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A Defect in the Mitochondrial Import of Mutant Mn-Superoxide Dismutase Produced in Sf21 Cells[1]

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Wild-type and several mutant human manganese superoxide dismutases (Mn-SODs) were produced in a baculovirus/insect cell system and characterized. The enzymatic activity of a homogenate of Sf21 cells, infected with baculovirus carrying wild-type Mn-SOD and grown in the conventional medium, was indistinguishable from that of control cells, but was augmented by supplementation with Mn^{2+}. The protein produced was largely imported into the mitochondria, as judged from the enrichment in the mitochondrial fraction, the mobility of the protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the results of N-terminal processing, which was confirmed by sequencing of the purified enzyme. However, a significant amount of precursor was also detected by an antibody raised against the human Mn-SOD signal peptide. While both Mn^{2+} and Fe^{3+} stimulated Mn-SOD accumulation within mitochondria, the active form was produced in the presence of submillimolar Mn^{2+} only. Amino acid substitutions at a signal peptide-cleavage site, His-Ser-Leu^4 to Pro-Met-Val^4, in the mature Mn-SOD prevented the processing of the precursor protein, and thus resulted in the accumulation of the precursor protein within mitochondria, as judged on immunostaining with an anti-Mn-SOD antibody. Mutant Mn-SODs with a truncated signal peptide or carboxyl region (8, 13, and 42 amino acid residues in the mature form) were barely solubilized, even with a nonionic detergent, and exhibited no activity, suggesting inappropriate folding of these mutant SODs. They were also susceptible to proteolytic degradation, while the wild-type and precursor forms were resistant. Thus, the baculovirus/insect cell expression system appears to be adequate for the analysis of mitochondrial import using intact cells as well as for the large scale production of active Mn-SOD.

Key words: baculovirus, manganese superoxide dismutase, mitochondrial import, signal peptide, site-directed mutagenesis.

Cells living under aerobic conditions have several systems for defending themselves against reactive oxygen species. Superoxide dismutases (SODs; EC 1.15.1.1) are enzymes that scavenge the superoxide radicals and protect cells against oxidative stress (*1*). *Escherichia coli* produces two similar types of SODs, Fe-SOD, a constitutively expressed form, and Mn-SOD, a form which can be induced by a variety of oxidative stimuli. These enzymes are highly homologous in their tertiary, as well as their primary structures, and therefore metal ions can be interchanged between the two enzymes, although Fe^{3+}-containing Mn-SOD exhibits no activity (*2*).

Eukaryotic cells contain three SOD isozymes, distributed in different regions of the cells (*3-5*). Mn-SOD is localized in the mitochondrial matrix (*6*) in the majority of eukaryotic cells and forms a homotetramer composed of 22 kDa subunits, each of which contains one Mn atom. Since the enzyme is synthesized in the cytoplasm and then imported into mitochondria, the precursor protein has a cleavable signal peptide which is essential for mitochondrial import (*7*). Human Mn-SOD, with the amino-terminal signal peptide deleted, has been produced in an active form in *E. coli* (*8*). Supplementation of the culture medium with Mn^{2+} was essential to obtain the active form of Mn-SOD, which, when produced in bacteria, forms a dimer.

The baculovirus system is a powerful system capable of

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Abbreviations: bp, base pairs; cDNA, complementary DNA; DTT, dithiothreitol; FCS, fetal calf serum; kDa, kilodalton(s); PAGE, polyacrylamide gel electrophoresis; PBS, 137 mM NaCl/2.67 mM KCl/1.47 mM KH_2PO_4/8.06 mM Na_2HPO_4, pH 7.4; APMSF, 3-amidinophenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Sf21, *Spodoptera frugiperda* 21; SOD, superoxide dismutase; WT, wild type.

producing large amounts of foreign proteins in insect cell cultures or larvae, in which post-translational modification similar to that which occurs in mammalian cells takes place (*9*). Several mitochondrial proteins, which are encoded by nuclear genes and are post- or co-translationally imported into mitochondria, have been overproduced in this system (*10-12*). While yeast ferrochelatase (*10*) and rat ornithine transcarbamylase (*11*) have been produced as active forms, bovine cytochrome P-450 (SCC), which requires heme as a cofactor, was imported into mitochondria but was inactive (*12*). This lack of activity could be due to either misfolding of this particular protein, as has been suggested (*12*), or to a short supply of the heme cofactor, as appears to be the case for endothelial type nitric oxide synthase (*13*). Since many mitochondrial enzymes require cofactors for their function, it was of interest to determine if supplementation of such cofactors to the medium would overcome this problem.

