Labeling of Two Different Regions of the Nucleotide Binding Site of the Uncoupling Protein from Brown Adipose Tissue Mitochondria with Two ATP Analogs†

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ABSTRACT: The nucleotide binding site of the uncoupling protein (UCP) from brown adipose tissue was mapped by photoaffinity labeling with 2-azidoadenosine 5′-triphosphate (2-azido-ATP) and by affinity labeling with 3′-*O*-(5-fluoro-2,4-dinitrophenyl)adenosine 5′-triphosphate (FDNP-ATP). Both analogs bind with high affinity and specificity to the UCP in intact mitochondria, as well as to the isolated solubilized protein. Reversible binding at 4 °C in the dark is competitively blocked by GTP. Like the natural ligands ATP and GTP, both analogs are capable of inhibiting the H^+/OH^- conductance of the UCP as measured in proteoliposomes with reconstituted UCP. 2-azido-ATP was incorporated into UCP in mitochondria in the presence of carboxyatractylate, while FDNP-ATP was inserted into isolated UCP by prolonged incubation at room temperature under pH variation. Both reactions can be blocked by GTP. The incorporation of 2-azido-ATP could be localized between residues 258 and 283 by cleavage with CNBr. Solid-phase sequencing of the homoserine-linked radioactive peptide indicated that the 2-azido-ATP was linked to threonine-263. The incorporation of FDNP-ATP could be assigned by cleavage with CNBr and alternatively with trypsin at a locus of covalent attachment between residues 238 and 255. On the basis of published data that no tyrosine participates in nucleotide binding of the UCP, the probable residue reacting with FDNP-ATP is cysteine-253. According to these results and the recently published experiments with 8-azido-ATP, the third hydrophilic domain of the triple-domain primary structure forms the nucleotide binding site between the two putative membrane helices α-5 and α-6. Within this region, the ATP seems to interact at the 2-adenine position with threonine-263 and at the 3′-OH group of the ribose with cysteine-253.

Brown adipose tissue mitochondria contain a unique membrane protein with an energy-dissipating function. This uncoupling protein (UCP)[1] located in the inner mitochondrial membrane is regulated by external purine nucleoside di- and triphosphates (Rafael et al., 1969; Nicholls, 1976). The localization of the nucleotide binding site of the UCP is of basic interest in understanding the structural arrangement of the protein. Photoaffinity labeling with 8-azido-ATP had been first used to detect the UCP in isolated mitochondria (Heaton et al., 1978). This analog was successfully applied to the isolated UCP to identify a part of the nucleotide binding site within the primary structure (Winkler & Klingenberg, 1992a).

To gain more detailed information about the structure of the protein, it was desirable to map the binding center with other nucleotide analogs, where a reactive or a photoactivatable group is located at different positions in the nucleotide backbone. In the present work, 2-azido-ATP and FDNP-ATP are used to covalently probe the binding center of UCP at two different positions.

2-Azido-ATP was used to study the ATP binding site of the AAC (Dalbon et al., 1988; Mayinger et al., 1989), a protein with some similarity to the UCP. As judged for the primary structure, UCP and AAC are thought to have a similar folding pattern (Aquila et al., 1985, 1987; Runswick et al., 1987). In contrast to 8-azido-ATP, the 2-substituted analog like the natural ATP possesses a preferred anti conformation of the purine with respect to the ribose (Czarnecki, 1984). However, treatment of the isolated AAC with 8-azido-ATP incorporates the label in the same region of the protein as 2-azido-ATP (Mayinger et al., 1989). FDNP-ADP and -ATP, ribose-modified affinity reagents, have been recently introduced to study the active site of the mitochondrial F_1-ATPase (Chuan & Wang, 1988).

Both 2-azido-ATP and FDNP-ATP were incorporated into the UCP in a GTP-sensitive manner, thus assuring the incorporation at the regulatory nucleotide binding site. Cleavage with CNBr allowed us to identify two different regions in the primary structure for the incorporation of each reagent. Moreover, the most likely residues for the attachment of the analogs could be determined. With these data, the position of the base and of the ribose moieties at the nucleotide binding site was inferred within the secondary folding pattern.

MATERIALS AND METHODS

$H_3{}^{32}PO_4$, [α-^{32}P]ATP, and [^{14}C]ATP were purchased from New England Nuclear. 1,5-Difluoro-2,4-dinitrobenzene was obtained from Aldrich. Cyanogen bromide was from Merck; TPCK-treated trypsin was from Boehringer. Sephacryl-100 was obtained from Pharmacia.

Brown adipose tissue mitochondria from cold-adapted hamsters were prepared as described by Lin and Klingenberg (1982).

Synthesis of Radiolabeled 2-Azido-ATP and FDNP-ATP. 2-Azido[α-^{32}P]ATP was synthesized as in Boulay et al. (1985), with the modifications described in Mayinger et al. (1989). The purified product usually had a specific activity of 5000

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[1] Abbreviations: AAC, ADP/ATP-carrier; UCP, uncoupling protein; 2-azido-ATP, 2-azidoadenosine 5′-triphosphate; FDNP-ATP, 3′-*O*-(5-fluoro-2,4-dinitrophenyl)adenosine 5′-triphosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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dpm/pmol and was diluted with nonradioactive 2-azido-ATP prior to use.

FDNP-[α-^{32}P]ATP and FDNP-[8-^{14}C]ATP were prepared starting from [α-^{32}P]ATP and [8-^{14}C]ATP according to the method described by Chuan and Wang (1988). The purification was conducted on silica gel plates from Merck. Differing from the published procedure, a mixture of 2-propanol, 1-butanol, and water (1:1:1) was used as the developing solvent. The radiolabeled FDNP-ATP (R_f = 0.44) was well resolved from nonreacted ATP (R_f = 0.27) and the major byproduct, 5-fluoro-2,4-dinitrophenol (R_f = 0.89). The labeled band was scraped off the plate and eluted with a mixture of methanol and water (1:1) and concentrated under reduced pressure. The yield of radiolabeled FDNP-ATP was usually 24–31%.

