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Irreversible Extrusion of the First Loop Facing the Matrix of the Bovine Heart Mitochondrial ADP/ATP Carrier by Labeling the Cys[56] Residue with the SH-Reagent Methyl Methanethiosulfonate[1]

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The effect of the SH-reagent methyl methanethiosulfonate (MMTS) on the ADP/ATP carrier of bovine heart mitochondria was studied under various conditions. MMTS labeled predominately Cys[56] in the first loop facing the matrix (loop M1), and the labeling inhibited ADP transport *via* the carrier. The transport inhibition was found to be due to fixation of the carrier in the m-state conformation. MMTS labeling was suggested not to affect ADP binding to its major binding site. These features were the same as those of another commonly used SH-reagent, *N*-ethylmaleimide (NEM). Although the van der Waals volume of the non-hydrogen-bondable methylthio group of MMTS is much smaller than that of the ethylsuccinimide group of NEM, modification of Cys[56] inhibited the interconversion between the m- and c-state conformation. The mechanism by which MMTS inhibited the transport activity is discussed in terms of stabilization of conformation of the loop M1.

Key words: ADP/ATP carrier, cysteine residue, methyl methanethiosulfonate, mitochondria, SH-reagent.

The mitochondrial ADP/ATP carrier mediates transport of ADP and ATP by interconversion between the c- and m-state conformations, in which the substrate-binding site of the carrier faces the cytosolic and matrix side, respectively. Carboxyatractyloside (CATR) and bongkrekic acid (BKA) inhibit the transport function by fixing the c- and m-state conformations, respectively (*1*, *2*). The carrier has three repeated domains with similar amino acid sequences, each consisting of a pair of transmembrane segments linked by a hydrophilic loop facing the matrix. These loops are referred to as the M1, M2, and M3 loops of the bovine heart mitochondrial carrier and contain Cys[56], Cys[159], and Cys[256], respectively (*3*). The carrier is thought to function as a dimer (*1*, *4–6*).

The 30-kDa bovine heart mitochondrial ADP/ATP carrier has four cysteine residues, Cys[56], Cys[128], Cys[159], and Cys[256]. We have studied the labeling of these cysteine residues of the carrier with various SH-reagents (*3–7*). The anionic SH-reagent eosin 5-maleimide (EMA) quickly and predominantly labels Cys[159], whereas electrically neutral *N*-ethylmaleimide (NEM) mainly labels Cys[56] and has very low affinities for Cys[159] and Cys[256] (*7*). In addition, bifunctional cross linkers as well as copper-*o*-phenanthroline [$Cu(OP)_2$] specifically induce cross-linking between two Cys[56] residues in a pair of M1 loops in the dimeric carrier (*4*, *5*). However, none of the reagents labels Cys[128]. Accordingly, we conclude that Cys[128] is located in the transmembrane segment, loop M1 containing Cys[56] is exposed to the matrix, and loops M2 and M3 containing Cys[159] and Cys[256], respectively, intrude into the membrane. The segment around Cys[159] is the major binding site of the transport substrates ADP and ATP (*2*, *3*, *6*). It is noteworthy that the extruded loop M1 is translocated to the membrane on conversion of the m-state conformation to the c-state conformation (*4*, *5*).

The SH-reagents used in these earlier studies label cysteine residues irreversibly. Another class of SH-reagents forms mixed disulfides with cysteine residues. These include Ellman's reagent [5,5'-dithiobis(2-nitrobenzoate)] and methyl methanethiosulfonate (MMTS). As the disulfide bridge is easily cleaved under mild conditions by reducing reagents such as dithiothreitol (DTT) and 2-mercaptoethanol, the SH-reagents of this latter class, when used in combination with reducing reagents, are expected to be useful for determining the states of cysteine residues. MMTS is electrically neutral and is one of the smallest SH-reagents known to date (*8*, *9*). It labels cysteine residues by introduction of a very small and non-hydrogen-bondable methylthio group (CH_3S-), as shown in Chart 1. Hence, labeling with MMTS minimizes the effect of steric hindrance, unlike

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Abbreviations: AAC, ADP/ATP carrier; $(AAC)_2$, dimeric form of AAC; BKA, bongkrekic acid; CATR, carboxyatractyloside; CBB, Coomassie Brilliant Blue R-250; $Cu(OP)_2$, copper-*o*-phenanthroline; DTT, dithiothreitol; EMA, eosin 5-maleimide; MMTS, methyl methanethiosulfonate; NACys, *N*-acetyl-L-cysteine; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TEM, transmission electron microscopy.

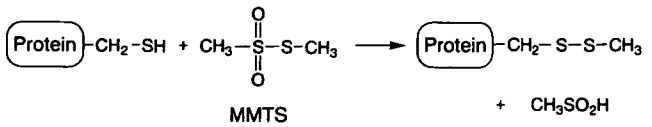

Chart 1. **Chemical modification of a cysteine residue with MMTS.**

labeling with bulky EMA or NEM. This paper deals with the labeling of the bovine heart mitochondrial ADP/ATP carrier with MMTS under various conditions.

