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Localization of the *N*-Ethylmaleimide Reactive Cysteine in the Beef Heart Mitochondrial ADP/ATP Carrier Protein†

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ABSTRACT: Alkylation of the ADP/ATP carrier protein in beef heart mitochondria by *N*-ethylmaleimide (NEM) results in inactivation of transport. One out of the four cysteinyl residues contained in 1 mol of carrier subunit of M_r 32000 is alkylated by NEM. The identification of the alkylated residue to Cys-56 has been achieved by chemical and enzymatic cleavages. The chemical cleavages included cleavages at the nonalkylated cysteinyl residues by cyanide at alkaline pH and at methionyl residues by cyanogen bromide. Enzymatic cleavage involved the use of trypsin and chymotrypsin; the resulting peptides were resolved by high-performance liquid chromatography. Analysis of a small size [^{14}C]NEM-labeled peptide obtained by tryptic and chymotryptic digestion of the [^{14}C]NEM-labeled carrier protein yielded the following amino acid sequence:

$$\begin{array}{c}[^{14}C]NEM\\ |\\ X_{51}\text{-}Gly_{52}\text{-}Ile_{53}\text{-}Ile_{54}\text{-}Asx_{55}\text{-}Cys_{56}\text{-}Val_{57}\text{-}Val_{58}\text{-}Arg_{59}\text{-}\end{array}$$

where X is probably a substituted lysine.

When ADP or ATP is added to isolated mitochondria at micromolar concentrations, the sensitivity of ADP/ATP transport to the inhibitory effect of *N*-ethylmaleimide (NEM)[1] increases markedly (Leblanc & Clauser, 1972; Vignais & Vignais, 1972). Concomitantly with inhibition of ADP/ATP transport, covalent binding of [^{14}C]NEM could be demonstrated (Vignais et al., 1975). It was postulated that the functioning of ADP/ATP transport could induce the unmasking of the SH group of a strategic cysteinyl residue in the carrier protein. The SH unmasking was prevented by uncoupling and conversely enhanced in respiring mitochondria developing a protonmotive force (Vignais & Vignais, 1972). The component of the protonmotive force that governs the SH unmasking was identified as the pH gradient (Michejda & Vignais, 1981). The reactivity of the membrane-bound ADP/ATP carrier to NEM is abolished by atractyloside (ATR) or carboxyatractyloside (CATR) and in contrast enhanced by bongkrekic acid (BA). The interest of these data lies in the fact that the ADP/ATP carrier protein can assume two conformations that are specifically recognized and trapped by ATR or CATR (CATR conformation) or by BA (BA conformation) and that the transition between the two conformations is facilitated by the substrates of the carrier, i.e., ADP or ATP (Block et al., 1983). One out of the four cysteinyl residues per carrier subunit (M_r 32000) reacts with NEM (Aquila et al., 1982a). The present report is a study of the localization of the reactive cysteine in the amino acid sequence of the carrier protein isolated from beef mitochondria.

Experimental Procedures

Chemicals and Enzymes. Atractyloside and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co. Guanidinium chloride, trifluoroacetic acid, succinic anhydride, and phenyl isothiocyanate were from Pierce; DABITC was from Fluka; acetonitrile, hydrochloric acid, and Coomassie blue R250 were from Merck; acrylamide and bis(acrylamide) were from Eastman Kodak. Na^{14}CN-(30–50 Ci/mol) and [^{14}C]-*N*-ethylmaleimide (20–40 Ci/mol) were from the Commissariate à l'Energie Atomique, Saclay, France. TPCK–trypsin was from Worthington and α-chymotrypsin from Miles Laboratories. All reagents used were of the purest grade commercially available.

Polyacrylamide Gel Electrophoresis. Electrophoretic separation of peptides was performed overnight in 20% acrylamide slab gels following the method described by Cabral & Schatz (1979) for resolution of peptides with molecular weights ranging from 20000 to 3000. The lyophilized peptide samples (20–30 μg) were dissolved first in 40 μL of 8 M urea, followed by 40 μL of a buffer consisting of 50% glycerol, 2% $NaDodSO_4$, 0.1 M sodium phosphate, pH 7.5, 7% β-mercaptoethanol, and traces of bromophenol blue.

^{14}C-Labeled peptides were detected by fluorography as described by Laskey & Mills (1975) after impregnation of the gel with an autoradiography enhancer (EN^{3}Hance) from New England Nuclear. The gels were dried and exposed for several weeks to a preflashed Fuji RX film at −70 °C with an intensifying Cronex screen.

