Fluctuation of the First Loop Facing the Matrix of the Mitochondrial ADP/ATP Carrier Deduced from Intermolecular Cross-Linking of Cys^{56} Residues by Bifunctional Dimaleimides†

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ABSTRACT: The effects of six thiol-specific cross-linker dimaleimides, in which the distance of the two maleimide groups ranged from 7.7 to 16.8 Å, on bovine heart mitochondria were studied at pH 7.2 and 0 °C. None of the dimaleimides affected mitochondrial proteins, but they caused significantly specific intermolecular cross-linking of the 30 kDa ADP/ATP carrier in submitochondrial particles. All the cross-links were found to be formed specifically between two Cys^{56} residues in the first loop facing the matrix, as we observed previously in intermolecular disulfide bridge formation catalyzed by copper *o*-phenanthroline [Majima, E., Ikawa, K., Takeda, M., Hashimoto, M., Shinohara, Y., and Terada, H. (1995) *J.Biol. Chem. 270,* 29548−29554]. The dimerization was dependent on the cross-linking span of the dimaleimides, being maximum with the dimaleimide having a span of about 12 Å. Cross-linking took place in the m-state carrier, but not in the c-state carrier, and inhibited ADP transport via the ADP/ATP carrier. We suggest that a pair of first loops with Cys^{56} residues in the dimer form of the m-state carrier fluctuates widely with a most probable distance between them of about 12 Å, and that this fluctuation modulates the transport activity of the ADP/ATP carrier.

The 30 kDa ADP/ATP carrier in the mitochondrial inner membrane mediates the specific transport of ADP and ATP from both the cytosolic and the matrix side. As this transporter is essentially important for oxidative phosphorylation, there have been many studies on its structural and functional properties (*1*−*4*). This ADP/ATP carrier is thought to consist of three homologous domains, in each of which two membrane-spanning regions are linked with a hydrophilic loop extending into the matrix (*5, 6*). These three loops are referred to as loops M1, M2, and M3 (*7*). This carrier is thought to function as a dimer, and the interconversion of its m-state and c-state conformations, in which the substrate binding site faces the matrix and cytosol, respectively, is essential for exhibition of its transport function (*1*−*6*).

There are four cysteine residues in the bovine heart mitochondrial ADP/ATP carrier (*8*). Of these, three cysteine residues, Cys^{56}, Cys^{159}, and Cys^{256}, reside in loop M1, M2, and M3, respectively, and Cys^{128} is located in the putative third membrane segment (*5*). From the reactivities of these cysteine residues in the carrier with the SH-reagents *N*-ethylmaleimide (NEM)[1] and eosin-5-maleimide (EMA), we found that loop M1 is exposed to the matrix space, loop M2 intrudes into the membrane, and loop M3 is located deep in the membrane (*9*). In addition, loops M1 and M2 are suggested to act as gates in the transport of ADP/ATP (*7, 10, 11*). The role of loop M3 remains unclear.

Recently, we reported that copper *o*-phenanthroline [$Cu(OP)_2$] specifically catalyzes disulfide bridge formation between two Cys^{56} residues in the functional dimer form of the m-state carrier, but not in the c-state carrier, suggesting that the location of the paired M1 loops changes greatly on interconversion between the c- and m-state conformations (*10*). Based on these results, we proposed a cooperative swinging loop model as the mechanism by which the carrier mediates the transport of ADP/ATP, the cooperative swinging of the three paired loops being responsible for the transport (*2, 7*).

For understanding the mechanism of transport function of the ADP/ATP carrier, it is of importance to characterize the state of loop M1 in relation to the transport function of the carrier, because change in its location in the inner mitochondrial membrane is suggested to take place on interconversion of the m- and c-state conformations of the carrier (*2, 10*). Therefore, we studied the cross-linking of the carrier with

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[1] Abbreviations: AAC, the 30 kDa monomeric ADP/ATP carrier; $(AAC)_2$, the 60 kDa cross-linked ADP/ATP carrier; BMH, 1,6-bismaleimidohexane; BMME, bis-maleimidomethyl ether; CBB, Coomassie Brilliant Blue R-250; $Cu(OP)_2$, copper *o*-phenanthroline; DMF, *N*,*N*′-dimethylformamide; DSI, disuccinimide-type adduct of dimaleimide with GSH; DTT, dithiothreitol; EMA, eosin-5-maleimide; GSH, reduced form of glutathione; MDPDM, *N*,*N*′-(4,4′-methylenediphenylene)dimaleimide; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; *o*-PDM, *N*,*N*′-*ortho*-phenylenedimaleimide; *m*-PDM, *N*,*N*′-*meta*-phenylenedimaleimide; *p*-PDM, *N*,*N*′-*para*-phenylenedimaleimide; SDS, sodium dodecyl sulfate; TFA, trifluoracetic acid.

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various bifunctional cross-linkers located various distances apart. Results showed that all the dimaleimide cross-linkers examined specifically formed intermolecular cross-links between two Cys[56] residues. Based on the results, a pair of M1 loops is suggested to fluctuate with the most probable distance between the two Cys[56] residues of about 12 Å.

