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Mapping of the Nucleotide-Binding Sites in the ADP/ATP Carrier of Beef Heart Mitochondria by Photolabeling with 2-Azido[α-^{32}P]adenosine Diphosphate†

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ABSTRACT: 2-Azido[α-^{32}P]adenosine diphosphate (2-azido[α-^{32}P]ADP) has been used to photolabel the ADP/ATP carrier in beef heart mitochondria. In reversible binding assays carried out in the dark, this photoprobe was found to inhibit ADP/ATP transport in beef heart mitochondria and to bind to two types of specific sites of the ADP/ATP carrier characterized by high-affinity binding (K_d = 20 μM) and low-affinity binding (K_d = 400 μM). In contrast, it was unable to bind to specific carrier sites in inverted submitochondrial particles. Upon photoirradiation of beef heart mitochondria in the presence of 2-azido[α-^{32}P]ADP, the ADP/ATP carrier was covalently labeled. After purification, the photolabeled carrier protein was cleaved chemically by acidolysis or cyanogen bromide and enzymatically with the *Staphylococcus aureus* V8 protease. In the ADP/ATP carrier protein, which is 297 amino acid residues in length, two discrete regions extending from Phe-153 to Met-200 and from Tyr-250 to Met-281 were labeled by 2-azido[α-^{32}P]ADP. The peptide fragments corresponding to these regions were sequenced, and the labeled amino acids were identified. As 2-azido-ADP is not transported into mitochondria and competes against transport of externally added ADP, it is concluded that the two regions of the carrier which are photolabeled are facing the cytosol. Whether the two photolabeled regions are located in a single peptide chain of the carrier or in different peptide chains of an oligomeric structure is discussed.

The mitochondrial ADP/ATP carrier is characterized by specific binding sites for the free forms of the natural substrates ADP and ATP. Two conformers of the ADP/ATP carrier exist, which can be differentiated by the binding of two categories of specific inhibitors, namely, atractyloside (ATR)[1] and carboxyatractyloside (CATR), on one hand, and bongkrekic and isobongkrekic acids (BA and isoBA) on the other [for review cf. Vignais et al. (1985)]. The ADP/ATP carrier molecule also contains amino acid residues whose modification results in inactivation of transport (Block et al., 1981). The beef heart ADP/ATP carrier is a protein of 297 amino acid residues whose sequence has been reported (Aquila et al., 1982). In this carier, a sequence has been identified, which spans residues Cys-159–Met-200 and is involved in ATR (CATR) binding (Boulay et al., 1983). Furthermore, the strategic cysteinyl residue that becomes exposed at the onset of transport and whose alkylation by *N*-ethylmaleimide inhibits the functioning of the carrier and prevents ATR binding, but not BA binding, has been assigned to position 56 (Boulay & Vignais, 1984). Mapping of the nucleotide-binding site(s) of the ADP/ATP carrier has not been reported so far. Such mapping requires the use of a photoprobe able to bind with high affinity and specificity to the carrier protein. Preliminary studies have shown that 2-azido[α-^{32}P]ADP binds reversibly and with high affinity to the ADP/ATP carrier in rat heart mitochondria in the absence of photoirradiation and competitively inhibits ADP transport (Dalbon et al., 1985). The ADP/ATP carrier from beef heart mitochondria can be prepared in large amounts, and a number of mapping studies have been conducted with this carrier (see above); for these reasons, we decided to use the beef heart mitochondrial ADP/ATP carrier for photolabeling studies. This paper describes the reactivity of 2-azido[α-^{32}P]ADP toward the ADP/ATP carrier in beef heart mitochondria and inverted

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[1] Abbreviations: ATR, atractyloside; CATR, carboxyatractyloside; BA, bongkrekic acid; 2-azido-ADP, 2-azidoadenosine diphosphate; DITC, *p*-phenylene diisothiocyanate; PTH, phenylthiohydantoin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; γ-AmNS-ATP, adenosine N^{γ}-(5-sulfo-1-naphthyl)triphosphoramidate; NEM, *N*-ethylmaleimide; NAP_4ATR, 4-azido-2-nitrophenyl aminobutyryl-ATR; Hse, homoserine lactone; EDTA, ethylenediaminetetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

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submitochondrial particles in the absence of photoirradiation and the mapping of the carrier nucleotide-binding site(s) covalently labeled with the photoprobe.

Experimental Procedures

Chemicals and Enzymes. $H_3{}^{32}PO_4$ was purchased from New England Nuclear. Cyanogen bromide was from Merck. Guanidinium chloride was obtained from Sigma Chemical Co. Acrylamide and *N,N'*-methylenebis(acrylamide) were from BDH. Sephadex G-75 superfine was from Pharmacia; Bio-Gel P-30 (400 mesh) was from Bio-Rad. DITC glass beads, 3-aminopropyl (aminoethyl) controlled-pore glass beads, trifluoroacetic acid, and PITC were from Pierce. Succinic and maleic anhydride were from Fluka. *Staphylococcus aureus* V8 protease was from Miles Laboratories. Carboxyatractyloside was from Boehringer. Bongkrekic acid was prepared as described (Lauquin & Vignais, 1976). All reagents used were of the purest grade commercially available.

Biological Preparations. Beef heart mitochondria were prepared according to the method of Smith (1967). Inverted submitochondrial particles were obtained by differential centrifugation after ultrasonic irradiation of beef heart mitochondria (Beyer, 1967; Lauquin et al., 1977). Prior to use, the particles were pretreated with 10 μM CATR, a specific inhibitor that binds to the cytoplasmic face of the carrier in mitochondria, to avoid interference of leaky vesicles and residual particles containing correctly oriented membranes having their CATR binding sites exposed to the outside.

Synthesis of 2-Azido[α-^{32}P]ADP. 2-Azido[α-^{32}P]ADP was synthesized as described by Boulay et al. (1985) except that the last step of purification by chromatography on Dowex 50W-X4 was replaced by a chromatography on a DEAE-cellulose DE52 (Whatman) column using a linear gradient of triethylammonium bicarbonate up to 0.5 M, pH 7.5. The specific activity of purified 2-azido[α-^{32}P]ADP was close to 2000 dpm/pmol.

Binding Assays. Binding of radiolabeled nucleotides to the nucleotide-binding sites of the adenine nucleotide carrier (specific binding) in beef heart mitochondria and inverted submitochondrial particles was assessed by the inhibitor chase method (Weidemann et al., 1970) with minor modifications. For mitochondria, the assay was carried out as described by Dalbon et al. (1985); the specifically bound 2-azido[α-^{32}P]-ADP or [^{14}C]ADP was chased with 10 μM CATR. Inverted submitochondrial particles were suspended at the final concentration of 10 mg of protein/mL in 125 mM KCl, 10 mM HEPES, and 1 mM EDTA, pH 7.2. Samples of 400 μL of this suspension were distributed into centrifuge tubes with increasing concentrations of the nucleotide tested. Incubation was for 60 min at 0–2 °C. The suspension in every tube was then divided in two fractions, one of which was kept as a control while the other was mixed with 10 μM BA, a specific inhibitor of the ADP/ATP carrier that binds to the matrix face of the carrier, and incubation was continued for an additional 30 min at 0–2 °C to allow complete release of specifically bound nucleotides. The incubation was terminated by centrifugation in a Beckman airfuge at 100000*g* for 10 min. The tube walls were rinsed once with incubation medium, and the pellets were solubilized with a mixture of 4% Triton X-100 and 0.5 M NaCl. The radioactivity was determined by liquid scintillation counting. The difference between the radioactivity incorporated into untreated and BA-treated submitochondrial particles was ascribed to specific nucleotide binding.

