Photolabeling Approach to the Study of the Topography of the Atractyloside Binding Site in Mitochondrial Adenosine 5′-Diphosphate/Adenosine 5′-Triphosphate Carrier Protein†

François Boulay,* Guy J. M. Lauquin, Akira Tsugita, and Pierre V. Vignais

ABSTRACT: The binding site of atractyloside (ATR), an impermeant inhibitor of the mitochondrial ADP/ATP carrier, has been investigated by the photolabeling technique. The photolabels used were long- and short-arm radioactive derivatives of ATR, namely, 6′-*O*-[3-[*N*-(4-azido-2-nitrophenyl)-amino]propionyl]atractyloside, 6′-*O*-[4-[*N*-(4-azido-2-nitrophenyl)amino]butyryl]atractyloside, and 6′-*O*-[*p*-azidobenzoyl)atractyloside. The photolabeling step was carried out on beef heart mitochondria. After the photolabeling, the covalently photolabeled ADP/ATP carrier protein was extracted by Triton X-100 and further purified by hydroxylapatite chromatography, acetone precipitation, and washing with a mixture of formic acid, ethanol, and ether. The peptide chain was cleaved at methionine and cysteine residues by specific chemical reagents. Cleavage of the carrier protein (M_r 32 000) at the methionine residues by cyanogen bromide yielded a large, 23 000-dalton segment, called CB1, that was radiolabeled and a number of unlabeled small fragments. Cleavage at cysteinyl residues by cyanide at alkaline pH involved the prior reaction of sulfhydryl groups with 5,5′-dithiobis(2-nitrobenzoic acid). Cyanide cleavage of the CB1 fragment, which contains three cysteinyl residues, resulted in the accumulation of a number of overlapping peptides and four nonoverlapping peptides, these latter being referred to as CN peptides. With both the long- and short-arm azido-ATR derivatives used, essentially only one of the CN peptides, with a M_r of about 4500, was found to be photolabeled; this peptide is situated at the C terminus of the CB1 fragment between cysteine-159 and methionine-200, according to the recently reported amino acid sequence of the ADP/ATP carrier protein [Aquila, H., Misra, D., Eulitz, M., & Klingenberg, M. (1982) *Hoppe-Seyler's Z. Physiol. Chem. 363*, 345–349]; the carrier protein contains 297 amino acid residues. These findings indicate that the ATR site in the membrane-bound ADP/ATP carrier protein is located near the center of the carrier molecule and suggest that this region is exposed to the cytosolic side of the inner mitochondrial membrane.

The ADP/ATP carrier protein is an intrinsic protein of the inner mitochondrial membrane, which most likely spans the width of the membrane. Its minimal molecular weight, on the basis of the recently reported amino acid sequence (Aquila et al., 1982), is close to 32 000. The region of the carrier protein that is exposed to the outside of the inner membrane in intact mitochondria can recognize and bind not only the substrates ADP and ATP but also the specific inhibitors atractyloside (ATR)[1] and carboxyatractyloside (CATR) (Vignais, 1976). Advantage can be taken of these binding features to explore the topography of the ATR and nucleotide binding site(s) in photolabeling experiments by using appropriate photoactivable derivatives of ATR and ADP or ATP. In this respect, azido derivatives of ATR and ADP were previously prepared, and their specific binding to the membrane-bound ADP/ATP carrier protein was assessed in mitochondria isolated from different species (Lauquin et al., 1976, 1978; Brandolin et al., 1979). An attempt to localize the ATR-binding site in the ADP/ATP carrier of beef heart mitochondria was previously reported (Boulay et al., 1979); this approach was based on photolabeling of beef heart mitochondria with an azido derivative of ATR and the subsequent cleavage of the isolated photolabeled carrier protein by cyanogen bromide at the peptide bonds of methionyl residues. Due to the fact that all of the seven methionyl residues in the carrier protein are localized close to the carboxyl end of the molecule, the CNBr cleavage resulted in the formation of a large peptide of M_r 23 000, called CB1, and a number of smaller peptides. The covalently bound azido-ATR was attached to the large CB1 fragment. Complementary experiments carried out with an azido derivative of ADP yielded similar results. Although these data did not allow a precise localization of the ATR site and the ADP/ATP site on the carrier protein, they showed convincingly that the photolabeling approach was a valid method to map the ATR site in the ADP carrier. Since there are four cysteinyl residues in the carrier protein, three of them being distributed along the CB1 fragment (Boulay et al., 1979), the possibility was envisaged to locate with a better resolution the ATR site by cleavage at cysteinyl residues. Here, we describe the fragmentation of the CB1 fragment by cyanylation of cysteinyl residues followed by alkaline treatment and the characterization of a photolabeled peptide of small size.

Experimental Procedures

Chemicals and Enzymes. Atractyloside and DTNB were obtained from Sigma Chemical Co. Guanidinium chloride, trifluoroacetic acid, and succinic anhydride were from Pierce; concentrated hydrochloric acid was from Fluka, acrylamide and bis(acrylamide) were from Eastman Kodak, Sephadex G-50 fine was from Pharmacia, Bio-Gel P-10 was from Bio-

† From the Laboratoire de Biochimie (CNRS/ERA 903 et INSERM U.191), Département de Recherche Fondamentale, Centre d'Etudes Nucléaires de Grenoble, France (F.B., G.J.M.L., and P.V.V.), and the European Molecular Biology Laboratory, 6900 Heidelberg, Federal Republic of Germany (A.T.). *Received August 6, 1982.* This work was supported in part by research grants from the Fondation pour la Recherche Médicale.

[1] Abbreviations: TPCK-trypsin, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin; $NaDodSO_4$, sodium dodecyl sulfate; ATR, atractyloside; CATR, carboxyatractyloside; DTNB, Ellman's reagent or 5,5′-dithiobis(2-nitrobenzoic acid); NAP_4, *N*-(4-azido-2-nitrophenyl)aminobutyric acid; NAP_3, *N*-(4-azido-2-nitrophenyl)aminopropionic acid; NAP_3-ATR, 6′-*O*-[3-[*N*-(4-azido-2-nitrophenyl)amino]-propionyl]atractyloside; NAP_4-ATR, 6′-*O*-[4-[*N*-(4-azido-2-nitrophenyl)amino]butyryl]atractyloside; Mops, 3-(*N*-morpholino)propanesulfonic acid; azidobenzoyl-ATR, 6′-*O*-(*p*-azidobenzoyl)atractyloside; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid; PPO, 2,5-diphenyloxazole; SH1–4, peptides obtained by cleavage at cysteinyl residues 1–4.