We (*14*) previously reported the overproduction of both wild-type and mutant Cu,Zn-SODs related to familial amyotrophic lateral sclerosis, and found that Cu supplementation is essential for obtaining an active form of the enzyme. In this paper, we report the overproduction of the wild type, as well as several mutant human Mn-SODs, in the baculovirus/insect cell system, and their characterization. The mutants are either a point mutant or contain deleted amino-terminal sequences.

EXPERIMENTAL PROCEDURES

Preparation of Mutant Mn-SOD Expression Constructs—Human Mn-SOD cDNA (*15*), a generous gift from Dr. Ye-Shih Ho (Duke University Medical Center), was subcloned into the *Eco*RI/*Pst*I site of pBluescript vector SK(+) (Stratagene) and used for mutagenesis. The wild-type and mutant Mn-SOD cDNAs created in this work are shown in Fig. 1. Amino acid substitutions in the cDNA at positions 2 to 4 (His-Ser-Leu to Pro-Met-Val), designated as MS1, were introduced by site-directed mutagenesis according to Kunkel (*16*) as described previously (*17*). Since the mutation in MS1 created an *Nco*I site after the codon for Ala-1 in the mutant protein, we prepared another mutant, designated as MS2, whose signal sequence was deleted but which contained a new initiator Met at position 2, by digestion with *Nco*I. To obtain an N-terminal deletion mutant, the 5′ region of the cDNA was deleted with exonuclease III/Mung bean nuclease, followed by ligation with a 12 mer *Nco*I linker to create an initiation codon. In order to select in-frame-mutants and to confirm the mutated sequences, sequencing of the single stranded DNA was carried out by the dideoxy sequencing method (*18*). Three deletion mutants, designated as MS3, MS4, and MS5, which lacked the amino-terminal 8, 13, and 42 amino acid residues, respectively, were selected.

Construction of the Recombinant Baculovirus Carrying the Wild-Type and Mutant Human Mn-SOD cDNAs—All mutant Mn-SOD cDNAs as well as the wild-type cDNA were ligated into the 5′-*Eco*RI and 3′-*Pst*I sites of baculovirus transfer vector pVL1393 (Invitrogen). Sf21 cells were grown in Grace's medium containing 10% FCS, 3.3 mg/ml yeastolate, 3.3 mg/ml lactoalbumin hydrolysate, and 50 μg/ml gentamicin (*19*). Transfer plasmid pVL-1393, carrying Mn-SOD cDNA, was purified by cesium

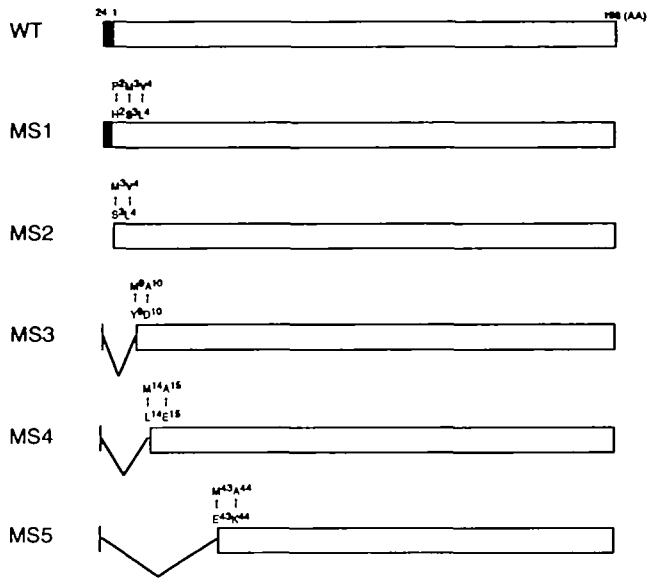

Fig. 1. **Schematic representation of the wild-type and mutant human Mn-SODs.** Black and white boxes indicate signal peptides essential for mitochondrial import and translated mature proteins, respectively. Amino acid substitutions and deletions are also indicated.

chloride ultracentrifugation, and 1-3 μg of DNA was cotransfected with 10 ng Baculogold DNA (Pharmingen) into Sf21 cells in FCS- and gentamycin-free medium using lipofectin (Gibco/BRL) for 24 h at 27°C. After 4 to 5 days incubation in the medium, which contained FCS and gentamycin, at 27°C, the recombinant virus was harvested. Since a lethal mutation had been introduced into the Baculogold DNA, further plaque purification was generally unnecessary. Viral infections were repeated 4-6 times to avoid spontaneous mutations of the coding sequences. The amplified virions were stored at 4°C until used.

Assaying of SOD Activity—One million cells, grown in a 3 cm dish and infected with baculovirus, were collected by centrifugation at 8,000 rpm for 5 min and then lysed by sonication in 100 μl of 2.5 mM potassium phosphate, pH 7.4. After centrifugation at 15,000 rpm for 15 min, the supernatant was collected and used to assay SOD activity, which was measured using the xanthine-xanthine oxidase/nitroblue tetrazolium system (*20*). Data are means ± SD for triplicate experiments.