MATERIALS AND METHODS

Reagents. The cross-linkers *o*-PDM, *m*-PDM, *p*-PDM, and MDPDM were purchased from Wako Pure Chemical Industries (Osaka), BMME was from Research Organics Inc. (Cleveland), and BMH was from Pierce (Rockford). These dimaleimides were dissolved in DMF before use. GSH was obtained from Wako, NEM from Nacalai Tesque (Kyoto), and CATR from Sigma (St. Louis). BKA was a gift from Prof. Duine (Delft University of Technology). Other reagents were of the highest grade commercially available.

Determination of Cross-Linking Spans of Dimaleimides. The initial conformer of dimaleimides was generated using an Insight II program (Biosym/MSI, San Diego), and the structures were optimized by molecular mechanics calculations with a CVFF force-field with a Discover program (Biosym/MSI). The distances between two maleimide moieties of the cross-linkers were determined as the averages of the bond lengths C(3)−C(3′), C(3)−C(4′), C(4)−C(3′), and C(4)−C(4′) of the most stable structures of the dimaleimides.

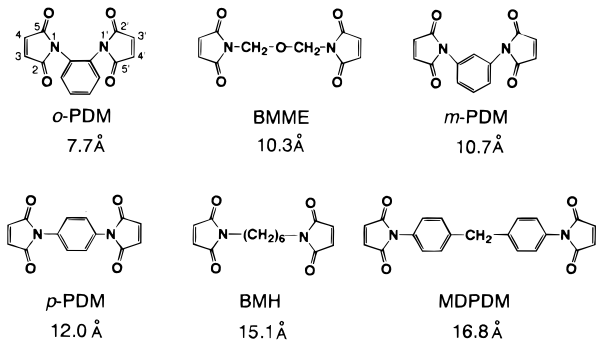
Preparations of Mitochondria and Submitochondrial Particles. Bovine heart mitochondria and their submitochondrial particles with 5 mM ATP incorporated were prepared as described previously (*9*). CATR-loaded submitochondrial particles were prepared from the mitochondria that had been incubated with 4 nmol of CATR/mg of protein at 25 °C for 10 min (*10*). 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.

Cross-Linking of the ADP/ATP Carrier by Dimaleimides. Bovine heart mitochondria and their submitochondrial particles (0.5 mg of protein) were suspended in 100 μL of medium consisting of 250 mM sucrose, 0.2 mM EDTA-2Na, 10 mM Tris-HCl buffer, pH 7.2, at 0 °C. The cross-linking reaction was started by addition of 1 μL of various concentrations of dimaleimide to suspensions of mitochondria or submitochondrial particles, and the reaction was terminated with 25 mM DTT. Mitochondria or the particles (12.5 μg of protein) were subjected to SDS−PAGE in 12% polyacrylamide gel by the method of Laemmli (*12*). After SDS−PAGE, the gel was stained with CBB, and the staining intensities of the bands were determined from their optical absorbance at 560 nm in a Shimadzu Chromatoscanner Model CS-9000. The effects of transport inhibitors of the carrier on the cross-linking reaction were examined by their preincubation with submitochondrial particles at 0 °C for 10 min.

Immunostaining of the ADP/ATP Carrier. Western-blotting of proteins in submitochondrial particles (10 μg of protein) was performed as described previously using antiserum against ADP/ATP carrier (*10*). An ECL reagent (Amersham) was used for detection of the ADP/ATP carrier.

ADP Transport via the Carrier in Submitochondrial Particles. The particles with incorporated 5 mM ATP (2 mg of protein/mL) were suspended in medium consisting of 250 mM sucrose, 0.2 mM EDTA-2Na, 5 μg/mL oligomycin, and 10 mM Tris-HCl buffer (pH 7.2). Then the suspension was incubated with various concentrations of dimaleimide cross-linker for 10 min at 0 °C, and cross-linking was terminated with DTT. ADP transport was started by addition of a final concentration of 20 μM [^{3}H]ADP (specific radioactivity, 200 μCi/μmol). Then, BKA (10 nmol/mg of protein) was added after 10 s to terminate the transport, and [^{3}H]ADP in the medium was promptly separated by chromatography on an AG1-X8 column (*9*). The amount of ADP incorporated was determined from the radioactivity in an Aloka scintillation counter, Model LSC-3500.

Chart 1. Structures of Dimaleimides Used in This Study[a]



[a] Cross-linking spans are shown below structures.

Analysis of Dimaleimide Adducts with Glutathione. Solutions of 50 μM dimaleimide were incubated for various periods with 100 μM GSH in 300 μL of 100 mM sodium phosphate buffer (pH 7.2) containing 0.2 mM EDTA at 0 °C. The reactions were terminated by changing the pH of the mixture to about pH 2 by addition of 200 μL of 500 mM phosphoric acid. Then, 100 μL samples of the mixtures were promptly subjected to reverse-phase HPLC on a column of TSK gel ODS-120T (0.46 × 15 cm, Tosoh, Tokyo) (*13*). HPLC was performed with a linear gradient of acetonitrile containing 0.05% TFA at a flow rate of 1.0 mL/min: *o*-, *m*-, and *p*-PDM, with a gradient of 18.0−31.5% for 15 min; BMME, with a gradient of 13.5−22.5% for 10 min; BMH, with a gradient of 18.5−40.5% for 25 min; and MDPDM, with a gradient of 22.5−49.5% for 30 min. Elution was monitored as absorbance at 210 nm.