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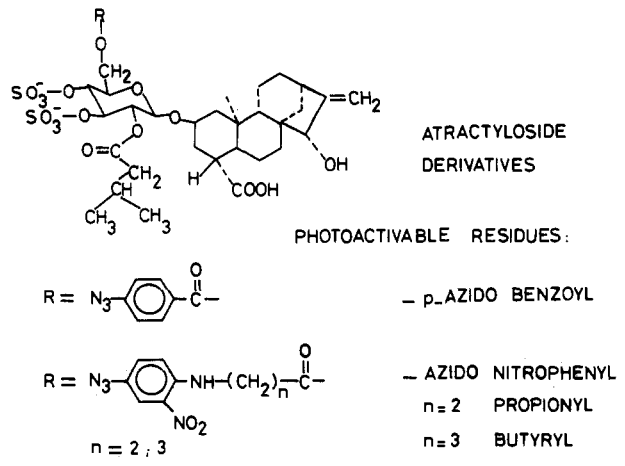

FIGURE 1: Atractyloside photolabels used to investigate the ATR binding site of the carrier protein. In *p*-azido[^{14}C]benzoyl-ATR, ^{14}C was located in the carboxyl group of benzoic acid. In the (azidonitrophenyl)propionyl-ATR (NAP_3-ATR) and the (azidonitrophenyl)butyryl-ATR (NAP_4-ATR), ^{3}H was located in the propionyl and butyryl arms. In all cases, the radioactive atoms were close to the photoreactive azido group (see Experimental Procedures). After covalent photolabeling of the carrier protein, the ester bond between the ATR residue and the rest of the photolabel (R–O–CH_2) was hydrolyzed at alkaline pH; the ATR residue was released, and the radioactive moiety of the photolabel remained attached to the protein.

Rad, and Coomassie blue R250 was from Merck. β-[2,3-$^3H_2$]Alanine (30 Ci/mmol) was from Amersham; 4-amino-[2,3-$^3H_2$]butyric acid (40 Ci/mmol), *p*-amino[^{14}C]benzoic acid (49 mCi/mmol), and Na^{14}CN (30–50 mCi/mmol) were from the Commissariat à l'Energie Atomique, Saclay, France. TPCK-trypsin was from Worthington, carboxypeptidases A and B were from Worthington, and carboxypeptidase P was from Takara-Shuzo (Japan). All reagents used were of the purest grade commercially available.

Synthesis of Radiolabeled Azido Derivatives of ATR. In the short- and long-arm azido-ATR derivatives described hereafter, the ^{3}H and ^{14}C atoms were located close to the photoreactive azido group, at distance from the ATR residue (Figure 1). The position of the radioactive atom on the photolabel, near the azido group, is quite advantageous, since this location allowed the removal, by alkaline hydrolysis, of the bulky ATR residue from the photolabeled carrier protein, without any loss of the covalent radiolabeling. This is illustrated under Results.

The short-arm azido-ATR used here was the UV-photoactivable azido[^{14}C]benzoyl-ATR. The preparation started with the synthesis of *p*-azido[^{14}C]benzoic acid from *p*-amino[^{14}C]benzoic acid (Hixson & Hixson, 1975). Coupling to ATR was carried out after activation of the carboxylic group of the *p*-azido[^{14}C]benzoic acid with *N,N'*-carbonyldiimidazole as described by Gottikh et al. (1970). Purification of azido-[^{14}C]benzoyl-ATR was performed with HPLC on a μBondapak C_{18} column (7.8 × 300 mm, 10 μm) with isocratic elution at a flow rate of 2 mL/min at room temperature. The solvent system used was methanol/1 M ammonium acetate/acetic acid/water (58:1:1:40 v/v). The product showed a retention time of 25 min. Its identification by mass spectrometry and its biological properties have been reported (Boulay et al., 1982).

[^{3}H]NAP_3-ATR and [^{3}H]NAP_4-ATR were long-arm derivatives of ATR, photoactivable by visible light. The initial preparation described by Lauquin et al. (1976) was refined in that the starting materials, namely, NAP_3 and NAP_4, and the final products, NAP_3-ATR and NAP_4-ATR, were purified by HPLC. In brief, 4-amino[^{3}H]butyric acid or β-[^{3}H]alanine in aqueous solution were diluted with the respective unlabeled compound to a specific radioactivity of about 10^9 dpm/μmol. To 100 μL containing 100 μmol of either ^{3}H-labeled compound was added an equimolar amount of (4-fluoro-3-nitrophenyl)azide in 400 μL of dimethyl sulfoxide. The mixture was made alkaline with 50 μL of triethylamine and stirred overnight at 60 °C (Levy, 1973). After acidification with 50 μL of acetic acid, the reaction products, i.e., NAP_3 or NAP_4, were separated by isocratic elution at a flow rate of 2 mL/min from a μBondapak C_{18} column (300 × 7.8 mm, 10 μm) at room temperature. The solvent system was methanol/acetic acid/water (50:2:48 v/v). The retention times for NAP_3 and NAP_4 were 36 and 49 min, respectively. Alternatively, NAP_3 and NAP_4 could be purified by descending chromatography on Whatman No. 3 filter paper, with a solvent system made of ethanol/butanol/1 M ammonium acetate/NH_4OH (40:20:40:1 v/v). The R_f values were 0.7 and 0.8 for NAP_3 and NAP_4, respectively. The coupling step consisting of esterification of the primary alcohol of the glucose moiety of ATR by the carboxylic groups of NAP_3 and NAP_4 was carried out in dimethylformamide previously dried over a 3-Å molecular sieve and calcium hydride after activation of the carboxyl groups by *N,N'*-carbonyldiimidazole as described by Gottikh et al. (1970). After being stirred in the dark for 15 min at room temperature, a mixture of ATR and triethylamine (1:1 mol/mol) (3-fold excess) was added dropwise within 2 min, and stirring was continued for 30 min. A longer contact did not improve the yield of coupling. The mixture was then evaporated in vacuo below 40 °C. The gummy residue was dispersed in 1 mL of distilled water and evaporated to dryness to remove the residual dimethylformamide. This procedure was repeated at least 3 times. The unreacted [^{3}H]NAP_3 or [^{3}H]NAP_4 was removed by several washings of the dry residue with chloroform. Finally, the residue was dissolved in 1 mL of methanol, and the solution was subjected to HPLC on a μBondapak C_{18} column. The isocratic elution was performed in methanol/1 M ammonium acetate/acetic acid/water (60:1:1:38 v/v) at room temperature. The retention times for [^{3}H]NAP_3-ATR and [^{3}H]NAP_4-ATR were 28 and 40 min, respectively. The final yield of recovery was around 15%. Purity was checked by TLC with a solvent system made of chloroform/methanol/acetic acid/water (55:20:2:1 v/v). The R_f value of NAP_3-ATR was 0.43, and that of NAP_4-ATR was 0.46. The products could be stored in methanol at −20 °C for several months without significant decomposition.