Subcellular Fractionation of Infected Sf21 Cells—Insect Sf21 cells grown in a 10 cm dish and infected with the virus carrying the wild-type, MS1, or MS2 Mn-SOD cDNA were harvested 3 days after infection. Forty million cells were lysed in 5 ml of buffer A (10 mM Tris-Cl, pH 7.4, 0.25 M sucrose, and 0.1 mM EDTA) and then centrifuged at 600 × *g* to precipitate nuclei and intact cells. The supernatant, containing crude mitochondria, was centrifuged at 8,000 × *g* for 10 min. The pellet was then resuspended in 10 ml buffer A and then the centrifugation was repeated. The final precipitate was suspended in buffer A and stored at −20°C until used.

Purification of Mn-SOD Proteins Produced in Sf21 Cells—Wild-type Mn-SOD, produced in Sf21 cells, was partially purified essentially as described previously (*21, 22*). Briefly, 5×10^7 Sf21 cells were infected with bacu-

lovirus carrying the wild-type Mn-SOD with 10 multiple infections for 1 h at 27°C, and then incubated for an additional 3 days in the medium described above, supplemented with 3 mM $MnSO_4$. Cells were collected, washed twice with TBS, and then suspended in 10 mM potassium phosphate, pH 7.8, containing 1 mM benzamidine. After sonication for 10 min, they were fractionated by ultracentrifugation at 100,000 × *g* for 1 h. The supernatant was then applied to a hydroxylapatite column preequilibrated with 100 mM potassium phosphate, pH 7.2. After extensive washing with the same buffer A, the bound proteins were eluted with a linear gradient of the pH 7.2 potassium phosphate buffer, from 100 to 400 mM.

Sequencing of the Amino-Terminus of Purified Mn-SOD—About 80 μg of the purified wild-type Mn-SOD was denatured in 7 M guanidine-HCl, 0.5 M Tris-HCl, and 10 mM EDTA, pH 8.5, reduced with 5 mg dithiothreitol, and then carboxymethylated with 12.5 μg of iodoacetate. After extensive dialysis against 50 mM Tris-HCl, pH 7.0, the alkylated protein was subjected to amino acid sequencing with an on-line analyzer (477A, Applied Biosystems) as its phenylthiohydantoin derivative.

Production of Anti-Signal Peptide Antibodies—A 24-mer oligopeptide, NH_2-Met-Leu-Ser-Arg-Ala-Val-Cys-Gly-Thr-Ser-Arg-Gln-Leu-Ala-Pro-Ala-Leu-Gly-Tyr-Leu-Gly-Ser-Arg-Gln-COOH, corresponding to the signal peptide of human Mn-SOD was synthesized by Ube Industries. A polyclonal antibody was raised by inoculation of peptide-conjugated keyhole limpet hemocyanin in Freund's complete adjuvant into rabbit lymph nodes.

SDS-PAGE and Immunoblotting—Protein concentrations were determined with a BCA kit (Pierce) (*23*) using bovine serum albumin as a standard. Proteins from Sf21 cells producing Mn-SODs were electrophoresed on a 12.5 or 15% SDS-gel according to Laemmli (*24*), and then stained with Coomassie Brilliant Blue G250. For immunoblotting, 10 μg of purified protein was transferred to a PVDF membrane using Trans-blot (Bio-Rad). After blocking in 4% skim milk overnight, the blot was incubated overnight at 4°C with a 1:500 dilution of the rabbit anti-signal peptide antibody or a 1:700 dilution of a goat anti-human Mn-SOD antibody (*25*). After washing of the PVDF membrane three times for 30 min each, the blot was incubated for 45 min with 1:2,000 diluted, peroxidase-conjugated anti-goat IgG (Cappel) at room temperature. The chemiluminescence method was employed to amplify the signal using an ECL kit (Amersham).

Immunostaining of Mn-SOD in Cells—Sf21 cells infected with recombinant baculovirus were scraped off dishes and sedimented by centrifugation at 1,500 rpm for 5 min. After washing of the cells with serum-free medium twice, they were suspended in serum-free medium and incubated for 30 min at 37°C with 0.5 μg/ml of rhodamine 123 (Molecular Probes), and then subjected to fluorescence microscopy (Provis, Olympus). To stain human Mn-SOD, the cells were fixed in 3% paraformaldehyde in PBS at 24°C for 1 h, washed 3 times with PBS, and then incubated overnight with the 1:500 diluted anti human Mn-SOD antibody in the permeabilization solution (0.2% Triton X-100, 3% BSA, and 0.05% sodium azide in 10 ml PBS) at 4°C. Samples were washed 3 times with PBS and then incubated with FITC-conjugated anti-rabbit IgG at room temperature for 2 h. After 3 washes with PBS, the cells were mounted on a slide glass and examined by fluorescence microscopy.