RESULTS

1. Cross-Linking of the ADP/ATP Carrier by Dimaleimide Derivatives. We examined the effects of six dimaleimide thiol cross-linkers on the ADP/ATP carrier in mitochondria and submitochondrial particles. Their chemical structures and the possible cross-link spans determined by molecular mechanics calculation are shown in Chart 1. The distances between reactive methines of the two maleimide rings of the energetically most stable conformations are taken as their cross-link spans. As all the cross-linkers except BMME and BMH are linked by the rigid aromatic ring(s) and thus regarded to change their conformations mainly by rotation of two maleimide rings around the bond connecting the aromatic ring(s), their cross-link spans are expected not to change greatly in solution. Although BMME and BMH with flexible spacers could change their cross-link spans more easily than the other dimaleimides, their changes should be

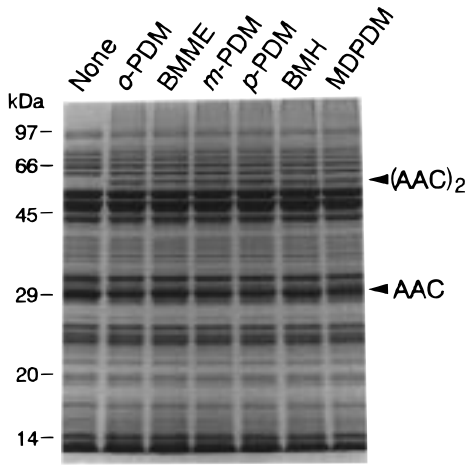


FIGURE 1: Effects of various dimaleimides on membrane proteins in bovine heart submitochondrial particles. Bovine heart submitochondrial particles (5 mg of protein/mL) in medium consisting of 250 mM sucrose, 0.2 mM EDTA-2Na, and 10 mM Tris-HCl buffer (pH 7.2) were incubated with various dimaleimides (50 μM) for 60 min at 0 °C, and then SDS−PAGE (12.5 μg of protein) was performed. Protein bands were stained with CBB.

2−3 Å at most due to trans-cis conversion of the spacer chain, which is the major conformational change of the spacers. The possibility of taking the shortest conformations in juxtaposition of the two maleimide rings was found to be energetically very unfavorable. Therefore, the values in Chart 1 should be the most possible cross-link spans in solution. These values agree well with those reported previously (*14*−*17*).

First, bovine heart mitochondria were incubated with 50 μM cross-linkers for 60 min at 0 °C and a physiological pH of 7.2, and the reaction was terminated by addition of excess DTT. The mitochondrial samples were solubilized with SDS and subjected to SDS−PAGE. No appreciable changes were observed in the protein bands on SDS−PAGE with these six cross-linkers. In contrast, distinct cross-links were formed with submitochondrial particles. As shown in Figure 1, with all the dimaleimides the 30 kDa protein band decreased with appearance of a 60 kDa band. No appreciable changes were observed in other protein bands. These 30 and 60 kDa bands were stained with antiserum against the carrier, so they were concluded to be due to the ADP/ATP carrier. In addition, we confirmed by amino acid sequence analysis that the 30 kDa protein was the ADP/ATP carrier (*9*). Therefore, intermolecular cross-linking was formed specifically between two ADP/ATP carriers, suggesting that the carrier has a dimeric form in the mitochondrial membrane.

Recently, we reported that $Cu(OP)_2$ causes similar specific intermolecular cross-linking between two Cys^{56} residues of the carrier in particles, but no cross-linking in mitochondria (*10*). In addition to the appearance of a 60 kDa band, a faint 28 kDa band was observed. As this protein band was stained with antiserum against the ADP/ATP carrier, it was concluded to be due to intramolecular cross-linking (*10*). However, no 28 kDa band was observed on incubation of the particles with dimaleimide cross-linkers even for more than 60 min, indicating that dimaleimide cross-linkers did not cause intramolecular cross-linking. Hereafter, we refer to the 30 kDa ADP/ATP carrier and the intermolecular cross-linked 60 kDa carrier as AAC and $(AAC)_2$, respectively.

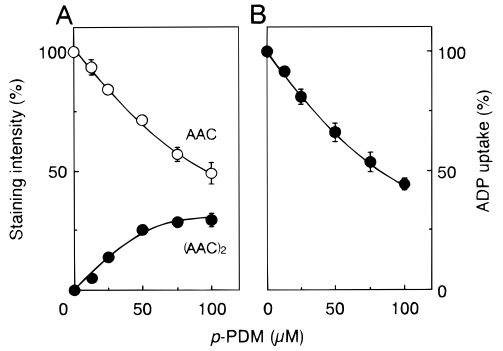


FIGURE 2: Changes in amounts of monomeric and dimeric ADP/ATP carrier (A) and ADP transport activity (B) with concentration of the cross-linker *p*-PDM. (A) Suspensions of bovine heart submitochondrial particles with incorporated ATP (5 mM) were incubated with various concentrations of *p*-PDM for 10 min at 0 °C, and the band intensities of AAC and $(AAC)_2$ on SDS−PAGE were determined. Other cross-linking conditions were as for Figure 1. (B) After termination of cross-linking by *p*-PDM, [^{3}H]ADP (final concentration 20 μM) was added, and ADP transport was terminated with BKA after 10 s. The transport activity of the carrier was determined from the radioactivity of the incorporated [^{3}H]ADP. The value for [^{3}H]ADP transported without cross-linking was 2.76 ± 0.18 nmol (mg of protein)$^{-1}$ min^{-1}. Results are means ± SD for three separate experiments.

