton-motive force that functions to drive the uptake of various ions and molecules from the milieu into the cell via chemiosmotic couplers known as porters [1]. The pma^+ is a polytopic integral membrane protein (IMP) with at least ten transmembrane spanning segments and with hydrophilic domains alternately exposed to the cytoplasmic and the exoplasmic sides of the membrane [2,3]. The amino terminus, and maybe the carboxyl terminus, is located on the cytoplasmic side of the membrane [4]. Properties of the pma^+ [5,6] are similar to those of the aspartylphosphoryl-enzyme intermediate family which comprise the pma^+ from *Saccharomyces cerevisiae*, H^+/K^+-ATPase from the gastric mucosa, Na^+/K^+-ATPase from the plasma membrane of eukaryotes, and the Ca^{2+}-ATPase from the sarcoplasmic reticulum and dissimilar to those of the proton-translocating F_1F_0-ATPase/ATP synthases of mitochondria, bacteria, and chloroplasts. Furthermore, a comparison of the amino acid sequences of the aspartylphosphoryl-enzymes revealed that several regions are highly conserved [2,3], suggesting that these serve essential functions common to these energy transducers. This is consistent with several models that postulated that this family of ATPases may function via similar mechanisms [7,8]. Monomer [9] of the hydrolytic moiety of the *N. crassa* pma^+ is capable of efficient proton translocation [10]. Since the pma^+ translocates only proton, this makes it the simplest of this family of ATPases.

Abbreviations: pma^+, plasma membrane H^+-ATPase; pma^+_{344}, a truncated pma^+ with the amino terminal 344 amino acids; nRM, *Neurospora crassa* rough endoplasmic reticulum microsomes; ER, endoplasmic reticulum; IMP, integral membrane protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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A goal of biological science is to understand not only the molecular mechanisms by which these cation-motive ATPases transduce the chemical energy of ATP hydrolysis into transmembrane gradients, but to understand also how the expression of these ATPases are regulated, how they are integrated into the membrane, and how they are sorted to the various intracellular compartments. Clearly, an important prerequisite to obtaining these goals is a system that is tractable to both biochemical and genetic manipulations. With the exception of *N. crassa* and *S. cerevisiae*, the other systems are less tractable to these methods. Since the pma^+ is the simplest of these cation-motive ATPases and more is known about the subunit composition of the *N. crassa* pma^+, it has been chosen as a model for investigating the integration of polytopic IMPs into microsomal membranes. In this paper, it is demonstrated that the in vitro synthesized pma^+ forms soluble, heterogeneous, high molecular weight aggregates, and it is integrated into microsomal membranes in a form that is unable to bind its substrates.

Experimental Procedures

Fractionation of the Translation Mixture. Translations of RNA transcripts in the *N. crassa* in vitro system were performed as described [11]. The assay was terminated by adding a 5-fold volume of 0.2 M Na_2CO_3 (pH 11.5). The mixture was incubated on ice for 10 min and, afterward, layered onto 150 μl of a 0.5 M sucrose cushion containing 0.1 M Na_2CO_3 (pH 11.5), 150 mM KOAc, 2 mM $Mg(OAc)_2$, and 1 mM DTT. Gradients were centrifuged in an airfuge (Beckman), using rotor type A-100/30, at 25 psi for 5 min, after attaining top speed. All procedurces were conducted at 4°C, unless otherwise indicated. Afterward, the applied volume and the sucrose cushion were removed, this is referred to as the soluble (S) fraction, except where noted. The pelleted microsomal membranes (P) were dissolved in 30 μl of 0.5 M Tris, 6.25% (w/v) SDS, and 10% (v/v) β-mercaptoethanol. The samples were incubated at ambient temperature for 1 h with occassional mixing. The soluble fraction was adjusted to 15% trichloroacetic acid (TCA), incubated on ice for 20 min, and centrifuged in a microfuge, placed in the cold room, for 10 min. The precipitated proteins were dissolved in the Tris/SDS/β-mercaptoethanol buffer and incubated as aforementioned.

Fractionation on sucrose gradient. Velocity sedimentation analyses were performed essentially as described [12] on 5-ml linear gradients consisting of 5–20% (w/w), or 10–40% (w/w), sucrose with 40 mM K^+-Hepes (pH 7.5), 0.1 M KOAc, 5 mM $Mg(OAc)_2$, 2 mM DTT, and 0.1% (v/v) Triton X-100 (where indicated). Fractions of approximately 0.4 ml were collected, by drops, from the top of the gradient, using a gradient former (Haake Buchler Instruments, Inc.). These were adjusted to 20% (w/v) TCA, and the precipitated proteins were treated as aforementioned. The method of McEwen [13] was used for estimating the sedimentation coefficient. The sucrose concentration of each of the collected fractions was measured by the Bausch & Lomb hand refractometer.

