Photoaffinity Labeling of Uncoupler Binding Sites on Mitochondrial Membrane

C. K. Ramakrishna Kurup* and D. Rao Sanadi

*Department of Cell Physiology, Boston Biomedical Research Institute,
Boston, Massachusetts, 02114 and
Department of Biological Chemistry, Harvard Medical School,
Boston, Massachusetts, 02115*

Abstract

^{3}H 2-azido-4-nitrophenol, a photoactive uncoupler, has been synthesized, and its uncoupling action on oxidative phosphorylation and its binding to the mitochondrial membrane have been studied. The uncoupler bound covalently to the mitochondrial membrane on photoirradiation was 3–4 times that bound reversibly in the absence of light. When irradiation was carried out in the presence of serum albumin, covalent binding was significantly depressed. The pattern of loss of ATP–P_i exchange activity with increasing amounts of the uncoupler suggests that serum albumin prevents the binding of the uncoupler to the functional sites as well. Polyacrylamide gel electrophoresis of photoaffinity labeled submitochondrial particles in the presence of sodium dodecyl sulfate revealed that a 9000 dalton peptide bound high levels of uncoupler. Other proteins in the molecular weight range of 20,000–40,000 and 55,000 were also labeled. Photolysis in the presence of serum albumin or ATP

ABBREVIATIONS

NPA = 2-azido-4-nitrophenol; DNP = 2,4-dinitrophenol; DCCD = N, N^1-dicyclohexyl-carbodiimide; AE particles = bovine heart submitochondrial particles prepared by treatment with NH$_4$OH and EDTA at pH 8.8; RCl = respiratory control index; BSA = bovine serum albumin.

* Present address: Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India.

decreased the covalent binding of the uncoupler to all the proteins, but particularly to the 20,000 dalton component. Soluble ATPase and the mitochondrial proteolipid purified from labeled mitochondria showed the presence of label.

Introduction

Since the discovery [1, 2] that aryl azides on photolysis give rise to highly reactive nitrenes, azido compounds have been used extensively for the photoaffinity labeling of the binding sites of enzymes [1, 3–8], antibodies [4, 9–11], carrier proteins [12–14], membranes [9, 15–17], and DNA [18]. Recently, it was shown by Hanstein and Hatefii [19] that the photoactive 2-azido-4-nitrophenol (NPA), a structural analog of the classical uncoupler 2,4-dinitrophenol, could be advantageously used to investigate uncoupler binding sites in mitochondria. They recognized the existence of high- and low-affinity sites for the reversible binding of the compound on mitochondrial inner membrane. On photoirradiation the compound was attached covalently to a protein which did not appear to be related to the soluble ATPase. In contrast to this, Bastos [20] reported that in yeast mitochondria, azido-ethidium bromide as well as DNP bind specifically to the smallest subunit of ATPase complex.   Evidence that the high-affinity binding sites for NPA are functionally involved in uncoupling rests primarily on the observation [19] that other uncouplers competitively inhibit the equilibrium binding of NPA to these sites. Similar correlation in covalent binding has not been established. We have attempted to evaluate the importance of these binding sites for mitochondrial function and photoaffinity binding of NPA to mitochondrial membranes. The results, unpublished hitherto, are presented and their implications discussed in this paper.

Materials and Methods

Synthesis of NPA

Since the preparation of NPA is not reported in the literature [See Reference 19] some details are given below: The starting material 2-amino-4-nitrophenol was diazotized and coupled with sodium azide. The azido compound thus produced was extracted into chloroform. The solution was washed with 1 N HCl, then repeatedly with water, dried, evaporated to a small volume, and left at −15°C when the azido-nitrophenol crystalized out as straw-colored needles (uncorrected mp 94–95°C). On thin-layer chromatography on silica gel the crystalline product moved as a single spot (R_f 0.6) with hexane–ether–chloroform

(1:1:1) as solvent. The starting material moved with R_f 0.2–0.3. In view of the light-sensitive nature of the compound, the above operations were carried out with minimal lighting. Details of the method will be supplied upon request.

The identity of the compound was established by the following criteria:

(i) Infrared spectrum (KBr) (Perkin-Elmer model 21) showed absorption bands at 3350 cm^{-1} (broad, phenolic $-OH$), at 2140 cm^{-1} (strong, N_3 asymmetric stretching), and at 1520 cm^{-1} and 1335 cm^{-1} ($-NO_2$).

(ii) Ultraviolet absorption spectrum (CHCl$_3$) (Cary model 15) showed maxima at 259, 292, and 331 nm. The starting material absorbed maximally at 258, 304, and 369 nm.