RESULTS

Overproduction of Wild-Type Mn-SOD in Sf21 Cells—Foreign genes inserted into the pVL1393 vector were expressed as nonfused proteins in insect cells. The expression of wild-type Mn-SOD in baculovirus-infected Sf21 cells was examined by SDS-PAGE and activity assaying. When the proteins within conditioned medium or cells were analyzed by SDS-PAGE at different times after viral infection, Mn-SOD was detected only in the cells (data not shown). Although distinct production of the Mn-SOD protein was observed in the soluble fraction of the cells grown in the conventional medium on SDS-PAGE, the SOD activity was the same as that of control cells without viral infection, indicating that the produced enzyme was inactive (Fig. 2A).

Since supplementation of the medium with Mn^{2+} ions is essential for the production of active Mn-SOD in *E. coli* (*8*), and Mn-SOD is structurally similar to bacterial Fe-SOD, the requirement of metal ions for the expression of SOD activity was examined. Among $MnCl_2$, $MnSO_4$, $FeCl_3$, and $FeSO_4(NH_4)SO_4$, both $MnCl_2$ and $MnSO_4$ increased the

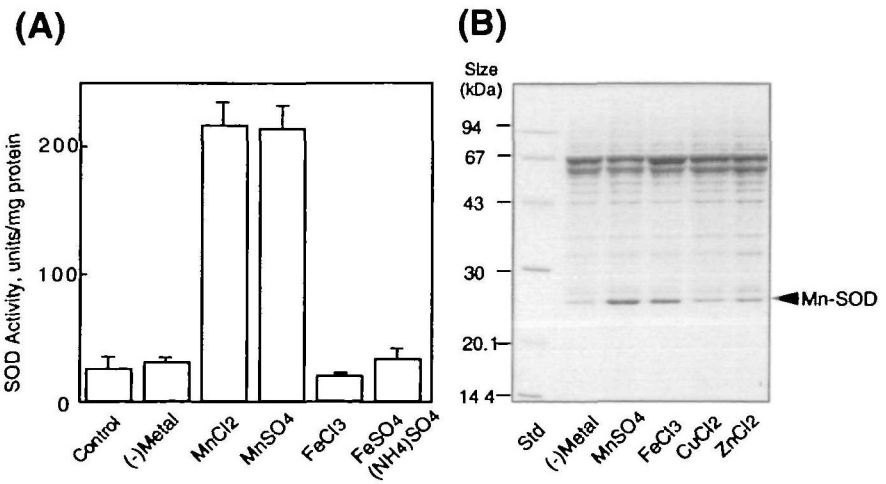

Fig. 2. **Effects of metal ions on wild type Mn-SOD activity and the levels of Mn-SOD proteins overproduced in Sf21 cells.** (A) After infection with baculovirus carrying the wild-type Mn-SOD cDNA, cells were incubated in medium supplemented with 1 mM of the metal salts indicated for 3 days. After sonication and centrifugation, SOD activities in the soluble fraction was measured and is shown as the mean ± SD for triplicate assays. (B) Mn-SOD was produced in the presence of several metal ions, as indicated, and 20 μg of total proteins was separated by 12.5% SDS-PAGE and then stained with Coomassie Brilliant Blue G250.

activity by approximately 10-fold over the control level. Fe^{3+}, but not Cu^{2+} or Zn^{2+}, increased Mn-SOD accumulation, probably by stabilizing it in mitochondria (Fig. 2B). Fe^{3+} failed to activate the Mn-SOD produced in Sf21 cells, although Mn^{2+} partially compensated for Fe^{3+} of the Fe-SOD from *Bacteroides fragilis* (*26*). When both Mn^{2+} (1 mM) and Fe^{3+} (1 mM) were added to the medium, SOD activity decreased, relative to that with Mn^{2+} alone (data not shown), suggesting that competitive binding of Fe to Mn binding site in the polypeptide occurred.

In order to determine the optimal Mn^{2+} concentration, virus-infected cells were grown in medium containing various concentrations of $MnSO_4$, and then the SOD activities of cellular extracts were measured (Fig. 3A). SOD activity increased in a $MnSO_4$ concentration-dependent manner and maximal activity was observed in the range of 0.3-3 mM $MnSO_4$. The Mn-SOD protein simultaneously accumulated in the cells as a function of the $MnSO_4$ concentration (Fig. 3B). This observation was quite different from in the case of Cu,Zn-SOD, whose production in Sf21 cells is unaffected by Cu or Zn ions (*14*).