N-Ethylmaleimide Covalent Labeling of the ADP/ATP Carrier. Fifty milligrams of beef heart mitochondria was suspended in 10 mL of an aerated sucrose buffer made of 0.25 M sucrose and 10 mM Hepes, pH 6.8, and supplemented with 100 μM ADP plus 20 μM BA. Additions of ADP and BA

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[1] Abbreviations: TPCK–trypsin, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin; $NaDodSO_4$, sodium dodecyl sulfate; ATR, atractyloside; CATR, carboxyatractyloside; BA, bongkrekic acid; DTNB, Ellman's reagent or 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NEM, *N*-ethylmaleimide; PITC, phenyl isothiocyanate; DABITC, 4-(dimethylamino)azobenzene 4′-isothiocyanate; DABTH, 4-(dimethylamino)azobenzene 4′-thiohydantoin.

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were dictated by their enhancing effect on the NEM reactivity of the membrane-bound carrier. To the suspension was added unlabeled NEM or [^{14}C]NEM at the final concentration of 500 μM, and this was allowed to react for 15 min at 25 °C. The reaction was stopped by a 5-fold dilution with an ice-cold buffer supplemented with 30 mM β-mercaptoethanol and 5 mM dithiothreitol. After 10 min, the mitochondria were sedimented for 10 min at 20000g and washed twice with 10 mL of the initial buffer. The NEM-labeled carrier protein was then extracted with 4% Triton X-100, 0.5 M NaCl, 1 mM EDTA, 30 mM β-mercaptoethanol, and 10 mM Hepes, pH 7.2 at 4 °C. The carrier protein was purified by hydroxylapatite gel chromatography, as described by Riccio et al. (1975) with some modification including delipidation with diethyl ether (Boulay et al., 1983).

The stoichiometry of [^{14}C]NEM incorporation in the purified BA–carrier complex was established on the basis of the specific radioactivity of [^{14}C]NEM, taking a molar extinction coefficient of 600 at 300 nm for NEM absorbance, and on the basis of the amount of protein as determined by the method of Lowry in the presence of 1% sodium dodecyl sulfate (Helenius & Simons, 1972). Under the above conditions, about 0.7 mol of [^{14}C]NEM was incorporated per mol of carrier protein. On the basis of the fact that each carrier subunit (M_r 32 000) contains one cysteinyl residue alkylated by NEM (Aquila et al., 1982a), it follows that 30% of the carrier molecules have all their SH residues in a free form. No attempt was made to increase the extent of alkylation by NEM in order to avoid unspecific labeling.

Chemical Fragmentation of the NEM-Alkylated ADP/ATP Carrier Protein. Cleavage at methionyl residues was performed as described by Gross (1967). It was used to prepare the large cyanogen bromide fragment called CB_1 which in the sequence of 297 amino acid residues of the beef heart ADP/ATP carrier protein extends from the N terminus to Met-200 (Figure 1). The CB_1 fragment of the [^{14}C]-NEM-alkylated carrier protein was obtained as follows. The [^{14}C]NEM-alkylated carrier protein (250 nmol) was dissolved in 1 mL of 7 M guanidinium chloride, 1 mM EDTA and 0.1 M sodium phosphate, pH 7.2, under a nitrogen stream and left in contact for 1 h at 37 °C with 5 mM dithiothreitol to allow full reduction. Unlabeled NEM was added in 3-fold excess with respect to the total amount of sulfhydryl groups to alkylate all free cysteinyl residues. The mixture was left to stand at 37 °C for 15 min, and the remaining free NEM was eliminated by reaction with 20 mM dithiothreitol. The alkylated protein was succinylated at room temperature by using small increments of succinic anhydride, the final concentration being 1000-fold excess over the free NH_2 groups of the protein; during the course of succinylation, the pH was carefully verified and maintained at 8.5 by controlled addition of 10 N NaOH. After the last addition of succinic anhydride, the solution was allowed to stand with stirring for 1 h, and the protein was desalted by passage of the solution through a column of Ultrogel ACA 202 (2.5 × 10 cm) equilibrated in 50 mM ammonium bicarbonate, pH 7.8. After freeze-drying, the desalted protein was taken up in 1 mL of 70% formic acid in the presence of a 500-fold excess of cyanogen bromide with respect to methionine. The cleavage by cyanogen bromide was performed in the dark at 37 °C for 24 h. Tryptamine at the final concentration of 1 mM was present to protect tryptophan against oxidation. Following cleavage, the mixture was freeze-dried and the residue taken up in 1 mL of 7 M guanidium chloride pH 7.8. The [^{14}C]NEM-alkylated CB_1 fragment was isolated by chromatography on Sephadex G-50 fine equilibrated in 50 mM ammonium bicarbonate, pH 7.8.