Digestion by trypsin. The samples in a final volume of 60 μl were incubated in an ice-water bath for 15 min. $CaCl_2$ and trypsin were added from stock solutions to give final concentrations of 0.25 mM and 50 μg/ml, respectively. The resultant samples were incubated for 5 min at 0°C. The reactions were stopped by adding soybean trypsin inhibitor from a stock solution to a final concentration of 200 μg/ml. This was followed by the addition of 75 μl of 25% (w/v) TCA. The precipitated proteins were treated as mentioned.

Other methods. The preparation of the *N. crassa* translation extract and microsomal membranes were as described [11]. The plasmid, pSPM8, containing the cDNA for the *N. crassa* pma^+ was a generous grift of Drs. S.M. Mandala and C.W. Slayman of the Department of Human Genetics at Yale University School of Medicine, New Haven, CN. For the production of pma^+, pSPM8 was restricted with the restriction endonuclease *Nde*I; for pma^+_{344}, pSPM8 was restricted with *Nco*I. The plasmid, pAB208 [11], which contains the yeast invertase cDNA was linearized with the restriction endonuclease *Eco*RI. The linerized DNA templates (100 μg/ml) in 300 μl were transcribed as described by the supplier of the SP6 RNA polymerase (Promega). The densities of the translated products were quantitated by scanning the individual lanes in an autoradiograph with a LKB 2222-020 Ultroscan XL Enhanced Laser Densitometer.

Materials. The sources of most of the materials have been previously described [11]. The other reagents were of the highest commercially available grade.

Results

The plasmid, pPSM8, containing the *N. crassa* pma^+ cDNA was linearized with the restriction endonuclease *Nde*I prior to transcription with SP6 RNA polymerase to prepare RNA transcripts for the full length pma^+. The pma^+ is an IMP with at least 10 putative transmembrane spanning segments (Fig. 1). The same plasmid was linearized with the restriction endonuclease *Nco*I to prepare truncated RNA transcripts encoding the amino terminal 344 amino acids of the pma^+ (pma^+_{344}): These have no termination codon. The resultant product contains the first three transmembrane spanning segments and 24 of the 32 amino acids of the fourth transmembrane spanning segment (Fig. 1). Re-

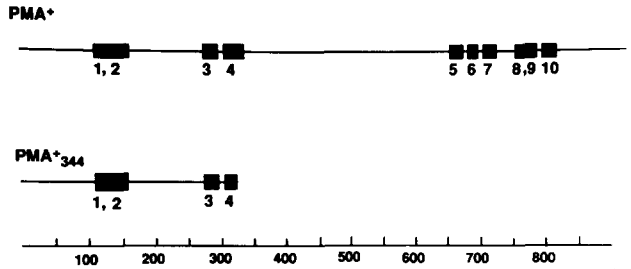

Fig. 1. Schematic diagrams of proteins encoded by pSP6M. The boxes represent putative transmembrane spanning segments. The amino acids sequence number is indicated along the horizontal axis.

sults from other laboratories [14,15] have demonstrated that truncated RNA transcripts without termination codon encoded for post-translationally integration-competent products. Therefore, this truncated version of the pma^+ was chosen for study because of the relative distance between the first two transmembrane spanning segments and the end of the polypeptide chain: eliminating interference from the bound ribosomes with the integration of the pma^+_{344} into the microsomal membranes.

When the RNA transcripts for the pma^+ were used to program the *N. crassa* in vitro system, a product with a $M_r \approx 99$ K was observed on SDS-PAGE (Fig. 2, lane 1). This is approximately the observed M_r for the pma^+ on SDS-PAGE. Additional proof to the identity of the product was obtained when it was immunoprecipitated