(iii) Nuclear magnetic resonance spectrum (CDCl$_3$) (Varian T–60, tetramethyl silane internal standard) showed signals at $\delta 6.23$ (s,1,phenolic–OH), 7.0 (m,1,aromatic) and 8.03 (m,2,aromatic).

(iv) High-resolution mass spectroscopy (CEC 110B double focusing, source 180°, standard 80°) showed high-intensity ion peaks at m/e 180.02811 corresponding to the composition $C_6H_4N_4O_3$ (calculated M$^+$ 180.02834) and M$-N_2$ at m/e 152.02291 (calculated 152.02219) (data courtesy of N.I.H. Mass Spectrometry Unit, Department of Chemistry, Massachusetts Institute of Technology).

Estimation of NPA

The compound could be conveniently estimated by its absorbance at 259 nm (ϵ 16.23 $\times$ 10^3 cm^{-1} mM^{-1}) in CHCl$_3$. The increase in absorbance was linear up to the concentration range tested (100 µM). The spectrum showed a characteristic trough at 278 nm and the absorbance 259–278 nm (ϵ 9.0 $\times$ 10^3 cm^{-1} mM^{-1}) was also linear with concentration. Since the presence of impurities decreased the latter absorbance, the ratio of the absorbance at 259 nm and 259–278 nm served as a measure of the purity of the compound.

Preparation of [^{3}H]NPA*

Tritiation was accomplished in N,N-dimethyl formamide by catalytic exchange (Pt black) with ^{3}H$_2$O. Labile tritium was removed *in vacuo* using ethanol. After removal of the catalyst, NPA was extracted into chloroform. The solution was purified as described earlier and chromatographed on

---

* Tritiation and removal of labile tritium were carried out by New England Nuclear, Boston, Massachusetts 02118.

silicic acid (sil-LC, Sigma Chemical Co.) column). The compound was eluted with chloroform, rechromatographed on silicic acid, and eluted with chloroform:hexane:ether (1:1:1). The solvents were removed by evaporation and NPA taken up in chloroform. This solution was at least 95% pure (spectroscopic) and showed only one spot on thin-layer chromatograms. It had a specific radioactivity of 11.8 mCi/mmole. All the operations described above were carried out in a darkened room.

Mitochondria

Rat liver mitochondria (twice washed) were prepared as described by Kurup et al. [21]. Heavy bovine heart mitochondria were prepared as described by Sanadi et al. [22]. Submitochondrial AE particles were prepared according to Lam et al. [23] except that sonic disruption was carried out at pH 8.8 and the submitochondrial particles were suspended in 0.25 M sucrose containing 10 mM Tris-SO$_4$, pH 7.5. The factor B used in these experiments was purified through the DEAE-cellulose stage [23]. About 40 mg of the Factor B protein was dialyzed against 2 liters of 10 mM Tris-SO$_4$, pH 7.5, 0.5 mM EDTA for 6 hr with one replacement of buffer and centrifuged at 150,000 × g for 30 min. Both AE particles and Factor B were tested using the energy-linked reduction of NAD$^+$ as the assay [24].

Assay Procedures

The reaction medium for the assay of ATP–^{32}P$_i$ exchange activity consisted of 100 mM Tris-SO buffer, pH 7.8, 10 mM potassium phosphate buffer, pH 7.8, 30 mM MgCl$_2$, 1.5 mM EDTA, 2.5 × 10^5 cpm ^{32}P$_i$ and 1 mg of particle protein. After incubation at 37°C for 5 min, the reaction was started by the addition of 24 mM ATP and incubated further for 5 or 10 min. The reaction volume was 1 ml. The reaction was arrested by the addition of 0.5 ml of 20% trichloroacetic acid and the mixture was processed as described by Lam and Yang [25]. Under these conditions the exchange activity was linear at least for 10 min and up to 1 mg of protein in the assay. No correction was applied for the ATPase activity of the particles. Protein was estimated by the biuret method [26], deoxycholate being used for solubilization. Inorganic phosphate was determined by the method of Fiske and Subbarow [27].