Cleavage at cysteinyl residues was carried out by cyanylation (Vanaman & Stark, 1970). The NEM-alkylated carrier protein was succinylated as described above and cleaved at the nonalkylated cysteinyl residues by reaction with cyanide in a two-step procedure as previously reported (Boulay et al., 1983). DTNB and NaCN or Na^{14}CN were allowed to react with the carrier protein, followed by alkaline treatment.

Tryptic–Chymotryptic Digestion of the NEM-Alkylated CB_1 Fragment. Equal amounts of trypsin and chymotrypsin in 50 mM NH_4HCO_3 and 50 μM $CaCl_2$, pH 8.5, were added to 150 nmol of the alkylated CB_1 fragment in a ratio of 1:50 (w/w). Incubation was carried out at 37 °C for 16 h; it was continued with an enzyme to protein ratio of 1:25 (w/w) for 6 h at 37 °C. The digest was freeze-dried. The residue was taken up in 500 μL of 50% trifluoroacetic acid, and 50-μL samples were directly used for fractionation. The peptides were separated and purified to homogeneity by high-performance liquid chromatography (HPLC). The Waters liquid chromatograph used for HPLC was equipped with the gradient delivery system M720, a Waters C_{18} μBondapak column (0.39 × 30 cm), and a Vydac 10TP RP column (0.39 × 25 cm) from Chrompak.

Amino Acid Composition and Sequencing. These operations were conducted on the [^{14}C]NEM-labeled peptides recovered by HPLC fractionation from the tryptic–chymotryptic digest. For amino acid analysis, an aliquot sample of the purified [^{14}C]NEM peptides was hydrolyzed in evacuated sealed tubes by treatment at 165 °C for 50 min with 200 μL of a mixture of concentrated HCl and trifluoracetic acid (2:1 v/v) containing 0.005% phenol (Tsugita & Scheffler, 1982). The amino acid analysis was performed with a Waters high-performance liquid chromatograph. Postderivatization of amino acids was achieved with *o*-phthalaldehyde; the derivatized amino acids were assayed with the 420 fluorescence detector from Waters. Proline was detected in a separate run by a postcolumn hypochlorite treatment at 62 °C for 2 min, prior to derivatization by *o*-phthalaldehyde.

Peptides obtained after digestion by trypsin and chymotrypsin were sequenced by the manual DABITC/PITC liquid-phase method with identification of the DABTH amino acids by chromatography on micropolyamide sheets (5 × 5 cm; F1700 from Schleicher & Schuell), as described by Chang et al. (1978). In our case removal of excess DABITC and PITC was performed by five successive extractions with a mixture of heptane and ethyl acetate (3:1 v/v). With this ratio, there was virtually no loss of peptide, which is not the case when extraction was performed with an heptane to ethyl acetate ratio of 2 to 1 (v/v) (Wittmann et al., 1980).

Results

Localization of the NEM Reactive Cys of the Beef Heart ADP/ATP Carrier after Chemical Cleavage. As shown in a previous work (Boulay et al., 1983) chemical cleavage of the beef heart ADP/ATP carrier protein at cysteinyl and methionyl residues yielded a series of peptide fragments. For the sake of clarity, the cleavage points and the generated peptides are illustrated in Figure 1. It is noteworthy that the four cysteinyl residues contained in the carrier protein are spread all over the sequence, whereas the seven methionyl residues are all located close to the C terminus (Boulay et al., 1979; Aquila et al., 1982b).

The [^{14}C]NEM-alkylated protein carrier obtained from mitochondria treated by [^{14}C]NEM in the presence of both ADP and bongkrekic acid was cleaved by cyanogen bromide,

