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Roles of Adjoining Asp and Cys Residues of First Matrix-Facing Loop in Transport Activity of Yeast and Bovine Mitochondrial ADP/ATP Carriers

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The mitochondrial ADP/ATP carrier (AAC) transports substrate by interconversion of its conformation between m- and c-states. The 1st loop facing the matrix (LM1) is extruded into the matrix in the m-state and is suggested to intrude into the mitochondrial membrane on conversion to the c-state conformation [Hashimoto, M., Majima, E., Goto, S., Shinohara, Y., and Terada, H. (1999) *Biochemistry* 38, 1050–1056]. To elucidate the mechanism of the translocation of LM1, we examined the effects of site-directed mutagenesis of two adjoining residues, Cys[56] and Asp[55] in the bovine type 1 AAC and Cys[73] and Asp[72] in the yeast type 2 AAC, on the substrate transport activity. We found that (i) replacement of the Cys by bulky and hydrophilic residues was unfavorable for efficient transport activity, (ii) the carboxyl groups of the Asp residues of the bovine and yeast AACs were essential and strictly position-specific, and (iii) hence, the mutation to Glu showed transport activity comparable to that of the native AACs. Based on these results, we discussed the functional role of LM1 in the transport activity of AAC.

Key words: ADP/ATP carrier, membrane protein, mitochondria, oxidative phosphorylation, site-directed mutagenesis.

Abbreviations: AAC, ADP/ATP carrier; bAAC1, bovine type 1 isoform of AAC; BKA, bongkrekic acid; CATR, carboxyatractyloside; MMTS, methyl methanethiosulfonate; NEM, *N*-ethylmaleimide; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; yAAC2, yeast type 2 isoform of AAC; Cys[56](B) & Asp[55](B), Cys[56] & Asp[55] of bAAC1, respectively; Cys[73](Y) & Asp[72](Y), Cys[73] & Asp[72] of yAAC2, respectively.

The ADP/ATP carrier (AAC) in the inner mitochondrial membrane mediates the transport of ATP synthesized by oxidative phosphorylation in the matrix to the cytoplasm for exchange with cytosolic ADP. As the AAC is essential for oxidative phosphorylation, considerable attention has been paid to its structural and functional properties (*1–3*). Transport of ADP and ATP via the AAC is known to be achieved by interconversion of the carrier between two distinct conformations known as c-state and m-state, in which the substrate recognition/binding site of the AAC faces the cytosol and matrix, respectively. These conformational states are fixed by the specific transport inhibitors carboxyatractyloside (CATR) and bongkrekic acid (BKA), respectively (*1–3*).

The AAC consists of about 300 amino acid residues comprising three repeat segments of about 100 amino acid residues each. Linking of the membrane-spanning segments is mediated by the hydrophilic segments, which are exposed to the aqueous cytosol or matrix space. Namely, the N and C termini and two loops (LC1 and LC2) are located on the cytosolic surface, and three loops (LM1 to LM3) are located on the surface of the matrix space, as illustrated in Fig. 1, which is based on the crystal structure of the bovine type 1 AAC (bAAC1) in its c-state conformation (*4*). This carrier is thought to function as a homodimer, the orientation of its two parts being in the same direction (*3*, *5–7*).

According to our previous studies on the characterization of bAAC1 and yeast type 2 AAC (yAAC2), loops on both membrane surfaces are important for the transport activity of AAC (*3*, *6–11*). In contrast to the loops on the cytosolic surface, those facing the matrix space have been characterized well. LM1 is suggested to function as a gate; LM2, to be the main substrate recognition site; and LM3, to be an auxiliary binding site for substrate, when ADP and ATP are transported from the matrix to the cytosol. We also found that LM1 is extruded into the matrix space in the m-state, and it is intruded into the membrane on conversion to the c-state (*7*, *9–11*). These structural features of LM1 mainly characterize both conformational states (*7*, *9–11*). Interestingly, LM1 is not directly associated with substrate binding (*6*, *11*).

bAAC1 has cysteine residues: Cys[128] is located in the 3rd membrane spanning segment, and Cys[56], Cys[159], and

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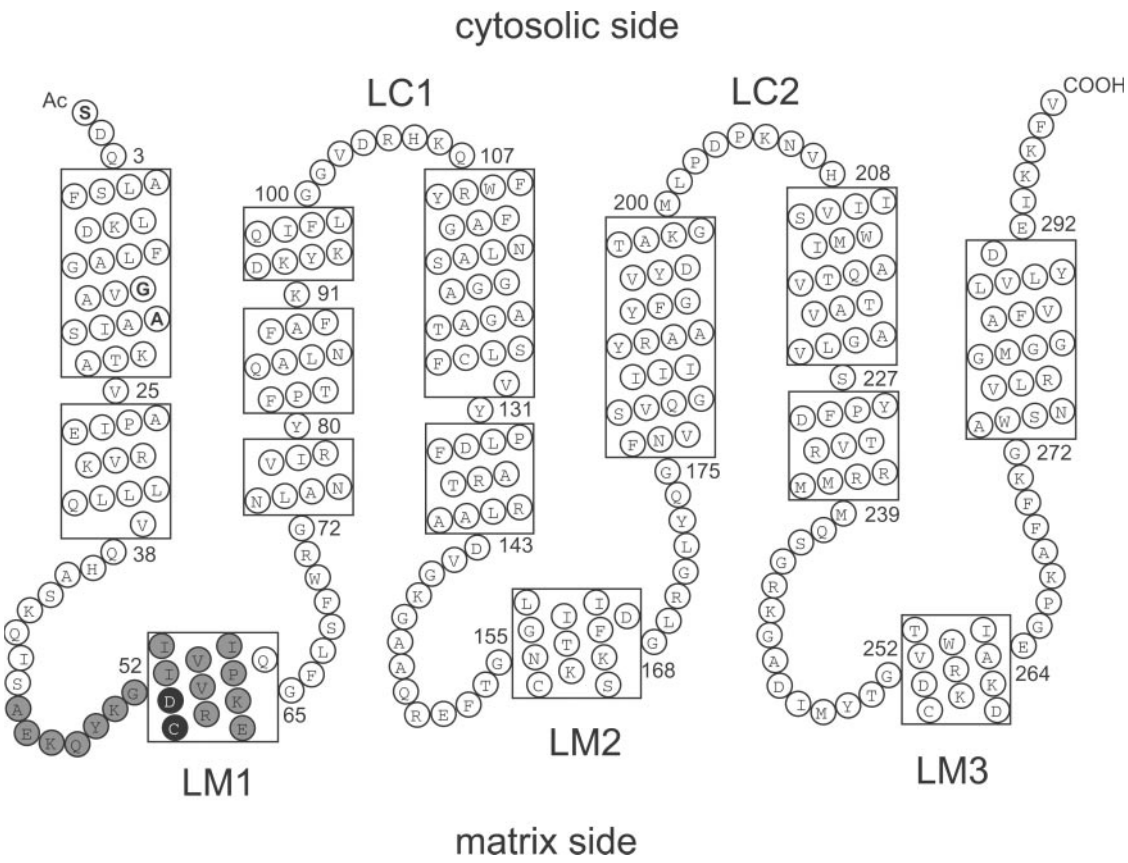