Assay of Reversible Binding of the Analogs to Mitochondria. The binding measurements were performed as follows: 100-μL portions of mitochondria (5 mg/mL) in 250 mM sucrose, 10 mM triethanolamine, and 1 mM EDTA, pH 6.5, were incubated with different concentrations (1–25 μM) of the analogs. After 30-min incubation at 4 °C, the samples were centrifuged at 100000*g* for 2 min. The tube walls were flushed once with incubation medium, and the pellets were solubilized with 1% SDS for scintillation counting. To evaluate specific binding to UCP, each sample was alternatively supplemented with 50 μM GTP for control. Subtraction of the control values with GTP from the total binding values without GTP was taken as GTP-sensitive binding to the UCP.

Assay of Reversible Binding of the Analogs to the Isolated UCP. These binding measurements followed the anion-exchange method described by Klingenberg et al. (1986). Fifty microliters of isolated UCP (0.23 mg/mL) in 10 mM Mes, 10 mM Pipes, and 0.5 mM EDTA, pH 6.0, was supplemented with 1–10 μM nucleotides followed by 30-min incubation at 4 °C. To remove nonbound nucleotides, the samples were rapidly filtered through 20 mg of wet Dowex 1 × 8, 200–400 mesh, in a Pasteur pipet and washed 2 times with 100 μL of water. The eluate and the combined washes were subjected to liquid scintillation counting.

Photoaffinity Labeling of Mitochondria with 2-Azido[α-^{32}P]ATP and Isolation of the Radiolabeled UCP. Thirty minutes prior to irradiation with UV light, the mitochondria (5 mg of protein) were incubated with 50 μM 2-azido[α-^{32}P]ATP at a specific activity of 2500 dpm/nmol in 400 μL of isolation medium. Control aliquots were preincubated alternatively with 1 mM GTP or 50 μM carboxyatractylate. The probes were flushed with N_2 and irradiated with a 90-W Hg lamp with a chloroform filter against the far-UV light as in Mayinger et al. (1989). After 8-min irradiation at 4 °C, the samples were centrifuged, and the pellet was washed 3 times by resuspension in the incubation medium.

For isolation of labeled UCP, the mitochondria were preincubated with 50 μM carboxyatractylate before irradiation was performed. The purification follows the procedure as described by Lin and Klingenberg (1982). Five milligrams of labeled mitochondria was solubilized in 250 μL of a medium containing 5% Triton X-100 together with 40 mM Na_2SO_4, 20 mM Mops, and 0.2 mM EDTA, pH 6.7. The resulting detergent: protein ratio was 2.5. After 30-min incubation, the mixture was centrifuged at 100000*g* for 30 min, and the supernatant was placed in 600 μL of wet hydroxyapatite in a Pasteur pipet. The column was eluted with 30 mM Na_2SO_4, 5 mM Mops, and 0.2 mM EDTA, pH 6.7, at room temperature, and the protein-containing fractions were pooled, precipitated with TCA/acetone (final concentration: 30% acetone, 5% TCA), and subjected to SDS–PAGE.

Isolated UCP was photolabeled with 2-azido[α-^{32}P]ATP following the procedure for 8-azido-ATP (Winkler & Klingenberg, 1992a). In these experiments, concentrations of up to 500 μM analog were used to yield maximal incorporation of label.

Affinity Labeling of Isolated UCP with FDNP-[α-^{32}P]-ATP and FDNP-[8-^{14}C]ATP. Isolated UCP (3 mg/mL) was incubated in 1 mL of isolation buffer and supplemented with a 5-fold excess of FDNP-[α-^{32}P]ATP (specific activity, 1000 dpm/mol) or FDNP-[8-^{14}C]ATP (specific activity, 150 dpm/nmol). After 30-min incubation at 4 °C, pH 6.0, the samples were adjusted to pH 8.0 and further incubated for 2 h at 25 °C followed by precipitation with TCA/acetone and three washings with 50% acetone.

Chemical Modification and Fragmentation of the Labeled UCP. Cleavage at the methionyl residues was performed in 80% formic acid overnight with CNBr in 500-fold excess over the methionines present (Gross, 1967). To remove phospholipids, the samples were diluted with water (1:5), extracted 3 times with an equal volume of ethyl acetate, and lyophilized. The peptides were analyzed using the gel system developed by Schägger and von Jagow (1987).

Prior to cleavage with trypsin, the protein was first carboxymethylated (Crestfield et al., 1963) and then citraconylated (Butler & Hartley, 1972) to delimit the cleavage to arginine residues. The labeled UCP was dissolved in 8 M guanidinium chloride, 500 mM Tris, and 2 mM EDTA, pH 8.2, under a stream of nitrogen. After reduction with dithioerythritol (150 mol/mol of protein) at 50 °C for 2 h in a nitrogen atmosphere, the solution was treated with iodoacetate (300 mol/mol of protein) at 4 °C for 30 min. The reaction was stopped by the addition of 5% thioglycolic acid, and the protein was precipitated by dilution with a 5-fold volume of water at 4 °C. After centrifugation, the pellet was dissolved in 6 M guanidinium chloride at 0 °C, and the pH was adjusted to 8.8 using a pH electrode. Citraconic anhydride (6 μL/mg of protein) was then added in five aliquots while the pH was maintained at 8.8 by the addition of NaOH. After 30-min incubation, the pH remained stable, indicating that the reaction was complete. The final solution was dialyzed 3 times against 2 L of water with 0.2% NH_4OH and then lyophilized.

The modified UCP was dissolved in 100 mM NH_4HCO_3, pH 7.9 (5 mg/mL), and incubated with TPCK-treated trypsin (1:100) for 2.5 h at 37 °C. The digestion was stopped by filtration through 10000 NMWL Millipore Ultrafree filters.

The cleavage products were separated by gel filtration using an S-100 column from Pharmacia. The column was eluted with 100 mM NH_4HCO_3, pH 7.9. The individual peptide-containing fractions were analyzed with the same gel system as above (Schaegger & von Jagow, 1987).

Solid-Phase Sequencing of the CNBr-Peptide Labeled with 2-Azido[α-^{32}P]ATP. The UCP labeled with 2-azido[α-^{32}P]-ATP was cleaved with CNBr as described above. The fragments were separated on SDS–PAGE that, without staining the gel, was cut into 2-mm-thick slices. The peptides were eluted as described by Winkler and Klingenberg (1992a). The coupling of the labeled peptide and the solid-phase Edman degradation were conducted according to Machleidt et al. (1986).