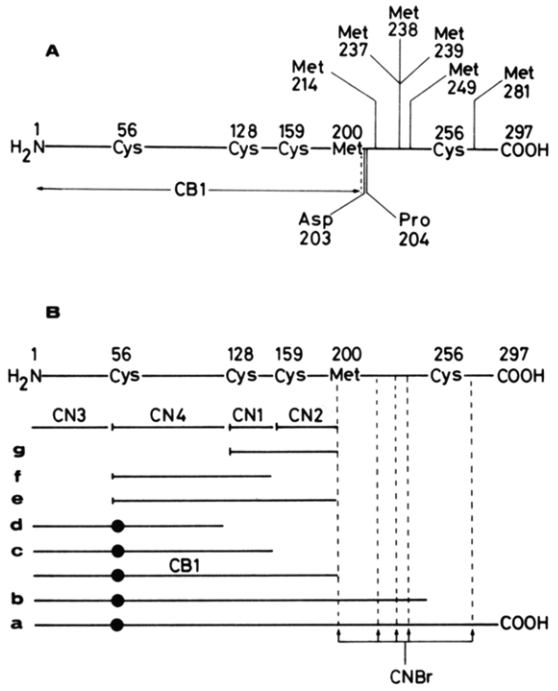

FIGURE 1: (A) Localization of cysteinyl and methionyl residues in the ADP/ATP carrier protein. The site of acidic cleavage Asp_{203}–Pro_{204} is also indicated (Aquila et al., 1982b). (B) Fragmentation of the beef heart carrier protein at cysteinyl residues by cyanide at alkaline pH and at methionyl residues by cyanogen bromide. As will be demonstrated in this paper, Cys-56 is alkylated by NEM (indicated by black circles). Cyanylation by $Na^{14}CN$ results in ^{14}C labeling of the fragmented peptides on the amino group side of the broken peptide bond. The N terminus of the carrier protein cannot be labeled by $Na^{14}CN$. Consequently all peptides arising from fragmentation of the carrier protein and terminated by the N terminus of the carrier protein cannot be labeled by $Na^{14}CN$. a, b, c, d, and CB_1 correspond to [^{14}C]NEM-labeled peptides, CN_1, CN_2, and CN_4 to nonoverlapping $Na^{14}CN$-labeled peptides after CNBr cleavage, and e, f, and g to overlapping $Na^{14}CN$-labeled peptides.

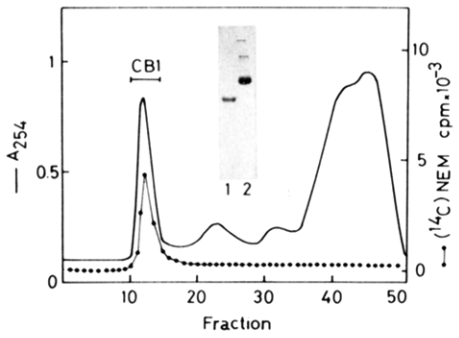

FIGURE 2: Isolation of the [^{14}C]NEM-labeled CB_1 fragment from the [^{14}C]NEM-labeled ADP/ATP carrier. The succinylated ADP/ATP carrier purified from mitochondria treated by (^{14}C)NEM in the presence of ADP and bongkrekic acid was cleaved by treatment with cyanogen bromide (cf. Experimental Procedures). The large CB_1 fragment resulting from cleavage (cf. Figure 1) was separated by chromatography on a column of Sephadex G-50 fine (90 × 1 cm) in 50 mM ammonium bicarbonate. Fractions of 2.5 mL were collected at a flow rate of 10 mL/h. The elution was followed at 254 nm with an LKB Uvicord 4701A. The radioactivity was counted by scintillation on 50-μL aliquots. (Inset) The homogeneity of the CB_1 peptide was checked by electrophoresis on a $NaDodSO_4$–polyacrylamide gel electrophoresis (10–20%) followed by staining with Coomassie Blue R250. (Track 1) CB_1; (track 2) ADP/ATP carrier.

and the resulting peptides were submitted to gel filtration. The pattern or radioactivity in the eluates is illustrated in Figure 2. The radioactivity was localized in the first peak which corresponds to the CB_1 peptide that extends from the N terminus to Met-200. No radioactivity was found in the smaller

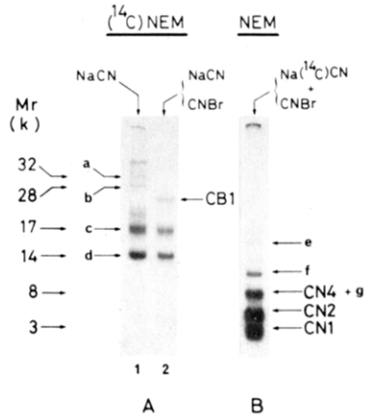

FIGURE 3: Identification of NEM-alkylated peptides arising by chemical cleavage of the ADP/ATP carrier protein. (A) Cleavage of the [^{14}C]NEM-labeled ADP/ATP carrier protein by cyanide or by cyanide followed by cyanogen bromide. The [^{14}C]NEM-alkylated carrier protein was isolated from beef heart mitochondria pretreated with ADP and BA and then incubated with [^{14}C]NEM (cf. Experimental Procedures). Peptides arising from cleavage were separated by $NaDodSO_4$–polyacrylamide gel electrophoresis. The radiolabeled peptides were revealed by fluorography on Fuji RX film at −70 °C after impregnation of the gel with EN^{3}Hance and drying. (Track 1) Fragmentation at cysteinyl residues by cyanylation; (track 2) subfragmentation of the cysteinyl peptides with cyanogen bromide. (B) Cleavage of the NEM-labeled ADP/ATP carrier protein by $Na^{14}CN$ followed by cyanogen bromide. The resulting peptides were separated by gel electrophoresis, and the radioactive peptides were revealed by fluorography as in (A).

peptides located between Met-200 and the C terminus. Thus, Cys-256 that is located in the short cyanogen bromide fragment Tyr-250–Met-281 is not alkylated by the [^{14}C]NEM. From this first assay, it is clear that the three remaining candidates for [^{14}C]NEM labeling are Cys-56, Cys-128, and Cys-159.