Effect of Mn^{2+} on Enzyme Produced in the Absence of Mn^{2+}—To assess the effect of post-supplementation of Mn^{2+} on the expression of SOD activity after infection, infected cells were grown in the conventional medium for 3 days, followed by supplementation with 1 mM $MnCl_2$ in the presence or absence of cycloheximide (Fig. 4). Three hours after the addition of Mn^{2+} to the medium, the cells exhibited about 60% of the SOD activity observed for the cells grown in medium supplemented with Mn^{2+} immediately after viral infection. The activity curves were composed of two phases; the fast phase, which was complete within 3 h after Mn^{2+} administration, was not blocked by cycloheximide. The slow phase, on the contrary, was partially blocked by cycloheximide. Taken together, these data suggest that a part of Mn-SOD produced in the Mn-deficient medium likely exists as a metal-free apoenzyme, which can be converted to the active enzyme by simple Mn^{2+} supplementation to the medium, as in the case of the bacterial system (*14*).

Subcellular Distribution of Overproduced Mn-SOD—Since Mn-SOD is a mitochondrial enzyme, infected cells were fractionated, in order to determine if the produced wild-type Mn-SOD is imported into the mitochondria. Figure 5 shows the protein contents of cellular fractions, as determined by SDS-PAGE. The mitochondrial fraction was enriched in Mn-SOD, compared with the crude cell homogenate, and its molecular size was comparable to that of the purified Mn-SOD from human liver, indicating that Mn-SOD is imported into mitochondria. Because the molecular size of the expressed protein on the gel was slightly higher than that of the purified protein from human liver, the N-terminal sequence of the purified enzyme was determined. Sequence analysis of the carboxymethylated protein revealed Lys-His-Xxx-Leu-Pro-Asp (Xxx, unidentified amino acid), the same sequence as reported for the N-terminus of human liver Mn-SOD in which Xxx is Ser (*27*). Thus, the Mn-SOD produced in Sf21 cells has the same sequence as the native protein.

Characteristics of Mutant Mn-SODs—The SOD activities, and the proteins examined by SDS-PAGE and immunoblotting of the total cellular homogenate are shown for the wild-type and mutant SODs in Fig. 6. When a mutant Mn-SOD with an amino acid substitution in proximity to

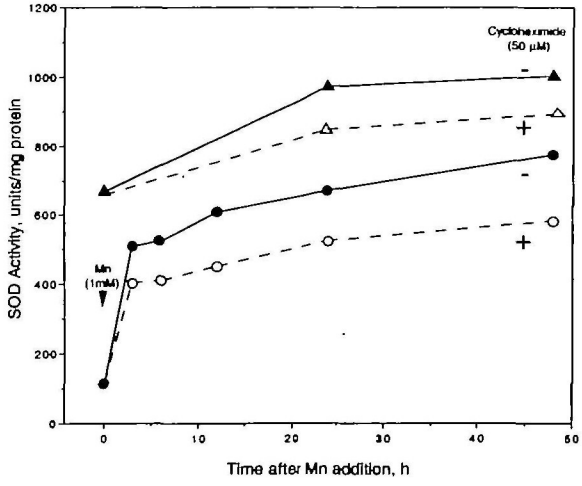

Fig. 4. **Effects of pre- and post-supplementation of $MnSO_4$ on activity of SOD overproduced in cells.** Sf21 cells were infected with baculovirus carrying the wild-type Mn-SOD and then grown for 3 days with (△, ▲) or without (○, ●) 1 mM $MnSO_4$ supplementation of the medium. $MnSO_4$ (1 mM) (○, ●) and/or cycloheximide (50 μM) (△, ○) was then added to the medium at time zero, and SOD activity was measured for triplicate cultures at each time point.