Figure 2A shows changes in the amounts of AAC and $(AAC)_2$ determined from the staining intensities of their protein bands on SDS−PAGE after incubation of the submitochondrial particles with various concentrations of the cross-linker *p*-PDM for 10 min at pH 7.2 and 0 °C. The amount of AAC decreased and that of $(AAC)_2$ increased with increase in the *p*-PDM concentration. Formation of $(AAC)_2$ attained almost a plateau level with more than 50 μM *p*-PDM, but AAC decreased steadily with *p*-PDM concentration. This was due to formation of higher aggregates, which remained in the stacking gel, as observed on cross-linking catalyzed by $Cu(OP)_2$ (*10*). Higher aggregates were not observed on treatment with up to 50 μM *p*-PDM for 10 min, but were formed on longer incubation with 50 μM *p*-PDM. Like *p*-PDM, the five other dimaleimides caused only intermolecular cross-linking and subsequent formation of higher aggregates. The mechanism of higher aggregate formation is not yet clear.

2. Effects of Various Reagents on Cross-Linking. Of the four cysteine residues, Cys^{56}, Cys^{128}, Cys^{159}, and Cys^{256} of the carrier, the SH-reagent NEM preferentially incorporates with Cys^{56} of the m-state carrier induced by BKA, but does not incorporate with any cysteine residues in the c-state carrier induced by CATR (*7, 10*). We reported from the reactivities of the SH-reagents EMA and NEM that BKA does not affect the conformation of the M1 loop containing Cys^{56}, and changes the conformations of the M2 and M3 loops (*7*).

We studied the effects of NEM, CATR, and BKA on the cross-linking of the carrier in submitochondrial particles by various dimaleimides at pH 7.2 and 0 °C. Figure 3 shows the effects of pretreatment with these compounds on the cross-linking by *p*-PDM (10 nmol/mg of protein) with the particles (5 mg of protein/mL) incubated for 60 min. Pretreatment of the particles (20 mg of protein/mL) with NEM (100 nmol/mg of protein) for 10 min completely inhibited cross-link formation. However, on pretreatment of the particles with BKA (10 nmol/mg of protein), cross-link

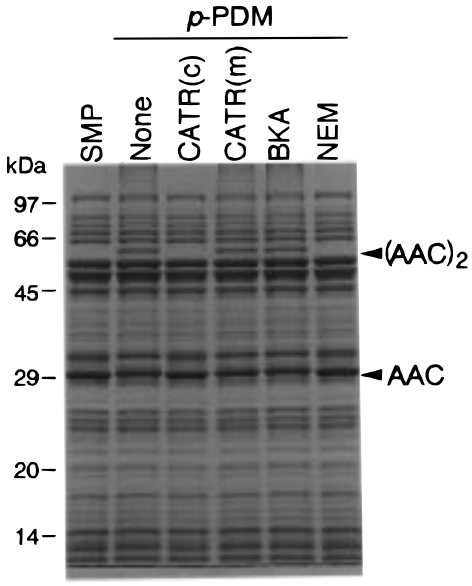


FIGURE 3: Effect of various compounds on cross-linking of bovine heart submitochondrial particles with *p*-PDM. Submitochondrial particles (20 mg of protein/mL) were preincubated with 200 μM CATR [CATR(m)], 200 μM BKA (BKA), and 2 mM NEM (NEM) for 10 min at 0 °C in the medium. Then, the submitochondrial suspension was diluted to 5 mg of protein/mL with the medium, and incubated with 50 μM *p*-PDM for 60 min at 0 °C. The particle samples were subjected to SDS−PAGE (12.5 μg of protein), and protein bands were stained with CBB. Other experimental conditions were as for Figure 1. SMP, submitochondrial particles without treatment with *p*-PDM; None, particles without incubation with test reagents; CATR(c), CATR-preloaded particles.

formation was similar to that without BKA treatment. These results showed that Cys^{56} residues were cross-linked by *p*-PDM. CATR (10 nmol/mg of protein) added from the matrix side did not have any effect on the cross-linking by *p*-PDM, and its addition from the cytosolic side completely inhibited cross-linking formation. These effects of NEM, CATR, and BKA were exactly the same as those observed with $Cu(OP)_2$ (*10*). Similar results were observed with five other dimaleimide cross-linkers (data not shown). Therefore, dimaleimides were concluded to cause cross-linking between two Cys^{56} residues. We confirmed by amino acid sequence analysis that Cys^{56} was modified with *p*-PDM, as with $Cu(OP)_2$ (*10*).