MATERIALS AND METHODS

Reagents—Sources of the reagents used in this study were as follows: MMTS, from Nacalai Tesque (Kyoto); NEM, from Aldrich (Milwaukee); CATR, from Sigma (St. Louis); EMA, from Molecular Probes (Eugene); $CuSO_4$ and *o*-phenanthroline, from Wako Pure Chemical Industries (Osaka); [^{14}C]NEM and [^{3}H]ADP, from Du Pont New England Nuclear Research Product (Wilmington); Sephadex G-50, from Pharmacia Biotech (Uppsala). BKA was a gift from Prof. Duine (Delft University of Technology).

Preparation of Bovine Heart Mitochondria and Submitochondrial Particles—Bovine heart mitochondria and submitochondrial particles with 5 mM ATP incorporated were prepared as described previously (*7*). CATR-preloaded submitochondrial particles were prepared from mitochondria that had been incubated with 4 nmol of CATR/mg of protein at 25°C for 10 min (*3*). The amounts of protein in mitochondria and submitochondrial particles were determined with a BCA protein assay kit (Pierce, Rockford) in the presence of 1% SDS using bovine serum albumin as a standard.

Treatment of Mitochondria and Submitochondrial Particles with MMTS—Unless otherwise noted, bovine heart mitochondria (1 mg of protein/ml) suspended in SPE medium consisting of 250 mM sucrose, 0.2 mM EDTA, and 10 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid)–NaOH buffer (pH 7.2) were preincubated with 50 μM ADP for 3 min at 25°C, then incubated with 1 mM MMTS for the indicated period at 25°C. Submitochondrial particles with incorporated ATP (20 mg protein/ml) suspended in STE medium consisting of 250 mM sucrose, 0.2 mM EDTA, and 10 mM Tris-HCl buffer (pH 7.2) were incubated at 0°C with 400 μM MMTS for the indicated period. Labeling of mitochondria and the particles was terminated by incubation with 50 mM *N*-acetyl-L-cysteine (NACys) for 30 min. Free NACys and MMTS-conjugated NACys were removed from mitochondria by centrifugation at 10,000 ×*g* for 1 min. The mitochondria were washed twice with STE medium, then suspended in STE medium containing 1 μg/ml oligomycin at 1 mg of protein/ml for measurement of ADP uptake. NACys was removed from the particles by gel chromatography on a Sephadex G-50 column eluted with ST or STE medium (*6*). When necessary, treatment with NEM was performed in a similar way.

ADP Transport Activity—Bovine heart mitochondria (1 mg of protein/ml) treated with MMTS for various periods at 25°C were incubated with 100 μM [^{3}H]ADP (specific radioactivity, 10 μCi/μmol) for 10 s at 0°C in STE medium containing 1 μg/ml oligomycin. ADP transport was terminated with 20 μM CATR. After centrifugation of the sample at 10,000 ×*g* for 1 min, the pellet was solubilized with 1% SDS. Submitochondrial particles (2 mg of protein/ml) pretreated with MMTS were suspended in STE medium containing 1 μg/ml oligomycin, and incubated with 20 μM [^{3}H]ADP (specific radioactivity, 200 μCi/μmol) for 10 s at 0°C. ADP transport was terminated with 20 μM BKA, and the [^{3}H]ADP remaining in the medium was promptly separated by chromatography on an AG1-X8 column (*7*). The amount of ADP incorporated into mitochondria or submitochondrial particles was determined from the radioactivity in an Aloka scintillation counter, model LSC-3500, as described previously (*7*).

Determination of NEM Bound to the Carrier—A suspension of submitochondrial particles (20 mg of protein/ml) was incubated with 2 mM [^{14}C]NEM (specific radioactivity 4 mCi/mmol) with or without MMTS for 10 min at 0°C, and the reaction was terminated with 50 mM NACys. The particles were then subjected to SDS-PAGE in 12% polyacrylamide gel by the method of Laemmli (*10*). The gel was dried, and the NEM bound to the proteins were determined with a Bio-imaging Analyzer BAS-1500 Mac (Fuji Film, Tokyo).

Cross-Linking of the Carrier by Cu(OP)$_2$—Cu(OP)$_2$ was prepared just before use by mixing $CuSO_4$ with *o*-phenanthroline in a molecular ratio of 1:2 in ST medium consisting of 250 mM sucrose and 10 mM Tris-HCl (pH 7.4) (*4*). Submitochondrial particles (4 mg of protein/ml) pretreated with MMTS were suspended in ST medium and incubated with 100 μM Cu(OP)$_2$ at 25 nmol of Cu(OP)$_2$/mg of protein for 10 min at 0°C. The reaction was terminated with 5 mM EDTA and 5 mM NEM. The particles were then subjected to SDS-PAGE in 12% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 (CBB), and the staining intensities of the bands of 30-kDa monomer and 60-kDa dimer of the ADP/ATP carrier were determined at 560 nm in a Shimadzu Chromatoscanner Model CS-9000 as described previously (*4*).