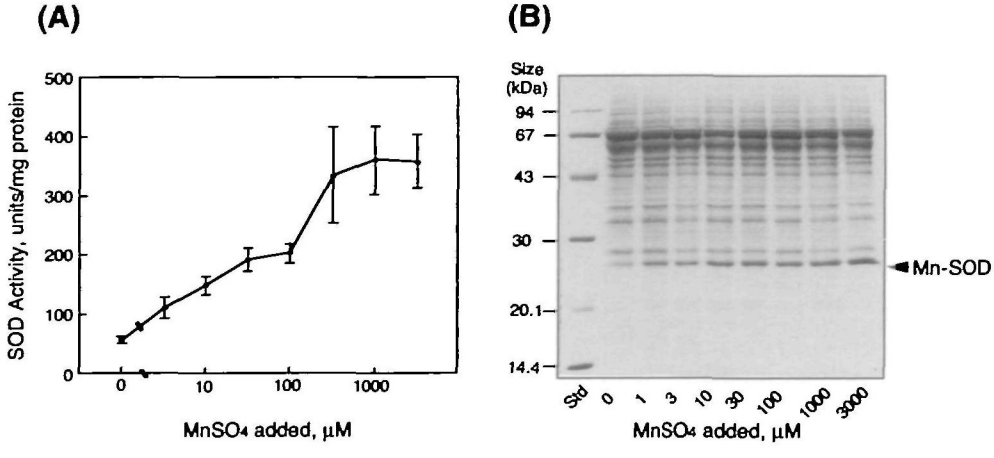

Fig. 3. **$MnSO_4$ concentration dependency of WT Mn-SOD activity and levels of the Mn-SOD protein.** (A) Essentially the same experiments as described in Fig. 2 were carried out in the presence of various concentrations of $MnSO_4$. SOD activities are shown as means ± SD for triplicate experiments. (B) Twenty micrograms of total proteins produced in cells grown in the presence of $MnSO_4$ was separated by 12.5% SDS-PAGE.

the cleavage site of the signal sequence, MS1, was expressed in Sf21 cells, the main band was higher than that of the wild type (Fig. 6C). MS1 has an intact signal sequence with three amino acid substitutions near the cleavage site (His-Ser-Leu^4 to Pro-Met-Val^4), which would be expected to lead to inhibition of translocation and accumulation of the precursor. MS1 was found to exist in a precursor form, on immunoblot analysis with an antibody against the human Mn-SOD signal peptide (Fig. 6D).

We also prepared a mutant, MS2, which lacked the signal peptide, as well as three mutant Mn-SODs, MS3, MS4, and MS5, with deletions at the N-terminus, and examined the properties of these mutant proteins. Figure 6B shows the SOD activities of homogenates of cells infected with baculovirus carrying the mutant cDNAs in the presence of 3 mM $MnSO_4$. In addition to the wild-type SOD, MS1 exhibited a significantly higher activity than the control cells. Because all of these mutants except for MS1 were synthesized without signal peptides, the proteins produced would not be expected to be imported into mitochondria. Inactive enzymes were precipitated, even on very low speed centrifugation, $1,000 \times g$ for 5 min, in the presence of 0.5% Lubrol, a non-ionic detergent, indicating that these proteins formed large aggregates with other cellular components (data not shown). It was also observed that these mutant SODs, but not the wild-type or MS1, were cleaved at specific sites in the cells.

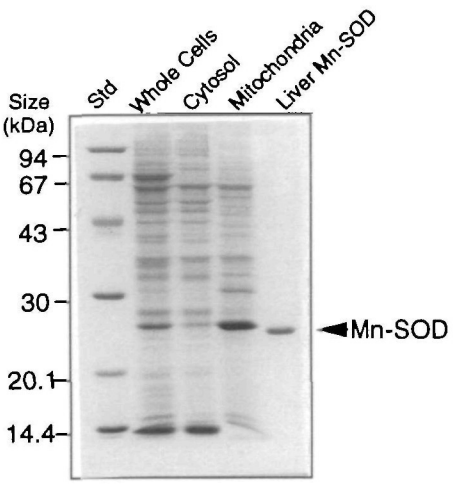

Fig. 5. **Subcellular localization of overproduced Mn-SOD.** After fractionation of infected cells, 6 μg of proteins from cell homogenates, the cytosolic fraction, and the mitochondrial fraction, and 0.4 μg of purified Mn-SOD from human liver were analyzed by SDS-PAGE.

Immunological Localization of the Wild-Type, MS1, and MS2 in Cells—We then carried out staining of mitochondria with rhodamine 123 and immunofluorescence detection of Mn-SOD in Sf21 cells to localize the Mn-SOD proteins (Fig. 7). Incubation of control cells (Fig. 7A) and ones infected with recombinant viruses (data not shown) with rhodamine 123 gave the same staining patterns. An antibody against human Mn-SOD stained the cells which produced wild-type SOD (Fig. 7B) more strongly than the control cells. Significant staining was observed as particles in the cytoplasm, a characteristic of mitochondrial localization. We also examined cells which produced mutants MS1 and MS2 (Fig. 7, C and D). The staining patterns were similar to those of cells which produced the wild-type SOD, except that even faint staining was also observed in the cytoplasm. Moreover, cellular fractionation followed by immunoblotting indicated that not only the wild-type SOD but also MS1 and MS2 were enriched in the mitochondrial fraction (Fig. 7E). A faint band of a lower molecular weight corresponding to the mature Mn-SOD was observed for the mitochondrial fraction of MS1. The SOD activity in mitochondrial fraction of MS was 21% of that of the wild-type SOD, while that of MS2 was only 8% (data not shown),