Analytical Procedures. Aqueous radioactive solutions (1.0-mL fractions) were dispersed in 10 mL of a scintillation fluid (Patterson & Greene, 1965), and the radioactivity was measured by liquid scintillation counting. Peptides resulting from cleavage of the ADP/ATP carrier protein were separated by polyacrylamide gel electrophoresis, using 20% acrylamide slab gels as described (Cabral & Schatz, 1979). For autoradiography, the gels were dried and exposed for several hours to a Fujix FX film at −70° C with a Cronex intensifying screen. The protein concentration was determined by the method of Zak and Cohen (1961) with addition of sodium dodecyl sulfate (Dulley & Grieve, 1975; Chin-Sun & Smith, 1975).

Covalent Photolabeling of Mitochondria. Beef heart mitochondria (250 mg) were first incubated for 15 min in darkness at room temperature in 35 mL of a medium consisting of 0.27 M sucrose, 10 mM HEPES, 1 mM EDTA, pH 7.2, and 20 μM 2-azido[α-^{32}P]ADP. Photoirradiation was carried out with xenon lamp XBO 1000 W/HS equipped with a parabolic reflector and placed at 30 cm. The power supply SVX 1000 (Müller Gmb, Moosinning, West Germany) was operated at 0.9 kW, as described (Boulay et al., 1985). After photoirradiation, the mitochondria were centrifuged for 10 min at 20000*g*. The pellet was resuspended in a fresh medium supplemented with 20 μM 2-azido[α-^{32}P]ADP, and the suspension was subjected to a second photoirradiation.

Purification and Chemical Modification of the 2-Azido-[α-^{32}P]ADP-Photolabeled ADP/ATP Carrier. The photolabeled carrier protein was extracted by dispersing the pellet of photoirradiated mitochondria in 4% Triton X-100, 0.5 M NaCl, 1 mM EDTA, and 10 mM MOPS, pH 7.2. The carrier protein was purified by hydroxylapatite gel chromatography as described by Riccio et al. (1975) with minor modifications, the amount of protein applied to the column being about 2 mg/mL of gel. The nonretained fraction (15–20 mg of protein) was concentrated by ultrafiltration through a PM 10 Amicon membrane and finally treated with 5 volumes of acetone at −20 °C overnight. The resulting precipitate was recovered by centrifugation and washed with acetone at −20 °C to remove the residual Triton X-100. The pellet was dissolved in 1.5 mL of formic acid. After solubilization, 4 mL of ethanol was added with 20 μL of 5 M NaCl. This was followed by addition of 20 mL of diethyl ether, and the temperature was lowered to −20 °C, resulting in the precipitation of carrier protein. Washing with diethyl ether was repeated twice, and the protein was finally freeze-dried. The radioactivity covalently bound to the carrier amounted to 10–15% of the radioactivity bound at saturation in darkness and displaceable upon addition of the specific inhibitor CATR.

For chemical modification, the photolabeled carrier protein was solubilized in 2 mL of 7 M guanidinium chloride, 1 mM EDTA, and 0.1 M sodium phosphate, pH 7.2, under a stream of nitrogen. Then cysteinyl residues were carboxamidomethylated as follows. After reduction with 3 mM dithiothreitol for 1 h at 37 °C, the protein was treated by 15 mM iodoacetamide for 15 min at room temperature, and the reaction was terminated by addition of 30 mM dithiothreitol. The carboxamidomethylated protein was succinylated at room temperature following the general procedure described by Klotz (1967). Succinic anhydride was added by increments, the final concentration being in a 1000-fold excess with respect to the free NH_2 groups of the protein. During the course of the reaction, the pH was maintained at 8.5 by addition of 10 N NaOH. After the final addition of succinic anhydride, the solution was allowed to stand with stirring for about 1 h. Finally the protein solution was desalted by passage through a column of Ultrogel ACA 202 (20 mL) equilibrated in 100 mM ammonium bicarbonate, pH 7.8. The eluted protein was

Table I: Binding Parameters of [^{14}C]ADP and 2-Azido[α-^{32}P]ADP to Beef Heart Mitochondria and Derived Inside-Out Submitochondrial Particles

	beef heart mitochondria			inside-out particles		
nucleotides	K_{d_1}[a] (μM)	K_{d_2}[a] (μM)	n[b] (nmol/mg of protein)	K_{d_1}[a] (μM)	K_{d_2}[a] (μM)	n[b] (nmol/mg of protein)
[^{14}C]ADP	4	65	1.3	5	70	1.4
2-azido[α-^{32}P]ADP	20	400	1.2	ND[c]	ND[c]	ND[c]

[a] The K_d values were determined from the Scatchard plots of the binding data (cf. Experimental Procedures). [b] n = total number of sites (high and low affinity). [c] ND = not determined because of too low binding.

used for either chemical or enzymatic cleavage.

Maleylation of the alkylated protein was used instead of succinylation when the ϵ-NH_2 group of lysine residues had to be restored for attachment to DITC glass beads prior to solid-phase sequencing. The procedure used for maleylation was the same as that described for succinylation except that temperature was maintained at 0 °C during the reaction (Butler & Hartley, 1972).

Fragmentation of Photolabeled Carrier Protein. The chemically modified carrier protein and the derived peptide fragments were cleaved at methionyl residues by an overnight treatment at 37 °C in 70% formic acid with a 500-fold excess of cyanogen bromide with respect to the number of methionyl residues (Gross, 1967). The reaction was terminated by addition of 5 volumes of distilled water, and the cleavage products were freeze-dried.

Acidolytic cleavage at the Asp–Pro bond was completed after a 60-h incubation at 37 °C of the carrier protein using 8–10 mg of protein in 1 mL of 70% formic acid.

V8 protease digestion of the carrier protein was performed overnight in 100 mM ammonium bicarbonate, pH 7.8 at 37 °C, using a protease to carrier protein ratio of 1/50 (w/w). To ensure efficient cleavage, digestion was continued for another 6 h with a further addition of V8 protease, using the same enzyme to carrier protein ratio.

Isolation of Peptide Fragments. The V8 protease fragments of the carrier protein were separated by chromatography on a 2.5 × 90 cm column of Sephadex G-75 superfine equilibrated in 6 M guanidinium chloride and 50 mM Tris, pH 7.8 at 25 °C. The column was eluted with the same medium. Fractions containing the bound radioactivity were pooled, concentrated on a YM5 Amicon membrane up to 2 mL, and then desalted by passage through a 12-mL column of Bio-Gel P6DG equilibrated in 80% formic acid.