Fig. 1. **Topology of bAAC1.** This model is based on the basis of the crystal structure of bAAC1 fixed in the c-state by CATR (*4*). Alpha-helical portions in the 6-transmembrane regions and in the loops facing matrix are shown by quadrilateral boxes. Residues in the gray circles represent a sequence including the consensus sequence Y-X-G-I-X-D-C-X-X-R (X: an arbitrary amino acid residue) shown in Fig. 2. Asp^{55} and Cys^{56} are shown by closed circles.

Cys^{256} are located in LM1, LM2, and LM3, respectively. The cysteine residue at position 56 of the LM1 of bAAC1 is conserved in all AAC isoforms (*12*), and this residue is thought to be important for the transport activity. Boulay and Vignais (*13*) first found that Cys^{56} of bAAC1 was the primary binding site of the SH-reagent *N*-ethylmaleimide (NEM) and that labeling of it caused loss of transport activity. Later, we determined the affinity of NEM for all four Cys residues of bAAC1 in the m-state quantitatively and found that it predominantly labeled Cys^{56}, but also labeled Cys^{159} and Cys^{256} to lesser extents and did not label Cys^{128} located in the membrane (*10*). When Cys^{56} of the m-state carrier was modified with various SH-reagents, the m-state conformation was stabilized and conversion to the c-state did not take place, leading to the loss of the transport activity (*10*, *11*, *14*). In contrast, SH-reagents did not label the c-state carrier at all (*7*, *9–11*, *14*).

Besides chemical modification of the carrier, mutation studies of the yAAC2, which is a dominant AAC isoform in *Saccharomyces cerevisiae*, provided important information about the role of Cys^{73} of yAAC2, which corresponds to Cys^{56} of bAAC1. The yAAC2 mutant in which Cys^{73} was replaced by Ser (C73S) showed considerable transport activity (*15–18*), suggesting that Cys^{73} of yAAC2 was not essential (*15–18*). However, the residues capable of hydrogen-bonding were suggested to be necessary for transport function (*15*). In contrast, we found recently that the Cys-less mutant, in which all four Cys residues of yAAC2 were replaced by Ala residues, was expressed well in mitochondria with efficient transport activity comparable to that of the native yAAC2, thus showing that Cys^{73} of yAAC2 and the corresponding Cys^{56} of bAAC1 are not essential (*19*). Hence, the possible hydrogen bonding of the Cys residue should not be associated directly with transport function.

Although Cys residues are not essential for transport activity of AACs, it is still not clear why the labeling of Cys^{56} of bAAC1 with NEM causes a loss of transport activity. As this should be of importance to understand the transport mechanism of AACs, we tried to solve this problem by site-directed mutagenesis of Cys^{56} of bAAC1 and Cys^{73} of yAAC2. In addition, we sought to understand the role of the adjoining acidic residue Asp^{55} of bAAC1 and Asp^{72} of yAAC2, which have not previously been received special attention.

For simplicity, Cys^{56} and Asp^{55} of bAAC1 are referred to herein as Cys^{56}(B) and Asp^{55}(B), respectively, and the corresponding residues of yAAC2, to Cys^{73}(Y) and Asp^{72}(Y), respectively. Mutations of Cys^{56}(B) and Asp^{55}(B) were performed by functional expression of bAAC1 in *S. cerevisiae* by replacing the first 11 residues of the N-terminal sequence of bAAC1 with the first 26 ones of yAAC2 (*20*); and this bAAC1, which was expressed efficiently, showed the same transport activity as that of the native bAAC1

(*20*). By this method, various mutants of bAAC1 could easily be prepared. In this paper, the bAAC1 in which the N-terminal region was replaced by that of the yAAC2 is referred to as bAAC1 unless otherwise stated, and the original bAAC1 is referred to as native bAAC1. The number of amino acid residues of mutated bAAC1 corresponds to that of the native sequence of bAAC1.

MATERIALS AND METHODS

Materials—The AAC-deficient yeast strain WB-12 (Matα, *ade2-1*, *leu2-3,112*, *his3-11,15*, *trp1-1*, *ura3-1*, *can1-100*, *aac1*::*LEU2*, *aac2*::*HIS3*) was prepared as described (*20*). In the genome, about two-thirds of the N-terminus of the coding region of yAAC1 and the entire coding region of yAAC2 were deleted. The AAC-expression vector pRS314-YA2P, which contains the intrinsic yAAC2 promoter region, was used as an expression promoter (*20*). Ex Taq polymerase was obtained from Takara (Tokyo), [^{14}C]ADP from DuPont-New England Nuclear (Wilmington), and CATR from Sigma (St.Louis). BKA was a gift from Prof. Duine (Delft University of Technology). Other materials and reagents were of the highest grade commercially available.