Measurement of Change in Optical Density—The change in optical absorbance of the freshly isolated bovine heart mitochondria (1 mg of protein/ml) in SPE medium (pH 7.2) containing 1 μg/ml oligomycin at 25°C was monitored continuously at 600 nm in a Shimadzu dual-wavelength spectrophotometer, model UV-3000. A mitochondrial suspension loaded with CATR was used as a reference compensate for the changes due to proteins other than the carrier. When the effect of BKA was examined, experiments were performed at pH 6.5, because BKA is more effective in weakly acidic conditions than at a neutral pH (*11*). Less than 5 μl volumes of various reagents were added to minimize the effect of dilution on the optical absorbance.

Transmission Electron Microscopy—Transmission electron microscopy (TEM) analyses of mitochondria treated with various reagents were carried out essentially as described previously (*12*). After treatment of bovine heart mitochondria (1 mg of protein/ml) with various test compounds in SPE medium (pH 7.2) containing 1 μg/ml oligomycin at 25°C, the suspension was centrifuged at 10,000 ×*g* for 1 min. The mitochondria were fixed with 2.5% glutaraldehyde and postfixed with OsO_4. After dehydration, the mitochondria were embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H-800MT electron microscope.

Treatment of the Particles with EMA—Submitochondrial

particles (20 mg of protein/ml) in STE medium were treated with 400 μM MMTS for 10 min at 0°C, and then diluted with STE medium to 2 mg of protein/ml. The particles were incubated with 20 μM EMA for various periods at 0°C, and the reaction was terminated with 50 mM NACys. The particles were then subjected to SDS-PAGE in 15% polyacrylamide gel. The intensity of the protein band labeled with EMA in the gel was determined from the fluorescent intensity on excitation at 530 nm in a Shimadzu chromatoscanner, CS-9000.

RESULTS

1. Effect on ADP Transport—We examined the effect of MMTS on ADP transport mediated by the ADP/ATP carrier. Bovine heart mitochondria (1 mg of protein/ml) preincubated with 50 μM ADP for 3 min were incubated with 1 mM MMTS for various periods at 25°C and pH 7.2 (Preincubation with ADP or ATP was necessary for inhibition of the ADP uptake by MMTS as described later on "*Effect on the conformational state of the carrier*"). Then NACys was added to terminate MMTS labeling. After removal of the external ADP and NACys, transport activity of the mitochondria was determined by incubation with 100 μM [^{3}H]ADP for 10 s at 0°C and pH 7.2. As shown in Fig. 1, MMTS inhibited transport of ADP effectively, and incubation for about 10 s was enough for 50% inhibition of the transport.

We next examined the effect of MMTS on submitochondrial particles. The particles (20 mg of protein/ml) with 5 mM incorporated ATP were incubated with 400 μM MMTS for various periods at 0°C and pH 7.2. After termination of the labeling, the particles (2 mg of protein/ml) were incubated with 20 μM [^{3}H]ADP for 10 s at 0°C and pH 7.2, then transport was terminated with 20 μM BKA. As shown in Fig. 2 (trace "MMTS"), MMTS inhibited ADP transport as in mitochondria, and its effect became greater with increase in the period of MMTS treatment. Almost complete inhibition of the transport was observed on incubation with MMTS for more than 30 min, and the period required for 50% inhibition of the transport by 400 μM MMTS was 3 min. The inhibitory effect on the particles appeared to be slower than that on the mitochondria shown in Fig. 1. However, this was because the effect of MMTS on the particles was examined under milder conditions such as at a lower MMTS concentration and lower temperature of 0°C. As these conditions were favorable for examination of the effect of DTT, we hereafter determined its effect under these conditions.

The mixed disulfide bond formed between cysteine residues and MMTS is cleaved by its reduction with DTT. As DTT may not penetrate the mitochondrial membrane, and it is likely to reduce the disulfide bridge located on the membrane surface, we examined its effect on submitochondrial particles. For this, we incubated the particles first with 400 μM MMTS for various periods at 0°C and pH 7.2, then with 50 mM DTT for 30 min at 25°C. The ADP transport activity of the carrier was then examined at 0°C. As shown in Fig. 2 (trace "MMTS/DTT"), DTT effectively restored the ADP transport activity of the particles preincubated with MMTS for less than 10 min. However, preincubation for 10 min or longer caused less restoration of the transport activity, and ADP transport was not recovered at all after preincubation with MMTS for 60 min.