RESULTS

Reversible Binding of 2-Azido[α-^{32}P]ATP and FDNP-[8-^{14}C]ATP to Mitochondria and the Isolated UCP. Specific

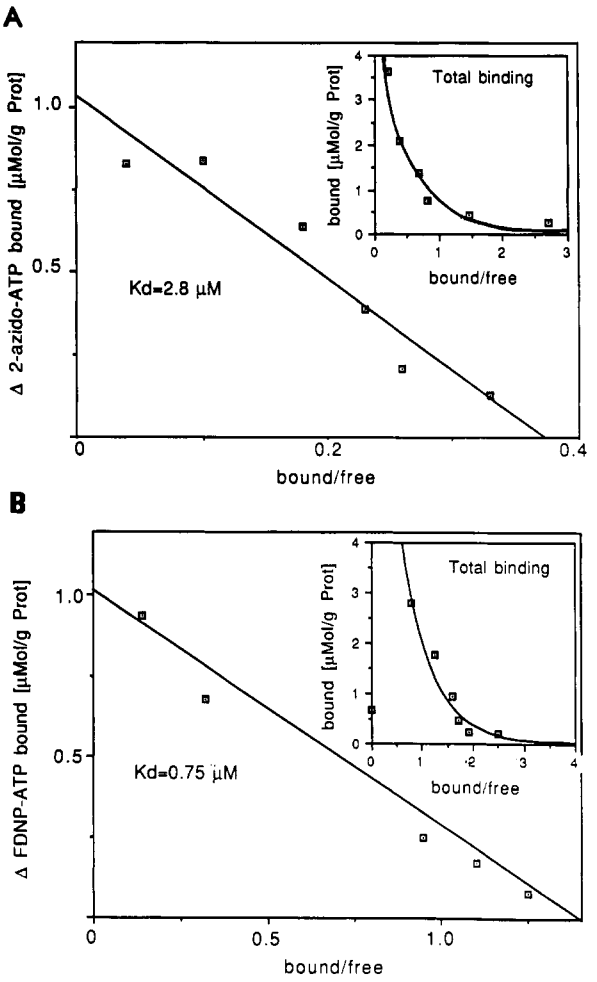

FIGURE 1: (A) GTP-sensitive binding of 2-azido-ATP to mitochondria. (B) GTP-sensitive binding of FDNP-ATP to mitochondria. Total binding is shown in the insert. GTP-sensitive binding was calculated by the difference of total binding and binding obtained with mitochondria preincubated with 50 μM GTP. The binding data were determined with freshly prepared mitochondria (4 mg/mL) using various concentrations of 2-azido[α-^{32}P]ATP or FDNP-[^{14}C]ATP at 0 °C, pH 6.0.

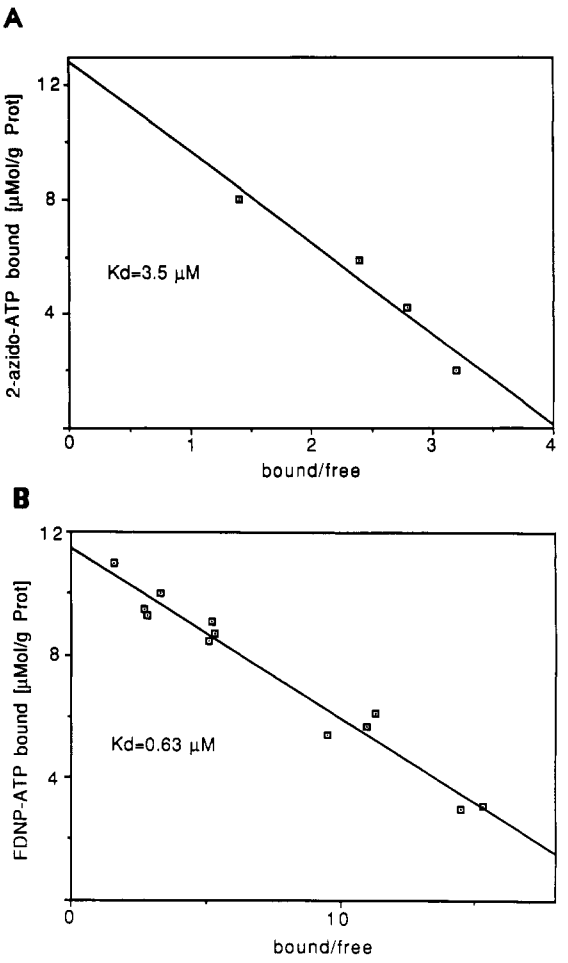

FIGURE 2: (A) Binding of 2-azido-ATP to isolated UCP. (B) Binding of FDNP-ATP to isolated UCP. The binding measurements were conducted by incubating 50-μL samples of the isolated UCP (0.2 mg/mL) with different concentrations of 2-azido[α-^{32}P]ATP or FDNP-[^{14}C]ATP at 0 °C, pH 6.0. The UCP–nucleotide complex was separated from free nucleotides by anion-exchange chromatography (Klingenberg et al., 1986).

labeling of the binding center requires that the affinity reagents bind with a good affinity and selectivity to the same site as the natural ligands. Therefore, the noncovalent binding to mitochondria of the employed analogs was first tested. Since azido compounds are light-sensitive, all binding studies with 2-azido-ATP were carried out in the dark.

The binding to mitochondria of 2-azido-ATP produces a curvilinear graph in the mass action analysis, indicating that the analog is bound to different sites on mitochondria, e.g., to the AAC in addition to UCP (Figure 1A). Nonspecific binding of 2-azido-ATP to mitochondrial membranes can also not be excluded. To determine the portion of 2-azido-ATP bound specifically to the UCP, binding of 2-azido-ATP was measured in the presence of GTP, because it is known that 2-azido-ATP but not GTP binds to the mitochondrial AAC (Dalbon et al., 1985; Weidemann et al., 1970). The GTP-sensitive binding, corresponding to the specific binding of 2-azido-ATP to UCP, is much smaller and produces a linear relation in the mass action plot with a maximum of 1.0 μmol/g of protein, which is similar to the binding of GTP to mitochondria (Rafael et al., 1972; Nicholls, 1976), and gives a K_D value of 2.8 μM (Figure 1A).