Binding of [^{3}H]NPA

Equilibrium binding was carried out in a medium containing 100 mM Tris-SO$_4$ buffer, pH 7.5, 30 mM MgCl$_2$, 1.5 mM EDTA, and 5 mg of

particle protein in a total volume of 5 ml. Other additions were made as indicated. After incubating at the desired temperature for 5 min, either the particles or [^{3}H]NPA were added and incubation was continued for 5 min. The particles were sedimented by centrifugation at 40,000 rpm for 30 min using a number 65 rotor of a Beckman model L-2 ultracentrifuge. The supernatant liquid was poured off and the tubes kept inverted over a filter paper for 15–30 min. The sides and mouth of the tubes were wiped free of any adhering fluid. The tubes were left overnight after the addition of 1 ml of Unisol and 0.1 ml of H_2O_2 (30%). Alkali (0.1 ml of 5 N KOH) was added where indicated. After the transfer of the contents into vials, the tubes were washed with 0.5 ml methanol. Unisol complement (10 ml) was added and the radioactivity determined within 2% error, after a delay of at least 2 hr, in a Beckman LS-230 liquid scintillation counter. To calculate the efficiency of counting, the samples were counted again after the addition of standard ^{3}H toluene. The efficiency was about 25%. The minimum radioactivity counted was about 20 times the background radiation (10–12 cpm).

Photoirradiation to induce covalent binding of NPA was carried out in the same system used for equilibrium binding. The tube was held partially immersed in the middle of a 1-liter beaker containing 5% wt/vol $CuSO_4$ solution. A slow stream of argon was bubbled through the contents of the tube (1 or 2 bubbles/second). The temperature of the $CuSO_4$ solution was maintained at 2°C using an immersion cooler. The light source (750 W, DDB-DDW lamp housed in a Beseler slide projector) was placed at a distance of 15 in. from the middle of the $CuSO_4$ solution. The light intensity was approximately 0.25 W/cm^2. During irradiation (5 min) there was no perceptible rise in temperature. The particles were sedimented as before, washed twice with 5 ml portions of 10 mM Tris-SO$_4$ buffer (pH 7.5) containing 5 mg BSA per ml, each time homogenizing in a Potter-Elvehjem homogenizer. One wash was found adequate to remove about 95% of the non-covalently-bound NPA. There was no measurable loss of protein by the wash. The particles were solubilized with Unisol and radio-activity was measured as described earlier.

Gel Electrophoresis

Acrylamide gels (10% acrylamide) with the base sensitive cross-linking agent ethylene diacrylate [28] were prepared essentially as described by Weber and Osborn [29]. The protein (AE particles) containing covalently bound NPA was digested with sodium dodecyl sulfate and mercapto-ethanol, applied on the gel (100 μg) and electrophoresed in 25 mM Tris-glycine buffer, pH 8.4. Ten gels (approximately 105–107 mm long) were

sliced with a lateral gel slicer (Ames Company) giving 68–70 slices per gel. The corresponding slices were combined, digested overnight with 0.5 ml Unisol, mixed with 2 ml methanol, and counted (5% error) after addition of 10 ml complement as described earlier. Two of the gels were stained with Coomasic Blue and scanned at 600 nm in a Gilford model 240 spectrophotometer with the model 2410 Linear Transport attachment.

Chemicals

Solutions of NPA were neutralized and kept frozen and protected from light. Thin-layer chromatographic plates (Baker-flex silica gel 1B) were obtained from Baker Chemical Company, Phillipsburg, New Jersey. Unisol and complement were purchased from Isolab, Inc., Akron, Ohio. Internal Standard ^{3}H toluene (National Bureau of Standards) was purchased from New England Nuclear, Boston, Massachusetts. Ethylene diacrylate was obtained from Monomer–Polymer Laboratory, Philadelphia, Pennsylvania.

Results

Binding of NPA

The characteristics of NPA binding to mitochondrial membranes were tested over a wide range of uncoupler concentration (up to 100 M), protein concentration (up to 22.5 mg in 5 ml), and period of incubation (up to 45 min). It was found that incubation for 5 min was adequate to establish equilibrium. Although increase in protein concentration resulted in increased binding of NPA, the studies reported here were done, unless specified otherwise, at a protein concentration of 1 mg/ml to simulate the experimental conditions under which the uncoupling action of the compound was studied. After equilibrium binding, the particles were sedimented at the same temperature at which the binding was carried out.

Several of the findings of Hanstein and Hatefi [19] have been confirmed in this study and other additional properties of the system were uncovered. The equilibrium binding of NPA to bovine heart mitochondria and sub-mitochondrial particles was biphasic with values for high-affinity binding (i.e., concentration of high-affinity sites) and dissociation constants in the range reported by them. In addition, the NPA level in the medium at which half the high-affinity sites were bound (3.5 nmol/mg protein) corresponded closely with that for half-maximal uncoupling (2.7 nmol/mg protein), as determined by release of controlled (state 4) respiration with glutamate-malate as substrates using rat liver mitochondria. This

correlation adds further support that NPA might bind, among other groups, the uncoupler site also. The binding sites in AE particles (0.67 mol/mg protein) were about three fold higher in concentration compared to the sites in heart mitochondria (0.22 nmol/mg), which would confirm that the uncoupler binding sites are located in the inner mitochondrial membrane [19]. In these experiments the high-affinity binding sites on the membrane were determined by the double reciprocal plot of NPA bound to NPA free as described by Hughes and Klotz [30].