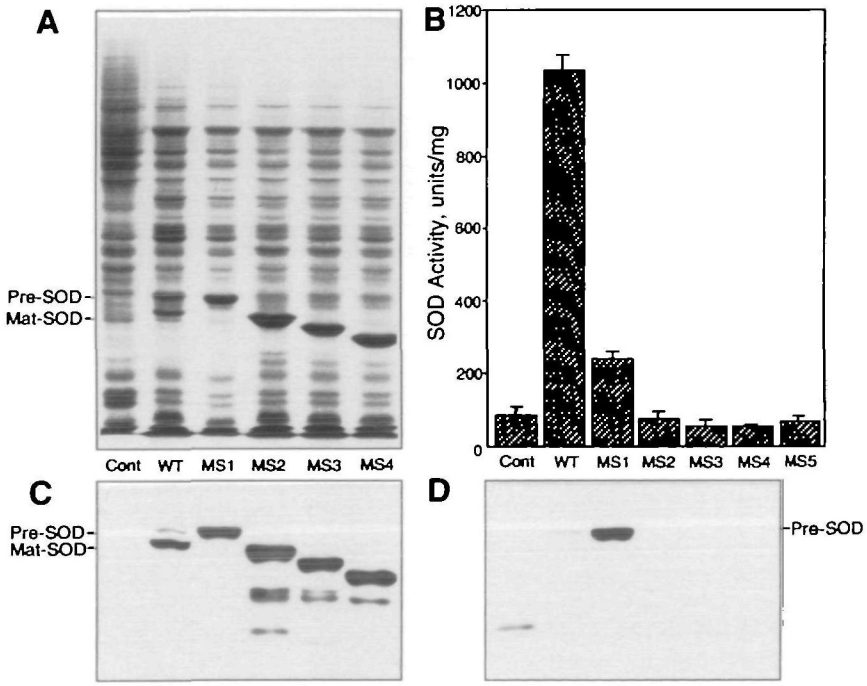

Fig. 6. **SDS-PAGE, immunoblotting, and activities of the wild-type and mutant Mn-SODs.** Twenty micrograms total proteins from cells infected with the wild-type and mutant cDNA-carrying baculovirus was analyzed by SDS-PAGE (A). The SOD activities of whole cell homogenates were also measured (B). Immunoblotting was carried out for the same samples transferred to a nitrocellulose membrane using the anti-human Mn-SOD antibody (C) and anti-signal peptide antibody (D). Cont, control cells; Pre-SOD, precursor form of Mn-SOD; Mat-SOD, mature form of Mn-SOD.