Binding experiments with FDNP-ATP also established a specific interaction with the membrane-bound UCP. Again, the curvilinear mass action relation of the total binding to mitochondria of FDNP-ATP indicates that more than one membrane component interacts with this analog. At 4 °C, FDNP-ATP binds reversibly in a GTP-sensitive manner to mitochondria with an extrapolated maximum of 1.1 μmol/g (Figure 1B). The K_D of 0.75 μM is in the same range as observed for the natural ligands.

Additional binding measurements were conducted using the isolated solubilized UCP. 2-Azido-ATP binds with a maximum of 12.9 μmol/g like GTP or ATP to the isolated protein (Figure 2A). The K_D of 3.5 μM is about 5 times higher than that for ATP. FDNP-ATP binds with the same high affinity as ATP to the isolated UCP with a K_D of 0.63 μM. The maximal binding was calculated to be 12 μmol/g (Figure 2B). To exclude unspecific binding of FDNP-ATP due to hydrophobic interaction of the fluorophenyl group with parts of the protein surface, competition experiments with GTP were performed (not shown). With 5 μM GTP, the binding of FDNP-ATP was decreased by 80%.

Another criterion for whether the reagents can substitute for natural ligands is the inhibition of the H^+ transport catalyzed by UCP. To that purpose, UCP was incorporated into phospholipid vesicles following an improved reconstitution method (Winkler & Klingenberg, 1992b). Both 2-azido- and FDNP-ATP inhibit the H^+ import by UCP as shown in Figure 3. For comparison, in the same experiment the inhibition by

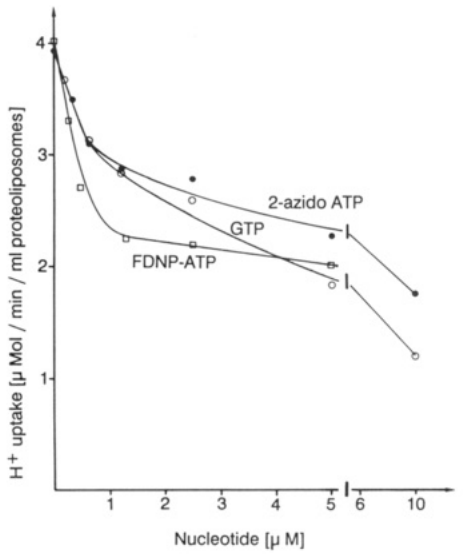

FIGURE 3: Inhibition of H^+ uptake of UCP reconstituted in phospholipid vesicles with FDNP-ATP, 2-azido-ATP, and GTP as control. Vesicles were loaded with 100 mM potassium phosphate, pH 7.5, and incubated in a medium of 0.5 mM Hepes, 0.5 mM Pipes, 0.2 mM EDTA, and 250 mM sucrose, pH 6.5, at 10 °C, with final concentrations of phospholipid of 3 mg/mL and protein 25 μg/mL. K^+ diffusion potential was generated by additions of 2 μM valinomycin, and the initial rates of H^+ uptake were recorded with increasing concentrations of FDNP-ATP and 2-azido-ATP [see also Winkler and Klingenberg (1992b)].

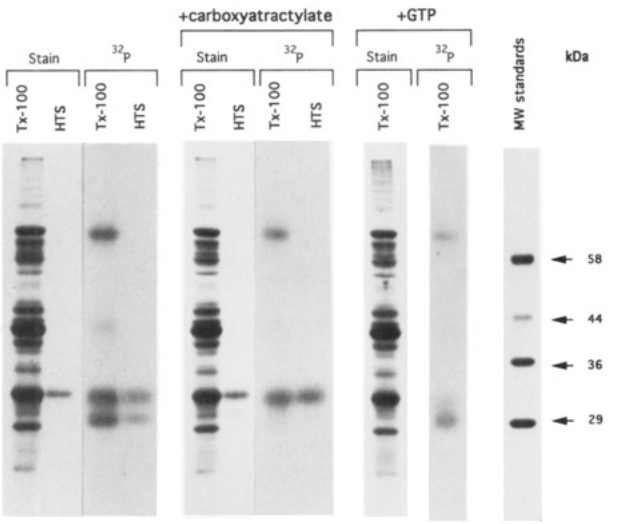

FIGURE 4: Photoaffinity labeling of brown adipose tissue mitochondria with 2-azido[α-^{32}P]ATP. Five milligrams of mitochondria was irradiated in the presence of 2-azido[α-^{32}P]ATP, extracted with Triton X-100, and, where indicated, treated with hydroxyapatide as described under Materials and Methods. The extracts were precipitated with TCA/acetone (final concentrations: 30% acetone, 5% TCA) and applied to SDS–PAGE.

GTP is given. The activity at 10 μM GTP can be regarded as the base level of the unspecific H^+ uptake rate. Both analogs are nearly as effective as GTP. FDNP-ATP inhibits to about 55% already at low concentration, whereas 2-azido-ATP is less effective and requires a higher concentration than GTP. We can conclude that both analogs bind with sufficient affinity to the regulatory nucleotide binding site of the UCP and are therefore promising tools for labeling experiments.

Photoaffinity Labeling of Mitochondria with 2-Azido[α-^{32}P]*ATP and Isolation of the Labeled UCP.* As reported recently, the binding center of the isolated UCP could be labeled with 8-azido[γ-^{32}P]ATP (Winkler & Klingenberg, 1992a). A problem in this study was the fact that a major amount of the reagent was incorporated unspecifically to phospholipids. To avoid this problem, we applied 2-azido-[α-^{32}P]ATP to freshly isolated mitochondria. Control experiments are necessary to ascertain the specificity of the labeling of the UCP in mitochondria. Photolabeling experiments were carried out alternatively in the presence of carboxyatractylate, a strong and specific inhibitor of the AAC, in the presence of GTP, or without addition of inhibitors.