Another way to localize the NEM-alkylated cysteinyl residue in the ADP/ATP carrier was by cleavage at the free (nonalkylated) cysteinyl residues. For this purpose, two parallel experiments were performed. In the first one, the carrier protein was alkylated by [^{14}C]NEM, and the ^{14}C-labeled carrier was cleaved with unlabeled NaCN at the nonalkylated cysteinyl residues; conversely, in the second experiment, the carrier protein was alkylated with unlabeled NEM and then cleaved with $Na^{14}CN$ at the nonalkylated cysteinyl residues.

In both cases, the ^{14}C-labeled peptides were detected by fluorography after $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 3). The results of the first experiment are illustrated by Figure 3A; the radioactive bands in track 1 indicate that the [^{14}C]NEM was mostly located in peptides c (M_r 17 000) and d (M_r 14 000); some ^{14}C radioactivity was also found in peptides a (M_r 32 000) and b (M_r 28 000). The faint radioactive band found above peptide a corresponded probably to a nondepolymerized material that accumulated at the interface of the 10–20% polyacrylamide gel. The radioactive band between peptides b and c was a nonspecific cleavage product resulting probably from the *O*-cyanylation of a serine or a threonine residue (Liao & Wadano, 1979).

The pool of cysteinyl peptides was further subjected to cleavage at methionyl residues by cyanogen bromide, yielding three radioactive bands (track 2, Figure 3A). Two of them correspond to the same peptides c and d found in track 1 of Figure 3A; the other one (M_r 23 000), which most likely arose from cleavage of peptides a and b, has the same molecular weight as the CB_1 fragment; its accumulation by cyanogen bromide cleavage of larger peptides is entirely consistent with

its identification as CB_1. A tentative assignment of peptides c and d, as suggested by the data of a previous paper (Boulay et al., 1983), is shown in Figure 1B; these peptides would extend from the N terminus of the carrier to the amino acid preceding Cys-128 and Cys-159, respectively.

In the second experiment, where the carrier had been first alkylated by unlabeled NEM and then cleaved by $Na^{14}CN$, the ^{14}C-labeled peptides were further cleaved by cyanogen bromide. By comparison with previously reported data (Boulay et al., 1983), the low molecular weight ^{14}C-labeled peptides visualized on the gel of Figure 3B could be readily identified as the nonoverlapping peptides CN_1, CN_2, and CN_4. On the other hand, peptides e, f, and g are overlapping peptides, as illustrated in Figure 1B. Peptides g and CN_4 which have the same molecular weight migrated at the same rate on the gel. As mentioned under Experimental Procedures, only 70% of the carrier molecules were alkylated; the remaining 30% are therefore expected to be cleaved by cyanylation at all cysteinyl residues. This explains the small accumulation of peptides e, f, and probably CN_4, although in the latter case accumulation of CN_4 was obscured by localization of g and CN_4 at the same place. Most importantly, the [^{14}C]NEM-alkylated peptides c and d of Figure 3A were not found in the gel of Figure 3B. This corroborates the hypothesis made above that the alkylated peptides c and d do contain the N terminus of the carrier protein; otherwise, they would have incorporated the ^{14}C radioactivity during the course of cleavage by $Na^{14}CN$. Peptide d most likely corresponds to the sequence from the N terminus of the carrier protein to the residue preceding Cys-128 and peptide c to the sequence from the N terminus to the residue preceding Cys-159. As the smaller molecular weight peptide, d, is alkylated by [^{14}C]NEM, it follows that the alkylated cysteinyl residue is Cys-56.