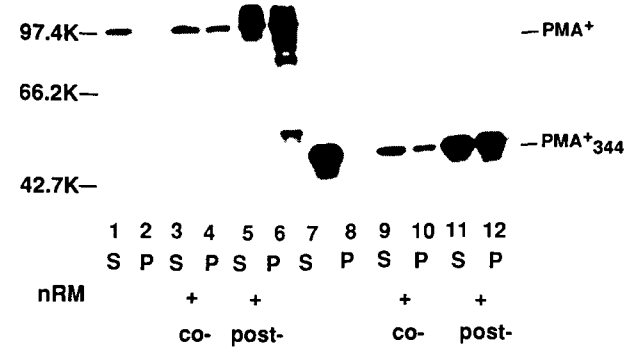

Fig. 2. Integration of pma^+ and pma^+_{344} into nRM in a *N. crassa* in vitro system. The RNA transcripts (20 ng/μl) for pma^+, or for pma^+_{344}, were translated in the *N. crassa* in vitro system in a final volume of 20 μl as described [11]. nRM were at a concentration of 3 A_{280} units/μl. For post-translational studies, emetine (1 mM) was added to stop protein synthesis, and the resultant mixture was incubated for 5 min before adding nRM. Afterward, the resultant mixtures were incubated for 20 min at 18°C. These were layered onto sucrose-0.1 M Na_2CO_3 (pH 11.5) cushions and fractionated into soluble (S) and membrane (P) fractions as outlined in Experimental Procedures. The final samples were analysed by SDS-PAGE, using 10% (w/v) acrylamide gel. After electrophoresis, the gel was placed in an aqueous methanol, acetic acid solution, transferred and soaked in Enlighting (NEN Research Products), dried, and exposed to Fuji film overnight at −80°C. The molecular weight markers are phosphorylase B, bovine serum albumin, and ovalbumin with M_r of 97.4K, 66.2K, and 42.7K, respectively.

by rabbit polyclonal antibodies against the purified *N. crassa* pma^+ (data not shown).

When microsomal membranes, containing mostly vesiculated endoplasmic reticulum (nRM) [11], were present co-translationally, approx. 40% of the pma^+ co-sedimented with the microsomal membranes, in the presence of 0.1 M Na_2CO_3 (pH 11.5), and the pma^+ that co-sedimented with the microsomal membranes migrated similarly as the soluble form on SDS-PAGE (lanes 3 and 4), suggesting that the pma^+ was unmodified by nRM in a way that can be detected by M_r differences: for example, by alterations in the M_r caused by cleavage of an amino terminal signal sequence or by the addition of asparagine-linked high mannose oligosaccharides. Similar results were obtained when the pma^+ was integrated into heterologous microsomal membranes [16]. Surpisingly, when nRM were added post-translationally, approximately 50% of the pma^+ co-sedimented with nRM, in the presence of 0.1 M Na_2CO_3 (pH 11.5) (lanes 5 and 6). Extraction of microsomal vesicles by Na_2CO_3 (pH 11.5) converts them into open sheets [17]. This allows for the extraction of the lumenal contents as well as peripherally associated proteins from the cytoplasmic and exoplasmic sides of the membrane. As noted, the pma^+ co-sedimented with nRM in the presence of 0.1 M Na_2CO_3 (pH 11.5). Also, the pma^+ was found associated with nRM after extraction of the mixture with 4 M urea, 25 mM EDTA, or 0.5 M KOAc (data not shown). Taken together, this suggests that the pma^+ had become an integral part of nRM. The results with the pma^+_{344} (Fig. 2, lanes 7–12) were similar to those obtained with the pma^+. Since the RNA transcripts for the pma^+_{344} have no termination codon, the pma^+_{344}, presumedly, should be physically associated with ribosomes as a peptidyl-tRNA complex. To gain insight into this possibility, the translation mixture containing the pma^+_{344} was fractionated on a linear sucrose gradient (Fig. 3A). At least 70% of the pma^+_{344} migrated in the positions of monosomes and polysomes. Whereas, if the tRNA analogue puromycin was added to the translation mixture before fractionation on the linear sucrose gradient, the position of the pma^+_{344} was shifted toward the top of the gradient (Fig. 3B), indicating its release from the ribosomes. Under similar conditions, the pma^+ was found in almost every fraction of the gradient, with approx. 50% within the first three fractions. Puromycin had no effect on this pattern (data not shown).

On 5–20% linear sucrose gradients, in the presence of 0.1 M KOAc, the pma^+ migrated with a sedimentation coefficient (S) of approx. 11 for the peak fraction (Fig. 4A). The sedimentation profile had a broad range in the region of 9–135. Whereas, the pma^+_{344} peaked in two positions in the sucrose gradient with S values of the peak fractions of approx. 9 and 15, respectively (Fig. 4B). This sedimentation profile had a broad range in the