Effect of ATP

It has been reported that ATP does not influence the binding of NPA to mitochondrial membrane [19]. In our experiments the amount of uncoupler bound to the membranes in the presence of ATP was
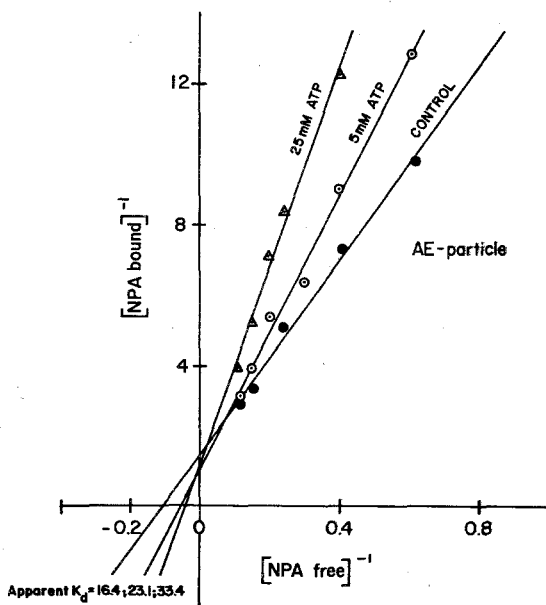

Figure 1. Effect of ATP on high-affinity binding of NPA to AE particles. To minimize ATP hydrolysis, the binding was carried out at 2°C. Other details were given in the Materials and Methods section. The specific binding for AE particles was calculated by subtracting the unspecific binding from total binding. Double reciprocal plots of NPA bound and free in the absence of ATP (●: control) and in the presence 5 mM (○) and 25 mM (△) ATP are given in the figure. The concentration of NPA is expressed in nmoles mg^{-1} protein. The unspecific binding of NPA to AE particles was 0.0275 nmoles/nmole of NPA added in the absence of ATP and 0.0250 nmole in the presence of 25 mM ATP. The apparent dissociation constant K_d (nmoles mg^{-1} protein) in the presence and absence of ATP is indicated.

consistently lower than the amount bound in its absence. In order to substantiate this phenomenon, the binding was studied at 2°C, at which temperature ATP hydrolysis is very low. Under these conditions, ATP (1–25 mM) did not appear to influence the nonspecific binding of NPA to rat liver mitochondria (0.0225 nmole NPA per mg protein). In the case of AE particles, the nonspecific binding decreased slightly, from 0.0275 per nmol of added NPA per mg protein (no ATP) to 0.0250 nmol in the presence of 25 mM ATP. The effect of varying the concentration of ATP on the specific binding of NPA to AE particles is illustrated by the double reciprocal plots presented in Fig. 1. In the presence of ATP both the apparent dissociation constant and the concentration of binding sites for NPA are altered. Replots of the slope versus ATP concentration appeared to be hyperbolic, suggesting the inhibition to be of the "partial competitive" type [31]. Similar results were obtained with rat liver mitochondria as well (data not given). The data suggest that the binding of ATP indirectly affects the binding of NPA.

Addition of ADP (20 mM) depressed equilibrium binding of NPA to AE particles about 25% while AMP (20 mM) had no effect. Ortho-phosphate did not have any influence on the binding of NPA to the membrane.

Photolabeling

There has been no detailed documentation of covalent binding of NPA to membrane. Under our conditions of photoirradiation, the amount of NPA covalently bound to the membrane depended both on the time of irradiation and the amount of NPA present in the medium. At low concentrations of NPA (1 μM) the amount bound appeared to be proportional to the time of irradiation and was maximum in 2 min. Prolonging the irradiation up to 10 min resulted in no further incorporation. At high concentrations of NPA (100 μM) the rate of incorporation was high for the first two min. Thereafter, the binding increased at a slower rate during the entire 10 min period tested (data not given).

In contrast to equilibrium binding, photoinduced covalent binding of the uncoupler increased linearly with its concentration in the medium over a wide range of 1–80 μM, indicating that photoinduced covalent binding is not confined to the high-affinity binding sites for the uncoupler. This was true in all the three systems that were tested.