The SDS–PAGE analysis of the Triton X-100 extract from labeled mitochondria revealed a protein band with an apparent molecular mass of about 32 kDa which is radioactively labeled in a GTP-sensitive manner (Figure 4), and which should correspond to UCP. A second labeled band of about 30 kDa can be attributed to AAC because the incorporation is blocked by carboxyatractylate. Since UCP easily forms a disulfide-linked dimer, a product with the double mass is often observed. The labeled band of higher molecular mass can be assigned accordingly to the cross-linked UCP, although the radioactive incorporation seems to be less GTP-sensitive.

The main problem of the isolation of the labeled UCP was its separation from the AAC, because apparently 2-azido-ATP-labeled AAC more easily passed the hydroxyapatite column than the nonlabeled portion of the AAC, which was largely adsorbed to the hydroxyapatite at room temperature (Figure 4). To suppress the reaction of 2-azido-ATP with AAC, carboxyatractylate-loaded mitochondria were employed for photoaffinity labeling. Solubilization of the membrane-bound photolabeled UCP in detergent and its purification are detailed under Materials and Methods. After the purification steps, 1.96 μmol of 2-azido-ATP/g of UCP was found to have been covalently incorporated. This corresponds to a 13% labeling of GTP-sensitive and therefore UCP-specific nucleotide binding sites in mitochondria.

Affinity Labeling of the Isolated UCP with FDNP-[α-^{32}P]*-ATP.* Labeling with FDNP-ATP of mitochondria yielded mostly aggregated reaction products (data not shown), and therefore no specific labeling of mitochondrial components could be attained in these experiments. As the FDNP-ATP was shown to bind specifically and with high affinity to the isolated UCP, the radiolabeled analog was applied to the solubilized protein. It is known that the binding affinity of nucleotides to UCP is strongly pH-dependent. The affinity of the UCP for binding nucleotides increases at lower pHs with a maximum at pH 6.0 (Klingenberg, 1984), while the nucleophilic substitution of the protein with the fluorophenyl group requires higher pH. Yet even at pH 6.0 only a negligible amount of reagent could be covalently incorporated into the UCP even with prolonged incubations and at 25 °C (Figure 5). Due to the poor interaction with UCP of nucleotides at pH of 6, advantage was taken of the very slow dissociation of the nucleotides from the UCP. The protein was first incubated with radiolabeled FDNP-ATP at 4 °C and pH 6.0 for 30 min. Then the pH was adjusted to 8.0, and the samples were further incubated at 25 °C (Figure 5). A pH of above 8.0 reduces the amount of covalently bound analog, since the protein obviously becomes unstable (results not shown). As shown in Figure 5, the covalent reaction could be nearly completely inhibited by the addition of GTP, proving that the FDNP-ATP was incorporated specifically to the binding center of the UCP.

Fragmentation of UCP Labeled with 2-Azido[α-^{32}P]*ATP.* For localization of the covalent attachment of both analogs, fragmentation of the labeled UCP was necessary. The important cleavage points of the protein and the resulting fragments are illustrated in Figure 10. With six methionyl

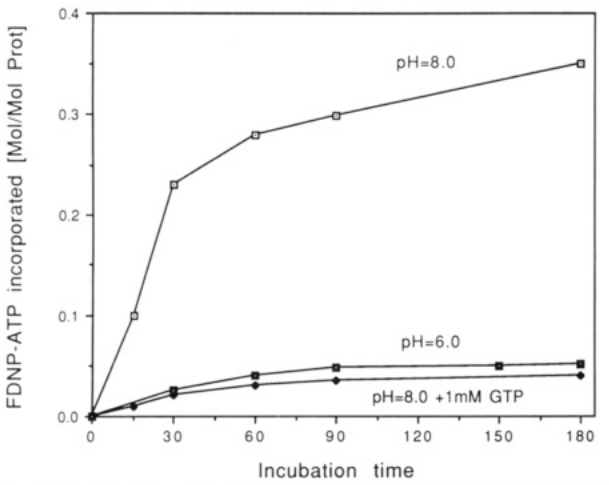

FIGURE 5: Kinetics of the covalent incorporation of FDNP-[α-^{32}P]-ATP into UCP. The protein (2 mg/mL) was incubated with stoichiometrical amounts of FDNP-[α-^{32}P]ATP. At the indicated time intervals, aliquots were precipitated with TCA/acetone (final concentrations: 30% acetone, 5% TCA), washed 3 times with 50% acetone, and resolubilized in 3% SDS for scintillation counting.

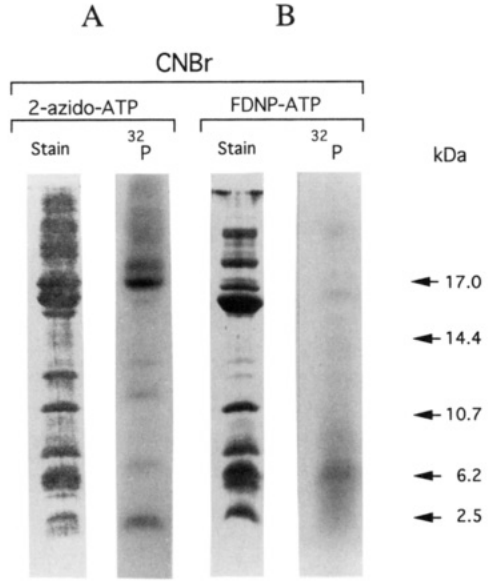

FIGURE 6: (A) Incorporation of 2-azido[α-^{32}P]ATP into UCP. Separation of CNBr-peptides by SDS–PAGE and corresponding autoradiography. The isolated UCP was incubated with 2-azido-[α-^{32}P]ATP (1.0 mol of label/mol of protein) for 3 min in the dark at 0 °C, pH 6.0. The sample was irradiated with a 90-W Hg lamp. After 10-min irradiation, the protein was precipitated with TCA/acetone, treated with CNBr, and applied to the gel system developed by Schägger et al. (1987). (B) Incorporation of FDNP-[α-^{32}P]ATP into UCP. Separation on SDS–PAGE of the fragments obtained by cleavage with CNBr and the corresponding autoradiography. UCP was incubated for 3 h with FDNP-[α-^{32}P]ATP at 25 °C, pH 8.0, precipitated with TCA/acetone, and subjected to CNBr cleavage. For separation of the CNBr-peptides, the Schägger gel system was used.

residues present in UCP, a maximum of seven CNBr fragments can be produced. The CNBr products are analyzed on an SDS gel system suitable for separating peptides with molecular mass below 4 kDa (Schägger & von Jagow, 1987).