Preparation of cDNAs of Coding Regions of bAAC1 and yAAC2—Double-stranded cDNAs encoding bAAC1 and yAAC2 were prepared as described earlier (*20*). To improve the expression level of bAAC1 in yeast cells, the DNA sequence encoding the first 11 amino acid residues from the N-terminus of bAAC1 was replaced by the corresponding region encoding 26 amino acid residues of yAAC2. This replacement did not cause any functional change in bAAC1 (*20*).

Site-Directed Mutagenesis—Mutagenesis was performed by the standard PCR method (*17*). For mutation of Cys^{56} (tgc) in bAAC1 and Cys^{73} (tgt) in yAAC2, the codons were changed to that of Gly (ggt), Ala (gct), Ser (tca), Asp (gac), Thr (acc), Val (gtc), Ile (atc), Met (atg) or Tyr (tac) with synthesized primers. For mutation of Asp^{55} (gat) in bAAC1 and Asp^{72} (gac) in yAAC2, the codons were changed to that of Glu (gaa), Ala (gct), Asn (aac), Met (atg), Tyr (tac), Ser (tca), Val (gtc) or Lys (aag).

Expression of AAC Mutants in Yeast Cells—The DNA fragment encoding yAAC2 or bAAC1 with or without a point mutation was introduced downstream of the yAAC2 promoter in pRS314-YA2P. These expression plasmids were introduced into WB-12 by the lithium acetate method (*21*). The transformants were selected on nutrient selection plates (*20*). For expression of AAC mutants, yeast cells were grown with adequate aeration at 30°C in YPGal medium consisting of 1% yeast extract, 2% bactopeptone, and 2% galactose. Cells were harvested when the optical density of the cell suspension at 600 nm had attained the value of 1.3–1.5. The mitochondria in yeast cells were isolated as described previously (*20*) and suspended in ST medium (250 mM sucrose and 10 mM Tris-HCl buffer, pH 7.4). The amount of proteins was determined with a BCA protein assay kit (Pierce) in the presence of 1% SDS, with bovine serum albumin used as a standard.

Determination of Expression Levels of AAC Mutants—Amounts of mutants of bAAC1 and yAAC2 expressed in yeast mitochondria were determined from the intensity of the immunostained AAC band on the gel of SDS-PAGE, as described previously (*20*). SDS-PAGE on 10% polyacrylamide gels was performed by the method of Laemmli (*20*), and immunostaining was carried out with antisera, one against a synthetic peptide having the sequence His^{39}–Ile^{60} of bAAC1, and the other directed against the sequence Asn^{53}–Lys^{75} of yAAC2. bAAC1 and yAAC2 purified by use of hydroxylapatite gels were used as standards (*20*).

Assay of ADP Transport Activity—The ADP transport activity of the transformed yeast mitochondria was determined essentially as described previously (*20*). Briefly, mitochondria (1 mg protein/ml) suspended in medium consisting of 250 mM sucrose, 1 mg/ml oligomycin, 0.2 mM EDTA-2Na and 10 mM Tris-HCl buffer (pH 7.2) in a total volume of 200 μl were incubated for 5 min. Then, [^{14}C]ADP (final concentration, 100 μM with the specific radioactivity of 36 kBq/μmol) was added, and the mixture was incubated at 0°C for 3 s and 10 s with yeast and bovine mitochondria, respectively. ADP transport was terminated with 20 μM CATR and 5 μM BKA, and the amount of [^{14}C]ADP incorporated was determined from its radioactivity measured in an Aloka liquid scintillation counter LSC-3500.

RESULTS

Growth Activities of Transformants in Medium with Glycerol as the Single Carbon Source—Cys^{56}(B) of the LM1 is a primary binding site of Cys residues of AACs for various SH-reagents, and the binding is achieved only in the m-state conformation. To know why the SH-labeling abolishes the transport activity and stabilizes the m-state conformation of AACs, we constructed mutants in which Cys^{56}(B) of the LM1 was replaced by Gly, Ala, Ser, Asp, Thr, Val, Met, Ile or Tyr by using cDNA of bAAC1 as a template for transformation of yeast strain WB-12, in which the intrinsic type 1 and 2 AACs had been disrupted (*20*). We also prepared similar transformants of yAAC2. First, we examined doubling times of transformants in YPGly medium, in which 3% glycerol was used as the single carbon source. In this medium, WB-12 cells were not able to grow; but the WB-12 transformed with yAAC2 or bAAC1 grew, with a doubling time of 4 or 8 h, respectively (Table 1). This difference may be associated with the fact that yAAC2 showed 4 times greater transport activity than bAAC1 (*20*). Thus, the effect of a mutation on the AAC function could be examined by measuring doubling times. As summarized in Table 1, the doubling time of the C56A and C56V mutants of bAAC1, which have small alkyl groups, was 8 h, the same as that of the parental bAAC1. In the case of C56S and C56T, bearing hydroxyl groups, the doubling time was 16 h; and for C56M and C56I, with bulky side chains, it was about 2 days. In addition, C56G, C56D, and C56Y did not grow at all in the YPGly medium. The effect of a mutation on the doubling time of the yAAC2 transformants was very similar to that on that of the corresponding transformants of bAAC1, although doubling times of yAAC2 transformants were shorter than those of bAAC1 transformants, as we found previously (*20*).