One may wonder whether, besides Cys-56, other cysteinyl residues in the CB_1 fragment, namely, Cys-128 and Cys-159, are also alkylated by NEM. This possibility is excluded by comparison of the radioactive bands in parts A and B of Figure 3. Peptides e, f, and g are visualized by autoradiography using $Na^{14}CN$ cleavage of the carrier alkylated by unlabeled NEM (Figure 3B). The same peptides obtained by cleavage with unlabeled NaCN of the carrier alkylated by [^{14}C]NEM would have given radioactive bands in the autoradiography of Figure 3A if they had incorporated [^{14}C]NEM. This is not the case. The chemical cleavage experiments therefore strongly suggest that the only alkylated cysteinyl residue in the membrane-bound carrier is Cys-56. This has been clearly established, as shown below, by enzymatic cleavage experiments and sequencing of the [^{14}C]NEM peptide.

Localization of the [^{14}C]NEM-Alkylated Cys of the ADP/ATP Carrier after Enzymatic Cleavage of the CB_1 Fragment. Figure 4 shows the elution pattern of fragments arising from cleavage of the [^{14}C]NEM-labeled CB_1 peptide by a mixture of trypsin and α-chymotrypsin (cf. Experimental Procedures). The ^{14}C radioactivity was localized in two peaks, I and II. The yield was 20% on the basis of the radioactive material loaded in the injection loop, a low but nonusual recovery in HPLC fractionation. The peptides contained in these peaks were further rechromatographed on a Vydac 10TP-RP column (Figure 5), yielding radioactive peaks I′ and II′, corresponding to purified peptides. The amino acid analysis of peptides I′ and II″ are shown in Table I. The two above chromatographic steps had to be run in the mentioned order to obtain the peptides in a sufficient degree of purity to undertake sequence analysis. The calculations were made on the basis of the integral number of two valine residues which is

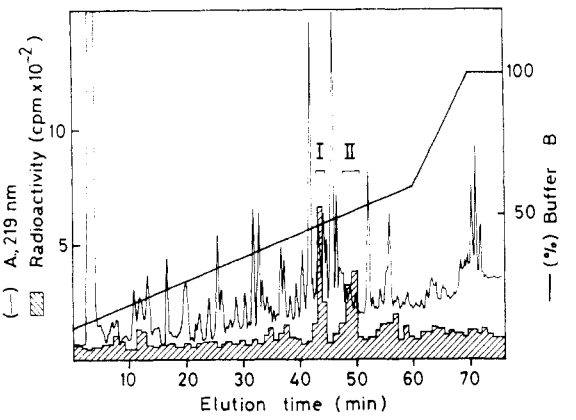

FIGURE 4: Separation of tryptic–α-chymotryptic fragments of the [^{14}C]NEM-labeled CB_1 peptide by HPLC. The [^{14}C]NEM-labeled CB_1 peptide (see Figure 2) was obtained by cyanogen bromide cleavage of the [^{14}C]NEM-labeled carrier protein. The CB_1 peptide was digested by a mixture of trypsin and α-chymotrypsin as described under Experimental Procedures. Peptides were resolved by HPLC at room temperature. The column used was a C_{18} μBondapak column (0.39 × 30 cm). At the onset, buffers A and B were in the proportion of 90% and 10%, respectively. Gradient was run at a flow rate of 1 mL/min as follows: from zero to 60 min, the percentage of buffer B was increased from 10 to 60%; from 60 to 70 min, it was increased from 60 to 100%. At the end of the operation, the column was washed for 10 min with buffer B. The eluted peptides were monitored at 219 nm. Two major radioactive peaks referred to as I and II were eluted at 43 and 48 min.

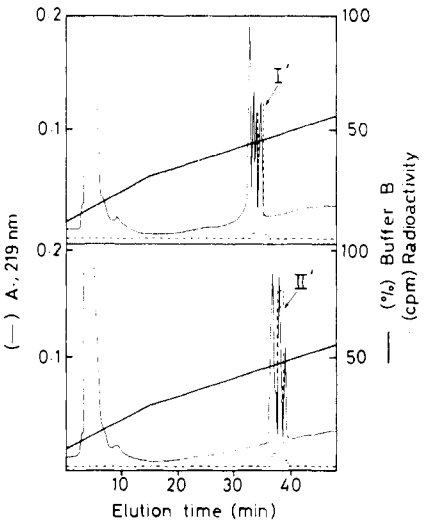

FIGURE 5: Subfractionation by HPLC of the radioactive fractions I and II collected in the experiment of Figure 4. The column used was a Vydac 10 TP-RP column (0.39 × 25 cm). Samples of the crude fraction I and fraction II were injected. At zero time, a mixture of 90% buffer A and 10% buffer B was applied to the column. From 0 to 15 min, the concentration in buffer B was increased from 10 to 30%. From 15 to 50 min, the concentration in buffer B was increased from 30 to 60%. The flow rate was maintained at 1 mL/min.

derived from the approximate value of one arginine residue per mole of peptide, as trypsin was used as the proteolytic enzyme. Interestingly, no significant difference between the amino acid composition of peptides I′ and II′ could be detected (Table I). This amino acid composition was quite similar to that of the sequence that extends from X being probably a substituted lysine (Aquila et al., 1982b). This sequence contains a cysteinyl residue at position 56.