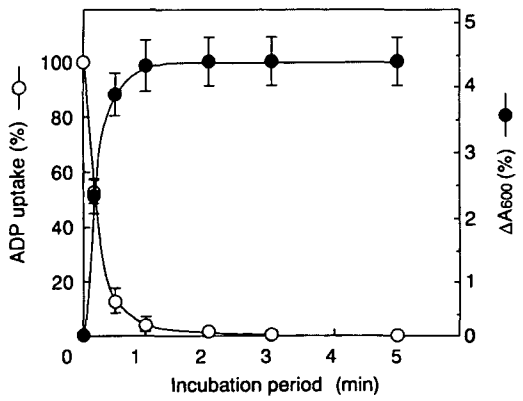

Fig. 1. **Effect of MMTS on ADP transport *via* the ADP/ATP carrier in bovine heart mitochondria.** A mitochondrial suspension (1 mg protein/ml) was preincubated with 50 μM ADP for 3 min at 25°C and pH 7.2, then treated with 1 mM MMTS for various periods. Labeling was terminated with NACys, and the labeled mitochondria (1 mg of protein/ml) in STE medium (pH 7.2) containing 1 μg/ml oligomycin were incubated with 100 μM [^{3}H]ADP for 10 s at 0°C. After termination of transport with 20 μM CATR, the amount of ADP transported was determined from the radioactivity of the incorporated [^{3}H]ADP. The amount of ADP incorporated without MMTS treatment was 1 μmol/mg of protein/min. Optical absorbance at 600 nm (ΔA_{600}) was determined with the mitochondrial suspension incubated with 1 mM MMTS before measurement of ADP transport, and is plotted as a function of the incubation period (see also legend to Fig. 5). Results are means ± SD for three separate experiments.

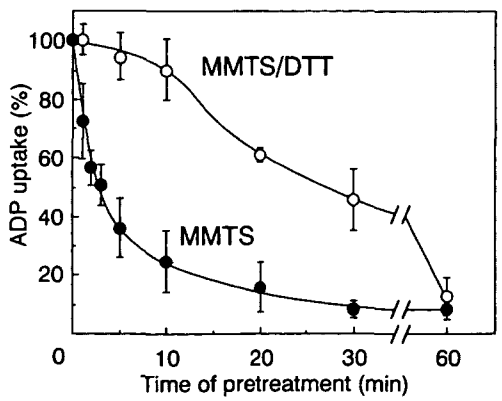

Fig. 2. **Effect of MMTS on ADP transport in submitochondrial particles.** To determine the effects of MMTS on the ADP transport (trace MMTS), bovine heart submitochondrial particles (20 mg of protein/ml) were preincubated with 400 μM MMTS for various periods at 0°C and pH 7.2, and labeling was terminated with NACys. The MMTS-treated particles (2 mg of protein/ml) were incubated with 20 μM [^{3}H]ADP for 10 s at 0°C and pH 7.2. To determine the effect of DTT on the ADP transport activity of the MMTS-treated submitochondrial particles (trace MMTS/DTT), the particles were pretreated with 400 μM MMTS for various periods and incubated with 50 mM DTT for 30 min at pH 7.2 and 25°C, then their ADP uptake was measured. In both cases, ADP transport was terminated with 20 μM BKA, and the amount of ADP transported was determined from the radioactivity of incorporated [^{3}H]ADP. The transport activity was expressed by the amount of ADP taken up by submitochondrial particles relative to that without treatment with MMTS (=2.81 ± 0.20 nmol/mg of protein/min). Results are means ± SD for three separate experiments.

As MMTS inhibited the ADP transport in both mitochondria and submitochondrial particles, MMTS was suggested to be membrane-permeable. MMTS probably labels the cysteine residue of the carrier in mitochondria from the matrix side after crossing the mitochondrial membrane. It might first label the cysteine residue exposed to the matrix side, and then the residues intruding into the membrane. The ineffectiveness of DTT in ADP transport of the particles treated with MMTS for 60 min showed that the modification of any one of the three cysteine residues in the loops facing the matrix inhibited ADP transport.

2. Effect on Cys^{56} in Loop M1—We tried to identify which cysteine residue in the ADP/ATP carrier was labeled with MMTS. The SH-reagent NEM is reported to label predominantly Cys^{56} of the carrier in both bovine heart mitochondria and the submitochondrial particles (*7*, *13*). We incubated the particles (20 mg of protein/ml) with 2 mM [^{14}C]NEM for 10 min at 0°C and pH 7.2, then subjected them to SDS-PAGE. As shown in Fig. 3A, NEM labeled proteins of 30, 32, and 34 kDa, and pretreatment of the particles with 400 μM CATR from the cytosolic side inhibited the labeling of 30-kDa protein with [^{14}C]NEM, whereas pretreatment with 400 μM BKA did not affect NEM labeling. The inhibition of NEM labeling with CATR was the result of intrusion of the exposed M1 loop of the ADP/ATP carrier into the membrane, but BKA does not affect the location of this loop (*3–5*). Therefore, the 30-kDa protein is the ADP/ATP carrier. As we found previously that more than 90% of the total NEM labeling of the carrier under identical experimental conditions was due to labeling of Cys^{56} (*7*), the labeling of the 30-kDa protein with [^{14}C]NEM in Fig. 3A should be due to labeling of Cys^{56}.