Effect of BSA

In the case of bovine heart mitochondria and AE particles, the amount of NPA bound covalently on photoirradiation was 4–5 times that bound

reversibly (equilibrium) at the same concentration of NPA. With rat liver mitochondria, however, there was better agreement between the two values at low concentrations of NPA (up to 5 μM). Even in this case the amount of NPA covalently bound at concentrations high enough to saturate the high-affinity binding sites (10 μM) was twice that bound reversibly. The results are similar to those of Ruoho et al. [14] on the photaffinity labeling of acetylcholine esterase with arylazides. They observed that nonspecific reaction of the photogenerated species with proteins can be reduced by the additions of "scavengers" like p-aminobenzoic acid or BSA. In our experiments, p-aminobenzoate (10 mM) was not effective, but BSA effectively depressed the incorporation of the label in all three systems. Equilibrium binding was also lowered in the presence of BSA by a factor of 4 at 10 mg/ml.

Irradiation in the presence of the ATP (25 mM) lowered the incorporation about 25% at all concentrations of NPA. Even in the presence of BSA, the incorporation was further depressed (20%) by ATP (25 mM).
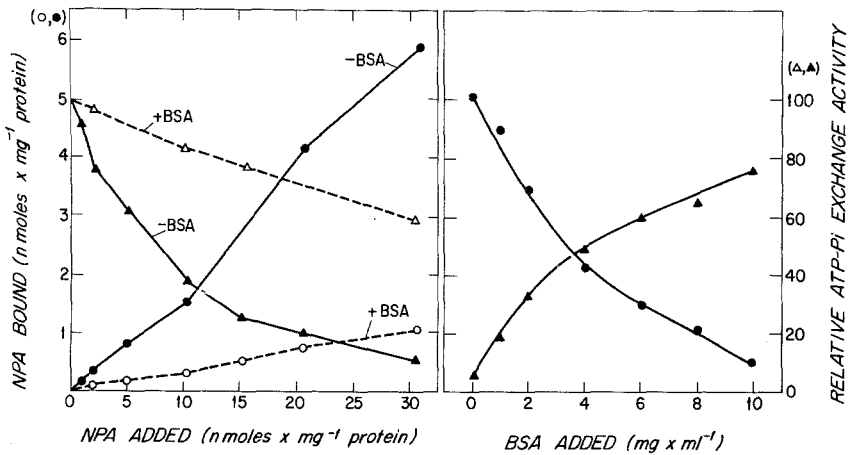

Figure 2. Left: Effect of photoinduced binding of NPA on ATP–P_i exchange activity of AE particles. Particles were incubated at 2°C with concentrations of NPA as indicated for 5 min and then irradiated with (O--O) or without (●—●) the addition of BSA (5 mg/ml for 2.5 min). The particles were washed once, suspended in 0.25 M sucrose, and the exchange activity was determined in the presence of factor B. The activity of particles irradiated with (△--△) and without (▲—▲) BSA is expressed as percent of control activity. The activity of the particle was 61 nmoles min^{-1} mg^{-1} protein. Right: Effect of irradiation with NPA in the presence of BSA on the ATP–P_i exchange activity of AE particles. Particles were incubated for 5 min at 2°C with 40 μM NPA, then BSA was added as indicated and they were irradiated for 2.5 min, washed once, and suspended in 1 ml 0.25 M sucrose. Activity was determined in the presence of Factor B. The exchange activity (▲—▲) is expressed as percent of controls (as in Fig. 2a). The bound NPA (●—●) was determined as described in the Materials and Methods section.

The relationship between the photoinduced covalent binding of NPA and the loss of ATP–P_i exchange activity is illustrated in Fig. 2. The covalent binding was found to be 3–5 times, and the inhibition of activity 2–3 times as high as that observed with equilibrium binding. However, as the concentration of NPA was increased, extraneous binding increased considerably. When irradiation was carried out in the presence of BSA, the covalent binding of NPA was decreased drastically, and the particles retained their activity to a large extent, indicating thereby that BSA prevented covalent binding of the uncoupler to the high-affinity site as well. The ability of BSA to prevent covalent binding of NPA to the functional site is demonstrated in Fig. 2 (right). With progressive increase in the concentration of BSA present during irradiation, the covalent binding of NPA decreased and the activity of the particles remained high.