Manual sequencing of the [^{14}C]NEM-labeled peptides I′ and II′ yielded the same amino acids (Table II). The ^{14}C radioactivity appeared at step 6 in full agreement with the expected release of Cys-56 at this step. Since the amino acid

Table I: Amino Acid Composition of [^{14}C]NEM-Labeled Peptides I′ and II′

amino acid residues	I′	II′
Asx	1.2[a] (1)[b]	1.1[a] (1)[b]
Thr	0.2 (0)	0.2 (0)
Ser	0.2 (0)	0.3 (0)
Glu	0.4 (0)	0.5 (0)
Pro[c]	0 (0)	0 (0)
Gly	1.3 (1)	1.4 (1)
Ala	0.4 (0)	0.6 (0)
Val	2.0 (2)	2.0 (2)
Met	0 (0)	0 (0)
Ile	1.6 (2)	1.7 (2)
Leu	0.5 (0)	0.6 (0)
Tyr	0 (0)	0 (0)
Phe	0.3 (0)	0.3 (0)
His	0 (0)	0.1 (0)
Lys	0.3 (0)	0.4 (0)
Arg	1.0 (10)	0.8 (1)

[a] Aliquot fractions of peaks I′ and II′ were hydrolyzed, as described by Tsugita & Scheffler (1982). Calculation was made on the basis of two residues of valine (see Results). The reported values are in moles of amino acids per mole of peptide. [b] Values found from sequence data. [c] Proline was determined in a special run by a postcolumn hypochlorite treatment at 62 °C for 2 min, before derivatization by *o*-phthalaldehyde and fluorescence detection. [d] Hydrolysis products of NEM were not detected in the analysis.

Table II: Sequence Analysis of the [^{14}C]NEM-Labeled Peptides I′ and II′[a]

residue	amino acid identification: peptide I′	amino acid identification: peptide II′	^{14}C (cpm/cycle)[b]: from peptide I′ (540 cpm)	^{14}C (cpm/cycle)[b]: from peptide II′ (680 cpm)
1	unknown[c]	unknown[c]	20	25
2	Gly	Gly	27	32
3	Ileu[d]	Ileu[d]	23	28
4	Ileu[d]	Ileu[d]	24	31
5	Asx[e]	Asx[e]	41	43
6	ND[f]	ND[f]	142	210
7	Val	Val	31	29
8	Val	Val	26	27
9	Arg	Arg	21	22

[a] For manual DABITC/PITC liquid-phase sequencing, 2.5 nmol of peptide was used. The DABTH-amino acids were dissolved in ethanol. An aliquot sample was used for identification by chromatography on micropolyamide sheets, and another sample was used for radioactivity counting. [b] The counted radioactivity corresponds to the total amino of ^{14}C-labeled DABTH-amino acid released in each cycle. [c] After back-hydrolysis of the thiazolinone derivative by acidic hydrolysis (Mendez & Lai, 1975), an unknown amino acid is eluted 0.5 min before lysine. [d] Established from the amino acid composition and amino acid analysis after regeneration of amino acids from their thiazolinone derivatives as described by Mendez & Lai (1975). [e] DABTH-asparagine was observed in nonnegligible amount at this step. [f] ND, not determined.

sequences of peptides I′ and II′ are the same, it is possible that the different elution patterns for the two peptides come from a different substituent at the level of X. In conclusion, the sequence data of the [^{14}C]NEM-labeled chymotryptic–tryptic peptide that was separated by HPLC clearly demonstrate the presence of Cys-56 alkylated by [^{14}C]NEM.

Discussion

One Amino Acid Residue Only in the Membrane-Bound ADP/ATP Carrier Is Alkylable by NEM, and This Residue Is Cys-56. The data presented in this paper indicate that NEM reacts specifically with one out of the four cysteinyl residues of the membrane bound ADP/ATP carrier protein. Alkylation by NEM is concomitant with loss of transport activity. Identification of the NEM reactive cysteine to Cys-56 has been achieved by examination of peptides arising from both chemical and enzymatic cleavages. Chemical cleavage, especially cleavage at those cysteinyl residues that were not