The peptides resulting from acidolytic cleavage or CNBr cleavage were fractionated by passage on a 2.5 × 70 cm column of Bio-Gel P30, (400 mesh) equilibrated in 70% formic acid at room temperature.

Determination of Amino Acid Sequence and Identification of Amino Acid Residues. Two different methods were used to immobilize peptide fragments of the carrier protein for solid-phase sequencing. Peptides resulting from CNBr cleavage were immobilized by the C-terminal homoserine residue, using the homoserine lactone procedure described by Horn and Laursen (1973), except that resin was replaced by 3-aminopropyl (aminoethyl) CPG-75 glass beads. The peptide was solubilized in 0.2 mL of anhydrous trifluoroacetic acid for 30 min at room temperature, which resulted in the quantitative conversion of the homoserine residue into the lactone. After drying, the peptide was redissolved in 20 mL of trifluoroacetic acid. This was followed by the successive additions of 0.5 mL of dimethylformamide and 0.1 mL of triethylamine. Activated beads (70 mg) were added, and the coupling reaction was allowed to proceed for 24 h at 55 °C. After the beads were washed with trifluoroacetic acid and methanol, the loaded beads were finally washed with 5 mL of methanol and 2 mL of diethyl ether. The yield of coupling was routinely in the range of 30–40%.

Coupling by lysine residues was performed with DITC-activated amino glass beads. The V15 peptide solution was first extensively dialyzed against 100 mM ammonium bicarbonate, pH 7.8, in order to remove guanidinium chloride. Demaleylation was achieved by lowering the pH to 3 with 50% formic acid and leaving the peptide in contact with this acid medium for 24 h at 37 °C. The solution was freeze-dried, and the peptide was attached to DITC beads as described by Chang (1979). Briefly, the peptide was solubilized in 50 μL of 10% sodium dodecyl sulfate, then 0.1 mL of 1 M sodium carbonate was added, and the pH was adjusted to 9 with 10 N NaOH. The sodium dodecyl sulfate concentration was lowered to 1% by addition of distilled water, and 50 μL 1-propanol was then added, followed by 70 mg of DITC beads. The coupling reaction was allowed to proceed overnight at 55 °C, and 80 μL of ethanolamine was added to block the unreacted isothiocyanate groups of the beads. Finally the beads were washed with 0.5 M sodium bicarbonate, water, and methanol. The yield of coupling was between 40 and 50%.

The amino acid sequence was determined with a solid-phase sequencer (Sequemat, Model 12, Watertown) using the single program recommended by Hoppe et al. (1986). About half of the peptides bound to beads could be sequenced. The amount of radioactivity recovered in the thiazolinone amino acids was determined by Cerenkov counting. The thiazolinone amino acids were next converted into stable phenylthiohydantoin (PTH) amino acid derivatives by a 15-min incubation with 1 M HCl in methanol. After drying for 30 min at 65 °C, the PTH derivatives of amino acids were taken up in 10 μL of acetonitrile, separated, and identified by thin-layer chromatography (TLC) on 10 cm × 10 cm silica plates (HPTLC-Fertig platten, Kieselgel 60 F_{254}) using chloroform/ethanol (98/2 v/v) as solvent (Hoppe & Sebald, 1980). A second chromatography step in the same direction with a solvent composed of chloroform and methanol (9/1 v/v) was used to separate the polar PTH amino acid derivatives.

Results

Reversible Binding of 2-Azido[α-^{32}P]ADP to the Membrane-Bound Carrier in Beef Heart Mitochondria and Inverted Submitochondrial Particles. The nucleotide analogue 2-azido[α-^{32}P]ADP was able to bind reversibly to beef heart mitochondria in the absence of photoirradiation. The Scatchard plots of the binding data were curvilinear. These plots were fitted to a two-site model, like those obtained previously with [^{14}C]ADP (Block & Vignais, 1984). High- and low-affinity K_d values for these sites of 20 and 400 μM, respectively, were determined (Table I). By comparison, the K_d values for ADP binding were 4 and 65 μM, respectively (Block & Vignais, 1984). As in rat heart mitochondria (Dalbon et al., 1985), 2-azido-ADP was not transported, and it reversibly inhibited ADP transport in beef heart mito-

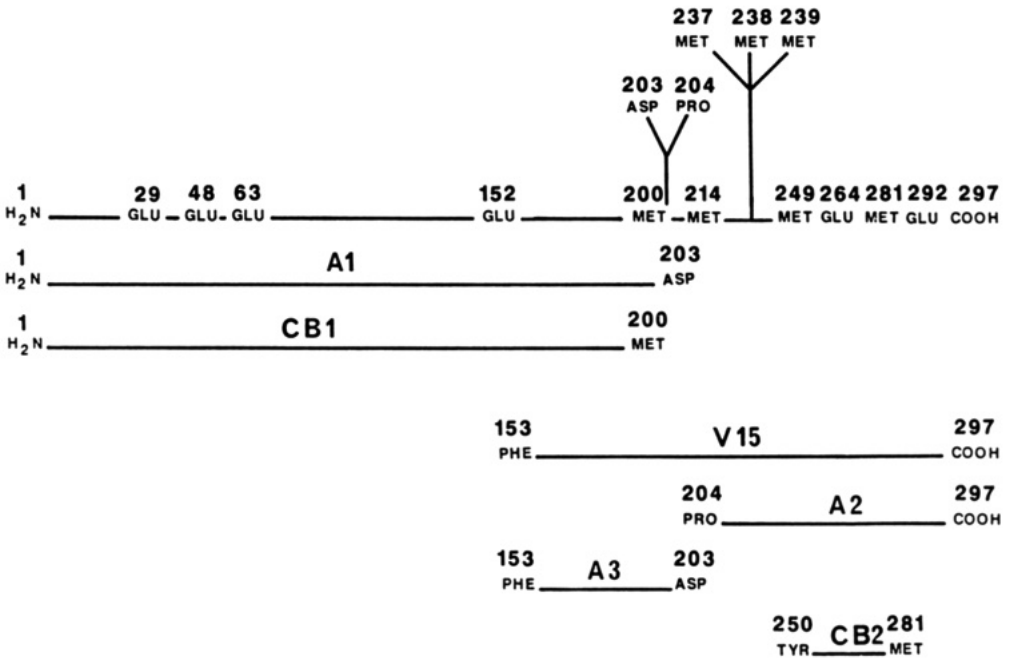

FIGURE 1: Scheme illustrating the localization of strategic amino acid residues and bonds susceptible to chemical and enzymatic cleavages in the ADP/ATP carrier. The cleavages described in the present study were at the Asp-203–Pro-204 bond (acidolytic cleavage), at the seven Met residues (CNBr cleavage), and at the Glu-152–Phe-153 bond (V8 protease cleavage). The photolabeled fragments (peptides A_1, A_2, A_3, CB_1, CB_2, and V15) are shown in the figure.

chondria (data not shown). Thus, two essential requirements for an effective photoprobe, namely, specific binding and high-affinity binding, are met when 2-azido-ADP interacts in darkness with sites on the ADP/ATP carrier. Binding experiments similar to those performed with beef heart mitochondria were carried out with inverted beef heart submitochondrial particles. The affinity of 2-azido[α-^{32}P]ADP for inverted particles was too low for an accurate determination of the binding parameters. Parallel experiments carried out with [^{14}C]ADP and the same preparation of inverted particles showed two classes of binding sites for ADP with K_d of 5 and 70 μM (Table I). In other words, whereas 2-azido-ADP interacts specifically and with high affinity with sites on the cytoplasmic face of the carrier, it does not recognize the matrix face of the carrier. Therefore, the photolabeling experiments described in the next section were carried out only with intact mitochondria. The mapping of nucleotide-binding sites by 2-azido[α-^{32}P]ADP will be discussed in terms of regions of the carrier accessible from the outside of the mitochondria.