Polyacrylamide Gel Electrophoresis. Overnight electrophoretic separation of peptides was performed in 20% acrylamide slab gels following the method described by Cabral & Schatz (1979) for resolution of peptides with molecular weights ranging from 20 000 to 3000. Rapid electrophoresis was performed under 40 mA for 4 h, in buffers prepared according to Laemmli (1970); the acrylamide composition remained the same as above. The lyophilized peptide samples were dissolved first in 8 M urea (40 μL for 20–30 μg of peptides), followed by another addition of 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- and ^{3}H-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.

Preparation of Beef Heart ADP/ATP Carrier Protein. The carrier protein was purified from beef heart mitochondria as

described by Riccio et al. (1975), with some modifications including washing with organic solvent to remove bound lipids. Routinely, 3 g of beef heart mitochondria was suspended in 400 mL of 0.12 M KCl, 1 mM EDTA, and 5 mM Mops, pH 6.8, with 20 mg of CATR. The suspension was left to stand for 10 min at room temperature. It was then centrifuged at 20000*g* for 15 min to sediment the mitochondria. The pellet was dispersed in 4% Triton X-100, 0.5 M NaCl, 1 mM EDTA, and 10 mM Mops, pH 7.2, and left to stand at 4 °C for 30 min. The lysate was centrifuged at 100000*g* for 30 min. The supernatant was recovered and chromatographed on a refrigerated column of hydroxylapatite (50 cm × 5 cm). The pass-through fraction (70–80 mg of protein) was concentrated and dialyzed on a PM-10 Amicon membrane. The concentrated solution (about 50 mL) was treated with 5 volumes of acetone at −20 °C overnight. The resulting precipitate was recovered by centrifugation and washed with 50 mL of acetone at −20 °C to remove the residual Triton X-100, and then the pellet was dissolved in 4 mL of 80% formic acid at 25 °C. After solubilization, 12 mL of ethanol was added with 50 μL of 5 M NaCl, followed by addition of 50 mL of diethyl ether, and the temperature was brought down to −20 °C to precipitate the protein. The washing step with diethyl ether was repeated twice, and the protein was freeze-dried.

Covalent Photolabeling. When the NAP derivatives of ATR were used, photoirradiation was performed with a 250-W Osram halogen reflector lamp. The ^{3}H-labeled NAP derivatives of ATR (40 nmol) were preincubated with the suspension of mitochondria (20 mg of protein) for 30 min in the dark in a standard medium consisting of 120 mM KCl, 5 mM Mops, and 1 mM EDTA, pH 6.8; the final volume was 10 mL. The mixture was introduced in a 50-mL flask that was rotated horizontally in an ice bath at a mean distance of 10 cm from the lamp; photoirradiation was applied for 30 min. After irradiation, the mitochondria were sedimented by centrifugation for 10 min at 20000*g* and washed once with 10 mL of the same medium as above. The photolabeled ADP/ATP carrier protein was then extracted and purified. On the other hand, photolabeling by azido[^{14}C]benzoyl-ATR required UV light that was provided by a 15-W Phillips UV germicidal lamp under an atmosphere of argon. The same standard medium as above was used; however, instead of being introduced in a flask, the mixture of photolabel and mitochondria was placed in a beaker with stirring, and a stream of argon was passed over the surface of the suspension.

Thionitrobenzoylation and Succinylation. The beef heart ADP/ATP carrier protein contains four sulfhydryl groups (Klingenberg et al., 1978; Boulay et al., 1979), which are accessible to and can be modified with DTNB according to the following procedure. The protein (8 mg) 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 for full reduction. A 6-fold excess of DTNB with respect to the total amount of sulfhydryl groups was added. The pH was maintained at 7.2 by dropwise addition of 0.1 N NaOH, and the mixture was left to incubate at 37 °C for 30 min.

The thionitrobenzoylated 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 large excess with respect to the free NH_2 groups of the protein (1000-fold) excess); the pH was carefully 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. The excess of free sodium succinate thus formed was removed by passing the protein solution through a column of Ultrogel ACA 202 (2.5 × 10 cm) equilibrated in 50 mM ammonium bicarbonate, pH 7.8.

In the case of the covalently photolabeled carrier protein, the protein was treated with 7 M guanidinium chloride, 1 mM EDTA, and 0.1 M NaOH at 45 °C for 15 min, after photolabeling and prior to succinylation, in order to hydrolyze the ester bond that links the ATR moiety to the remaining ^{3}H or ^{14}C portion of the photolabel. By this procedure, the bulky charged residue of ATR was therefore removed from the labeled protein, and the protein remained tagged, with [^{3}H]NAP_4, [^{3}H]NAP_3, or [^{14}C]benzoic acid. These small-size residues, in contrast to the whole molecule of azido-ATR, introduced negligible perturbation in protein migration by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Cyanogen Bromide Cleavage. Cleavage of the peptide bonds in the TNB-modified and succinylated carrier protein at the methionyl residues was performed in 70% formic acid, with a 500-fold excess of cyanogen bromide with respect to methionine (Gross, 1967). Tryptamine at the final concentration of 1 mM was added to protect tryptophan against oxidation. A large peptide fragment of M_r 23 000 (CB1) together with smaller peptides were found to accumulate. The TNB-modified and succinylated CB1 fragment was isolated by gel filtration in 50 mM ammonium bicarbonate, pH 7.8, on a column of Sephadex G-50 (1.5 × 90 cm).

Cyanylation of Sulfhydryl Groups, Followed by Alkaline Cleavage. Complete cyanylation of sulfhydryl groups in protein can be achieved under denaturing conditions either with 2-nitro-5-(thiocyano)benzoic acid (Jacobson et al., 1973) or by a two-step procedure with DTNB and NaCN (Vanaman & Stark, 1970). In the present work, the latter procedure offered the advantage of protecting sulfhydryl groups during succinylation and cleavage by cyanogen bromide. On the other hand, the use of Na^{14}CN resulted in labeling of cysteinyl residues and allowed easy detection of the peptide fragments. For cyanylation, the lyophilized carrier protein or CB1 fragment (5–10 mg) was dissolved in 1 mL of 7 M guanidinium chloride, 1 mM EDTA, and 0.1 M sodium phosphate buffer, pH 7.2, and cyanylation was achieved by addition of 50 μL of 0.1 M Na^{14}CN (2.10^7 dpm/μmol). A yellow color developed rapidly. After 30 min at 35 °C, an excess of cold NaCN was added to ensure complete cyanylation of sulfhydryl groups, and the incubation was continued for another 30 min. The solution of cyanylated carrier protein or CB1 fragment was desalted by gel filtration on a 1 × 10 cm column of Ultrogel ACA 202 equilibrated in 0.015 M $NaHCO_3$/Na_2CO_3, pH 9.8, and it was left to incubate at 37 °C for 24 h. The mixture was then neutralized with 6 N HCl.