3. Effect of Cross-Linking on ADP Transport. The effect of cross-linking on ADP transport via the ADP/ATP carrier in submitochondrial particles was examined at pH 7.2 and 0 °C. Suspensions of particles containing 5 mM ATP were incubated with various concentrations of *p*-PDM for 10 min. After termination of the cross-linking reaction, [^{3}H]ADP (final concentration 20 μM) was added and incubated for 10 s. Then, BKA was added to terminate the transport, and the ADP transport activity of the particles was determined from the radioactivity of the incorporated [^{3}H]ADP. As shown in Figure 2B, inhibition of ADP transport proceeded with increase in *p*-PDM concentration in a manner very similar to that of the decrease in the amount of AAC (Figure 2A), showing that cross-link formation inhibited ADP transport.

As reaction of Cys^{56} with the SH-reagent NEM inhibited transport activity of the carrier (*7, 18*), we expected that formation of the conjugated monomeric carrier (1:1 complex between a *p*-PDM and a carrier) would inhibit ADP transport.

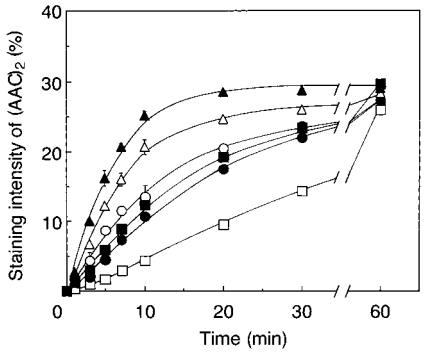


FIGURE 4: Time course of intermolecular cross-linking of the ADP/ATP carrier with various dimaleimides. Submitochondrial particles were incubated with 50 μM dimaleimides for various periods at 0 °C under the conditions described in the legend of Figure 1. Relative cross-link formation was determined from the band intensity of $(AAC)_2$ on SDS−PAGE, taking the initial band intensity of monomeric carrier as a reference. Results are means ± SD for three separate experiments. Formation of $(AAC)_2$ on the ordinate is shown by its relative band intensity stained with CBB on SDS−PAGE. Open circles, *o*-PDM; closed circles, BMME; open triangles, *m*-PDM; closed triangles, *p*-PDM; open squares, BMH; closed squares, MDPDM.

However, the results in Figure 2 show that inhibition of ADP transport proceeded in parallel with a decrease in the amount of monomeric carrier. Thus, the conjugated monomeric carrier was suggested not to be involved in the AAC bands on incubation of the particles with dimaleimides. This was due to quick conversion of the conjugated monomeric carrier to the cross-linked dimerized carrier, $(AAC)_2$, as described later (see section 5).

4. Time-Dependent Cross-Link Formation by Dimaleimides in the Submitochondrial Particles. Next, we determined the time dependence of cross-linking formation by the dimaleimide cross-linkers. Submitochondrial particles were incubated with 50 μM dimaleimides for various periods at pH 7.2 and 0 °C, and the progress of cross-linking was determined by measuring the intensity of CBB-stained bands due to AAC and $(AAC)_2$ on SDS−PAGE. Figure 4 shows results on the time-dependent formation of $(AAC)_2$. Cross-linking proceeded first rapidly and then gradually, attaining a plateau level with all the cross-linkers. Although the rate of $(AAC)_2$ formation was dependent on the cross-linker, the cross-linkings with all the dimaleimides attained the same plateau level in about 60 min.

Accordingly, progress of cross-linking can be evaluated quantitatively from the rate of $(AAC)_2$ formation, $v(AAC)_2$, determined from the slope of the first linear portion of the plot for time-dependent $(AAC)_2$ formation, when no higher aggregates have yet been formed. The $v(AAC)_2$ values increased in the order BMH < BMME < MDPDM < *o*-PDM < *m*-PDM < *p*-PDM. The relative values of $v(AAC)_2$ of all dimaleimides are summarized in Table 1. There is about 7.5-fold difference between the smallest $v(AAC)_2$ of BMH and the greatest $v(AAC)_2$ of *p*-PDM.

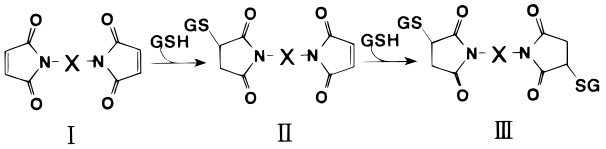
5. Reactivities of Dimaleimides with Glutathione in Solution. As shown in Figure 4, cross-linking formations between two Cys^{56} residues were dependent on the dimaleimides. Formation of $(AAC)_2$ is governed by at least (1) reactivity of the maleimide moiety in the cross-linkers with cysteine residues and (2) the span between the two maleimide moieties of the cross-linkers. In this study, we examined the

Table 1: Initial Rates of Intermolecular Cross-Linking of the ADP/ATP Carrier by Dimaleimide Cross-Linkers, and Rates of Their Disuccinimide-Type Adduct Formation with GSH

cross-linker	cross-link span (Å)[a]	$v(AAC)_2$[b]	v(DSI)[c]	$v(AAC)_2$/v(DSI)
o-PDM	7.7	78 ± 12	152 ± 13	0.51
BMME	10.3	38 ± 3	68 ± 8	0.56
m-PDM	10.7	86 ± 8	104 ± 5	0.83
p-PDM	12.0	100 ± 9	100 ± 9	1
BMH	15.1	13 ± 3	19 ± 4	0.68
MDPDM	16.8	50 ± 2	84 ± 19	0.60