Gel Electrophoresis

In order to identify the proteins involved in the binding of NPA, AE particles after irradiation with NPA (20 µM) were subjected to polyacrylamide gel electrophoresis in the presence of SDS. The distribution of proteins and radioactivity is shown in Fig. 3. Even though most of the protein bands carry the label, a major part of NPA appeared to be associated with two proteins of approximate molecular weight 9000 and 20,000, respectively. Two other proteins (approximate molecular weight 13,000 and 55,000) carried less, but significant, amounts of label. When photolysis was carried out in the presence of ATP (50 mM), the binding of NPA to these proteins decreased. The decrease appeared to be greater in a 20,000-dalton polypeptide, but was also seen distinctly in the 55,000- and 9000-dalton peptides. When irradiation was carried out in the presence of BSA (5 mg/ml), the covalent binding of NPA to all proteins was decreased by about 50%. Even in the presence of BSA, ATP lowered the binding of NPA, particularly to the 20,000-dalton protein (Fig. 3). When BSA in the medium was increased to 10 mg/ml, the binding of NPA was lowered by 75%. Even in this case, the effect of ATP in decreasing the binding of the uncoupler to the 20,000 molecular weight protein could be distinguished (data not shown). When the concentration of NPA during irradiation was lowered to 10 µM, the radioactivity bound to the protein was lowered. However, the general pattern of distribution was not altered. De-lipidated particles also yielded the same pattern.
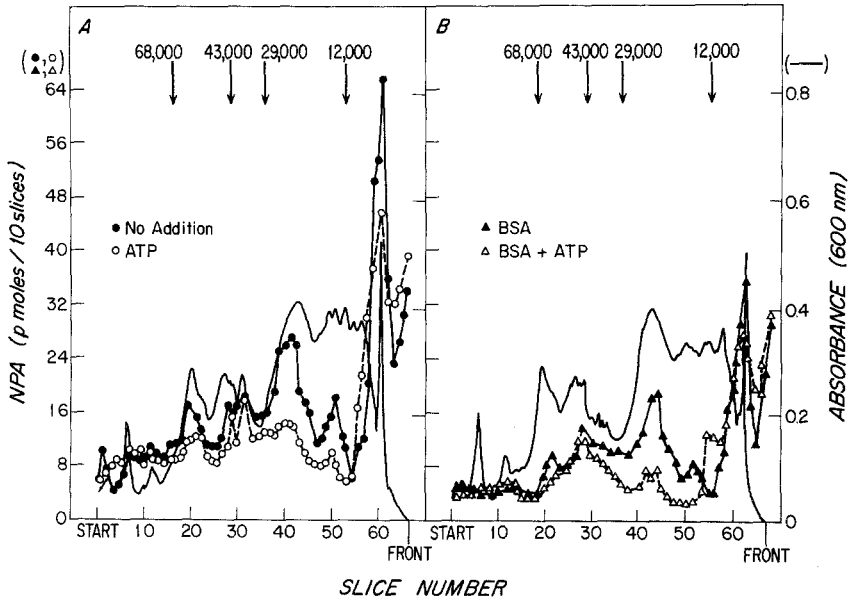
Figure 3. Polyacrylamide gel electrophoresis of AE particles containing covalently bound NPA. Particles were irradiated in the presence of 20 µM NPA either alone (●—●) or in the presence of 50 mM ATP (O - - O), 5 mg/ml BSA (▲ - - ▲), and ATP and BSA (△ - - △). The particles were washed, electrophoresed, and sliced, and radioactivity in 10 slices was determined as described in the Materials and Methods section. The protein bands were stained with Coomassie blue and scanned at 600 nm (—). The molecular weights of the protein bands calculated from the mobility of standard proteins (BSA, ovalbumins, carbonic anhydrase, and cytochrome c) electrophoresed under the same conditions is indicated by arrows. The values given in the ordinate are for 10 replicate slices counted together.

DCCD-Binding Protein

From AE particles containing covalently bound NPA, the DCCD-binding proteolipid was isolated by treatment with chloroform–methanol and ether precipitation [32, 33]. On polyacrylamide gel electrophoresis with sodium dodecyl sulfate present, the protein moved as a single band, the mobility being the same as that of the small-molecular-weight protein (9000) in Fig. 3. The radioactivity also corresponded with the protein band (data not presented). From AE particles containing covalently bound NPA (5.2 nmoles mg^{-1} protein), the soluble ATPase was isolated by treatment with chloroform [34]. It contained 6.4 nmoles of covalently bound NPA $\times$ mg^{-1} protein. The ATPase isolated similarly from the AE particles irradiated with NPA in the presence of ATP (5.2 nmoles bound NPA per mg particle protein) carried 3.6 nmoles of NPA per mg ATPase protein.

These results show that the uncoupler binds to ATPase also, and that the binding is slightly decreased by the presence of ATP, which is consistent with the data in Fig. 1.