Expression Levels of AAC Mutants in Yeast Mitochondria—Next, we determined the amounts of AAC mutants in yeast mitochondria by observing the immunostaining intensities of AAC after Western blotting with the antibodies against His^{39}–Ile^{60} of bAAC1 and

Table 1. **Biological properties of transformants of Cys mutants of bAAC1 and yAAC2.**

bAAC1			yAAC2		
Mutant	Doubling time (h)[a]	AAC expression (nmol/mg of protein)[b]	Mutant	Doubling time (h)[a]	AAC expression (nmol/mg of protein)[b]
bAAC1[c]	8	0.60 ± 0.03	yAAC2[c]	4	0.72 ± 0.05
C56G	N.G.[d]	0.33 ± 0.03	C73G	N.G.[d]	0.40 ± 0.04
C56A	8	0.62 ± 0.05	C73A	4	0.77 ± 0.02
C56S	16	0.52 ± 0.02	C73S	8	0.55 ± 0.03
C56D	N.G.[d]	0.36 ± 0.03	C73D	N.G.[d]	0.35 ± 0.03
C56T	16	0.49 ± 0.01	C73T	4	0.69 ± 0.06
C56V	8	0.63 ± 0.04	C73V	4	0.75 ± 0.05
C56M	44	0.44 ± 0.05	C73M	48	0.40 ± 0.03
C56I	50	0.45 ± 0.04	C73I	45	0.36 ± 0.05
C56Y	N.G.[d]	0.33 ± 0.04	C73Y	N.G.[d]	0.33 ± 0.03

[a]Determined from the optical density at 600 nm of the culture medium YPGly with aeration at 30°C. The host WB-12 cells did not grow because of the deficiency of oxidative phosphorylation due to lack of AAC genes (*20*). [b]Expressed AAC amount in mitochondria isolated from the transformants cultured in YPGal medium at 30°C with aeration. Proteins of the isolated mitochondria were subjected to SDS-PAGE and Western blotting analysis. The immunostained intensities were detected in an ATTO Model AE-6900 image analyzer. Values are means ± SD of at least 3 runs. [c]Parental carrier. [d]No growth.

Table 2. **Transport rates of ADP (V_{ADP}) of mitochondria from Cys mutants of bAAC1 and yAAC2, and physicochemical properties of side-chains.**

Substituting amino acid residue	V_{ADP}[a] (nmol ADP/nmol AAC/min)		Physicochemical properties of residues	
	bAAC1 (Cys^{56})	yAAC2 (Cys^{73})	Side-chain volume (Å)[b]	Hydropathy index[c]
Cys(WT[d])	22.4 ± 2.5	86.7 ± 6.7	43	2.5
Gly	N.D.[e]	N.D.[e]	6	–0.4
Ala	21.9 ± 1.8	83.4 ± 7.6	25	1.8
Ser	13.5 ± 2.6	40.2 ± 5.1	31	–0.8
Asp	N.D.[e]	N.D.[e]	49	–3.5
Thr	13.8 ± 1.0	82.4 ± 8.1	52	–0.7
Val	26.8 ± 1.6	88.3 ± 5.1	61	4.2
Met	4.8 ± 2.2	4.4 ± 2.5	83	1.9
Ile	4.4 ± 1.9	3.9 ± 1.9	83	4.5
Tyr	N.D.[e]	N.D.[e]	101	–1.3

[a]Isolated yeast mitochondria (1 mg protein/ml) of transformants of the bAAC1 mutants and yAAC2 mutants were suspended in medium containing 1 μg/ml oligomycin, and then 100 μM [^{14}C]ADP (specific radioactivity, 37 kBq/μmol) was added. After incubation for various periods at 0°C, ADP uptake was terminated with 20 μM CATR and 5 μM BKA. The amount of [^{14}C]ADP incorporated was determined from its radioactivity, and the initial rate of ADP transport was determined (*24*). Values are means ± SD of 3 separate experiments. [b]van der Waals volumes calculated by using parameters in Refs. *22* and *23*. [c]Values determined by Kyte and Doolittle (*26*). [d]Parental carrier. eNot detected.

Asn^{53}–Lys^{75} of yAAC2 (*20*). As shown in Table 1, the bovine mutants C56A and C56V showed the same expression level as the parental bAAC1, while the other AAC mutants showed lower amounts. The yAAC2 mutants showed expression trends similar to those of bAAC1 mutants. Namely, C73A and C73V were expressed to the same level as the parental yAAC2, and the other mutants showed lower expression. It is noteworthy that expression levels of yAAC2 mutants were in general greater than those of corresponding bAAC1 mutants, a reflection of the more efficient expression of yAAC2 than bAAC1 in yeast mitochondria.

Transport Activities of AAC Mutants—Next, we determined the ADP transport activities of the AAC mutants. Isolated transformed mitochondria in which bovine or yeast AAC mutants were expressed were incubated with radiolabeled ADP at 0°C for 10 or 3 s, respectively, and the ADP uptake was terminated with the substrate transport inhibitors CATR (20 μM) and BKA (5 μM). Complete inhibition of the transport activity was confirmed, under identical conditions, when we examined native bAAC1 and yAAC2 and all the transformed mitochondria (data not shown). The amounts of ADP incorporated into mitochondria in 10 and 3 s were taken as the initial velocity of ADP transport for transformed bovine and yeast mitochondria, respectively (*20*). As shown in Table 2, the transport activities of C56A and C56V mutants of bAAC1 were the same as that activity of parental bAAC1. Transport activities of C56S and C56T and those of C56M and C56I were about 60% and 25%, respectively, of the activity of the parental bAAC1. The transformants C56G, C56D, and C56Y, which did not grow in the medium containing glycerol, showed no transport activity.

The yAAC2 transformants showed similar tendencies to the bovine AAC transformants. Namely, the transport activities of the C73A and C73V transformants were the same as that of the parental yAAC2, and that of C73S was about 45% of that of yAAC2. No transport was observed for

transformants C73G, C73D and C73Y, which did not grow in the medium containing glycerol. A difference in the transport activity between yeast and bovine AAC mutants was observed only with Thr mutants; i.e., the transport activity of C73T(Y) was about the same as that of yAAC2, but that of the corresponding bovine mutant C56T was less than that of bAAC1, being similar to the activity of C56S(B). The transport activity of C73S(Y) was in consistent with the result reported by Klingenberg *et al.* (*15*). It is noteworthy that the transformants that did not grow in the medium containing glycerol did not transport ADP.

The effect of the mutations could be analyzed in terms of bulkiness and hydrophobic nature of the substituting amino acid residue. As shown in Table 2, the mutation of Cys^{56}(B) and Cys^{73}(Y) to bulkier residues, such as Met, Ile or Tyr, abolished the transport activity. The mutants with side chain residues having greater van der Waals volumes than 80 $Å^3$, determined by molecular mechanics calculation (*22*, *23*), showed a significantly reduced transport activity, suggesting that the inhibition of the transport activity by mutation to bulkier residues was due to steric hindrance.