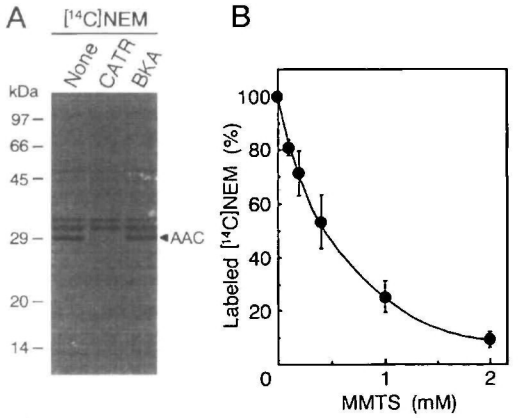

Fig. 3. **[^{14}C]NEM-labeling of membrane proteins in bovine heart submitochondrial particles (A) and the effect of MMTS on [^{14}C]NEM-labeling of the 30-kDa ADP/ATP carrier (B).** A: Autoradiograms of [^{14}C]NEM-labeled mitochondrial proteins on SDS-PAGE gel are shown. Suspension of the particles (20 mg of protein/ml) were preincubated with 400 μM CATR or BKA for 10 min at 0°C and pH 7.2, then treated with 2 mM [^{14}C]NEM for 10 min. Samples (10 μg of protein) were subjected to SDS-PAGE. None, without addition of CATR and BKA. AAC, the ADP/ATP carrier. B: Suspension of submitochondrial particles (20 mg of protein/ml) was incubated concomitantly with 2 mM [^{14}C]NEM and various concentrations of MMTS at 0°C and pH 7.2. After 10 min, the samples (10 μg of protein) were subjected to SDS-PAGE, and radioactivity of the 30-kDa carrier band labeled with [^{14}C]NEM was determined by a BAS-1500 Mac. Results are means ± SD for three separate experiments.

We next examined the effects of MMTS on the NEM labeling. Various concentrations of MMTS were added to a suspension of submitochondrial particles concomitantly with 2 mM [^{14}C]NEM at 0°C and pH 7.2. After 10 min, the labeling was terminated with excess NACys and the particles were subjected to SDS-PAGE. Figure 3B shows change in the intensity of the carrier band labeled by [^{14}C]NEM as a function of the MMTS concentration. Labeling with NEM decreased hyperbolically with increase in the concentration of MMTS, suggesting that MMTS labeled Cys^{56} in competition with NEM. The concentration of MMTS required for 50% inhibition of NEM labeling was determined to be about 400 μM. As 2 mM NEM was employed for the labeling, the efficiency of MMTS for labeling the carrier was at least five times that of NEM. As both SH-reagents modified the same Cys^{56} residue covalently, the more efficient effect of MMTS was due to its quicker labeling of the Cys^{56} residue.

We reported previously that Cu(OP)$_2$ specifically catalyzes disulfide bridge formation between two Cys^{56} residues of a pair of carriers in the membrane to give a band of a 60-kDa carrier, (AAC)$_2$, on SDS-PAGE (*4*). Therefore, we examined whether MMTS inhibited formation of the dimeric carrier linked by a disulfide bridge. For this, the submitochondrial particles were treated with 400 μM MMTS for various periods, then the particles were incubated with 100 μM Cu(OP)$_2$ for 10 min at pH 7.4 and 0°C. Without MMTS pretreatment, as shown by the results on SDS-PAGE of "none" in Fig. 4, Cu(OP)$_2$ decreased the intensity of the CBB-stained 30-kDa band of the monomeric ADP/ATP carrier (band AAC), and caused formation of the 60-kDa dimeric carrier [band (AAC)$_2$] (*4*). Pretreatment of the particles with 400 μM MMTS suppressed the formation of (AAC)$_2$, as in its inhibitory effect on ADP transport shown

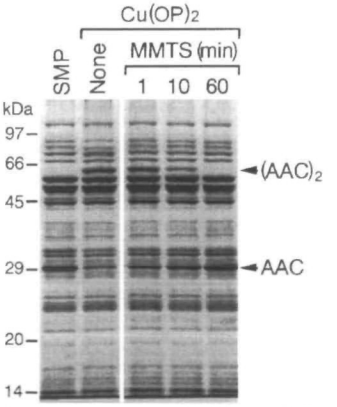

Fig. 4. **Effect of MMTS on cross-linking of the ADP/ATP carrier catalyzed by Cu(OP)$_2$.** Submitochondrial particles (20 mg of protein/ml) were preincubated with 400 μM MMTS for various periods at 0°C and pH 7.2. The particles (4 mg of protein/ml) were then treated with 100 μM Cu(OP)$_2$ for 10 min at 0°C and pH 7.4. The cross-linking reaction was terminated with 5 mM EDTA and 5 mM NEM, and samples were subjected to SDS-PAGE under non-reducing conditions. The results of SDS-PAGE of mitochondrial membrane proteins are shown. SMP, the particles without treatments with MMTS and Cu(OP)$_2$; None, the particles treated with Cu(OP)$_2$ without pretreatment with MMTS. The time (min) shown on the electrophoreogram is the preincubation period with MMTS. AAC represents the monomeric ADP/ATP carrier, and (AAC)$_2$, the dimeric carrier.

in Fig. 2, and $(AAC)_2$ formation was inhibited almost completely by preincubation for 30 min and longer (data not shown). These results again showed that MMTS labeled Cys[56].