The cleavage products were further purified by gel filtration on a 60 × 1 cm column of Bio-Gel P-10 equilibrated with 50% formic acid. Cyanylation of the radiophotolabeled protein was carried out under the same conditions as above.

Tryptic Map of Photolabeled Carrier Protein. Prior to tryptic attack, the photolabeled carrier protein was citraconylated to facilitate solubilization. Citraconylation was carried out on protein samples of about 800 μg in 0.4% $NaDodSO_4$ and 200 mM *N*-ethylmorpholine, pH 8.5, at room temperature. To ensure full citraconylation, ten fractions of 3 μL of citraconic anhydride were added, the pH being returned to 8.5 after each addition. Sodium dodecyl sulfate was removed by precipitation with ethanol, and the pellet was washed twice with ethanol. The pellet was redissolved in 500 μL of 100 mM *N*-ethylmorpholine buffer, pH 7.5, and the solubilized protein

was subjected to tryptic digestion at 37 °C with TPCK-trypsin. A trypsin:protein ratio of 1:50 (w/w) was used for the first 16 h, followed by a second period of 6 h at a trypsin:protein ration at 1:25. After being freeze-dried, the protein digest was taken up in 500 μL of 40% acetic acid and let stand at 20 °C overnight for decitraconylation. After a further step of freeze drying, the protein was redissolved in 20 μL of electrophoresis buffer (see below), and the insoluble residue was removed by centrifugation in a Beckman airfuge. An aliquot of 5 μL of supernatant was spotted onto a 10 × 10 cm cellulose thin-layer plate (F 1440, Schleicher and Schüll) and subjected to electrophoresis at 20 V/cm for 1 h at 4 °C in pyridine/acetic acid/H_2O (100:3.5:900 v/v), pH 6.5. Ascending chromatography in butanol/pyridine/acetic acid/H_2O (30:20:6:24 v/v) was performed in the second dimension.

Amino Acid Composition and Determination of Carboxyl-Terminal Amino Acids. Purified peptides (about 5 μg) arising from cleavage of the ADP/ATP carrier protein were hydrolyzed by treatment with 200 μL of a mixture of concentrated HCl and trifluoroacetic acid (2:1 v/v) containing 0.005% phenol, at 166 °C for 25 or 50 min in evacuated sealed tubes (Tsugita & Scheffler, 1982). The amino acid analysis was performed with a Durrum D500 amino acid analyzer. The C-terminal sequence was determined by digestion with carboxypeptidase A or (and) B pretreated with diisopropyl fluorophosphate or with carboxypeptidase P. Peptides (2 nmol) were dissolved in 50 μL of 0.1 M pyridine–formate buffer, pH 2.5, for carboxypeptidase P and 0.1 M sodium bicarbonate buffer, pH 8.5, for the other carboxypeptidases. The amount of carboxypeptidase used was 5 μg, and the digestion was carried out for 3–12 h at 37 °C. The digests were dried and analyzed directly with the amino acid analyzer (Tsugita et al., 1979).

Results

Evidence That [^{3}H]NAP_4-ATR Binds to a Specific Region of ADP/ATP Carrier Protein. When the azido derivative of a substrate is made to react with the specific enzyme, the nitrene generated upon photoirradiation can potentially react with amino acid residue(s) at/or in the vicinity of the active site of the enzyme (Knowles, 1972). The experiment described hereafter shows that covalent photolabeling of the ADP/ATP carrier protein by NAP_4-ATR is restricted to a limited sequence of amino acids in the peptide chain of the carrier.

Heart mitochondria were photoirradiated in the presence of [^{3}H]NAP_4-ATR; the covalently photolabeled ADP/ATP carrier protein after extraction and purification was subjected to extensive trypsin digestion, and the tryptic fragments were separated on a cellulose plate as described under Experimental Procedures. The fluorographic analysis of the peptide map revealed a single radioactive spot (Figure 2). Ninhydrin staining showed no coincidence between the radioactive spot and the ninhydrin-positive peptides. The radioactive peptide was not contaminated with [^{3}H]NAP_4-ATR or [^{3}H]NAP_4, since these compounds were found to migrate to the front of the solvent in the chromatography step; most probably, the radioactive spot was a tryptic peptide of the carrier protein, specifically photolabeled with [^{3}H]NAP_4-ATR. Absence of coincidence between the [^{3}H]NAP_4-ATR labeled peptide and any of the ninhydrin peptide spots may be explained on the basis that the yield of photolabeling is low and that the photolabeled peptide has different electrophoretic and chromatographic mobilities, due to the electric charge and the bulky character of the bound NAP_4-ATR. The finding that the tryptic digest of the photolabeled carrier protein contained only one radioactive spot was consistent with the labeling of a restricted portion of the peptide chain of the carrier protein and therefore encouraged further investigations to characterize this peptide portion.

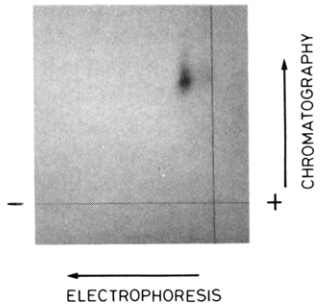

FIGURE 2: Autoradiography of the peptide map of a tryptic digest of the ADP/ATP carrier protein photolabeled by [^{3}H]NAP_4-ATR. Digestion by trypsin and separation of the tryptic peptides by electrophoresis followed by chromatography were as described under Experimental Procedures. The cellulose plate was sprayed with PPO in diethyl ether (20% w/v) and placed on a Fuji RX film at −70 °C for 2 months.