In addition, the results of Table 2 show that mutation of Cys^{56}(B) and Cys^{73}(Y) to the residues with side chain volumes of less than 80 $Å^3$ did not always result in efficient transport activity. Namely, Gly, Ser, Asp, and Thr mutants showed lower or no transport activities, although side chain volumes of these residues are less than 80 $Å^3$. As the hydropathy indices of these residues were all negative, and as those of Ala and Val mutants of the bovine and yeast AACs, which showed efficient ADP transport comparable to their own parental AACs, were positive, a hydrophilic nature would appear to be unfavorable for efficient transport function. It is noteworthy that the relative transport activity of the Thr mutant of yAAC2 was greater than that of the corresponding bAAC1 mutant. The reason is not clear at present, but it could be due to a different location of the OH group of Thr mutants in yAAC2 and bAAC1, as reflected by the fact that the homology of the amino acid sequence of LM1 between bovine and yeast AACs is about 46% (*12*).

Mutation of the Asp Residue Adjoining the Cys Residue in LM1—We compared the amino acid sequences predicted from the cloned cDNAs of the middle region of LM1 of 36 AAC isoforms of various animals, plants, and eukaryotic microorganisms (*12*). We found that the homology of this region was high in mammalian AACs (about 90%), lower between mammalian and yeast AACs (46%), and much lower between mammalian and plant AACs. However, there was a consensus sequence, Y-X-G-I-X-D-C-X-X-R (X: an arbitrary amino acid residue), in most AACs, as shown in Fig. 2. As the Cys residue is always linked to the Asp residue, except in plant AACs, in which it is linked to a Glu residue, we examined a role of the Asp residue adjoining to the Cys residue, namely, Asp^{55}(B) and Asp^{72}(Y).

We prepared transformants of bAAC1 in which Asp^{55} was mutated to Glu (D55E), Ala (D55A) or Asn (D55N). The doubling time of the D55E transformant in YPGly medium containing glycerol as its carbon source was the same as that of the parental bAAC1. In contrast, transformants D55A and D55N did not grow (data not shown). As

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          *  **  **   *
bAAC1  -46AEKQYKGIIDCVVRIPKE63-
yAAC2  -63LDRKYAGILDCFKRTATQ80-
yAAC1  -53LDTRYKGILDCFKRTATH70-
yAAC3  -52LDKKYSGIVDCFKRTAKQ69-
ncAAC  -53LDRRYNGIIDCFKRTTAD70-
hAAC1  -46AEKQYKGIIDCVVRIPKE63-
hAAC2  -46ADKQYKGIVDCIVRIPKE63-
hAAC3  -46AEKQYKGIIDCVVRIPKE63-
bAAC2  -46ADKQYKGIVDCIVRIPKE63-
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Fig. 2. **Sequences of hydrophobic regions around Cys^{56}(B) and Cys^{73}(Y).** Amino acid sequences of various AACs around Cys residues corresponding to Cys^{56}(B) including the consensus sequence Y-X-G-I-X-D-C-X-X-R (X: an arbitrary amino acid residue) are shown according to Ref. *12*. In the figure, b, bovine; y, yeast (*Saccharomyces cerevisiae*); h, human; nc, *Neurospora crassa*. The sequence of bAAC1 is shown by gray circles in Fig. 1. Asterisks show the conserved residues in the Y-X-G-I-X-D-C-X-X-R (X: an arbitrary amino acid residue) sequence.

summarized in Table 3, the expression level of the D55E mutant was the same as that of bAAC1, but D55A and D55N showed levels of about 42% and 47%, respectively, of that of bAAC1. In addition, the D55E mutant showed the same ADP transport activity as bAAC1, but D55A and D55N did not transport ADP. The transformants in which Asp^{55}(B) of the carrier was replaced by Met, Tyr, Lys, Ser or Val did not grow in the medium containing glycerol (data not shown), thus suggesting that these mutants did not transport ADP. These results clearly show that the carboxyl groups of Asp and Glu residues are essential for transport function of bAAC1. Similar results were obtained with corresponding yAAC2 mutants.

To know the position specificity of the Asp residue, we next replaced the Cys residue by an Asp residue in bovine and yeast mutants in which the original Asp residue had been replaced with Ala. As Cys residues at position 56 of bAAC1 and position 73 of yAAC2 are functionally compatible with Ala residues, we thought that Ala mutants would be more useful to analyze the position specificity of Asp residues in both AACs by eliminating the possible involvement of Cys residues in the hydrogen bonding and salt bridge formation. Hence, we prepared the bovine mutant D55A/C56D, in which Asp^{55} and Cys^{56} were mutated to Ala and Asp residues, respectively, and yeast double mutant D72A/C73D. These mutants were expressed quite well in the transformed mitochondria, as shown in Table 3. However, neither double mutant could transport ADP via the carrier (Table 3). These results suggest that the location of the carboxylic acid groups of Asp^{55} of bAAC1 and Asp^{72} of yAAC2 in their original position is essential for the transport function of AAC.

DISCUSSION

The LM1 of bAAC1 containing Cys^{56} exhibits a significant conformational change between two distinct conformational states known as m-state and c-state (*3*, *7*, *10*). We found that Cys^{56} could be an effective residue responding to conformations of m- and c-state. Namely, the residue is

Table 3. **Expression levels in mitochondria and ADP transport activities of Asp mutants.**

bAAC1			yAAC2		
Mutant	AAC expression (nmol/mg of protein)[a]	V_{ADP}[b] (nmol ADP/nmol AAC/min)	Mutant	AAC expression (nmol/mg of protein)[a]	V_{ADP}[b] (nmol ADP/nmol AAC/min)
bAAC1[c]	0.60 ± 0.12	22.4 ± 2.5	yAAC2[c]	0.72 ± 0.05	86.7 ± 6.7
D55E	0.60 ± 0.58	24.8 ± 3.2	D72E	0.77 ± 0.08	83.4 ± 8.5
D55A	0.28 ± 0.03	N.D.[d]	D72A	0.33 ± 0.06	N.D.[d]
D55N	0.28 ± 0.03	N.D.[d]	D72N	0.35 ± 0.05	N.D.[d]
D55A/C56D	0.22 ± 0.03	N.D.[d]	D72A/		
C73D	0.33 ± 0.02	N.D.[d]			