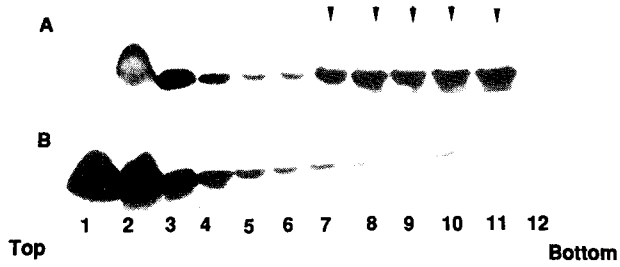

Fig. 3. Pma$^{+}_{344}$ association with ribosomes on a linear sucrose gradient. (A) An 100 μl translation mixture with pma$^{+}_{344}$ was layered onto a 5 ml 10–40% sucrose gradient containing 40 mM K^+-Hepes (pH 7.5), 100 mM KOAc, 5 mM $Mg(OAc)_2$, 0.1% (v/v) Nikkol, and 2 mM DTT. (B) Before layering onto a gradient, puromycin was added to a final concentration of 2 mM, and the resultant mixture was incubated for 10 min at 18°C. The gradients were centrifuged in a SW 50.1 rotor (Beckman) at 115000× g_{av} for 3 h at 4°C. The gradients were fractionated and analysed as outlined in Experimental Procedures. The samples were analysed by SDS-PAGE, using 12.5% (w/v) acrylamide. The arrow heads mark the positions of monosomes and polysomes.

region of 6–15 S. Approx. 70% of the aggregates of the pma$^{+}_{344}$ was in the region of 13–15 S. These gradients did not contain visible pellets or any immunoprecipitable radioactivity from the bottom of the tubes (not shown). Since the pma^{+} is a large hydrophobic protein and since little is known about the behavior of these large IMPs in the aqueous milieu of these in vitro systems, a well characterized secretory protein was also examined under identical conditions. Yeast invertase has been studies for many years and has been well characterized [18]. The RNA transcripts for the yeast invertase were translated in the *N. crassa* in vitro system, and the resultant mixture was subsequently analysed on a linear sucrose gradient. Invertase migrated as a board peak, with an *S* value of the peak fraction of approx. 7 (Fig. 4C). The RNA transcripts for invertase

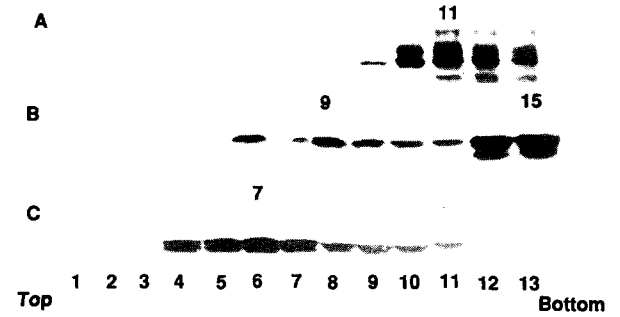

Fig. 4. Velocity sedimentation behavior of pma^{+}, pma$^{+}_{344}$, and invertase on 5–20% linear sucrose gradients. A translations mixture (100 μl) containing either pma^{+} (A), pma$^{+}_{344}$ (B), or invertase (C) was layered onto a 5 ml 5–20% sucrose gradient. Before layering onto a gradient, the mixture with pma$^{+}_{344}$ was adjusted to 2 mM puromycin and was incubated for 10 min at 18°C. The gradients were centrifuged, fractionated, and analysed as outlined in Experimental Procedures. Samples were analysed by SDS-PAGE, using 10% (w/v) acrylamide gel. The numbers above each indicate the sedimentation coefficient of the peak fraction.