Figure 6A shows the CNBr cleavage products of UCP labeled with 2-azido[α-^{32}P]ATP. In the Coomassie-stained gel, a considerable amount of uncleaved protein is found. The autoradiography pattern for CNBr-cleaved UCP labeled with 2-azido[α-^{32}P]ATP (Figure 6A) is identical with that obtained in experiments with 8-azido[γ-^{32}P]ATP (Winkler & Klingenberg, 1992a). The main portion of radioactivity is located at a fragment with an apparent molecular mass of about 2.5 kDa. This corresponds either to CB6 or to the C-terminal fragment CB7. A differentiation between the incorporation to CB6 and CB7 is difficult as the CB6 appears to migrate faster despite its larger molecular mass (Winkler & Klingenberg, 1992a). A further complication observed with 8-azido[γ-^{32}P]ATP is a small upward shift on the gel of the radioactive peptide bands. The labeled band with molecular mass 20 kDa is a combination of partially uncleaved peptides because a total cleavage would yield fragments only with a molecular mass below 12 kDa.

Fragmentation of UCP Labeled with FDNP-[α-^{32}P]ATP. CNBr cleavage was also applied to the FDNP-[α-^{32}P]ATP-labeled UCP. The incorporation of FDNP-ATP was found to be specifically delimited to a band with an apparent molecular mass of 6.1 kDa, which should correspond to the fragment of CB4 (Figure 6B). The combined CB6 + CB7 fragment with a molecular mass of 5.6 kDa can be excluded as a carrier for the label, due to the absence of radioactivity in the single CB6 and CB7 fragments.

Alternatively the labeled UCP was fragmented by cleavage with thermolysin. For the proteolytic cleavage, the hydrophobic membrane protein was brought into a water-soluble form by carboxymethylation followed by citraconylation. The labeled UCP was then cleaved by thermolysin. The resulting peptide mixture was analyzed by reversed-phase HPLC using RP-18 and RP-8 columns. Two radioactive peptide peaks could be detected within the bulk of other cleavage products (not shown). Even after two subsequent purification steps using different elution methods, we did not succeed in isolating the labeled peptides in a purity sufficient for sequencing.

To define more precisely the labeling of FDNP-[α-^{32}P]-ATP within the primary structure, another dissection of the labeled UCP was used. The method of choice was the digestion with trypsin, which hydrolyzes peptide bonds only at arginyl residues, when all lysines are protected by modification with citraconic anhydride. Up to 13 single fragments should be produced, with the majority in a molecular mass range below 3 kDa. Fortunately, the labeled CNBr fragment CB4 overlaps with two tryptic fragments of higher molecular weight: T10 with molecular mass 6.1 kDa and T11 with molecular mass 4.2 kDa (Figure 10). For analysis of these fragments on SDS–PAGE, the peptide mixture was separated by gel filtration on Sephacryl S-100 prior to application to the above-mentioned gel system. Because of the tendency of low molecular weight fragments to be washed out during the staining and destaining steps, the gels were autoradiographed immediately after electrophoresis. Comparison of the labeled band with molecular weight standards shows that the radioactivity is concentrated in the 4.2-kDa fragment T11 and that no radioactivity is present in the 6-kDa fragment (Figure 7).

The overlap of CB4 with T11 between phenylalanine-239 and arginine-255 contains 2 amino acids within the 16 residues which are capable of a reaction with FDNP-ATP, namely, tyrosine-247 and cysteine-253. Published data suggest that a tyrosyl residue is not involved in nucleotide binding because the suppression of GTP binding after reaction with tetranitromethane of UCP was not due to a modified tyrosine, as shown by amino acid analysis (Rial & Nicholls, 1986). We investigated the influence of this reagent on the binding to UCP of FDNP-ATP. Tetranitromethane treatment reduces the binding of FDNP-ATP in a noncompetitive way (Figure 8), similar to the results reported for GTP binding. Therefore, we propose that cysteine-253 and not tyrosine-247 is the residue for attachment of the FDNP-ATP.

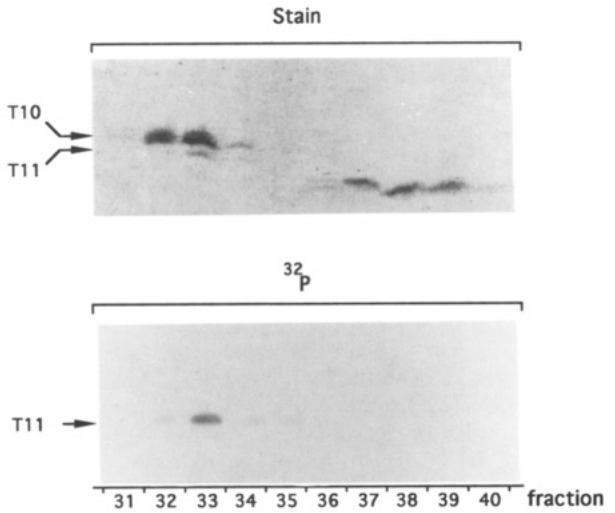

FIGURE 7: Tryptic fragments of the UCP labeled with FDNP-[α-^{32}P]ATP. SDS–PAGE and corresponding autoradiography. For specific cleavage at arginyl residues, the labeled protein was citraconylated prior to treatment with trypsin. The resulting peptide mixture was separated by gel filtration on Sephacryl S-100. The peptide-containing fractions were applied to the Schägger gel system.

Solid-Phase Sequencing of the CNBr Fragment Labeled with 2-Azido[α-^{32}P]ATP. The CNBr peptide labeled with 2-azido[α-^{32}P]ATP could be coupled to glassbeads via homoserine lactone with a yield of 86%. This fact excludes the CB7 as a carrier of the radioactivity, because it contains no methionine at its C-terminus.

This leaves CB6 as the labeled peptide, the same as in the experiments with 8-azido-ATP (Winkler & Klingenberg, 1992a). Similarly as mentioned there, no amino acid could be identified during the Edman degradation and the radioactivity was monitored only because of the poor yield of labeled peptide by gel electrophoresis. A large amount of radioactivity was released during the first cycles of Edman degradation probably due to hydrolysis of the label. Nevertheless, a distinct radioactive peak could be detected at the seventh step, which corresponds to threonine-263 in the sequence of CB6 (Figure 9).