3. Effect on Conformational State of the Carrier—It is important to know whether labeling of Cys[56] with MMTS affects the interconversion of the carrier between the c-state and m-state. The interconversion of these two conformations is easily observable by monitoring the optical absorbance of the suspension, because it is accompanied by a large morphological change of mitochondria that induces turbidity change of the mitochondrial suspension (*11*, *14*, *15*). Figure 5A shows typical traces of the change in the optical absorbance at 600 nm (ΔA_{600}) due to conformation-dependent turbidity change of a bovine heart mitochondrial suspension at pH 6.5 and 25°C. At this pH, BKA is more effective than at a neutral pH value (*11*), and freshly isolated mitochondria mostly showed the c-state conformation with low optical density due to the absence of the adenine nucleotide in the external medium (*15*). When ADP was added at a final concentration of 50 μM, ΔA_{600} promptly increased due to induction of the m-state conformation by transport of ADP to the matrix space, and soon attained a plateau level. At this optical level, the populations of both m-state and c-state mitochondria were equilibrated in the suspension (*6*). Addition of BKA (final concentration, 20 μM) instantly increased ΔA_{600} due to conversion of all the c-state mitochondria to m-state mitochondria, and subsequent addition of CATR (20 μM) did not affect ΔA_{600}, because fixation of the m-state conformation by BKA is not reversed by CATR (*6*). In contrast, c-state mitochondria fixed by CATR were insensitive to BKA treatment in the presence of ADP (discontinuous curve in Fig. 5A).

The effect of MMTS was examined using a mitochondrial suspension after addition of ADP. As shown in Fig. 5B, 1 mM MMTS caused instant increase in ΔA_{600} in association with conversion of the c-state conformation to the m-state conformation. ΔA_{600} finally attained the same level as that induced by ADP plus BKA shown in Fig. 5A. Essentially the same extent of optical change was observed at pH 7.2 (data not shown). CATR did not affect the m-state mitochondria induced by MMTS. Addition of MMTS to the mitochondrial suspension prior to ADP did not cause any optical change, because the conversion of the c-state mitochondria to the m-state did not take place in the absence of ADP. Therefore, MMTS labeling fixed the m-state conformation of the carrier.

To confirm the above results, we analyzed of the effects of various reagents, namely, CATR, BKA, and MMTS, on the morphology of the ADP-treated mitochondria, in which m-state and c-state mitochondria were present (see Fig. 5), by transmission electron microscopy (TEM). As shown in Fig. 6, mitochondria treated with MMTS in the presence of ADP took the condensed configuration as in BKA-induced m-state mitochondria, unlike the loose configuration of the c-state mitochondria induced by CATR. Therefore, MMTS, in the presence of ADP, was confirmed to fix the m-state conformation.

We next examined whether the induction of the m-state conformation by MMTS labeling causes inhibition of ADP transport. We determined ΔA_{600} of the mitochondrial suspension under identical conditions to those of ADP transport shown in Fig. 1. Namely, bovine heart mitochondria (1 mg of protein/ml) were preincubated with 50 μM ADP for 3 min, then incubated with 1 mM MMTS for various periods at pH 7.2 and 25°C. ΔA_{600} was measured at intervals and plotted as a function of the incubation period with MMTS, as shown in Fig. 1. Increase in ΔA_{600} due to conversion to the m-state conformation was well correlated with inhibition of ADP transport, showing that fixation of the m-state conformation by MMTS was directly associated with inhibition of ADP transport.

4. Effect on the Binding Site of Adenine Nucleotides—The divalent anionic maleimide fluorescent SH-reagent EMA has similar structural characteristics to ADP and ATP and labels predominantly the Cys[159] residue in competition with ADP and ATP. Therefore, the domain around Cys[159] in the M2 loop is regarded as the major binding site of the ade-

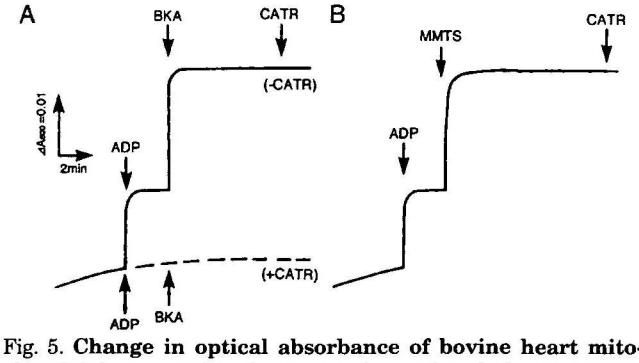

Fig. 5. **Change in optical absorbance of bovine heart mitochondrial suspension due to conformational change of the ADP/ATP carrier.** Optical absorbance at 600 nm (ΔA_{600}) of the suspension of bovine heart mitochondria (1 mg of protein/ml) in a total volume of 2.5 ml was monitored continuously at 25°C and pH 7.2. When BKA was used (A), the experiments were carried out at pH 6.5. Upward deflection of the absorbance is due to condensed cristae of the m-state mitochondria, and downward deflection to loosened cristae of the c-state mitochondria (*11*, *14*). After standing the mitochondrial suspension in the presence of oligomycin (1 μg/ml) for 5 min, various compounds were added. Concentrations of the reagents: ADP, 50 μM; CATR, 20 μM; BKA, 20 μM; MMTS, 1 mM. In A, the result for CATR added before addition of ADP is shown by a discontinuous line.