Cleavage of the ^{14}C Cyanylated ADP/ATP Carrier Protein and the CB1 Fragment. As shown by Vanaman & Stark (1970), the unsymmetrical disulfide made by condensation of a cysteinyl residue in a protein and the TNB moiety of DTNB readily reacts with cyanide to release TNB. This is followed at alkaline pH by cleavage of the peptide bond involving the amino group of the cysteinyl residue and the formation of an iminothiazolidin ring by condensation of the free amino and thiol groups of the cysteinyl residue with cyanide. Using [^{14}C]cyanide results in incorporation of ^{14}C radioactivity in the cysteinyl residue, which allows the localization of the cysteinyl residues in a polypeptide chain and the identification of the peptide fragments in which the N-terminal amino acid residue is a cysteine.

Cyanylation with Na^{14}CN was applied to both the ADP/ATP carrier protein (M_r 32 000 according to sequence data; Aquila et al., 1982) and the CB1 fragment (M_r 23 000) obtained from the carrier protein by cyanogen bromide cleavage [cf. Boulay et al. (1979)]. It may be recalled that all methionyl residues in the ADP/ATP carrier protein are located in the C-terminal region and that cyanogen bromide cleavage generates a number of small peptides and a large fragment CB1 terminated at the C terminus by homoserine (replacing methionine). Incubation at pH 9.8 of the ^{14}C cyanylated carrier protein and the CB1 fragment resulted in accumulation of a number of ^{14}C-labeled and unlabeled peptides that were separated by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 3). Coomassie blue stained both ^{14}C-labeled and unlabeled peptides (tracks 1 and 4, Figure 3). The ^{14}C-labeled peptides were revealed by autoradiography (tracks 2 and 3, Figure 3). The unlabeled peptides (difference between tracks 1 and 2 for the carrier protein and between tracks 3 and 4 for the CB1 fragment) corresponded to the shortest cysteinyl peptide fragment SH1, containing the N terminus of the carrier protein and CB1, and to overlapping (partially cleaved) cysteinyl peptides starting from the N terminus of both molecules. The M_r of SH1 was about 6000. Two of the overlapping cysteinyl peptides were shared by the carrier protein and CB1; they are SH2 ($M_r \simeq$ 14 000) and SH3 ($M_r \simeq$ 17 000). In the case of the carrier protein, a supplementary overlapping cysteinyl peptide, SH4 ($M_r \simeq$ 27 000), was detected.

Six main ^{14}C-labeled peptides arising from alkaline cleavage of the ^{14}C cyanylated carrier protein were revealed by autoradiography (track 2, Figure 3). They will be referred to as

Table I: Amino Acid Composition of CN Peptides of CB1 Segment

amino acid residues	CN1 peptide (AA128-158)		CN2 peptide (AA159-200)		CN3 peptide (AA1-55)		CN4 peptide (AA56-127)		CB1 segment (AA1-200)	
	nearest integer[a]	sequence data[b]	nearest integer[a]	sequence data[b]	nearest integer[a]	sequence data[b]	nearest integer[a]	sequence data[b]	nearest integer[a]	sequence data[b]
Asp	3	3	3	3	3	3	6	6	15	15
Thr	2	2	2	2	1–2	1	3	2	9	7
Ser	1	0	2	2	5	5	3	3	10	10
Glu	2	2	2	2	7	7	5	5	16	16
Pro	1	1	0	0	1	1	2	2	5	4
Gly	4	4	5	6	4–5	3	8	8	21	21
Ala	5	5	3	3	9	9	8	8	25	25
Val	2	2	3	3	4	4	3–4	4	15	13
Ile	0–1	0	3	5	4	5	3	3	11	13
Leu	3	3	2	2	6	6	6	6	17	17
Tyr	1	1	3	4	1	1	3	3	9	9
Phe	3	3	2	3	2	2	6	8	16	16
His	0	0	0	0	1	1	1	1	2	2
Lys[c]	nd	1	nd	3	nd	5	nd	5	nd	14
Arg	3	3	2	2	1	1	4	5	11	11
Met	0	0	Hse	1	0	0	0	0	Hse	1
Trp[d]	0	0	0	0	0	0	2	2	3	2
Cys[e]	1	1	1	1	0	0	1	1	3	3

[a] The nearest integer was based on the number of amino acid residues determined after 25 and 50 min of hydrolysis carried out as described by Tsugita & Scheffler (1982). Calculation was made on the basis of the M_r determined by gel electrophoresis and the number of leucyl residues from the sequence data (Aquila et al., 1982). [b] Number of amino acid residues obtained from the amino acid sequence as reported by Aquila et al. (1982). [c] Lysine was not expressed since the lysyl residues in the ADP/ATP carrier protein were succinylated. [d] Tryptophan analysis was made in 3 N mercaptoethanesulfonic acid (Penke et al., 1974) and adapted to a nanomolar scale (K. Maeda and A. Tsugita, unpublished results). [e] Cysteinyl residues of CB1 were carboxymethylated.

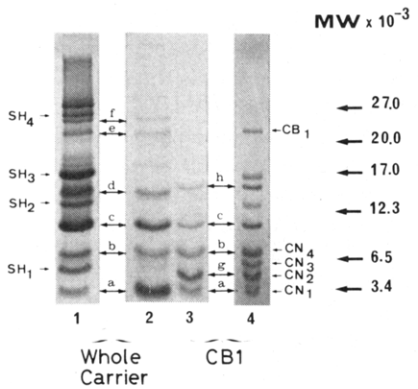

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of ^{14}C S-cyanylated peptides. The ADP/ATP carrier protein and the CB1 fragment were cyanylated with $Na^{14}CN$, and the peptide bonds at the level of cyanylated cysteinyl residues were cleaved at alkaline pH. The peptides were separated by gel electrophoresis as described by Cabral & Schatz (1979). All peptides arising by cleavage (radiolabeled and unlabeled) were stained by Coomassie blue (carrier protein, track 1, and CB1 fragment, track 4). The radiolabeled peptides were revealed by fluorography (carrier protein, track 2, and CB1 fragment, track 3). The following proteins were used for calibration: triosephosphate isomerase (M_r 27 000), trypsin inhibitor (M_r 20 000), myoglobin (M_r 17 000), cytochrome *c* (M_r 12 300), aprotinin (M_r 6500), and insulin B (M_r 3400). Unlabeled overlapping peptides carrying the N-terminal region of the carrier protein and the CB1 fragment were designated as SH1, SH2, SH3, and SH4. The Coomassie blue stained peptide from the carrier protein, designated by (d) was absent in the cyanylated products of the CB1 fragment. The peptides CN1, CN2, CN3, and CN4 are nonoverlapping peptides.

a–f, in order of increasing molecular weights. The same procedure of cyanylation performed on CB1 generated a similar labeling pattern for the small peptides a–c (track 3, Figure 3); the three large peptides d–f that accumulated after cleavage of the cyanylated carrier protein were not found in the cleavage products of CB1, which, on the other hand, contained two supplementary peptides, g and h, (track 3, Figure 3). Peptide g and its parent peptide, CB1, were both terminated by a homoserine residue (this residue was formed during cyanogen bromide cleavage of the carrier protein to give CB1). This indicates that peptide g is located in the C-terminal region of CB1 (see Table I).