DISCUSSION

In the present work, we succeeded in defining two different sites within the structure of UCP which are associated with the nucleotide binding center by using 2-azido-ATP, where the covalent linkage is at the adenine, and FDNP-ATP, with a covalent linkage at the ribose.

During this work, a number of unforeseen difficulties were encountered from the stage of labeling until the identification of the cleavage peptides. Despite extensive efforts, it was technically impossible to achieve cleavage of the UCP suitable for HPLC purification of labeled peptides and subsequent sequencing. Although in the laboratory great expertise in fragmenting the UCP has been developed for the complete amino acid sequencing (Aquila et al., 1985), in this work the instability of the linkage between the nucleotide analogs and the protein and the previously described difficulties inherent to the UCP (Winkler & Klingenberg, 1992a) prevented the application of standard procedures for HPLC separation and for sequencing of the labeled peptides. This could be overcome by the fortunate segregation of the major incorporation of both 2-azido-ATP and FDNP-ATP in small but different CNBr fragments, which could be precisely identified.

The labeling of UCP with the covalently binding ATP analogs takes advantage of the specific interaction with the

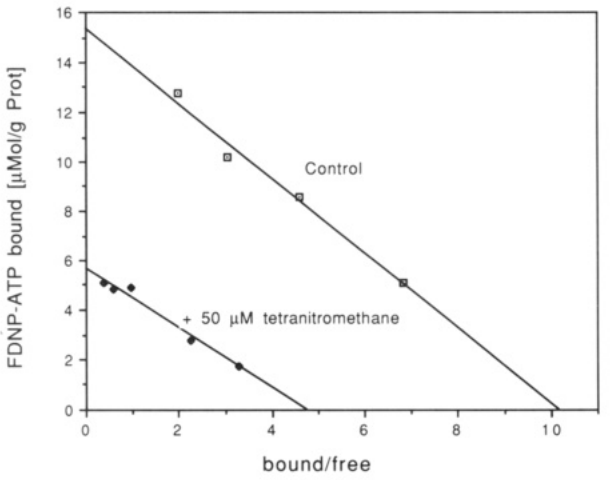

FIGURE 8: Inhibition with tetranitromethane of FDNP-ATP binding to the isolated UCP. The UCP was preincubated with tetranitromethane for 30 min at 0 °C, pH 6.0. The binding measurements were conducted by incubating 50-μL samples of the modified UCP (0.2 mg/mL) with different concentrations of FDNP-[^{14}C]ATP at 0 °C, pH 6.0. The UCP–nucleotide complex was separated from free nucleotides by anion-exchange chromatography (Klingenberg et al., 1986).

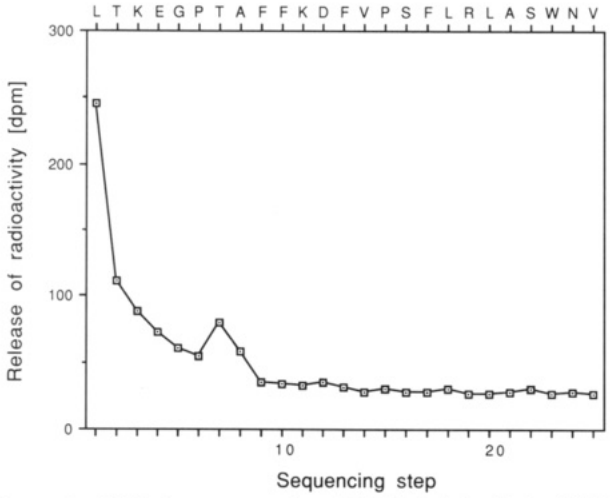

FIGURE 9: Solid-phase sequencing of CB6 labeled with 2-azido[α-^{32}P]ATP. The peptide was eluted from the gel and coupled covalently to glassbeads via homoserine lactone. The sequencing was conducted by A. Esterl and W. Machleidt as described in Machleidt et al. (1986).

regulatory nucleotide binding site of UCP. 2-Azido-ATP and particularly FDNP-ATP bind with a higher specificity and affinity to UCP than 8-azido-ATP. All analogs were ascertained by competition studies to bind to the same site as ATP or GTP.

In a previous paper (Winkler & Klingenberg, 1992a), the photolabeling of UCP with 8-azido-ATP was reported. A major portion of 8-azido-ATP was shown to be located in a well-delimited region between residues 258 and 279 close to the C-terminal section of UCP. Also for 2-azido-ATP, the cleavage with CNBr allowed the delimination of the incorporation to the CB6 peptide between residues 258 and 279 (Figure 10), i.e., the same region as with the 8-azido derivative. To attain specific labeling, 2-azido-ATP was applied to intact mitochondria. It can be assumed that in mitochondria all UCP molecules are in a more intact and better defined conformational state with respect to the nucleotide binding center. As a consequence, the delimitation of the 2-azido-ATP incorporation into the CB6 region was better defined than with 8-azido-ATP. Defining by solid-phase sequencing the amino acid modified covalently with 2-azido-ATP was

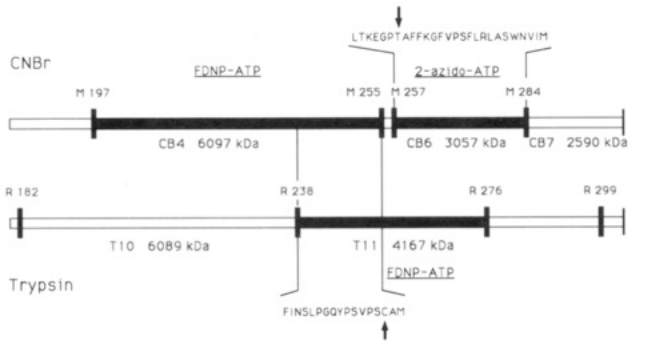

FIGURE 10: Scheme illustrating the fragmentation of the UCP by CNBr and by trypsin and localization within the primary structure of the labeling by FDNP-[α-^{32}P]ATP and 2-azido[α-^{32}P]ATP.

found to be difficult. In spite of limitations, threonine-264 could be the likely candidate for the reaction with this analog.