[a]Amount of AAC expressed in mitochondria isolated from the transformant cells. Values are means ± SD determined for 3 different preparations. [b]Initial rate of ADP uptake *via* the AAC mutant determined from the amount of ADP incorporated into mitochondria on incubation for 10 s for bAAC1 mutants and 3 s for yAAC2 mutants at 0°C. These values were taken as initial transport rates (*20*). Values are means ± SD determined for 3 different preparations. [c]Parental carrier. [d]Not detected.

susceptible to various SH-reagents (*7*, *9*–*11*) and it is easily oxidized to form a disulfide bridge with the counterpart Cys^{56} residue in a functional dimeric carrier in the m-state, whereas no labeling or disulfide bridge formation is observed in the c-state (*7*). Hence, AAC conformation is likely to be loose in the m-state, but be compact in the c-state. In the m-state, Cys^{56} is located in the aqueous environment, and it is suggested to be transferred into the hydrophobic region on conversion to the c-state (*6*, *9*, *11*). This translocation may be caused by rotation or disentanglement of the alpha-helix of the LM1, as observed with the membrane-spanning region (*25*) and the 1st loop facing the cytosol (*8*). In addition, the finding that the labeling of Cys^{56}(B) with SH-reagents always abolishes transport activity of the AAC (*10*, *11*) suggests an important role of the amino acid residue at this position, although the Cys residue itself is not essentially important.

According to the crystal structure of the c-state bAAC1 (*4*), LM1 forms an amphipathic cluster on the membrane surface between TM1 and TM2 in such a way that LM1 is localized closer to TM1 (Fig. 3, A and B, depicted according to Ref. *4*). The Cys^{56} residue in the amphipathic short alpha-helical chain between Ile^{53} and Glu^{63} (see Fig. 1) is located close to the well-conserved bulky hydrophobic residues Val^{31}, Leu^{34}, and Leu^{35} in the hydrophobic space (Fig. 3, A and B). On conversion of the m-state carrier to the c-state, Cys^{56}(B) and Cys^{73}(Y) have to be translocated from the aqueous phase to the hydrophobic region of the amphipathic cluster (Figs. 3, A–C). However, steric hindrance makes it difficult for the bulky side chain at positions 56 of bAAC1 and 73 of yAAC2 to intrude into the hydrophobic space. Hence, the carrier fails to achieve the c-state conformation, and the transport activity is abolished. We found that a side-chain volume of less than 80 $Å^3$ was favorable for transport activity. Hence, the inhibition of the ADP transport previously found by labeling of Cys^{56}(B) in the m-state carrier with the SH-reagents NEM and methyl methanethiosulfonate (MMTS) (*10*, *11*) would have been due to bulkier modification of Cys^{56}(B), because the van der Waals volumes of the Cys residues modified with NEM and MMTS are 152 $Å^3$ and 91 $Å^3$, respectively. In contrast, the decrease or complete loss of transport activity observed on replacement of Cys^{56}(B) and Cys^{73}(Y) by Asp, Ser or Thr could be due to the hydrophilic nature of these residues, which would be unfavorable for translocation to the hydrophobic space. In addition, loss of transport activity of the Gly mutants could be due to flexible rotation of the α-carbon of Gly, which should be unfavorable for fixed conformation.

Asp^{55} of bAAC1 is conserved in all AAC isoforms except plant AACs, in which the Asp residue is substituted with Glu (*12*). The compatibility of Asp and Glu residues is consistent with our present finding that mutation of Asp^{55}(B) and Asp^{72}(Y) to Glu did not affect transport activity. These residues are strictly position-specific, and their carboxyl groups are essential for transport function of AACs. In the crystal structure of the c-state bAAC1 (*4*), Asp^{55} is located on the outer surface of the amphipathic cluster of LM1 (Fig. 3, A and B) and forms a charged bridge with Arg^{59}, which is conserved in all AACs (*12*), as is shown in Fig. 3C. The salt bridge between Asp^{55} and Arg^{59} is thought to be responsible for the compact conformation of LM1 in the c-state conformation. Accordingly, NEM is inaccessible to Cys^{56} in the c-state conformation. In contrast, the conformations around Cys^{56}(B) and Cys^{73}(Y) in m-state carriers are expected to be so flexible that NEM is able to label these Cys residues. It is interesting to note that mutation of Asp^{55}(B) and Asp^{72}(Y) to Glu did not affect transport activity, although the side chain of Glu is longer and hence more flexible than that of Asp residue. As this salt bridge should be essential for stabilization of the hydrophobic space of the LM1 in the c-state carrier, it is possible that, in the Glu mutants in the c-state, the Glu also forms a salt bridge with the flexible side chain of Arg. At present, it is not clear why the Glu residue is more favorable than the Asp in plant AACs. Possibly, the alpha-helical hydrophobic space of the LM1 in plant AACs is wider than those in mammalian and yeast AACs. The distance between the Glu or Asp and the Arg may become longer by the bending of the alpha-helix in the wider hydrophobic space. Hence, Glu is more advantageous than Asp in forming the salt bridge with Arg in plant AACs. This possibility can be examined by mutation of the Glu residue to Asp residue in plant AACs.