Attention was paid to the small peptides obtained by alkaline cleavage, after cyanylation of CB1. These small peptides revealed by Coomassie blue staining (unlabeled and labeled) were referred to as CN1 ($M_r \simeq 3500$), CN2 ($M_r \simeq 4500$), CN3 ($M_r \simeq 6000$), and CN4 ($M_r \simeq 8000$); the ^{14}C-labeled ones were CN1, CN2, and CN4. The unlabeled one, CN3, was apparently identical with SH1. The ^{14}C-labeled peptides are those terminated by an imino[^{14}C]thiazolidine residue arising from cyclization of S-cyanylated [^{14}C]cysteine. Clearly, the peptides CN1, CN2, and CN4 stained by Coomassie blue are equivalent to the radiolabeled peptides a, g, and b revealed by autoradiography. The unlabeled peptide, CN3, was devoid of cysteinyl residue; thus it can be placed at the N terminus of the CB1 fragment. Higher molecular weight peptides obtained from CB1 were most likely overlapping fragments, in which one or even several cysteinyl peptide bonds remained uncleaved.

Localization of Covalently Bound [^3H]NAP_4-ATR in Cleavage Products of Photolabeled ADP/ATP Carrier Protein: Comparative Labeling Studies with [^3H]NAP_3-ATR and Azido[^{14}C]benzoyl-ATR. In preliminary experiments dealing with photolabeling, three parallel control assays were performed to check the effect of bound NAP_4-ATR on the electrophoretic mobility of the photolabeled peptides obtained by cyanide cleavage. In the first one, cyanylation followed by alkaline treatment was carried out on the ^3H-photolabeled carrier as described in the former section, except that unlabeled NaCN was used. In the second assay, the ^3H-photolabeled carrier protein was first treated with 0.1 N NaOH for 15 min at 45 °C, as detailed under Experimental Procedures, in order to cleave the ester bond between the ATR residue and [^3H]NAP_4 in bound [^3H]NAP_4-ATR; the carrier protein still covalently labeled by [^3H]NAP_4 was finally subjected to cyanylation with unlabeled NaCN, followed by cleavage at alkaline pH. In the third assay, alkaline cleavage after reaction

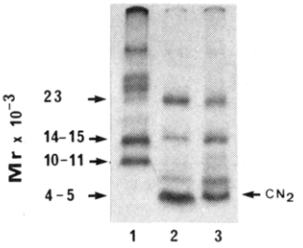

FIGURE 4: Fluorography of $NaDodSO_4$–polyacrylamide slab gel electrophoresis of ^3H peptides obtained from [^3H]NAP_4-ATR-photolabeled carrier protein by different cleavage sequences. The sequence cleavage was either cyanylation and alkaline treatment (track 1), cyanogen bromide cleavage followed by cyanylation and alkaline treatment (track 2), or cyanylation and alkaline treatment followed by cyanogen bromide cleavage (track 3).

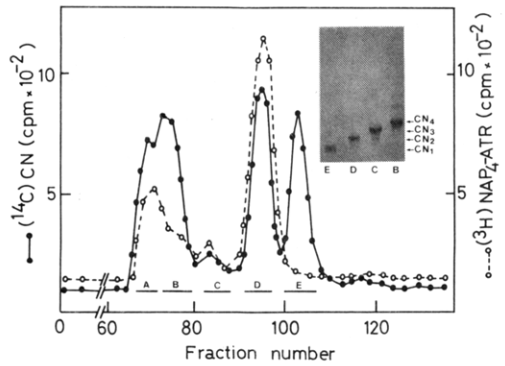

FIGURE 5: Bio-Gel chromatography of peptides obtained by alkaline cleavage of the ^{14}C cyanylated CB1 fragment recovered from [^3H]NAP_4-ATR-photolabeled carrier protein. The photolabeled carrier protein was first treated with 0.1 M NaOH for 15 min at 45 °C to remove the ATR moiety of the covalently bound [^3H]-NAP_4-ATR. The resulting protein covalently labeled with [^3H]NAP_4 was cleaved by cyanogen bromide, and the CB1 fragment was subjected to alkaline cleavage after cyanylation with $Na^{14}CN$. The cyanylated peptides (10 mg) were separated by chromatography on a column of Bio-Gel P-10 (1 × 60 cm) and eluted at a flow rate of 5 mL/h. The counts per minute are for 30-μL aliquots taken out of 600-μL samples. Fractions 73–80 (peak B), 83–88 (peak C), 92–98 (peak D), and 101–108 (peak E) were pooled, and their peptide homogeneity was checked by electrophoresis on a $NaDodSO_4$–polyacrylamide gel (10–20%), followed by staining with Coomassie blue R250 (inset). ^{14}C radioactivity (●); ^3H radioactivity (○).

with $Na^{14}CN$ was carried out on a sample of carrier protein that had not been photolabeled. In the three cases, the peptide fragments obtained by cyanide cleavage were separated by $NaDodSO_4$ slab gel electrophoresis, and the ^3H- and ^{14}C-labeled products were revealed by fluorography (not shown). In the first assay, ^3H radioactivity was concentrated in two peptides of M_r 10000–11000 and 14000–15000; in the second assay, two ^3H-labeled peptides were also found, with M_r close to 10000 and 14000, virtually similar to the M_r of two major ^{14}C-labeled peptides found in the third assay. A careful examination of the radioactive bands in the tracks of the slab gel showed that the two labeled peptides in track 1 (first assay) migrated more slowly than the corresponding peptides in tracks 2 and 3 (second and third assay). Obviously, this slower migration was due to the peptides carrying the bulky and charged ATR residue. As a consequence, in all experiments bearing on the localization of covalently bound derivatives of ATR, namely, [^3H]NAP_4-ATR, [^3H]NAP_3-ATR, and azido[^{14}C]benzoyl-ATR, in the carrier protein, the photolabeled carrier was first treated at alkaline pH, as mentioned above, to remove the ATR residue; under these conditions, the protein remained tagged with the radioactive moiety of the photolabel. Following this procedure and using [^3H]NAP_3-ATR and azido[^{14}C]benzoyl-ATR as photolabels, the same pattern of photolabeling in the cleavage products of the carrier protein was obtained as that with [^3H]NAP_4-ATR.