[a] Cross-link span determined by molecular mechanics calculation of the most stable structure of the dimaleimide cross-linker. [b] Relative initial rate of intermolecular cross-link formation, determined from the linear portion of the time-dependent increase of band intensity of $(AAC)_2$ on SDS−PAGE per minute, between Cys^{56} residues of the ADP/ATP carrier in bovine heart submitochondrial particles mediated by dimaleimide cross-linkers at pH 7.2 and 0 °C. Values are means ± SD for 3 separate experiments. [c] Relative rate of disuccinimide-type adduct (DSI) formation, determined from increase in amount of DSI per second, between dimaleimides and GSH at pH 7.2 and 0 °C. Values are means ± SD for 3 separate experiments.

Chart 2. Possible Adduct Formation of Dimaleimide Cross-Linkers with GSH[a]



[a] The reaction scheme is according to our previous report on adduct formation of NEM and EMA with various SH compounds (*13*). X indicates the spacer of the cross-linker; I, dimaleimide cross-linker; II, monosuccinimide-type adduct of dimaleimide with a GSH molecule; III, disuccinimide-type adduct of dimaleimide with two GSH molecules.

conformational characteristics of loop M1 by studying cross-link formation by dimaleimide cross-linkers with various distances between their two maleimide moieties. For this, cross-link formation had to be determined under conditions giving the same reactivity of cross-linkers with cysteine residues.

To examine whether there was any difference in the reactivities of cross-linkers with SH-groups, we determined the extents of adduct formation between various dimaleimides and the reduced form of glutathione (GSH) at pH 7.2 and 0 °C. According to the adduct formation of NEM and EMA with SH-compounds (*13*), the reaction should proceed as shown in Chart 2, in which X refers to the spacer of dimaleimides. According to Chart 2, one of the maleimide groups of the cross-linker (I) reacts with GSH, forming a monosuccinimide-type adduct (II), and then the other free maleimide group of II interacts with GSH, forming the final disuccinimide-type conjugate III. We incubated 50 μM dimaleimides with 100 μM GSH in phosphate buffer (pH 7.2) at 0 °C for various periods and terminated the reactions by addition of excess phosphoric acid to bring the solution to a nonreactive pH of about 2. Then, we promptly subjected the reaction mixtures to reversed-phase HPLC on an ODS column eluted with acetonitrile containing TFA at about pH 2, in which no adduct formation took place.

The mixture of *p*-PDM and GSH at the nonreactive pH of 2.0 gave two single elution peaks of *p*-PDM and GSH monitored at 210 nm, indicating that these compounds were pure (data not shown). We examined the adduct formation between them at pH 7.2 and 0 °C by monitoring changes in the elution profiles due to compounds I, II, and III with time. The time-dependent changes in their peak areas showed a typical sequential reaction. Formation of II was so quick that its rate of formation could not be determined. However, formation of adduct III proceeded more slowly, showing that the second reaction was the rate-limiting step in sequential adduct formation of 1 mol of *p*-PDM with 2 mol of GSH (data not shown). Therefore, the rate of formation of III represents the affinity of *p*-PDM for GSH. These features were common to all dimaleimides except BMH, with which formation of II was measurably slow.

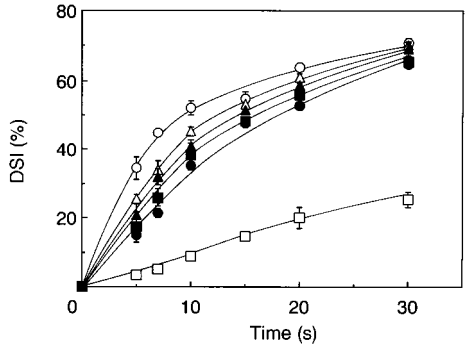


FIGURE 5: Time courses of adduct formation between various dimaleimides and GSH. The formations of disuccinimide-type adducts (DSI) of various dimaleimides and GSH were determined as described in the text. Results are means ± SD for three separate experiments. The formation of DSI of each dimaleimide is shown as its peak area at the given times relative to that at 3 min, where all the dimaleimide was converted to its DSI and only an elution peak due to DSI was observed. Open circles, *o*-PDM; closed circles, BMME; open triangles, *m*-PDM; closed triangles, *p*-PDM; open squares, BMH; closed squares, MDPDM.

Figure 5 shows the time courses of formation of disuccinimide-type adduct III with various cross-linkers. In all cases, formation of adduct III proceeded first quickly, and then gradually, showing that formation of disuccinimide-type adducts with various dimaleimides proceeded similarly to that with *p*-PDM. From the linear portions of the initial increases in formation of III, we determined the apparent rates of formation of the disuccinimide-type adduct (DSI), v(DSI). As summarized in Table 1, the v(DSI) values of various cross-linkers were not the same, being greatest with *o*-PDM and smallest with BMH, possibly due to difference in their electronic structures (*19*). The v(DSI) values were not correlated well with cross-link formation of the ADP/ATP carrier [$v(AAC)_2$] shown in Table 1, because the two Cys^{56} residues should be located at a certain distance, while GSH molecules rotate freely in solution.