Photolabeling Experiments with the ADP/ATP Carrier of Beef Heart Mitochondria. Chemical and Enzymatic Fragmentation of the Photolabeled Carrier. As reported previously (Dalbon et al., 1985), when beef heart mitochondria are photoirradiated in the presence of 2-azido[α-^{32}P]ADP, only one protein, which is located in the mitochondrial membrane, is photolabeled; its apparent molecular weight is close to 32000. On the basis that (1) the covalent photolabeling was prevented by preincubation of the mitochondria with CATR, a strong and specific inhibitor that binds to the cytoplasmic face of the ADP/ATP carrier, (2) ADP transport in mitochondria is reversibly inhibited by 2-azido-ADP in the dark, and (3) the molecular weight of the beef heart ADP/ATP carrier is close to 32000 (Aquila et al., 1982), it was concluded that the mitochondrial protein photolabeled by 2-azido[α-^{32}P]ADP is the ADP/ATP carrier.

Solubilization of the membrane-bound photolabeled carrier in detergent and its purification are detailed under Experimental Procedures. After carboxymethylation, followed by succinylation or maleylation, the carrier protein was subjected

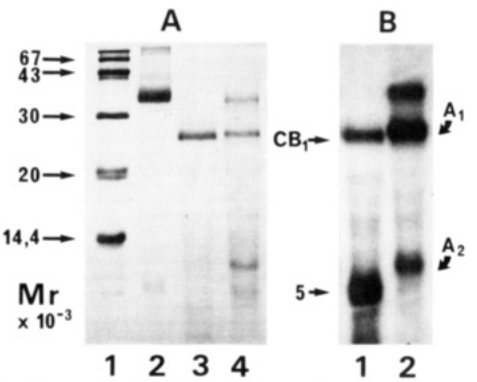

FIGURE 2: Separation by $NaDodSO_4$–polyacrylamide gel electrophoresis of the peptide fragments resulting from the CNBr cleavage and acidolytic cleavage of the photolabeled ADP/ATP carrier. (A) Staining by Coomassie Blue: Track 1 corresponds to molecular weight markers, track 2 to the purified photolabeled ADP/ATP carrier after chemical modification, track 3 to the CNBr cleavage products of the photolabeled carrier, and track 4 to the peptides resulting from acidolytic cleavage. (B) Autoradiography: Lanes 1 and 2 of the autoradiography correspond to lanes 3 and 4 of the stained gel, respectively.

to fragmentation. For the sake of clarity, the cleavage points of the carrier protein and the resulting fragments are illustrated in Figure 1. Attack of peptide bonds at methionyl residues 200, 214, 237, 238, 239, 249, and 281 by CNBr generated a large peptide referred to as CB_1 of M_r close to 23000 and a number of small peptides (Boulay et al., 1979) (Figure 2A, lane 3). Treatment with 70% formic acid resulted in the cleavage of the acid-labile bond Asp-203–Pro-204 and accumulation of two peptides A_1 and A_2, whose molecular weights assessed by $NaDodSO_4$–polyacrylamide gel electrophoresis were approximated to 23000 and 10000, respectively (Figure 2A, lane 4). Because of the tendency of low M_r peptides to be washed out during the course of the staining and destaining steps, the gels were autoradiographed immediately after electrophoresis. Under these conditions, the large CNBr fragment of M_r 23000 (CB1) and a small CNBr cleavage product of M_r close to 5000 were found to be radiolabeled.

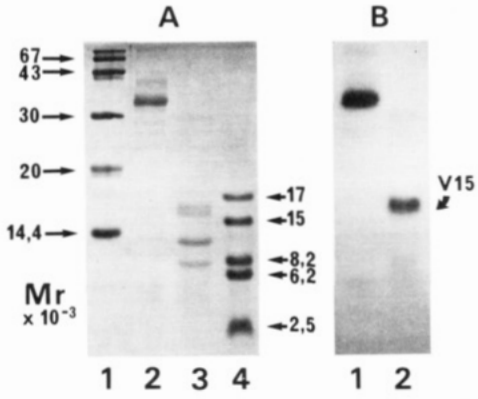

FIGURE 3: Separation by $NaDodSO_4$–polyacrylamide gel electrophoresis of the cleavage products obtained by treatment of the photolabeled ADP/ATP carrier with the V8 protease. (A) Staining by Coomassie Blue: Tracks 1 and 4 correspond to molecular weight markers; track 2 corresponds to the purified photolabeled carrier after chemical modification, and track 3 corresponds to the peptide fragments of the photolabeled carrier after treatment with V8 protease. (B) Autoradiography: Lanes 1 and 2 of the autoradiography correspond to lanes 2 and 3 of the stained gel, respectively.

Likewise, the two peptides arising from acidolytic cleavage of the carrier protein were photolabeled (Figure 2B, lanes 1 and 2).

Further dissection of the ADP/ATP carrier benefited from the use of the glutamic acid specific *S. aureus* V8 protease. Digestion of the photolabeled carrier with V8 protease yielded four peptides with apparent M_r of 15 500, 15 000, 10 000, and 7000 (Figure 3A, lane 3). The autoradiography of the gel revealed a labeled band comprising most likely the two peptides of M_r 15 500 and 15 000 (Figure 3B, lane 2). The M_r 15 000 peptide might be derived by proteolysis from the M_r 15 500 peptide, as discussed later. For convenience, the term V15 was ascribed to the peptides corresponding to the two juxtaposed bands of M_r 15 500 and 15 000. The cleavage products in the V8 protease digest were chromatographed on a column of Sephadex G-75 superfine (cf. Experimental Procedures). The pattern of absorbancy at 280 nm and bound radioactivity in the eluted fractions is illustrated in Figure 4. The material contained in these fractions was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (insert of Figure 4). Peaks 1 and 2 only were labeled. Peak 2 (fractions 145–161) contained peptide V15. The material of peak 3 (fractions 169–182) and peak 4 (fractions 188–195) was represented by unlabeled peptides of apparent M_r 10 000 and 7000, respectively. Peak 1 consisted of aggregates of the three above peptides.