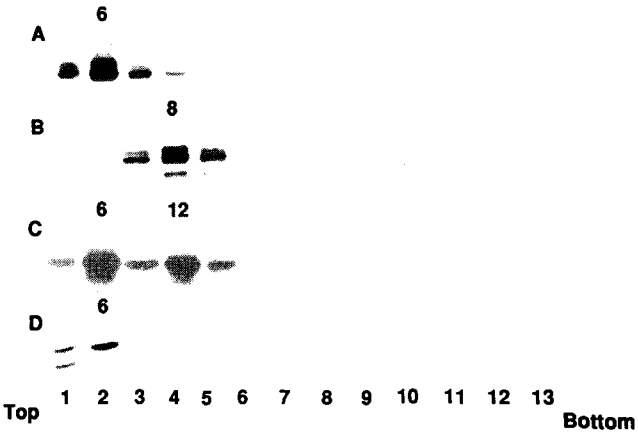

Fig. 5. Velocity sedimentation behavior of Triton X-100 solubilized pma^{+} and pma$^{+}_{344}$. To a translation mixture (100 μl) with pma^{+}, or pma$^{+}_{344}$, nRM was added to 5 A_{280} units/μl, and the resultant mixture was incubated for 20 min at 18°C. Afterward, this mixture was layered onto 200 μl of 0.5 M sucrose, 40 mM K^+-Hepes (pH 7.5), and 2 mM DTT. This was centrifuged in a TLA-100 rotor (Beckman) at 245070× g_{max} for 6 min at 4°C. The applied volume and 50 μl of the top most part of the sucrose layer were removed and used as the soluble fraction (B, D). This was adjusted to 1% Triton X-100 and 0.3 M KOAc. The pelleted microsomal membranes (A, C) were resuspended in 100 μl of 1% Triton X-100, 6% (v/v) glycerol, 40 mM K^+-Hepes (pH 7.5), 0.3 M KOAc, 5 mM $Mg(OAc)_2$, 2 mM DTT, 0.5 mM PMSF, and 0.5 μg/μl each of pepstatin A, chymostatin, antipain, and leupeptin. Samples were incubated at 4°C for 20 min and, afterward, were centrifuged, using the TLA-1 00 rotor, as aforementioned. The resultant supernatants were applied to individual 5 ml 10–40% sucrose gradient. The gradients were centrifuged, fractionated, and analysed as outlined in Experimental Procedures. The samples were analysed by SDS-PAGE, using 10% (w/v) acrylamide gel. The numbers above each indicate the sedimentation coefficient.

encoded for the secretory and the constitutive forms [19]. These form dimers of identical subunits [20,21]. As previously demonstrated [11], the top band of the pair in Fig. 4C is the secretory form; the bottom, the constitutive form. Both forms migrated with a sedimentation profile distributed in the region of 4–8 S (Fig. 4C). In addition, the former formed higher aggregates which extented its sedimentation profile to near the bottom of the gradient.

The nRM integrated pma^{+} was separated from the soluble form by centrifugation through a sucrose cushion containing physiological concentration of salts. The pelleted membranes were resuspended in a buffer containing 0.3 M KOAc and 1% Triton X-100. The resultant mixture was layered on a linear 10–40% sucrose gradient containing 0.1 M KOAc and 0.1% Triton X-100, the solubilized pma^{+} migrated with a S value of the peak fraction of approx. 6 (Fig. 5A). The resultant soluble fraction was adjusted to 1% Triton X-100 and 0.3 M KOAc and was run on sucrose gradients containing 0.1% Triton X-100. The *S* value of the peak fraction of the soluble form was approx. 8 (Fig. 5B). In contrast, when the membrane integrated form of pma$^{+}_{344}$ and its soluble form were treated similarly, the former peaked

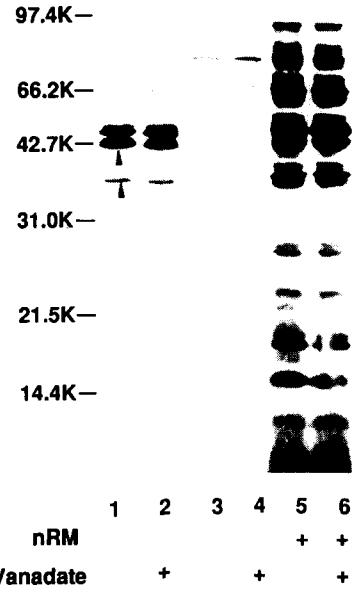

Fig. 6. SDS-PAGE analyses of the effects of trypsin on the soluble and the nRM integrated forms of pma$^+$. The post-translational integration of pma$^+$ into nRM and the fractionation of the resultant mixture were conducted as outlined in the legend to Fig. 5. The applied volume was removed from the gradient and used as the soluble fraction. The pellet was resuspended in 100 μl of a buffer containing 40 mM K$^+$-Hepes (pH 7.5), 120 mM KOAc, 3 mM $Mg(OAc)_2$, 6% (v/v) glycerol, 2 mM DTT, and 0.5 μg/μl of chymostatin. For the data in lanes 1 and 2, a translation mixture (100 μl) with pma$^+$ was desalted by chromatography on a 1-ml column containing Sephacryl S-200 as described [26]. The void volume (200 μl) was divided (100 μl) and used in the trypsin assay. The resultant samples were incubated with trypsin and were subsequently analysed as outlined in Experimental Procedures. The number of cpm/μl added per digestion for samples in lanes 1 and 2 were approx. 30% less than for the others samples. The samples were resolved by SDS-PAGE, using 12.5% (w/v) acrylamide gel. The molecular weight markers are phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme with M_r of 97.4K, 66.2K, 42.7K, 31.0K, 21.5K, and 14.4K, respectively.

in two positions in the gradient, with S values of the peak fractions of approx. 6 and 12, respectively (Fig. 5C). The closeness of the peaks in Fig. 5C is due to the steepness of this gradient. The resultant soluble form of the pma^+_{344} showed only one peak with an S value of the peak fraction of approx. 6 (Fig. 5D).