The M_r 10000–11000 and 14000–15000 peptides contained methionyl residue(s), since they generated, by cyanogen bromide cleavage, smaller ^3H-labeled peptides (tracks 2 and 3 compared to track 1, Figure 4). Whatever the order of the treatments performed on the photolabeled carrier protein, i.e., cyanylation followed by cyanogen bromide cleavage or the reverse, the cleavage products obtained exhibited the same pattern. In both cases, the $M_r \simeq 4500$ peptide was preferentially ^3H labeled. At this point, it may be recalled that a peptide of similar molecular weight, referred to as CN2, was obtained by cyanylation of CB1 followed by alkaline cleavage. The larger peptides found in the autoradiography, some of which were only slightly labeled (Figure 4), probably corresponded to overlapping fragments, as will be discussed later (see Figure 6B).

Isolation of Photolabeled Peptide by Gel Filtration. The data in Figure 5 illustrate a double-labeling experiment in which the [^3H]NAP_4-ATR-labeled CB1 peptide was cyanylated by $Na^{14}CN$ and then cleaved at alkaline pH. Using filtration on Bio-Gel P-10, it was possible to separate the four small peptides corresponding to the CN1, CN2, CN3, and CN4 fragments shown in Figure 3. Most of the covalently bound ^3H radioactivity was recovered in peptide CN2 of $M_r \simeq 4500$, in good agreement with the data of $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 4). As shown in Figure 5, the A pool (fractions 67–71) was slightly photolabeled; it consisted of aggregates and overlapping peptides that were unresolved by Bio-Gel filtration. Essentially similar results showing the preferential labeling of peptide CN2 were obtained with the carrier protein photolabeled with azido-[^{14}C]benzoyl-ATR and [^3H]NAP_3-ATR.

Amino Acid Analysis of the CN Peptides Resulting from Cleavage of CB1. The amino acid composition of peptides CN1, CN2, CN3, and CN4 is given in Table I. The amino acid content was first approximated from the apparent molecular weight of the peptides determined by $NaDodSO_4$–polyacrylamide gel electrophoresis; it was then recalculated, on the basis of integral numbers of leucine residues. It is consistent with that obtained from the amino acid sequence (Aquila et al., 1982). Only a few minor differences were noticed: for example, the amino acid analysis of peptides CN2, CN3, CN4, when compared to the sequence data, showed a lack of isoleucine, tyrosine, and phenylalanine residues. This can be explained by the hydrophobic sequences Ile-Ile, Ile-Phe, or Ile-Ile-Ile-Tyr, which may resist acidic cleavage. Another difference concerned a serine residue, which was found by amino acid analysis (Table I) and was absent in the amino acid sequence reported by Aquila et al. (1982). In our protein samples, lysine residues were succinylated and therefore could not be determined.

Digestion of peptides CN1 and CN2 by carboxypeptidase A revealed that the terminal sequences were Leu-Gly-Asn-COOH and Gly-Hse-COOH, respectively. Peptide CN2 contained the same C terminus as CB1, namely, homoserine (that was formed from methionine, during CNBr cleavage). Digestion by carboxypeptidase P revealed Asp as the C terminus of CN3 (carboxypeptidases A and B had only a sluggish effect on CN3). The C-terminal sequence Thr-Ser-Leu-COOH was found in CN4 with carboxypeptidase A. All these data were in good agreement with the amino acid sequence reported by Aquila et al. (1982). On the basis of structural data, i.e., carboxyl-terminal ends of the peptides and CN3 carrying the N-terminal end of CB1, and on the molecular

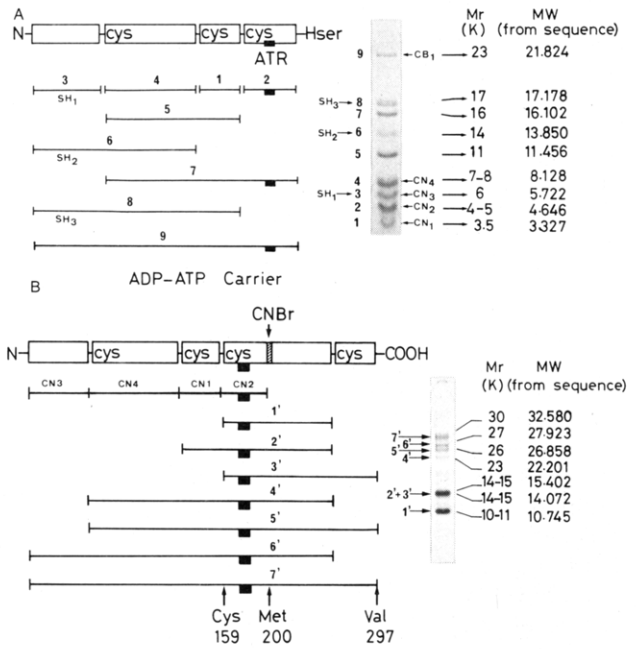

FIGURE 6: (A) Alignment of cysteine cleavage peptides in CB1 fragment of photolabeled carrier protein. SH1 is the shortest N-terminal peptide obtained by cyanylation. The other peptides, SH2 and SH3, obtained by cyanylation also start from the N terminus and are overlapping peptides. The C terminus of the CB1 fragment is terminated by a homoserine residue (see text). Peptides revealed by Coomassie blue staining are referred to by number from 1 to 9 in order of increasing molecular weights. The position of the photolabeled peptide CN2 is indicated by a dark rectangle. (B) Complete alignment of cysteine cleavage peptides in photolabeled ADP/ATP carrier protein. Complementary to the data of (A) showing the peptide SH1 and the overlapping peptides SH2 and SH3, this diagram shows the cleavage site at the fourth cysteinyl residue, close to the C terminus of the carrier protein, and the localization of the photolabeled peptides obtained by cleavage of the photolabeled carrier protein at cysteinyl residues. Azido ^{3}H-labeled peptides are visualized by fluorography of the NaDodSO$_4$–polyacrylamide gel and are referred to by numbers from 1′ to 7′, in order of increasing molecular weight. Band 7′ corresponds to the uncleaved ADP/ATP carrier protein and band 6′ to the overlapping cysteinyl peptide SH4.

weight of the unlabeled overlapping (partially cleaved) fragments SH1, SH2, and SH3 of CB1, alignment of the nonoverlapping CN peptides of the CB1 fragment was unequivocally deduced (Figure 6A).

As shown in a preceding section, the photolabeled peptides of M_r 14000–15000 and 10000–11000 obtained by cyanide cleavage of the carrier molecule could be cleaved again by cyanogen bromide to release the photolabeled CN2 peptide; the alignment of these peptides in the whole amino acid sequence of the carrier protein is illustrated in Figure 6B.