The N-terminal sequencing of V15 was performed after demaleylation and coupling to DITC glass beads. The sequence found, NH_2-Phe-Thr-Gly-Leu-Gly-Asn-Cys-Ile-Thr-, was identical with that present in the middle region of the ADP/ATP carrier protein, beginning with Phe-153. Moreover, the molecular weight of V15 determined by $NaDodSO_4$–polyacrylamide gel electrophoresis was compatible with the presence of 140–150 amino acid residues. In conclusion, the M_r 15 500 component of V15 most likely extends from Phe-153 to the C-terminal end of the carrier protein, i.e., Val-297, and the M_r 15 000 component probably arises by cleavage of the M_r 15 500 species at Glu-292 by V8 protease, with release of the pentapeptide Ile-293–Val-297.

A more precise assignment of bound radioactivity to given amino acid residues in V15 was achieved by acidolytic cleavage at the Asp-203–Pro-204 bond. Prior to cleavage, V15 was desalted as described under Experimental Procedures. The two peptides A_2 and A_3 generated by acidolytic cleavage of

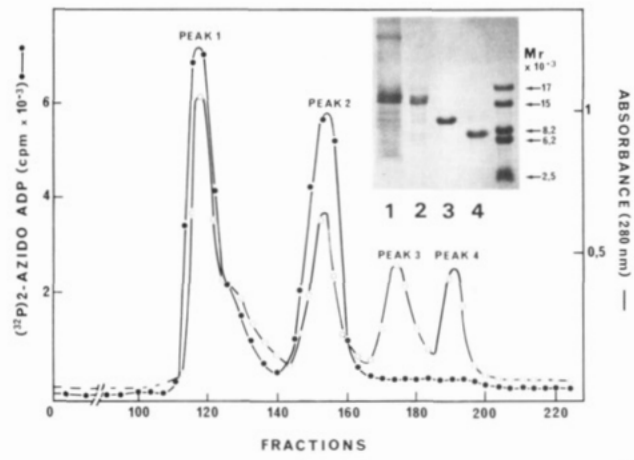

FIGURE 4: Elution profile of peptide fragments arising from cleavage of the photolabeled ADP/ATP carrier with the V8 protease. The photolabeled carrier protein (500 nmol) was cleaved with the V8 protease for 24 h, as described under Experimental Procedures. The resulting peptides were separated by chromatography on a 2.5 × 90 cm column of Sephadex G-75 superfine. The column was eluted with 6 M guanidinium chloride and 50 mM Tris, pH 7.8 at 25 °C, with a flow rate of 17 mL/h. One-milliliter fractions were collected, and radioactivity was measured on 10-μL aliquots. Fractions 110–140 (peak 1), 145–161 (peak 2), 169–182 (peak 3), and 188–195 (peak 4) were pooled, and their homogeneity was tested by $NaDodSO_4$–polyacrylamide gel electrophoresis followed by Coomassie Blue staining (insert). Lanes 1–4 correspond to peaks 1–4, respectively. The track on the right corresponds to molecular weight markers.

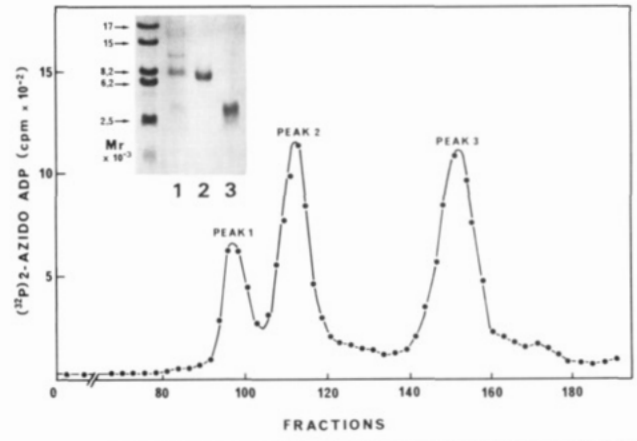

FIGURE 5: Elution profile of peptide fragments arising from acidolytic cleavage of the photolabeled peptide V15. An aliquot of 150 nmol of photolabeled peptide V15 was incubated for 60 h at 37 °C in 70% formic acid. The resulting peptide fragments were subjected to chromatography on a 2.5 × 70 cm column of Bio-Gel P30 (400 mesh). The column was eluted with 70% formic acid at room temperature with a flow rate of 4 mL/h. One-milliliter fractions were collected, and 10 μL was withdrawn for radioactivity counting. Fractions 95–105 (peak 1), 106–121 (peak 2), and 143–160 (peak 3) were pooled, and aliquots were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis followed by Coomassie Blue staining (see insert). Lanes 1–3 correspond to peaks 1–3, respectively. The track on the left corresponds to molecular weight markers.

V15 were fractionated by chromatography on Bio-Gel P30 in 70% formic acid and recovered in peaks 2 and 3 (Figure 5). Their molecular weights assessed by $NaDodSO_4$–polyacrylamide gel electrophoresis were 8000 and 4000, respectively (insert of Figure 5). Both peptides were radiolabeled. Peak 1 was made of aggregates. Clearly, the labeled peptide of M_r 8000 (peptide A_2) extended from proline residue 204 to Val-297 (or Glu-292), and the labeled peptide of M_r 4000 (peptide A_3) spanned the sequence Phe-153–Asp-203 (Figure 1).

Attempts to fragment peptide A_3 by proteolytic attack at

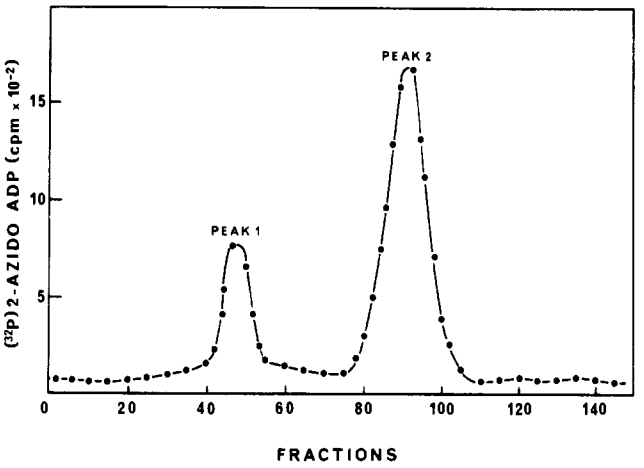

FIGURE 6: Elution profile of the radiolabeled peptides obtained by CNBr cleavage of the photolabeled segment Pro-204–Val-297 (peptide A_2) of the ADP/ATP carrier. The Pro-204–Val-297 peptide was prepared by acidolytic cleavage of photolabeled V15. Fifty nanomoles of peptide A_2 in 70% formic acid was treated by CNBr in the dark for 24 h at 37 °C, using a 500-fold excess of CNBr with respect to the methionine residues present in the peptide. The resulting fragments were separated by chromatography on a 2.5 × 60 cm column of Bio-Gel P30 (400 mesh); the column was eluted with 70% formic acid at room temperature with a flow rate of 5 mL/h. One-milliliter fractions were collected, and radioactivity was assayed on 10-μL fractions.

the level of arginyl residues by a number of proteases including trypsin, arginine endoprotease (Schenkein et al., 1977), clostripain (Gilles et al., 1979), or thrombin were unsuccessful, and it was therefore decided to subject the whole A_3 peptide to solid-phase sequencing in order to identify the photolabeled amino acid residues (see next section). On the other hand, peptide A_2, which was twice larger than peptide A_3, was digested by CNBr to obtain a radiolabeled fragment of shorter size, readily amenable to Edman degradation.