Though the relative transport activities of Cys and Asp mutants of bovine and yeast AACs were in general very similar, the C73T yeast mutant showed high transport activity, being comparable to that of yAAC2, in contrast to the lower transport activity of the C56T mutant of bAAC1. This difference could be due to the fact that the hydrophobic space in LM1 of yAAC2 is somewhat wider than that of bAAC1, leading to easier intrusion of the

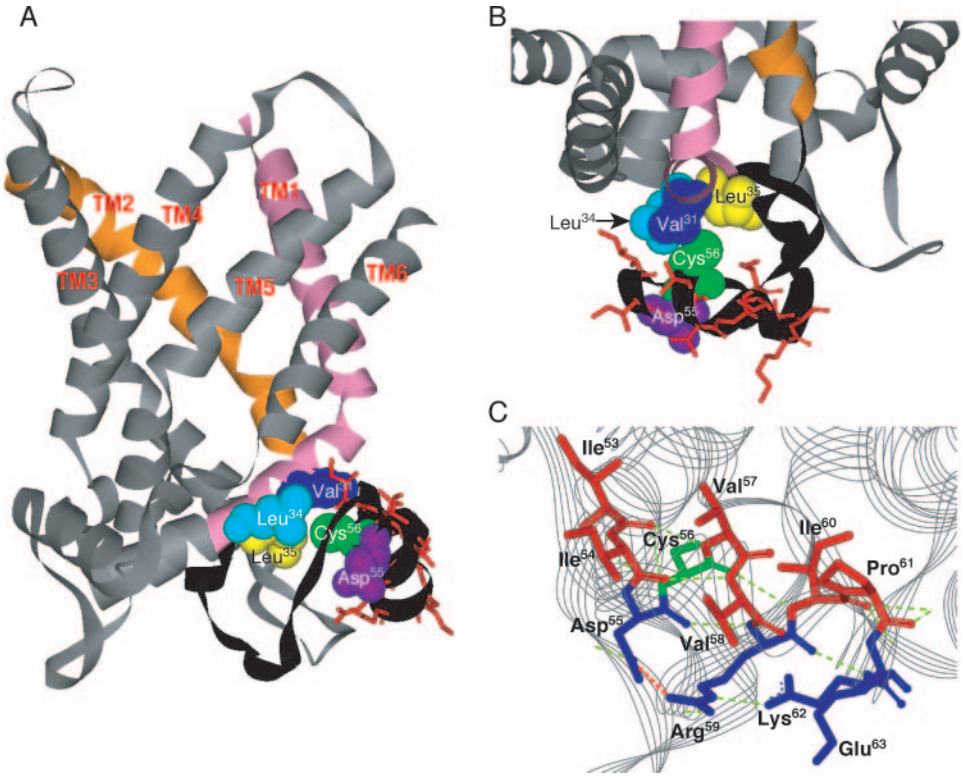

Fig. 3. **Crystal structure of bAAC1 fixed in the c-state conformation by CATR.** A: Whole AAC structure, in which Cys^{56} and Asp^{55} of LM1 (loop in black) are shown in green and purple, respectively. The residues Val^{31}, Leu^{34}, and Leu^{35}, located close to Cys^{56}, are shown in blue, sky blue, and yellow, respectively. The short alpha-helix of LM1 is shown by red wires. Transmembrane alpha-helices are indicated as TMs. Of these, TM1 and TM2 are shown in pink and orange, respectively. B: Structure of LM1 seen from a different angle from that in A. Amino acid residues and secondary structure are as in A. C: Short alpha-helical region between Ile^{53} and Glu^{63}. The hydrophobic and hydrophilic residues are shown in red and blue, respectively. Cys^{56} is shown in green; and hydrogen bonding between Asp^{55} and Arg^{59}, by a red broken line. The figures were drawn according to the crystal structure (*4*) downloaded from a protein data bank (PDB, http://www.rcsb.org/pdb/) by the use of ViewerLite.

mutated Thr residue in the hydrophobic space. The greater hydrophobicity of the Thr residue compared with the Ser residue should be favorable for this translocation; and, in fact, the transport activity of C73T was greater than that of C73S of yeast mutants. However, the corresponding mutants of bAAC1 showed similar activities. In addition, the consistently lower transport activity of the C56S and C56T mutants of bAAC1 could have been due to the fact that the hydrophilic OH groups of the Ser and Thr residues could be more disadvantageous for their translocation into the hydrophobic space than those of corresponding residues of yAAC2.

In conclusion, LM1 takes a loose conformation in the m-state and it forms a compact amphipathic cluster on the membrane surface close to TM1 in the c-state conformation. For organization of the stable amphipathic cluster of LM1, Cys^{56}(B) and Cys^{73}(Y) have to be located close to the hydrophobic residues inside the cluster in the c-state conformation. This location should be helpful for stabilization of the amphipathic cluster. Hence, any hydrophobic residue with a molecular volume less than 80 $Å^3$ could replace the Cys residue. The loss of transport activity of the m-state carrier by labeling with SH-reagents such as NEM (*10*, *11*) could be due to inhibition of the conversion of the flexible m-state carrier to the compact c-state by steric hindrance on modification of Cys residues with the bulky SH-reagents.

In contrast, the carboxyl group of Asp^{55}(B) is located on the surface of the amphipathic cluster of LM1 and forms a salt bridge with Arg^{59}. This salt bridge should be important in formation of a compact conformation of the amphipathic cluster. Hence, Asp^{55}(B) was replaceable with a Glu residue, but its location was strictly position-specific. As in bAAC1, Asp^{72}(Y) is expected to form a hydrogen bond with Arg^{76}(Y).

The alanine residue next to the initial methionine residue of the translation product was taken as the 1st residue of bAAC1, because the initial methionine is removed post-translationally from the amino acid sequence (*27*). As no post translational event has been reported for yAAC2, its initial methionine was taken as the 1st residue.