6. Feasibility of Cross-Link Formation of the Carrier at the Same Affinity of Dimaleimides toward Cysteine Residues. From the results on the adduct formations of various dimaleimides with GSH, their reactivities with SH-compounds were found to be different. Therefore, the rates of cross-link formation of the ADP/ATP carrier mediated by dimaleimides shown in Figure 4 consisted of at least reactivity with cysteine residues and the effect of the cross-linking span of the dimaleimides. The value of $v(AAC)_2$/v(DSI) is regarded as representing the feasibility of cross-linking between two Cys^{56} residues located at a certain distance under conditions in which the reactivities of dimaleimides with SH-groups are the same. The values of

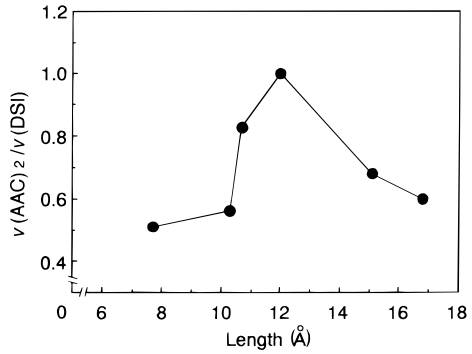


FIGURE 6: Dependence of cross-linking of Cys^{56} residues of the ADP/ATP carrier in submitochondrial particles on the cross-link span of bifunctional dimaleimides with the same reactivities. The values of $v(AAC)_2/v(DSI)$ are those listed in Table 1.

$v(AAC)_2/v(DSI)$ with dimaleimides are summarized in Table 1.

Figure 6 shows the dependence of $v(AAC)_2/v(DSI)$ on the cross-linking span. The value increased significantly at more than about 11 Å, and was maximal at about 12 Å (with *p*-PDM), decreasing at above 12 Å. The change above 12 Å seemed to be more gradual than that below 12 Å. Our findings that $Cu(OP)_2$ catalyzes formation of disulfide bridges between two Cys^{56} residues show that these residues are able to become close. Accordingly, a pair of Cys^{56} residues in M1 loops in the dimeric ADP/ATP carrier are suggested to fluctuate widely between a very close distance and at least 17 Å, with a most probable distance of about 12 Å.

DISCUSSION

As cysteine residues are the most reactive amino acid residues with chemical reagents in proteins, their locations and states in proteins have been studied extensively by their reactivities with SH-reagents (*9, 10, 20*). Dimaleimide sulfhydryl cross-linkers are effective for elucidating the geometric organization of oligomeric proteins and membrane proteins (*14*−*17*). In fact, topologies of subunits in oligomeric proteins such as ribosomal subunits (*14, 15*), myosin (*16*), and phosphorylase kinase (*17*) have been determined from differences in cross-link formation with dimaleimides having various cross-link spans.

In this study, we examined the effects of six dimaleimide derivatives with cross-link spans of 7.7−16.8 Å on membrane proteins of bovine heart mitochondria and submitochondrial particles at pH 7.2 and 0 °C. Although these dimaleimide cross-linkers did not have appreciable effects from the cytosolic side, they induced specific cross-linking of two ADP/ATP carriers from the matrix side. Unlike the action of dimaleimides on various oligomeric proteins, in which formation of pairs of cross-linked proteins is strictly dependent on the cross-link spans of dimaleimides, all the dimaleimides used in the present study formed a specific cross-link between Cys^{56} residues of M1 loops in the ADP/ATP carrier, but no other sets of cross-links, although loops M2 and M3 face the matrix space like the M1 loop. Therefore, the M2 and M3 loops are located in the membrane at positions inaccessible to all the cross-linkers. This finding is consistent with our previous report that $Cu(OP)_2$ mediates cross-link formation predominantly between Cys^{56} residues (*10*). In addition, we confirmed that the ADP/ATP carrier functions as a homodimer, as suggested previously (*10, 21, 22*).

The specific transport inhibitor BKA, which fixes the m-state conformation, did not affect cross-link formation, but CATR, which fixes the c-state conformation, completely inhibited formation of cross-links when added from the cytosolic side. Therefore, loop M1 containing Cys^{56} is exposed to the matrix space in the m-state conformation, and it is not accessible to cross-linkers in the c-state conformation, possibly due to its translocation in the membrane region as suggested in our previous report (*7, 10*). This possibility is supported by the reports that proteinases easily digest loop M1 in the m-state, but not in the c-state, carrier (*23, 24*).

Cross-linking, in terms of the same affinity to SH-groups, increased with increase in the cross-linking span, being maximal with *p*-PDM having a cross-link span of 12 Å, and decreasing at above 12 Å. The level of cross-link formation by the longest span of 16.8 Å (MDPDM) was similar to that of BMME with a 10.3 Å span, being about half that of *p*-PDM. We have reported that formation of a specific S−S linkage between Cys^{56} residues is catalyzed by $Cu(OP)_2$ (*10*), so the Cys^{56} residues of M1 loops in the dimeric ADP/ATP carrier could be located close to each other. Therefore, two M1 loops are suggested to fluctuate between a very close distance and a distance of at least 17 Å, with a most probable distance of 12 Å in the m-state conformation. At present, the physiological significance of a distance of 12 Å is not clear, but this may be related to the maximal length of 11−12 Å of ADP and ATP (*25*−*27*).