Localization of Photolabeled Amino Acid Residues in Peptides A_2 and A_3. As peptide A_2 (Pro-204–Val-297) contains six methionyl residues, an obvious approach to localize the photolabeled amino acid residues was fragmentation by CNBr treatment. As well as proceeding to near completion, this approach has the advantage to generate a C-terminal homoserine lactone that can be used for peptide attachment for amino acid sequencing. The CNBr cleavage products of peptide A_2 were applied to a Bio-Gel P30 column in 70% formic acid, and the column was eluted with the same solvent. Radioactivity was recovered in two peaks (Figure 6): Peak 2 contained a small-size peptide referred to as CB_2, while peak 1 comprised a small proportion of CB_2 and aggregates of undetermined fragments. Peptide CB_2 was sequenced after attachment to 3-aminopropyl (aminoethyl) glass beads using the homoserine lactone procedure (cf. Experimental Procedures). The N-terminal residue of CB_2 was found to be Tyr-250, and its C-terminal residue was Met-281.

Figure 7A shows the amount of radioactivity released at each step of the Edman degradation of the photolabeled peptide CB_2 and the nature of the corresponding amino acid residues. The shape of the curve indicated a steady fractional release of radioactivity at each step of the sequence analysis, over which two discrete peaks of radioactivity were clearly observed at steps 5 and 10. The unmodified amino acids released at steps 5 and 10 were identified as Val and Lys by TLC (cf. Experimental Procedures). It was therefore concluded that photolabeling by 2-azido[α-^{32}P]ADP was at the level of Val-254 and Lys-259. In the insert of Figure 7A is shown the histogram of distribution of released ^{32}P radioactivity after deduction of the background radioactivity and correction for the yield of recovery at each step of the Edman degradation estimated to be 95% (Hoppe et al., 1986). Two discrete regions of CB_2 around Val-254 and Lys-259 are photolabeled. The same labeling pattern of CB_2 was obtained from two independent photolabeling experiments.

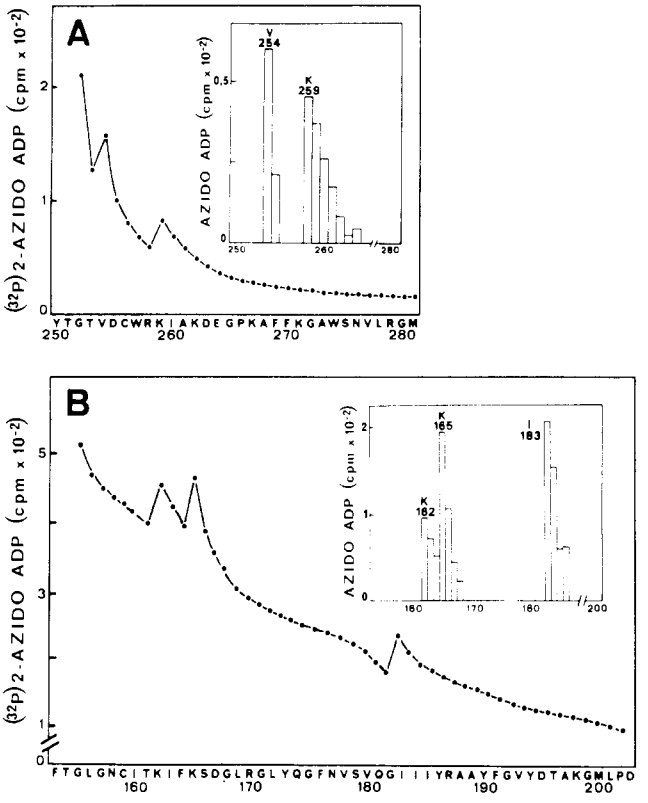

FIGURE 7: Edman degradation of peptide CB_2 (A) and peptide A_3 (B) covalently labeled by 2-azido[α-^{32}P]ADP. The peptides were immobilized and sequenced in an automated solid-phase sequenator as described under Experimental Procedures. Sixty percent of the released radioactivity was recovered in the first two cycles of the Edman degradation for peptide CB_2 and 15% for peptide A_3. Two peaks of radioactivity emerged from the background of radioactivity, corresponding to cycles 5 and 10 for the Edman degradation of peptide CB_2. Three peaks of radioactivity corresponding to cycles 10, 13, and 31 were found for A_3. The unmodified PTH amino acids released at each cycle were identified by thin-layer chromatography. The peaks of radioactivity were found to correspond to V-254 and K-259 in CB_2 and K-162, K-165, and I-183 in A_3. In the inserts are shown the histograms of distribution of ^{32}P radioactivity peaks after deduction of background radioactivity and correction for a repetitive yield of 95% for each cycle of the Edman degradation (Hoppe et al., 1986). The same profiles of release of radioactivity were obtained in two independent photolabeling experiments.

For sequence analysis, the photolabeled peptide A_3 (Phe-153–Asp-203) was demaleylated and attached to DITC glass beads via its NH_2 groups. As for peptide CB_2, the shape of the radioactivity curve (Figure 7B) was indicative of nonspecific release of covalently bound 2-azido[α-^{32}P]ADP. Three radioactive peaks emerged from the background radioactivity at positions 10, 13, and 31 (Figure 7B, insert). At these steps of the sequencing, PTH corresponding to unmodified Lys-162, Lys-165, and Ile-183 was identified by TLC. The release of the two lysine residues could not be differentiated from the release of peptides attached to DITC glass beads via Lys-162 and Lys-165. Therefore, a different strategy of coupling was used. As peptide A_3 contained a single methionine residue, Met-200, its treatment by CNBr was expected to yield the labeled peptide Phe-153–Hse-200 and the unlabeled tripeptide Leu-201–Pro-202–Asp-203. So, coupling by the Hse-200 of peptide Phe-153–Hse-200 to 3-aminopropyl (aminoethyl) glass

beads was carried out. Although the yield of coupling was low for undetermined reasons, the two lysine residues Lys-162 and Lys-165 as well as Ile-183 were effectively recovered with the attached 2-azido[α-^{32}P]ADP, corroborating the results of sequence analysis after attachment to DITC beads mentioned above. This experiment was repeated twice with similar results.