Discussion

The peptide mapping data presented in this paper show convincingly that a number of arylazido derivatives of ATR, where the azido group is carried by a long or short arm (NAP$_4$-ATR, NAP$_3$-ATR, and azidobenzoyl-ATR), bind to a restricted region of the ADP/ATP carrier protein corresponding to the cysteinyl peptide of $M_r \simeq 4500$, referred to as CN2 (Figure 6A,B). This region was identified as the C-terminal region of CB1, which was previously characterized as the large cyanogen bromide cleavage product of M_r 23000 obtained from the carrier protein (Boulay et al., 1979). In the sequence recently reported by Aquila et al. (1982), the CN2 peptide was located between cysteine-159 and methionine-200; its exact molecular weight is 4646. A more precise localization of the bound photolabel could not be achieved at present, because of the insolubility of the CN2 peptide and difficulty of cleavage by trypsin. As a general rule, alkaline cleavage of a S-cyanylated protein generates at each cleavage point an iminothiazolidine group that blocks the terminal NH_2 group (Degani & Patchornik, 1971, 1974); therefore, the sequential Edman degradation cannot be performed to find at which step the radioactive labeling is released. To free the blocked NH_2 terminus in the peptide fragments arising by cyanylation of the carrier protein, we used W6 Raney nickel (Otieno et al., 1978); however, this method was ineffective in our hands.

As pointed out by Hixson & Hixson (1975), there are limitations to the method of photolabeling to investigate binding sites in macromolecules. In particular, random labeling is sometimes encountered, e.g., as a result of the rather low reactivity of arylnitrenes: because of their noninstantaneous reactivity, a sufficient length of time may elapse between their generation upon photoirradiation and their covalent binding, which explains why they may bind to amino acid residues at some distance from the active site at which they were originally produced. In the specific case of the membrane-bound ADP/ATP carrier protein, random photolabeling was not observed; instead, a restricted locus of the carrier protein was found to be labeled, as previously discussed. Moreover, it is of interest that, whatever the length of the arm that connects the ATR moiety to the photoreactive azido, the same region of the carrier protein was probed. Comparing the photolabeling data obtained with the ADP/ATP carrier protein with those of other proteins makes it clear that the geometry of the protein and the structure of the probe may be critical for mapping studies. The high affinity of ATR for the carrier protein and an appropriate topography and reactivity of the ATR binding region in the membrane-bound carrier protein may be favorable factors that are responsible for selective photolabeling of the ATR site. Staros (1980) and Nielsen & Buchardt (1982) reported that nitrenes react preferentially with nucleophilic groups in proteins, for example, hydroxyl groups, imidazole groups, and sulfhydryl groups. It is noteworthy that the CN2 segment of the carrier protein that binds the nitreno derivatives of ATR contains a number of serine, threonine, and tyrosine residues that are potential reactive sites for nitrene.

In line with the topography of the ATR site, most interesting is the recent report by Saraste & Walker (1982) concerning the possible arrangement of the peptide chain of the ADP/ATP carrier, as predicted from the localization of hydrophobic and hydrophilic segments of the peptide chain and the distribution of repetitive sequences. Saraste and Walter suggested that two hydrophilic segments of the peptide chain (residues 137–170 and residues 234–263) are located on the same side of the membrane in a hydrophilic environment at/or near the surface of the membrane. The CN2 peptide (residues 159–200) that contains the ATR site consists of a hydrophilic sequence (residues 159–170) and a hydrophobic sequence (residues 171–200). The latter is most likely buried in the lipid core of the membrane. Because the ATR site in the membrane-bound carrier is directed to the outside (see the introduction), it is inferred that the hydrophilic sequence from residues 159 to 170 is located on the outer side of the inner mitochondrial membrane.

It is probable that the active ADP/ATP carrier unit is made up of two subunits (Hackenberg & Klingenberg, 1980; Brandolin et al., 1980) and that the binding of one molecule of ATR per dimer results in full inhibition of transport (unpublished data). The photolabeling data reported here, how-

ever, provide no indication as to whether photolabeling occurs on only one subunit of the dimer, as could be expected for a half of the site mechanism, or whether both subunits of the dimer are covalently photolabeled.

Registry No. ATR, 17754-44-8; NAP_3-ATR, 83876-80-6; NAP_4-ATR, 83876-81-7; azidobenzoyl-ATR, 83876-82-8; adenine nucleotide translocase, 9068-80-8.

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Glycosylation, ADP-Ribosylation, and Methylation of *Tetrahymena* Histones†

Beatriz Levy-Wilson*

ABSTRACT: We have examined some of the postsynthetic modifications that occur in macronuclear histones from *Tetrahymena thermophila*. When purified macronuclei are incubated with [^{32}P]NAD^+, histones H1, H2A, H2B, and H3 are ADP-ribosylated. Furthermore, histones H1, H2A, H2B, and H3 contain fucose and mannose residues as evidenced by the incorporation of [^{3}H]fucose and by the specific binding to these proteins of gorse seed lectin and concanavalin A. Finally, our studies on incorporation of methyl groups into histones show that histone H2A, together with the related nonhistone protein A24, is methylated in *Tetrahymena*.

Histones are fundamental components of nucleosomes, the building blocks of chromatin structure in eucaryotes [for a review, see Kornberg (1977)]. Two each of histones H2A, H2B, H3, and H4 are associated with 140 base pairs of DNA to constitute the nucleosome core particle. The fifth histone, H1, is found bound to DNA in the internucleosomal "linker" region. Even though the bulk of the DNA in most eucaryotic cells is organized into nucleosomes, there is ample evidence that nucleosomes in transcriptionally active chromatin have a different structure from those in inactive chromatin. These differences include the presence in active nucleosomes of high mobility group proteins (HMG's) in stoichiometric amounts in relation to the inner histones (Hutcheon et al., 1980; Egan & Levy-Wilson, 1981), the lack of methylation of DNA sequences around active genes (McGhee & Ginder, 1979), and a high level of histone acetylation in transcriptionally active nucleosomes (Levy-Wilson et al., 1979; Nelson et al., 1980).

To further understand the structural prerequisites of transcriptionally active chromatin domains, we have examined the occurrence of a variety of postsynthetic modifications in hi-

† From the Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92717. *Received August 30, 1982.* This work was supported by a grant from the National Institutes of Health (to B.L.W.).

* Address correspondence to California Biotechnology, P.O. Box 51650, Palo Alto, CA 94303.

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