As the cross-links mediated by dimaleimides and the disulfide bridge catalyzed by $Cu(OP)_2$ (*10*) inhibited the transport activity of the carrier, fluctuation of M1 loops in the dimeric carrier is suggested to be decisively important in the transport function of the ADP/ATP carrier. Possibly, cross-linking locks the m-state conformation, thus inhibiting conversion to the c-state conformation. As a result, no ADP transport takes place. From the amino acid sequence of the carrier (*8*), loop M1 is rich in positive amino acid residues. Fluctuation of loop M1 may be favorable for attraction of the polyvalent negatively charged transport substrates ADP/ATP, of which maximal width is about 11−12 Å, facilitating access of the substrate anion to its primary binding domain in loop M2 (*11*). Therefore, a pair of M1 loops could function as a gate in the transport by fluctuating widely from a very close distance apart to a distance of at least 17 Å with a most probable distance of about 12 Å. The fluctuation of paired M1 loops should be favorable for their swings in cooperation with the M2 and M3 loops in the conversion of m-state carrier to the c-state carrier, as we proposed previously as a cooperative swinging loop model for the mechanism by which the ADP/ATP carrier mediates the transport of ADP/ATP (*2, 7*).

REFERENCES

1. Klingenberg, M. (1989) in *Anion Carriers of Mitochondrial Membranes* (Azzi, A., et al., Eds.) pp 169−181, Springer-Verlag Berlin, Heidelberg.
2. Terada, H., and Majima, E. (1997) *Prog. Colloid Polym. Sci. 106*, 192−197.
3. Klingenberg, M. (1993) *J. Bioenerg. Biomembr. 25*, 447−457.

4. Brandolin, G., Le Saux, A., Trezeguet, V., Lauquin, G. J. M., and Vignais, P. V. (1993) *J. Bioenerg. Biomembr. 25*, 459−472.
5. Klingenberg, M. (1989) *Arch. Biochem. Biophys. 270*, 1−14.
6. Walker, J. E. (1992) *Curr. Opin. Struct. Biol. 2*, 519−526.
7. Majima, E., Shinohara, Y., Yamaguchi, N., Hong, Y.-M., and Terada, H. (1994) *Biochemistry 33*, 9530−9536.
8. Aquila, H., Misra, D., Eulitz, M., and Klingenberg, M. (1982) *Hoppe-Seyler's Z. Physiol. Chem. 363*, 345−349.
9. Majima, E., Koike, H., Hong, Y.-M., Shinohara, Y., and Terada, H. (1993) *J. Biol. Chem. 268*, 22181−22187.
10. Majima, E., Ikawa, K., Takeda, M., Hashimoto, M., Shinohara, Y., and Terada, H. (1995) *J. Biol. Chem. 270*, 29548−29554.
11. Majima, E., Yamaguchi, N., Chuman, H., Shinohara, Y., Ishida, M., Goto, S., and Terada, H. (1998) *Biochemistry 37*, 424−432.
12. Laemmli, U. K. (1970) *Nature 227*, 680−685.
13. Majima, E., Goto, S., Hori, H., Shinohara, Y., Hong, Y.-M., and Terada, H. (1995) *Biochim. Biophys. Acta 1243*, 336−342.
14. Chang, F. N., and Flaks, J. G. (1972) *J. Mol. Biol. 68*, 177−180.
15. Walleczek, J., Martin, T., Redl, B., Stöffler-Meilicke, M., and Stöffler, G. (1989) *Biochemistry 28*, 4099−4105.
16. Wells, J. A., Knoeber, C., Sheldon, M. C., Werber, M. M., and Yount, R. G. (1980) *J. Biol. Chem. 255*, 11135−11140.
17. Nadeau, O. W., Sacks, D. B., and Carlson, G. M. (1997) *J. Biol. Chem. 272*, 29196−26201.
18. Boulay, F., and Vignais, P. V. (1984) *Biochemistry 23*, 4807−4812.
19. Knight, P. (1979) *Biochem. J. 179*, 191−197.
20. Van Iwaarden, P. R., Driessen, A. J. M., and Konings, W. N. (1992) *Biochim. Biophys. Acta 1113*, 161−170.
21. Riccio, P., Aquila, H., and Klingenberg, M. (1975) *FEBS Lett. 56*, 133−138.
22. Hackenberg, H., and Klingenberg, M. (1980) *Biochemistry 19*, 548−555.
23. Brandolin, G., Boulay, F., Dalbon, P., and Vignais, P. V. (1989) *Biochemistry 28*, 1093−1100.
24. Marty, I., Brandolin, G., Gagnon, J., Brasseur, R., and Vignais, P. V. (1992) *Biochemistry 31*, 4058−4065.
25. Cini, R., Burla, M. C., Nunzi, A., Polidori, G. P., and Zanazzi, P. F. (1984) *J. Chem. Soc., Dalton Trans.*, 2467−2476.
26. Sabat, M., Cini, R., Haromy, T., and Sundaralingam, M. (1985) *Biochemistry 24*, 7827−7833.
27. Protein Data Bank, Brookhaven National Laboratory, Upton, New York.

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