It is noteworthy that a large percentage of bound radioactivity was released during the first two cycles of the Edman degradation, predominantly at the first one (cf. legend of Figure 7). This released radioactivity might be due to loosely attached peptides. Alternatively, unstable photolabeled amino acid derivatives might have decomposed during the first cycle of the Edman degradation. It should also be recalled that about half of the peptides attached to the beads are not sequenced. Therefore, the possibility cannot be excluded that the steady release of bound radioactivity observed throughout the Edman degradation might be due to acid hydrolysis of the probe attached to the nonsequenced peptide.

Despite the limitations peculiar to the technique used, it can be safely concluded that two regions of the ADP/ATP carrier are labeled by 2-azido[α-^{32}P]ADP. The first one extends from Phe-153 to Met-200, with the probe predominantly attached to Lys-162, Lys-165, and Ile-183. The second region spans residues Tyr-250–Met-281, with Val-254 and Lys-259 being predominantly labeled.

Discussion

Reversible Binding Asymmetry of 2-Azido-ADP to Mitochondrial Membranes. In the absence of photoirradiation, 2-azido-ADP binds reversibly to intact heart mitochondria; the curvilinear Scatchard plots of the binding data were fitted to a two-site model. A high-affinity binding site with a K_d of 20 μM was calculated, the curvilinearity of the plots being explained by a second site of low affinity or by negative interactions between sites. Specific binding of 2-azido-ADP was demonstrated by its displacement by CATR and its ability to reversibly inhibit ADP transport.

An interesting finding was the inability of 2-azido-ADP to bind to inverted submitochondrial particles, demonstrating that the specificity of the matrix face of the carrier is more strict than that of the outer face. A possible artifact inherent in the particles used was excluded, as the same preparation of submitochondrial particles was able to bind ADP with high affinity (Table I). The binding asymmetry of 2-azido-ADP, i.e., recognition of and interaction with the cytoplasmic face of the ADP/ATP carrier but not with the matrix face, is not unique. In screening experiments, wherein a number of nucleotides were assayed for their ability to bind to the ADP/ATP carrier and to be transported in mitochondria and inverted submitochondrial particles (Block & Vignais, 1984), an analogue of ATP, γ-AmNS-ATP, was found to exhibit properties very similar to those found for 2-azido-ADP, namely, absence of transport, curvilinear Scatchard plots for binding to mitochondria, and no apparent binding to inverted submitochondrial particles.

In brief, the present work on the reversible binding of 2-azido-ADP corroborates the previous statement that nucleotides fall into three classes, depending on their binding characteristics and their ability to be transported by the mitochondrial carrier (Block & Vignais, 1984): (1) nucleotides that bind and are transported, for example, the natural nucleotides ADP and ATP, for which Scatchard plots of the binding data are curvilinear; (2) nontransportable nucleotides that bind with typical rectilinear Scatchard plots, for example, naphthoyl-ADP(ATP) and 8-bromo-ADP(ATP); and (3) nontransportable nucleotides that bind with curvilinear Scatchard plots, for example, γ-AmNS-ATP and 2-azido-ADP. As previously discussed, in the case of 2-azido-ADP and γ-AmNS-ATP, in spite of binding properties similar to those of ADP and ATP, the transport is not achieved; conceivably, the overall conformational change assumed by the carrier upon binding of γ-AmNS-ATP or 2-azido-ADP is not equivalent to that induced by ADP or ATP binding and is therefore unable to initiate transport.

ADP Binding Sites of the Carrier Protein Compared to the Substrate Site of Nucleotide-Binding Proteins. A number of proteins capable of binding mono- and dinucleotides have in common a highly flexible glycine-rich sequence that is supposed to interact with the phosphate groups of bound nucleotides (Möller & Amons, 1985). This consensus sequence is not present in the ADP/ATP carrier protein.

A weak homology was reported to exist between the sequence Ser-275–Val-297 of the ADP/ATP carrier protein and other nucleotide-binding proteins like the β subunit of mitochondrial F_1-ATPase, adenylate kinase, and phosphofructokinase (Walker et al., 1982). This weakly homologous sequence of the ADP/ATP carrier does not coincide, however, with the two sequences photolabeled by 2-azido[α-^{32}P]ADP.

The uniqueness of the nucleotide-binding site of the ADP/ATP carrier, compared to that of other nucleotide-binding proteins, may reside in the function itself of the protein. In fact, whereas the ADP/ATP carrier transports in a specific manner free ADP and ATP (and not the metal-complexed forms of these nucleotides) (Brandolin et al., 1981) and delivers the nucleotide, unmodified, to the other side of the mitochondrial membrane, the mononucleotide-binding proteins are in general able to bind the Mg^{2+}-complexed form of ATP or GTP for further hydrolysis, a function that could be related to interaction of their glycine-rich sequence with the phosphate chain of nucleotides (Möller & Amons, 1985).

The identification of two segments of the ADP/ATP carrier photolabeled by 2-azido-ADP described in the present work can be added to two other strategic loci previously identified by our group, namely, the ATR binding site (Boulay et al., 1983) and the Cys-56 residue that reacts with permeant alkylating reagents when the carrier is in the BA conformation (Boulay & Vignais, 1984). These data are assembled in Figure 8A. In the same figure is given a possible arrangement of the polypeptide chain of the ADP/ATP carrier, based on the hydropathy plots reported by Runswick et al. (1987) and on our experimental results (Figure 8B). This model includes five α helices of about 25 amino acid residues that are postulated to cross the mitochondrial membrane and three extramembrane loops that extend from amino acids 28 to 105, 137 to 170, and 234 to 266. In heart mitochondria, residue Cys-56 reacts only with hydrophobic, permeant SH reagents (Boulay & Vignais, 1984). It is therefore suggested that Cys-56 is exposed to the matrix face of the mitochondrial membrane. Cys-56 belongs to the loop 28–105, which comprises the hydrophobic segment 64–90, sufficient in length to cross the membrane. Yet, as mentioned by Saraste and Walker (1982), this segment contains several acid and basic residues, and for this segment to be buried in the membrane, charge neutralization by ion pairing would be required.

Finally, the prediction that the N terminus of the ADP/ATP carrier protein is exposed to the cytoplasmic face of the mitochondrial membrane is derived from results of recent experiments from this laboratory utilizing an immunochemical approach (manuscript in preparation).