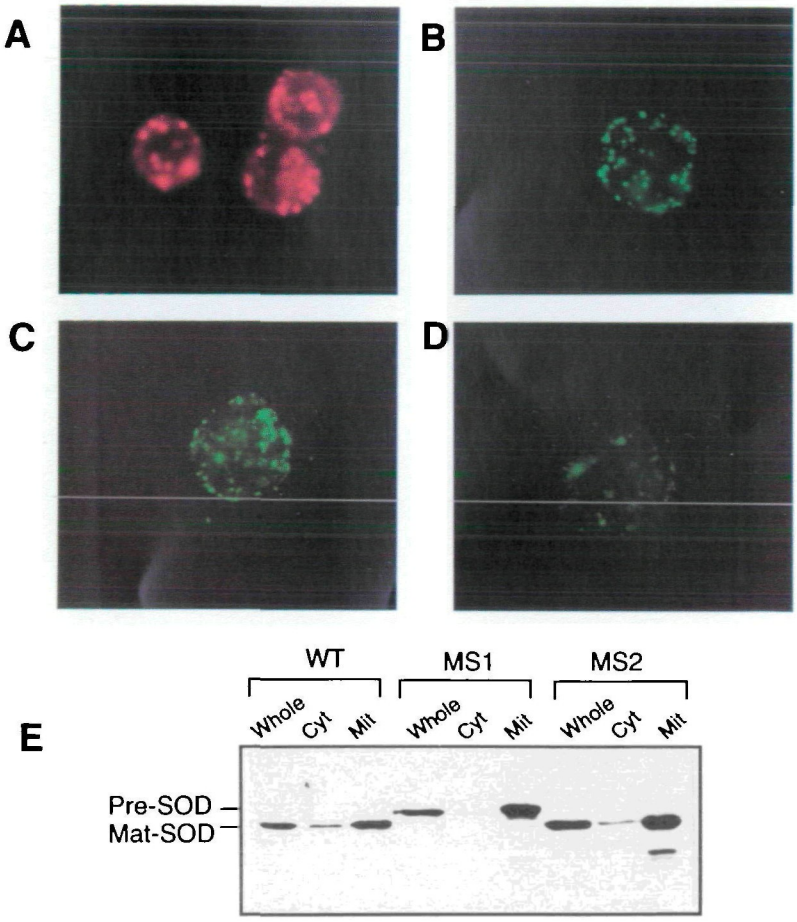

Fig. 7. **Immunofluorescence staining and subcellular fractionation of cells overproducing the wild-type, MS1, and MS2 Mn-SODs.** Cells infected with the wild-type, MS1, and MS2, as well as control cells, were stained with 0.5 μg/ml rhodamine 123. The result for only control cells is shown in (A). After infection of cells with baculovirus carrying wild-type (B), MS1 (C), and MS2 (D) cDNA, immunofluorescence staining was carried out with an antibody against human Mn-SOD as the primary antibody. A FITC-conjugated anti-rabbit antibody was used to visualize the immunoreactive site. ×1,000. Immunoblot analysis of the wild-type, MS1, and MS2 Mn-SOD in whole cellular extracts (Whole), and cytosolic (Cyt) and mitochondrial (Mit) fractions was also performed.

indicating that a part of MS1 existed as an active form. These observations suggest that MS1 and MS2 are mainly localized in the mitochondria, although it is not clear whether they exist outside or inside of the mitochondrial membrane.

DISCUSSION

The baculovirus expression system involves insect cells, which are not precisely the same as mammalian cells, but are known to exhibit similar posttranslational modifications including glycosylation and phosphorylation (*9*). Proteins are produced in large amounts in this system, and it is a convenient system for analyzing the compartmentalization and functions of proteins whose content is too low to be visualized within ordinary cells. Using this system, we were able to overproduce the wild-type Mn-SOD and to purify it. Supplementation of Mn^{2+} to the medium was essential for obtaining SOD activity, indicating that the protein produced in cells grown in the conventional medium was an apoenzyme. In addition, Mn^{2+} enhanced the accumulation of the protein within mitochondria (Figs. 3 and 5), probably by stabilizing it through the formation of a conformation which is stable as to proteolytic cleavage. Although Fe^{3+} did not stimulate SOD activity, it stabilized Mn-SOD, although to a lesser extent than Mn^{2+}, probably due to the structural similarity of Mn-SOD to bacterial Fe-SOD (*2*). Thus, Mn^{2+} appears to be involved in both the formation of the catalytic center and the increase in the half life of the enzyme within mitochondria.

Mutant MS1, which had 3 amino acid substitutions at the signal peptide-cleavage site, was accumulated within cells as a precursor (Fig. 6). This precursor form was found to bind to mitochondria, as shown by an immunofluorescence study (Fig. 7). In the case of mutant MS2, the signal peptide was removed and, therefore, it would not be expected to be imported into the mitochondria. This signal peptide-depleted Mn-SOD would have remained in the cytoplasm as an inactive form even though only the two N-terminal amino acid sequences were different from those of the mature protein. It is well known that a molecular chaperone is required for some proteins to fold properly and to retain competency for mitochondrial import after biosynthesis in the cytoplasm (*28*). Since a part of the translated MS1 could be processed to the mature and active form, the correctly folded MS2 would retain enzymatic activity. In the bacterial expression system, active Mn-SOD can be produced from a signal peptide-depleted cDNA which has a structure quite similar to that of the one used here, obtained by simply adding Mn^{2+} to the growth medium (*8*). Because the mitochondrial chaperone is evolutionarily homologous to bacterial one, the bacterial chaperone may facilitate in the correct folding of Mn-SOD in bacterial cells, and therefore makes it possible to produce active Mn-SOD in bacteria without the signal peptide. The cytoplasmic chaperone, however, would not allow the mutant to fold correctly. Immunostaining of Mn-SOD in Sf21 cells showed a staining pattern consistent with the mitochondrial

distribution. This suggests that information for binding to mitochondria is present not only in the signal peptide but also in other parts of the protein. MS3, MS4, and MS5, which contain truncated N-terminal sequences, became insoluble and exhibited no SOD activity, suggesting that these mutant SODs were improperly folded in the absence of the mitochondrial chaperone, even though they were small in size. Thus, it is conceivable that a mitochondria- or bacteria-type molecular chaperone is essential for proper folding of this protein in the insect cell system.

We also found that a significant amount of precursor was present in wild-type Mn-SOD producing cells (Fig. 6C). This may be due to saturation of the capacity for mitochondria to handle them. Quite recently, paraquat, a herbicide which produces reactive oxygen species, was found to suppress the processing of the precursor form of Mn-SOD produced in Sf9 cells (*29*). It is generally believed that the processing of the precursor for a mitochondrial protein is too rapid to be detected under normal conditions. However, as in case of paraquat, a disease might exist which is caused by a defect in the processing of Mn-SOD. This eukaryotic expression system would provide a system suitable for investigating the mitochondrial import of proteins as well as the production of large amounts of proteins for other purposes.

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