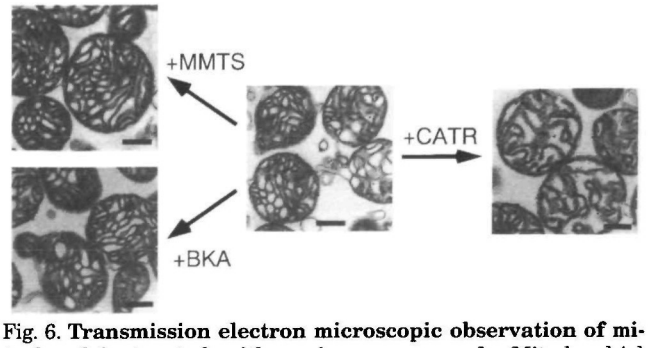

Fig. 6. **Transmission electron microscopic observation of mitochondria treated with various compounds.** Mitochondrial samples used for determination of the ΔA_{600} values as described in the legend to Fig. 5 were incubated first with 50 μM ADP for 1 min, then with 20 μM CATR for 2 min, 20 μM BKA for 3 min, or 1 mM MMTS for 3 min at 25°C. These incubation times were enough for mitochondria to take completely either the m-state or c-state conformation as judged from the ΔA_{600} change of the suspension (see Fig. 5). *Mitochondrial samples for transmission electron microscopic* analysis were prepared as described in Ref. *12*. Bars indicate 1.0 μm.

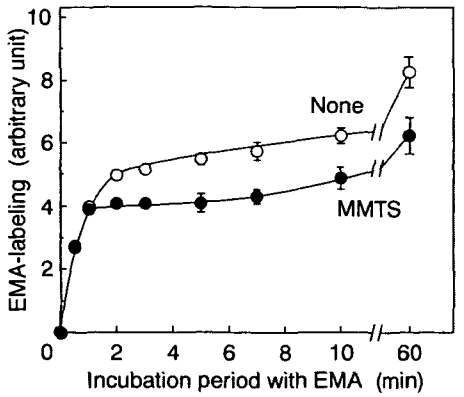

Fig. 7. **Effect of MMTS on EMA-labeling with Cys^{159} in the ADP/ATP carrier of bovine submitochondrial particles.** Submitochondrial particles (20 mg of proteins/ml) were treated with 400 μM MMTS for 10 min at pH 7.2 and 0°C. The particles (2 mg protein/ml) were then incubated with 20 μM EMA for various periods at 0°C and pH 7.2, and the reaction was terminated with 50 mM NACys. The particles were subjected to SDS-PAGE, and the fluorescent intensity (arbitrary units) of the EMA-labeled band due to the carrier on the gel was determined. Results are means ± SD for five separate experiments.

nine nucleotides (*3*, *6*). To know whether MMTS affects binding of the transport substrates, we examined the effect of MMTS on the EMA labeling of Cys^{159}. Submitochondrial particles (20 mg of protein/ml) were preincubated with 400 μM MMTS for 10 min at pH 7.2 and 0°C, and the treated particles (2 mg of protein/ml) were further incubated with 20 μM EMA for various periods at 0°C and pH 7.2. After SDS-PAGE, the fluorescent intensity of the 30-kDa band due to the carrier labeled with EMA was determined.

Without MMTS pretreatment, EMA labeling proceeded quickly for the first 1 min, then became slower, as shown in Fig. 7. According to our previous results, EMA labels Cys^{159} almost completely on incubation for 1 min, and on further incubation it labels Cys^{56} and Cys^{256} (*7*). Therefore, the quick labeling with EMA was due to labeling of Cys^{159}, and the subsequent slow labeling was due to the labeling of Cys^{56} and Cys^{256} (*7*).

When the particles were pretreated with MMTS, EMA labeling proceeded in the same manner as that without MMTS treatment for the first 1 min, and then did not proceed further for about 7 min. After incubation for more than 7 min, EMA labeling increased slightly. These results showed that MMTS did not affect the EMA labeling of Cys^{159} in a short incubation period. The inhibition of EMA labeling by MMTS on further incubation with EMA was due to MMTS labeling of Cys^{56}, and the subsequent slight increase was due to slow EMA labeling of Cys^{256} (*7*). These results showed that MMTS did not label Cys^{159} and Cys^{256} upon incubation for at least 10 min, being consistent with the efficient recovery by DTT of the ADP transport activity of the particles treated with MMTS for less than 10 min shown in Fig. 2, under which conditions MMTS labeled Cys^{56} predominantly. Hence, the binding of ADP and ATP to their major binding site was suggested not to be affected by MMTS.