Discussion

The technique of affinity labeling rests on the principle that by virtue of its stereospecific complementarity, the labeling reagent combines specifically and reversibly with the receptor site [35]. Since compounds that uncouple oxidative phosphorylation bear little structural similarities to one another, presumably steric factors would play a minor role in the binding of the uncoupler at the functional site on the mitochondrial membrane. In the chemical tagging of these sites, therefore, the danger of nonspecific binding would be greater than in the reactions with enzymes, antibodies, etc., which are characterized by a high degree of structural specificity in receptor–ligand interactions. This is amply illustrated by the fact that the binding of the uncoupler does not reach saturation even at concentrations many times in excess of those required to uncouple oxidative phosphorylation. This phenomenon was first observed in 1956 by Jacobs et al. [36], who used cadmium ion as an uncoupler and ^{115}Cd for the binding studies. Three observations, however, suggest that NPA binding to uncoupling sites does indeed occur. They are (a) the presence of high-affinity binding sites for NPA, (b) the competitive inhibition of high-affinity binding by other uncoupling agents [19], and (c) the good correlation between the concentrations for half-maximal uncoupling in AE particles and for binding of half the high-affinity sites.

   Our observation that the photoinduced covalent binding of NPA is many times higher than equilibrium binding resembles the findings of Ruoho et al. [4], that photolytic covalent binding of azido compounds to acetylcholine esterase in erythrocyte membrane preparations was 1000 times as high as equilibrium binding. Similar results were obtained in antibody labeling as well. We have shown that scavenging agents like BSA help to depress the covalent binding of the reagent (Fig. 3). From our results it would also appear that the chances of specific labeling of the mitochondrial uncoupler binding sites by NPA are increased by using low levels of proteins, low concentrations of NPA, and short periods of irradiation. The present studies were carried out with attention to these considerations. It should be noted that the danger of scavenging may also arise when photolysis is carried out at higher mitochondrial protein concentration [19], and may possibly account for differences in the results with respect to binding of the 9000-dalton polypeptide. The judicious use of low concentrations of BSA may also prove helpful.

The 20,000-dalton polypeptide, which shows high affinity for NPA, has yet to be characterized. The smaller, 9000-dalton polypeptide appears to be the same as the DCCD-binding protein, a subunit of mitochondrial oligomycin-sensitive ATPase. NPA binding to the 55,000-dalton peptide, probably a subunit of soluble ATPase, is of interest from the observations of Cantley and Hammes [37], who demonstrated that at least one specific binding site for DNP is present on the ATPase, and that the presence of the uncoupler influences the binding of ATP to the enzyme.

The pattern of distribution of radioactivity presented in this paper (Fig. 3) may be contrasted with the report of Hanstein and Hatefi [19], who found that the bulk of the NPA was bound to the larger polypeptide (30,000 daltons), and that of Bastos [20] that the covalent binding of azido-ethidium bromide is confined to the smaller polypeptide (molecular weight 9000). It may, however, be stated that the electrophoretic pattern presented by Hanstein and Hatefi [19] shows significant radioactivity in the small peptide region (molecular weight 9000) as well. When longer gels were used for electrophoresis, Hatefi et al. [38] observed significant radioactivity accumulating in the low-molecular-weight region (7000–9000) where no protein band was observed. This may indicate that the content of this protein in the membrane or its affinity to dyes may be much less than of proteins of higher (30,000) molecular weight.

It is of considerable interest that three proteins (ATPase, 20,000-dalton peptide, and the Beechey proteolipid) bind the uncoupler in significant amounts, and the binding to at least the latter two, if not all three, is reduced in the presence of ATP. Two of them, ATPase and proteolipid, are concerned with the process of energization of the membrane system by ATP.