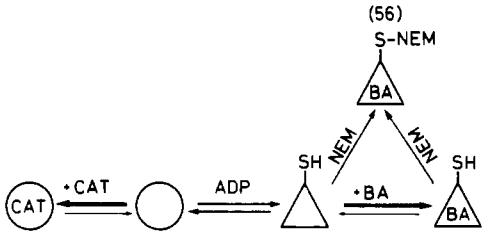

FIGURE 6: Scheme illustrating the unmasking of Cys-56 in the BA conformation of the carrier and the stabilization of the BA conformation by covalent binding of NEM to the unmasked SH. In the presence of ADP (or ATP), the carrier is postulated to assume two conformations, namely, the CATR conformation (○) recognized by CATR, and the BA conformation (△) recognized by BA. The CATR and BA conformations are trapped by binding of CATR and BA. The BA conformation is characterized by the unmasked Cys-56 which is accessible to NEM.

alkylated by NEM, provided evidence that one cysteinyl residue only in the amino acid sequence of the ADP/ATP carrier protein is alkylated by NEM. Unambiguous identification of the alkylated cysteinyl residue was obtained by analysis of the sequence of a short [^{14}C]NEM-alkylated peptide formed by cleavage with a mixture of trypsin and α-chymotrypsin. The peptide, X_{51}-Gly_{52}-Ile-Ile-Asx-Cys_{56}-Val-Val_{58}-Arg, which is rather hydrophobic, is itself included in a large hydrophilic segment extending from Val-37 to Gln-64 (Saraste & Walker, 1982). The folding of the small hydrophobic segment Gly-52–Val-58 to form a pocket inside the large hydrophilic segment (Val-37–Gln-64) may explain the inhibitory effect of membrane-permeant SH reagents like NEM, fuscin, and *N*-(*N*-acetyl-4-sulfamoylphenyl)maleimide on ADP/ATP transport (Vignais & Vignais, 1972) and the lack of reactivity to nonpermeant SH reagents. Another explanation for the selective inhibition by membrane-permeant SH reagents is that the hydrophilic segment Val-34–Gln-64 is exposed to the matrix space of mitochondria.

The amino acid residue X in the above sequence is possibly related to lysine. In fact after acid hydrolysis of the corresponding thiazolinone as described by Mendez & Lai (1975), an amino acid residue of unknown nature was eluted just 0.5 min before lysine. In an independent work, X was tentatively assigned to methyllysine (Aquila et al., 1982b).

What Is the Significance of the NEM Reactivity of Cys-56 in the Membrane-Bound ADP/ATP Carrier When Transport Is Functioning? The ADP- or ATP-induced reactivity of Cys-56 to NEM is inhibited by ATR or CATR (Vignais & Vignais, 1972); in contrast it is enhanced by isoBA (Block et al., 1981). This is fully consistent with the two conformations that the ADP/ATP carrier can assume during transport, namely, the CATR and BA conformations (Block et al., 1983). The fact that NEM added to mitochondria inhibits ATR or CATR binding (Vignais & Vignais, 1972), but not BA binding (Lauquin & Vignais, 1976), might be explained by the higher reactivity or the unmasking of Cys-56 in the BA conformation followed by alkylation of Cys-56 with NEM (Figure 6). Thus, by covalent binding of NEM, the carrier is trapped irreversibly in the BA conformation, with concomitant loss of its transport function. It is noteworthy that the same cysteinyl residue, namely, Cys-56, is alkylated by NEM, whether the mitochondria had been incubated with ADP (unshown data) or ADP plus BA. In other words, Cys-56 is accessible to NEM whether the carrier protein is in a reversible BA conformation in the absence of added BA or trapped in a stabilized BA conformation in the presence of added BA. Under uncoupling conditions, even in the presence of ADP or ATP, the reactivity of the carrier to NEM is strongly diminished (Vignais &

Vignais, 1972). This might be explained by a modification in the lipid environment of the carrier protein which would make Cys-56 less accessible to NEM. The NEM reactivity of Cys-56 is not the only manifestation of conformational changes assumed by the carrier protein in response to the addition of ADP or ATP. Marked changes in the intrinsic fluorescence have been shown to occur upon addition of ADP or ATP to the isolated carrier in detergent (Brandolin et al., 1981).

There are a number of sequence homologies between the primary structure of the beef heart ADP/ATP carrier and that of the *Neurospora crassa* carrier (Arends & Sebald, 1984). In particular, the same amino acid sequence extending from Gly-52 to Cys-56 in the beef heart carrier occurs in the corresponding region of the *Neurospora* carrier. Because of the ADP/ATP-induced reactivity of Cys-56 to NEM in the membrane-bound carrier from heart, it is possible that the corresponding cysteinyl residue in the homologous region of the *Neurospora* carrier may also be subject to an increassed reactivity upon addition of ADP or ATP to *Neurospora* mitochondria.

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