As demonstrated, the nRM integrated pma$^+$ was not modified by nRM in a way that could be detected by M_r differences. To determine, however, if the pma$^+$ was integrated into the nRM as a active enzyme, nRM integrated pma$^+$ was treated with trypsin. Previous reports have demonstrated that the pma$^+$ gives distinct trypsin cleavage patterns as a function of the pma$^+$ ligands present during trypsin digestion [22]. If the pma$^+$ was integrated into nRM as an active enzyme, it should binds its ligands and give the characteristic trypsin cleavage patterns. The results of such an experiment are depicted in Fig. 6. The soluble pma$^+$ was rapidly degraded by trypsin (Fig. 6, lane 3): similar to what is observed for the solubilized native pma$^+$ [22]. Unlike the latter, the rapid degradation of the soluble pma$^+$ by trypsin was unaltered by MgATP and vanadate (lane 4). The trypsin cleavage pattern of the nRM integrated pma$^+$ differ from the soluble form, for it is more resistant to trypsin digestion (lane 5). This pattern was also unaltered by the presence of MgATP and vanadate (lane 6). The trypsin cleavage patterns of the co- or post-translationally nRM integrated pma$^+$ were identical (data not shown). As mentioned, the pma$^+$ was integrated into nRM post-translationally (Fig. 2); after desalting of the translation mixture, the pma$^+$ became incompetent to integrate into nRM post-translationally (unpublished data). To gain some insight into the reason for this, the velocity sedimentation profile was determined for the pma$^+$ in the desalted mixture. The S value was approximately the same as that observed for the pma$^+$ that was in the undesalted mixture (data not shown). However, the form of pma$^+$ in the desalted mixture was more resistant to trypsin digestion than the integration-competent form (lanes 1 and 3), but less than the nRM integrated form (compare lanes 1 and 5). Again, MgATP and vanadate had no effect on the trypsin cleavage pattern (lane 2). The trypsin cleavage products of the pma$^+$ in the desalted mixture differ from what was observed for the form of the pma$^+$ that was integrated into nRM (see arrowheads in lane 1). This was interpreted to suggest that the former had assumed an altered tertiary, or quaternary, structure.

Discussion

The integration of the *N. crassa* pma$^+$ into microsomal membranes was examined using an in vitro system developed from *N. crassa*. The nRM integrated products were insensitive to extraction by 0.1 M Na_2CO_3 (pH 11.5), 4 M urea, 25 mM EDTA, or by 0.5 M KOAc. Peripherally associated membrane proteins are extracted by 4 M urea (24); whereas, peripheral membrane and luminal proteins are extracted by 0.1 M Na_2CO_3 (pH 11.5). The insensitivity of the nRM integrated pma^+_{344} to extraction by EDTA, high salts, or a combination thereof, indicated that the integrity of the ribosomes was not essential for its association with the membrane. Bovine opsin [25], Ca^{2+}-ATPase [26], and other IMPs [27] are not extracted from the membrane by these treatments; accordingly, these extraction procedures are used as criteria for determining if a membrane associated protein is integral or not. By these criteria, therefore, the pma$^+$ was integrated into and not peripherally associated with the microsomal membranes.

The in vitro synthesized pma$^+$ formed heterogeneous, high molecular weight aggregates on sucrose gradients. The formation of these aggregates could be triggered by the need to minimize interaction of the

hydrophobic areas on the pma^+ with water. In support of this notion, Triton X-100, a mild, nondenaturing detergent, reduced the size of the aggregates. Triton X-100 solubilizes various IMPs without loss of their biological activities and without disrupting their quaternary structures [28,29], but this detergent binds to the solubilized IMPs [30]. Therefore, the reduction in the *S* value of pma^+ observed in the presence Triton X-100 could be caused by the formation of the pma^+-Triton X-100 mixed micelles, which would give a higher Stokes radius. Alternatively, the pma^+ aggregates could be maintained by weak forces which were disrupted by Triton X-100. In the absence of knowledge about the pma^+ specific volume, its molecular shape, and the number of bound detergent molecules, it was impractical to try to determine the exact number of molecules contained in these aggregates. Furthermore, it was impractical to try to determine if the aggregates were homooligomers, because of the small quantity of the pma^+ synthesized in vitro. Comparisons with standards run under similar conditions suggest that the M_r of the aggregates were between that of a dimer and a trimer of pma^+; they were roughly 30% larger than a monomer of pma^+, in the presence of Triton X-100; whereas, when pma^+ was solubilized from the microsomal membranes by and was fractionated in the presence of Triton X-100, the M_r corresponded roughly to a monomer of the pma^+. Regardless of the accuracy of these numbers, the *S* value of the pma^+ was clearly reduced in the presence of Triton X-100, and this was further reduced for the pma^+ that was solubilized from the microsomal membranes by Triton. The peak fraction of invertase, when analysed under similar conditions, migrated as a dimer. The secretory form also formed aggregates that migrated with higher *S* values. It is not clear if these are either homooligomers or aggregates of invertase complexed with some nonradioactive components in the translation mixture. The latter could be putative proteinaceous components required for the targeting of invertase to the ER or prevent the acquisition of a stable tertiary structure [31]. For example, hsp70 maintains polypeptide in translocation-competent conformations [31]. Since the *N. crassa* in vitro translation mixture is a crude extract, it most likely contain homologus of hsp70. This could explain why pma^+, a hydrophobic membrane protein and insoluble in aqueous buffered solution [32], was soluble under these conditions: Complexed with extrinsic component(s), the pma^+ could be maintained in solution until it is associated with the microsomal membranes. To prove these points convincingly, however, will require additional experiments beyond the scope of the present paper.