DISCUSSION

In this study, we examined the labeling of cysteine residues of the bovine heart ADP/ATP carrier with MMTS, which has been widely employed for characterization of cysteine residues of various proteins (*8*, *9*, *16*, *17*). We found that MMTS predominantly labeled Cys^{56} of the carrier from both the cytosolic and matrix side very effectively. Possibly, MMTS added to the cytosolic side of mitochondria labeled Cys^{56} from the matrix side after crossing the bilayer membrane. A wide magnitude of fluctuation of the exposed M1 loop in the m-state conformation (*5*) should be advantageous for Cys^{56} to trap MMTS effectively. The labeling caused fixation of the m-state conformation, resulting in inhibition of ADP transport. The labeling with MMTS did not affect the affinity of EMA for Cys^{159} in loop M2, which constitutes the major binding site of the transport substrates. As the structural features of EMA are very similar to those of ADP and ATP (*6*), MMTS labeling was suggested not to affect substrate binding. These results were essentially the same as those with the well studied SH-reagent NEM (*7*). It is not known why NEM-labeling fixes the m-state conformation, leading to inhibition of transport activity of the carrier, but does not affect binding of the transport substrates or the transport inhibitor BKA to the carrier (*7*, *13*, *18*).

The van der Waals volume of the methylthio group of 31.3 Å^3 computed by molecular mechanics (MM) calculation (*19*) is much smaller than that of the ethylsuccinimide group (80.2 Å^3) of NEM, and the molecular size of the modified *S*-methylthio-cysteine is about the same as that of methionine. Therefore, labeling with MMTS of the ADP/ATP carrier corresponds to the replacement of Cys by Met with regard to molecular size. It is noteworthy that even a slight increase in the molecular size of the Cys^{56} by MMTS labeling locked loop M1 in the m-state conformation.

The importance of Cys^{56} in the transport activity is suggested by the fact that this cysteine residue is conserved in almost all ADP/ATP carriers (*20*). However, this cysteine residue was found not to be essential in site-directed mutagenesis studies (*20*, *21*). Namely, the type 2 ADP/ATP carrier of *Saccharomyces cerevisiae* retained the transport activity on replacement of Cys^{73}, which corresponds to Cys^{56} of the bovine heart carrier, by Ser (C73S mutation), although the transport activity was reduced (*21*). Therefore, fixation of the m-state conformation of the bovine heart ADP/ATP carrier by labeling of Cys^{56} with NEM, and subsequent inhibition of the transport activity, are thought to be due either to steric hindrance or to disruption of the hydrogen bonding by introduction of the bulky and non-hydrogen-bondable ethylsuccinimide group (*22*). As modification of Cys^{56} with MMTS caused only a slight increase in the molecular size, steric hindrance does not appear to be responsible for inhibition of the transport activity. In addition, it is known that the SH-group of cysteine participates in hydrogen-bonding far less frequently than the OH-group of serine (*23*, *24*). As C73S mutation reduced the transport activity, it is highly possible that the Cys^{73} in the yeast mitochondrial carrier and Cys^{56} in the bovine heart mitochondrial carrier are not in fact associated with hydrogen-bonding. Therefore, an alternative possibility should be considered.

There are several bulky hydrophobic residues (Tyr^{50}, Ile^{53}, Ile^{54}, Val^{57}, and Val^{58}) and some charged residues (Lys^{51}, Asp^{55}, Arg^{59}) in the segment around Cys^{56} in the M1 loop (*25*). These hydrophobic residues could form a hydrophobic pocket, with Cys^{56} in the middle (*7*, *13*), and the charged residues could take part in electrostatic interaction. For the wide magnitude of translocation of loop M1 from the extruded location to the inner side of the membrane on conversion from the m-state to the c-state, the conformation of M1 loop should be elegantly regulated by cooperative formation and breakage of various intra- and inter-loop interactions, such as hydrophobic interactions, electrostatic interactions and hydrogen bonding caused by access or loss of the transport substrates. Extreme stabilization or destabilization of these interactions will terminate the transition.

Serine is less hydrophobic than cysteine, so C73S mutation of the yeast carrier may diminish the interaction of this residue with the possible hydrophobic pocket of loop M1. This could be a reason why the transport activity of C73S was decreased. In addition, as the hydrophobic nature of Cys^{56} of the bovine heart mitochondrial carrier increased greatly by introduction of a methylthio group, fixation of the m-state conformation of the bovine heart carrier by MMTS labeling can be regarded as being due to high stabilization of the hydrophobic pocket. In this respect, it is noteworthy that the allosteric conversion of the R-state that supports enzymic activity of the M2 isozyme of pyruvate kinase was inhibited by stabilization of the hydrophobic region containing Cys^{423} by introduction of the more hydrophobic group to Cys^{423} with MMTS labeling and also by replacement with more hydrophobic Leu (*9*).

In conclusion, we think that higher stabilization of the hydrophobic pocket in loop M1 caused by insertion of the hydrophobic methylthio group into the hydrophobic pocket is directly associated with fixation of the m-state conformation by MMTS labeling. It is possible that relatively low hydrophobic Cys^{56} is suitable for well balanced stabilization of conformation of loop M1, which supports conversion of the m-state to the c-state conformation.

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