References

1. G. W. J. Fleet, R. R. Porter, and J. R. Knowles, *Nature (London)*, 224 (1969) 511–512.
2. J. R. Knowles, *Acc. Chem. Res.*, 5 (1972) 155–160.
3. G. W. J. Fleet, J. R. Knowles, and R. R. Porter, *Biochem. J.*, 128 (1972), 499–508.
4. A. E. Ruoho, H. Keiffer, P. E. Roeder, and S. J. Singer, *Proc. Natl. Acad. Sci. USA*, 70 (1973) 2567–2571.
5. M. Yoshika, J. Lifter, C. L. Hew, C. A. Converse, M. Y. K. Armstrong, W. H. Konigsberg, and F. F. Richards, *Biochemistry*, 12 (1973) 4679–4685.
6. C. L. Hew, J. Lifter, M. Yoshika, F. F. Richards, and W. H. Konigsberg, *Biochemistry*, 12 (1973) 4685–4689.
7. J. Lifter, C. L. Hew, M. Yoshika, F. F. Richards, and W. H. Konigsberg, *Biochemistry*, 13 (1974) 3567–3571.
8. F. F. Richards, J. Lifter, C. L. Hew, M. Yoshika, and W. H. Konigsberg, *Biochemistry*, 13 (1974) 3572–3575.
9. H. Keifer, J. Lindstrom, E. S. Lennox, and S. J. Singer, *Proc. Natl. Acad. Sci. USA*, 67 (1970) 1688–1694.
10. S. C. Hixon and S. S. Hixon, *Biochemistry*, 14 (1975) 4251–4255.
11. A. H. Pomerantz, S. A. Rudolph, B. E. Haley, and P. Greengard, *Biochemistry*, 14 (1975) 3858–3862.
12. J. A. Katzenellenbogen, H. J. Johnson, Jr., and H. N. Myers, *Biochemistry*, 12 (1973) 4085–4092.
13. M. Erecinska, J. M. Vanderkooi, and D. E. Wilson, *Arch. Biochem. Biophys.*, 171 (1975) 108–116.
14. D. F. Wilson, Y. Miyata, M. Erecinska, and J. M. Vanderkooi, *Arch. Biochem. Biophys*, 171 (1975) 104–107.
15. C. E. Guthrow, H. Rasmussen, D. J. Brunswick, and B. S. Cooperman, *Proc. Natl. Acad. Sci. USA*, 70 (1973) 3344–3346.
16. J. V. Staros, B. E. Haley, and F. M. Richards, *J. Biol. Chem.*, 249 (1974) 5004–5007.
17. B. E. Haley, *Biochemistry*, 14 (1975) 3852–3857.
18. S. C. Hixon, W. E. White, Jr., and K. L. Yielding, *Biochem. Biophys. Res. Commun.*, 66 (1975) 31–35.
19. W. G. Hanstein and Y. Hatefi, *J. Biol. Chem.*, 249 (1974) 1356–1362.
20. R. N. Bastos, *J. Biol. Chem.*, 250 (1975) 7739–7746.
21. C. K. R. Kurup, H. N. Aithal, and T. Ramasarma, *Biochem. J.*, 116 (1970) 773–779.
22. D. R. Sanadi, R. L. Pharo, and L. A. Sordahl, *Methods Enzymol.*, 10 (1967) 297–302.
23. K. W. Lam, J. B. Warshaw, and D. R. Sanadi, *Arch. Biochem. Biophys.*, 119 (1967) 477–484.
24. K. W. Lam, D. Swann, and M. Elzinga, *Arch. Biochem. Biophys.*, 130 (1969) 175–182.
25. K. W. Lam and S. S. Yang, *Arch. Biochem. Biophys.*, 133 (1969) 366–372.
26. A. G. Gornall, G. J. Bardawill, and M. M. David, *J. Biol. Chem.*, 177 (1949) 751–766.
27. C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, 66 (1925) 375–400.
28. G. L. Choules and B. H. Zimm, *Anal. Biochem.*, 13 (1965) 336–344.
29. K. Weber and M. Osborn, *J. Biol. Chem.*, 244 (1969) 4406–4412.
30. T. R. Hughes and I. M. Klotz, *Methods Biochem. Anal.*, 3 (1956) 265–299.
31. I. H. Segel, *Enzyme Kinetics*, John Wiley & Sons, Inc., New York (1975) p. 185.

32. K. J. Cattell, I. G. Knight, C. R. Lindop, and R. B. Beechey, *Biochem. J.,* **117** (1970) 1011–1013.
33. K. J. Cattell, C. R. Lindop, I. G. Knight, and R. B. Beechey, *Biochem. J.,* **125** (1971) 169–177.
34. R. B. Beechey, S. A. Hubbard, P. E. Linnet, A. D. Mitchell, and E. A. Munn, *Biochem. J.,* **148** (1975) 533–537.
35. S. J. Singer, *Adv. Prot. Chem.,* **22** (1967) 1–54.
36. E. E. Jacobs, M. Jacob, D. R. Sanadi, and L. R. Bradley, *J. Biol. Chem.,* **223** (1956) 147–156.
37. L. C. Cantley and G. Hammes, *Biochemistry,* **12** (1973) 4900–4904.
38. Y. Hatefi, W. G. Hanstein, Y. Galante, and D. L. Stiggal, *Fed. Proc.,* **34** (1975) 1699–1706.