When solubilized from the plasma membrane by lysolecithin and run on gradients containing deoxycholate, the pma^+ migrates as a homooligomers consisting of six monomers [33,34]. Whereas, the Triton X-100 solubilized nRM integrated pma^+ migrated with a *S* value which corresponded roughly to a monomer of the pma^+. A possible explanation for these differences could be caused by the different lipid compositions of the plasma membrane and the endoplasmic reticulum [35]: This could influence the rate of aggregation of the pma^+. Alternatively, the observed difference could be that the nRM integrated pma^+ had not assumed its native conformational state, and this could effect its aggregation. The trypsin cleavage patterns of the nRM integrated pma^+ suggest that the latter may be the most likely possibility. Vanadate is a transition state analogue of the pma^+ dephosphorylation reaction [36] and an inhibitor of the pma^+ hydrolysis of ATP [22]. It is clear that enzymes display their greatest affinity for analogues of the transition states of the reactions they catalyzes [37]. The structure of these imitates the structure the substrate molecule assumes in the transition state enzyme complex. This enzyme-inhibitor complex, therefore, reflects a central role in the catalytic cycle. So, if pma^+ is active in nRM, it should bind vanadate, in the presence of MgATP, and restrict its degradation by trypsin to a form with a $M_r \approx 95K$ [22]: This was not observed, suggesting that the pma^+ was catalytically inactive. Reconstituted monomer of the hydrolytic moiety of the pma^+ is capable of efficient proton translocation [10], demonstrating that the hydrolytic moiety is the only subunit required for the energy transduction reaction. It is possible, however, that other component(s) not required for the catalytic activity of pma^+ may be required for the proper integration of the hydrolytic moiety of the pma^+ into nRM. For instance, the β-subunit of the $Na + /K^+$-ATPase is essential for the integration and the acquisition of a functional conformation of the α-subunit [38,39]. Alternatively, the pma^+ may require post-translational modification(s). The inability to conformationally mature in the nRM could be caused by the inability of the nRM to perform the required modification(s). If these are not performed by the nRM, then the acquisition of the pma^+ native conformational state could occur later during its passage through the secretory pathway. Where in the secretory pathway the pma^+ acquires its native conformation or whether another component(s) is required for the proper integration of the pma^+ into microsomal membranes will be the subject of future research.

The velocity sedimentation behavior of the nRM integrated pma^+_{344} formed two peaks in the sedimentation profiles, similar to its soluble counterpart (compare Figs. 4B and 5C). The exact reasons for the aggregation of the pma^+_{344} in the microsomal membrane are not apparent. It could be that the aggregation of this unphysiological form of the pma^+ could be a physiological signal to prepare it for subsequent degradation [40]. Since the pma^+_{344} contains a partial transmembrane spanning segment (see Fig. 1), an alternative interpreta-

tion could be that this causes retention of the pma^{+}_{344} to putative component(s) required for integration into the membrane, or to the targeting component(s), or prevents its proper solvation by the lipids in the membrane.

Obviously, a problem in interpreting these observations is the lack of data from in vivo studies on the early events in the biogenesis of the pma^{+}. The current view of the secretory pathway [41] suggests that before termination of protein synthesis the nascent polypeptide chain of an IMP is targeted to the ER, where protein synthesis continues. This suggests that the pma^{+} would not exist in the cytosol in vivo. It is curious, however, that a mechanism do exist in *N. crassa* that is able to maintain a polytopic IMP, after termination of protein synthesis, in a soluble form until it is associated with the membranes. The fact that these aggregates interact with the integration machinery suggests that a mechanism exist that can accommodate heterogeneous, high molecular weight aggregates of the IMP which is to be integrated. Insights into these intriguing observations should provide information about how polytopic IMPs are integrated into the lipid bilayer.