Among a wide variety of other ATP analogs, FDNP-ATP proved to be a surprisingly good ligand for UCP. Obviously, the substitution at the 3′-OH group of the ribose does not interfere with the binding at the nucleotide binding center. This would agree with the good binding reported for DAN-ATP (Klingenberg et al., 1986). However, differently from DAN-ATP, FDNP-ATP also inhibits the H^+ transport by UCP similarly to ATP or GTP.

With FDNP-ATP, it was possible to use the trypsin cleavage in addition to CNBr cleavage because the FDNP-ATP survived the relatively harsh procedures of citraconylation and carboxymethylation, which are prerequisites for a well-defined trypsin cleavage at the arginine residues only. The incorporation of FDNP-ATP is precisely confined to the fragments CB4 and T11 (Figure 10). The higher selectivity of FDNP-ATP toward some amino acid residues, compared to the less discriminating nitrene linkage during photoaffinity labeling, might be used to suggest cysteine-253 as the most probable candidate within the overlapping region defined by the CNBr and trypsin cleavage.

In contrast to the AAC, which binds nucleotides preferentially in the anti conformation (Mayinger and Klingenberg, unpublished results), the UCP interacts with a similar affinity also with the syn conformer 8-azido-ATP. This indicates that the minor fraction of anti conformation in equilibrium with the syn conformation of 8-azido-ATP is not responsible for the binding of this analog. Consequently, we conclude that UCP does not distinguish between the syn conformation and anti conformation of nucleotides and that, accordingly, the 8-azido-ATP is bound in the syn conformation. Although the distance between C-2 and C-8 in the purine ring is close to 6 Å, both 2- and 8-azido-ATP in their opposite conformation expose the azido group to the same site with respect to the ribose. This is in agreement with the comparative unspecificity of the UCP toward the base moiety in the natural ligands such as the nearly identical affinity for the adenine and guanine nucleotides (Klingenberg, 1984).

Different from other nucleotide binding proteins, UCP and AAC contain no glycine-rich region homologous to the "nucleotide binding loop" (Walker et al., 1982; Möller & Amons, 1985; Fry et al., 1986). The uniqueness of the nucleotide binding site of these mitochondrial transport proteins is also illustrated by the fact that free nucleotides, i.e., not the Mg^{2+}-complexed form, act as ligands. Therefore, it is not surprising that the binding center of UCP does not contain previously reported characteristic structural features of nucleotide binding sites in other proteins.

For UCP, the sidedness of the binding center is clearly defined, since the protein interacts with cytosolic nucleotides

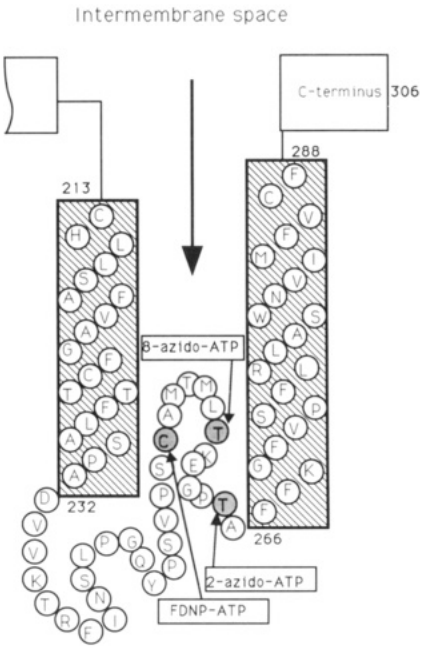

FIGURE 11: Proposed transmembrane arrangement of the third repeat domain within the triplicate structure of UCP. The positions of the likely amino acids covalently modified by FDNP-[α-^{32}P]ATP, by 2-azido[γ-^{32}P]ATP (this paper), and by 8-azido[γ-^{32}P]ATP (Winkler & Klingenberg, 1991) are emphasized.

only. Consequently, the labeled parts of the protein, namely, the hydrophilic part of the third domain, have to be accessible from the cytosolic side. It was suggested by partial cleavage with trypsin of the membrane-bound UCP that the 10 C-terminal amino acids protrude to the cytosol (Eckerskorn & Klingenberg, 1987; Klingenberg & Appel, 1989). On the basis of models of the secondary structure (Aquila et al., 1985, 1987; Klingenberg et al., 1987; Runswick et al., 1987), the binding center at the hydrophilic center of the third repeat domain seems to be located more at the matrix side of the protein. This can be reconciled with the labeling results by assuming that this region of UCP is folded through the membrane and forms a nucleotide binding pocket within the transmembranal arrangement of the protein (Figure 11).

An intrusion into the membrane area of the central hydrophilic sections within each repeat domain was first proposed on the basis of probing the accessibility of the lysine residues of the bovine heart AAC with the membrane-impermeable pyridoxal phosphate (Bogner et al., 1986). The three central hydrophilic loops were suggested to line the translocation channel. Because of the similarity between UCP and AAC, a transmembrane localization of the nucleotide binding center is also suggested for UCP. Moreover, in AAC, the central hydrophilic section, within the second repeated domain, contained the incorporated azidonucleotides (Dalbon et al., 1988; Mayinger et al., 1989). Again, this part of the AAC has to be accessible for the azidonucleotides from the cytosolic side and probably also forms a hydrophilic pocket in the center of this protein in accordance with the results from the pyridoxal phosphate probe (Bogner et al., 1986).

In UCP which is assumed to have evolved within the mitochondrial carrier family, the locus of the nucleotide binding site seems to be partially conserved within the tripartite domain structure. However, it exhibits different specificity for nucleotide binding. As previously pointed out, in UCP the lower base specificity reflects a smaller intrinsic binding energy of the nucleotide than in AAC (Klingenberg, 1985). In UCP, the nucleotide binding produces an inhibition of the

H^+/OH^- transport, whereas in AAC the bound nucleotide activates its own transport by inducing a large conformational change associated with the translocation. This requires a larger interaction energy which is reflected in the higher specificity of the AAC toward the base portion of nucleotides.

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Registry No. ATP, 56-65-5; Thr, 72-19-5; Cys, 52-90-4.