Three other sets of topographical data from literature deserve comments. The first one concerns the labeling of the

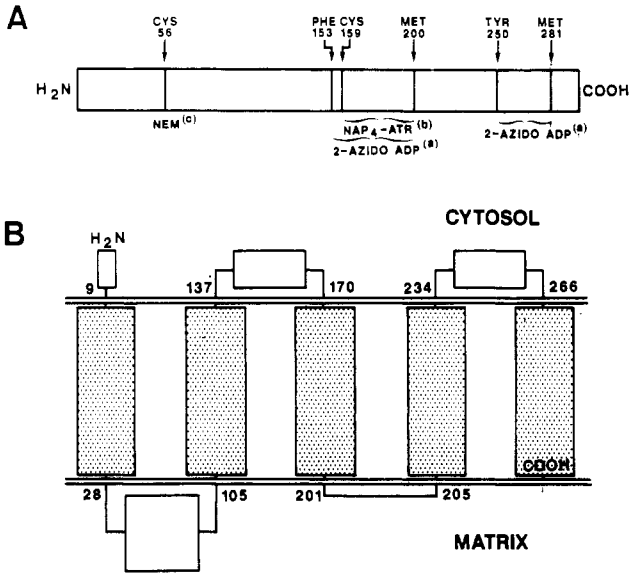

FIGURE 8: (A) Scheme illustrating the localization of strategic regions in the beef heart ADP/ATP carrier molecule. Data from (a) this paper, (b) Boulay et al. (1983), and (c) Boulay and Vignais (1984). (B) Possible arrangement of the peptide chain of the beef heart ADP/ATP carrier in the mitochondrial membrane. The membrane spanning α helices are represented by dotted boxes and the intervening extramembrane segments by open boxes.

ADP/ATP carrier in beef heart mitochondria with eosin-5-maleimide (Müller et al., 1984). Cys-256 was reported (without experimental details) to be the preferred labeled cysteinyl residue, and the binding site for eosin-5-maleimide was postulated to overlap the CATR binding site on the cytoplasmic side of the carrier. This conclusion is in accordance with the scheme of Figure 8B. From another study (Torok & Joshi, 1985), it was concluded that, upon reaction of the ADP/ATP carrier with copper *O*-phenanthroline, an intramolecular disulfide bridge was formed; this bridge was localized between Cys-159 and Cys-256, which is consistent with the arrangement of the peptide chain illustrated in Figure 8B. The third set of data concerns chemical modification of lysine residues of the ADP/ATP carrier in mitochondria and inverted submitochondrial particles by radiolabeled pyridoxal phosphate in the presence of sodium borohydride (Bogner et al., 1986). It was concluded that Lys-42, Lys-48, and Lys-146 were oriented to the matrix face of the carrier, whereas Lys-198 and Lys-205 were exposed to the cytoplasmic face. Scrutiny of the labeling data indeed indicated that Lys-42 and Lys-48 of the carrier in inverted submitochondrial particles were significantly labeled by tritiated pyridoxal phosphate, a result in accordance with the model proposed here in Figure 8B. However, the assigned orientations for Lys-22, Lys-146, Lys-198, and Lys-205 were questionable, due to the very small difference between the amount of bound pyridoxal phosphate in mitochondria and inverted submitochondrial particles. Furthermore, in the model proposed by Bogner et al. (1986), the segment 234–265 is shown to traverse the phospholipid bilayer in spite of its hydrophilic nature (Saraste & Walker, 1982).

Photolabeling of the ADP/ATP Carrier by 2-Azido-ADP vs Photolabeling by Azido-ATR. The evidence presented here, based on photolabeling of the mitochondrial ADP/ATP carrier by 2-azido[α-^{32}P]ADP in beef heart mitochondria, identifies two contact points between the photoprobe and the carrier protein, which correspond to sequences Phe-153–Met-200 and Tyr-250–Met-281, respectively. As 2-azido-ADP was not transported into mitochondria, it is clear that the two regions of the carrier molecule were accessible to the photoprobe from the outside.

ATR is a nonpermeant competitive inhibitor. When added to mitochondria, it binds to the cytosolic face of the ADP/ATP carrier and inhibits ADP/ATP transport by trapping the carrier molecules in the CATR conformation [for review cf. Vignais et al. (1985)]. Photolabeling assays performed with radiolabeled derivatives of arylazido-ATR, namely, azidobenzoyl-ATR and NAP_4-ATR, indicated that both photoderivatives of ATR bind to a fragment of the carrier protein spanning Cys-159–Met-200 (Boulay et al., 1983). This fragment corresponds to the central part of the sequence of the carrier molecule. One of the two regions of the carrier photolabeled with 2-azido[α-^{32}P]ADP was the same as that photolabeled by azido-ATR, a result consistent with the competitive inhibition of ADP transport by ATR.

The second photolabeled region spanning Tyr-250–Met-281, located close to the C-terminal end of the carrier molecule, is intriguing. The two photolabeled sequences might be present in the same peptide chain lining a crevice, which would correspond to the binding site. Photolabeling of two peptide segments by an azido group in position 2 of the purine ring of ADP supposes a loose adjustment and thereby a significant mobility of the purine ring of the nucleotide in the binding site. Although plausible, this hypothesis does not take into account the presence of high- and low-affinity binding sites (or cooperative interactions between sites) in the ADP/ATP carrier. The presence of two binding sites of high and low affinities in the same monomer is definitely excluded on the basis of binding data (Brandolin et al., 1982; Block & Vignais, 1984). The remaining alternative is based on the postulate that a functional ADP/ATP carrier consists of several protomers. As summarized in the first section under Discussion, 2-azido-ADP belongs to that class of nontransportable nucleotides which bind to the cytoplasmic sites of the ADP/ATP carrier with negative cooperativity. The different conformations assumed by two interacting protomers of a carrier oligomer upon binding of 2-azido-ADP implicitly include different arrangements of the peptide chain at the two cytoplasmic binding sites. It is therefore conceivable that upon photoirradiation the azido group of the photoprobe might label covalently two distinct regions in the two protomers.

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Structure Elucidation of the Blood Group B Like and Blood Group I Active Octaantennary Ceramide Tetracontasaccharide from Rabbit Erythrocyte Membranes by Two-Dimensional ^{1}H NMR Spectroscopy at 600 MHz†

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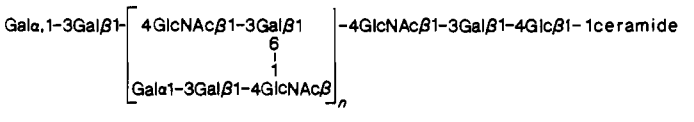
ABSTRACT: The primary structure of the ceramide tetracontasaccharide (**1**) from rabbit erythrocyte membranes has been determined with the aid of 600-MHz two-dimensional phase-sensitive correlated, "totally correlated" (TOCSY, homonuclear Hartmann–Hahn), relayed coherence transfer, triple quantum filtered, and nuclear Overhauser enhancement ^{1}H NMR spectra. It was shown that obtaining subspectra of the constituent sugar residues from a totally correlated spectrum and assigning the resonances occurring in these subspectra by analyzing the relevant cross-peaks in phase-sensitive correlated spectra is the most efficient way for establishing complex oligosaccharide structures. This analysis has shown **1** to be the highest homologue of the multiantennary neolactoglycosphingolipids of the following general formula with $n = 7$:



In previous papers from our laboratories the isolation, structure determination, and properties of the biantennary (Hanfland et al., 1981), triantennary (Dabrowski et al., 1984), and tetra- and pentaantennary (Hanfland et al., 1988) neolactoglycosphingolipids of the general formula given under Abstract have been described. It was pointed out that blood group I activity of these compounds increases along with increasing branching of their oligosaccharide skeleton. Recently, we reported on the isolation of hexa-, hepta-, and octaan-

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