References

1 Mitchell, P. (1973) Bioenergetics 4, 63–91.
2 Addison, R. (1986) J. Biol. Chem. 261, 14896–14901.
3 Hager, K.M., Mandala, S.M., Davenport, J.W., Speicher, D.W., Benz, E.J., Jr. and Slayman, C.W. (1986) Proc. Natl. Acad. Sci. USA 83, 7693–7697.
4 Mandala, S.M. and Slayman, C.W. (1988) J. Biol. Chem. 263, 15122–15128.
5 Addison, R. and Scarborough, G.A. (1981) J. Biol. Chem 256, 13165–13171.
6 Goffeau, A. and Slayman, C.W. (1981) Biochim. Biophys. Acta 639, 197–223.
7 Mitchell, P. (1981) in Oxygen, Fuels, and Living Matter, Part 1 (Semenza, G., ed.), pp. 1–160, John Wiley and Sons, Chichester.
8 Dame, J.B. and Scarborough, G.A. (1981) J. Biol. Chem. 256, 10724–10730.
9 Goormaghtigh, E., Chadwick, C.C. and Scarborough, G.A. (1986) J. Biol. Chem. 261, 7466–7471.
10 Scarborough, G.A. and Addison, R. (1984) J. Biol. Chem. 259, 9109–9114.
11 Addison, R. (1987) J. Biol. Chem. 262, 17031–17037.
12 Martin, R.G. and Ames, B.N. (1961) J. Biol. Chem. 236, 1372–1379.
13 McEwen, C.R. (1967) Anal. Biochem. 20, 114–149.
14 Perara, E., Rothman, R.E. and Lingappa, V.R. (1986) Science 232, 348–352.
15 Mueckler, M. and Lodish, H.F. (1986) Nature 322, 549–552.
16 Aaronson, L.R., Hager, K.H., Davenport, J.W., Mandala, S.M., Chang, A., Speicher, D.W. and Slayman, C.W. (1988) J. Biol. Chem. 263, 14552–14558.
17 Fujiki, Y., Hubbard, A.L., Fowler, S. and Lazarow, P.B. (1982) J. Cell Biol. 93, 97–102.
18 Lampen, J.O. (1971) in The Enzymes (Boyer, P.D., ed), Vol. 5, pp. 291–305, Academic Press, New York.
19 Carlson, M. and Botstein, D. (1982) Cell 28, 145–154.
20 Trimble, R.B. and Maley, F. (1977) J. Biol. Chem. 252, 4409–4412.
21 Taussig, R. and Carlson, M. (1983) Nucleic Acids Res. 11, 19434–1954.
22 Addison, R. and Scarborough, G.A. (1982) J. Biol. Chem. 257, 10421–10426.
23 Reference deleted.
24 Gilmore, R. and Blobel, G. (1985) Cell 42, 497–505.
25 Friedlander, M. and Blobel, G. (1985) Nature 318, 338–343.
26 Anderson, D.J., Mostov, K.M. and Blobel, G. (1983) Proc. Natl. Acad. Sci. USA 80, 7249–7253.
27 Singer, T. (1974) Annu. Rev. Biochem. 43, 805–833.
28 Helenius, A. and Simons, K. (1972) J. Biol. Chem. 247, 3656–3661.
29 Tzagoloff, A. and Penefsky, H.S. (1971) Methods Enzymol. 22, 219–230.
30 Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta. 415, 29–79.
31 Rothman, J.E. (1989) Cell 59, 591–601.
32 Scarborough, G.A. (1988) Methods Enzymol. 157, 574–579.
33 Addison, R. and Scarborough, G. (1981) J. Biol. Chem. 256, 13165–13171.
34 Chadwick, C.C., Goormaghtigh, E. and Scarborough, G.A. (1987) Arch. Biochem. Biophys. 252, 348–356.
35 Bowman, B.J., Borgeson, C.E. and Bowman, E.J. (1987) Exp. Mycol. 11, 197–205.
36 Macara, I.G. (1980) Trends Biochem. Sci. 5, 92–94.
37 Lienhard, G.E. (1973) Science 188, 149–157.
38 Okamoto, C.T., Karpilow, J.M., Smolka, A. and Forte, J.G. (1990) Biochim. Biophys. Acta 1037, 360–372.
39 Cayanis, E., Bayley, H. and Edelman, I.S. (1990) J. Biol. Chem. 265, 10829–10835.
40 Rose, J.K. and Doms, R.W. (1988) Annu. Rev. Cell Biol. 4, 258–288.
41 Walter, P. and Lingappa, V.R. (1982) Annu. Rev. Cell Biol. 2, 499–516.