REFERENCES

1. Pedersen, P.L. (1993) An introduction to the mitochondrial anion carrier family. *J. Bioenerg. Biomembr.* **25**, 431–434
2. Klingenberg, M. (1989) Molecular aspects of the adenine nucleotide carrier from mitochondria. *Arch. Biochem. Biophys.* **270**, 1–14

3. Terada, H., and Majima, E. (1997) Important role of loops in the transport activity of the mitochondrial ADP/ATP carrier. *Progr. Colloid Polym. Sci.* **106**, 192–197
4. Pebay-Peyroula, E., Dahout-Gonzalez, C., Kahn, R., Trezeguet, V., Lauquin, G. J., and Brandlin, G. (2003) Structure of mitochondrial ADP/ATP carrier in complex with carboxyatractyloside. *Nature* **426**, 39–44
5. Klingenberg, M. (1980) The ADP-ATP translocation in mitochondria, a membrane potential controlled transport. *J. Membrane Biol.* **56**, 97–105
6. Majima, E., Yamaguchi, N., Chuman, H., Shinohara, Y., Ishida, M., Goto, S., and Terada, H. (1998) Binding of the fluorescein derivative eosin Y to the mitochondrial ADP/ATP carrier: characterization of the adenine nucleotide binding site. *Biochemistry* **37**, 424–432
7. Majima, E., Ikawa, K., Takeda, M., Hashimoto, M., Shinohara, Y., and Terada, H. (1995) Translocation of loops regulates transport activity of mitochondrial ADP/ATP carrier deduced from formation of a specific intermolecular disulfide bridge catalyzed by copper-*o*-phenanthroline. *J. Biol. Chem.* **270**, 29548–29554
8. Kihira, Y., Majima, E., Shinohara, Y. and Terada, H. (2005) Cysteine labeling studies detect conformational change in region 106–132 of the mitochondrial ADP/ATP carrier of *Saccharomyces cerevisiae*. *Biochemistry* **44**, 184–192
9. Hashimoto, M., Majima, E., Goto, S., Shinohara, Y., and Terada, H. (1999) 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. *Biochemistry* **38**, 1050–1056
10. Majima, E., Koike, H., Hong, Y.-M., Shinohara, Y., and Terada, H. (1993) Characterization of cysteine residues of mitochondrial ADP/ATP carrier with the SH-reagents eosin-5-maleimide and *N*-ethylmaleimide. *J. Biol. Chem.* **268**, 22181–22187
11. Hashimoto, M., Majima, E., Hatanaka, T., Shinohara, Y., Onishi, M., Goto, S., and Terada, H. (2000) Irreversible extrusion of matrix facing first loop of the bovine heart mitochondrial ADP/ATP carrier by labeling of Cys^{56} residue with the SH-reagent methyl methanthiosulfonate. *J. Biochem.* **127**, 443–449
12. Firoe, C., Trezeguet, V., Le Saux, A., Roux, P., Dianoux, A.C., Noel, F., Lauquin, G.J.M., Brandolin, G., and Vignais, P.V. (1998) The mitochondrial ADP/ATP carrier: Structural, physiological and pathological aspects. *Biochimie* **80**, 137–150
13. Boulay, F. and Vignais, P.V. (1984) Localization of the *N*-ethylmaleimide reactive cysteine in the beef heart mitochondrial ADP/ATP carrier protein. *Biochemistry* **23**, 4807–4812
14. Aquila, H., Eiermann, W., and Klingenberg, M, (1982) Incorporation of N-ethylmaleimide into the membrane-bound ADP/ATP translocator. Isolation of the protein labeled with *N*-[3H]ethylmaleimide. *Eur. J. Biochem.* **122**, 133–139
15. Klingenberg, M., Gawaz, M., Douglas, M.G., and Lawson, J.E. (1992) Mutagenized ADP/ATP carrier from *Saccharomyces* in *Molecular Mechanism of Transport* (Quagliarello, E. and Palmieri, F., eds.) pp. 187–195, Elsevier Science Publishers B.V., Amsterdam
16. Nelson D.R. (1996) The yeast ADP/ATP carrier. Mutagenesis and second-site revertants. *Biochim. Biophys. Acta* **1275**, 133–137
17. Nelson, D.R., Lawson E.L., Klingenberg, M and Douglus M.G. (1993) Site-directed mutagenesis of the yeast mitochondrial ADP/ATP translocator. *J. Mol. Biol.* **230**, 1159–1170
18. Klingenberg, M. and Nelson, D. R. (1994) Structure-function relationships of the ADP/ATP carrier. *Biochim, Biophys. Acta* **1187**, 241–244
19. Hatanaka, T., Kihira, Y., Shinohara, Y., Majima, E., and Terada, H. (2001) Characterization of loops of the yeast mitochondrial ADP/ATP carrier facing the cytosol by site-directed mutagenesis. *Biochem. Biophys. Res. Commun.* **286**, 936–942
20. Hashimoto, M., Shinohara, Y., Majima, E., Hatanaka, T., Yamazaki, N., and Terada, H. (1999) Expression of bovine heart mitochondrial ADP/ATP carrier in yeast mitochondria significantly enhanced expression by replacement of the N-terminal region of the bovine carrier by the corresponding region of the yeast carriers. *Biochim. Biophys. Acta* **1409**, 113–124
21. Schiestl, R.H. and Gietz, R.D. (1989) High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr. Genet.* **16**, 339–446
22. Moriguchi, I., Kanda, Y., and Komatsu, K. (1976) Use of van der Waals volume in structure-activity studies. *Chem. Pharm. Bull.* **24**, 1799–1806
23. Richareds, F.M. (1974) The interpretation of protein structures: Total volume, group volume distributions and packing density. *J. Mol. Biol.* **82**, 1–14
24. Majima, E., Takeda, M., Miki, S., Shinohara, Y., and Terada, H. (2002) Close location of the first loop to the third loop of the mitochondrial ADP/ATP carrier deduced from cross-linking catalyzed by copper-o-phenanthroline of the solubilized carrier with Triton X-100. *J. Biochem.* **131**, 461–468
25. Kihira, Y., Iwahashi, A., Majima, E., Terada, H., and Shinohara, Y. (2004) Twisting of the second transmembrane alpha-helix of the mitochondrial ADP/ATP carrier during the transition between two carrier conformational states. *Biochemistry* **43**, 15204–15209.
26. Kyte, J. and Doolittle, R.F. (1982) A sample method for displaying the hydrophathic character of a protein,. *J. Mol. Biol.* **157**, 105–132
27. Aquila, H., Misra, D., Eluitz, M., and Klingenberg, M. (1982) Complete amino acid sequence of the ADP/ATP carrier from beef heart mitochondria. *Hoppe-Seyler's Z. Physiol. Chem.* **363**, 345–349