Characterization and Localization of Mitochondrial Uncoupler Binding Sites with an Uncoupler Capable of Photoaffinity Labeling*

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SUMMARY

2-Azido-4-nitrophenol (NPA) is a potent, water-soluble uncoupler of oxidative phosphorylation. It is photochemically active and capable of covalent labeling of the mitochondrial components presumably concerned with the uncoupling process. Acrylamide gel electrophoresis of NPA-labeled mitochondria digested with sodium dodecyl sulfate and mercaptoethanol showed that 40% of NPA radioactivity was associated with protein bands in the molecular weight region of 20,000 to 30,000. Equilibrium binding studies with NPA, under nonphotolytic conditions, have shown that bovine heart mitochondria contain 0.56 ± 0.13 nmole of a uniformly reacting uncoupler binding site per mg of protein. The binding of NPA to these sites is competitively inhibited by other uncouplers, but not by antimycin A, rutamycin, valinomycin, or arsenate. The uncouplers tested against NPA were sodium azide, 2,4-dinitrophenol, pentachlorophenol, carbonylcyanide m-chlorophenylhydrazone, and 5-chloro-3-t-butyl-2′-chloro-4′-nitrosalicylanilide. The uncoupler binding sites are located in the inner membrane, and do not appear to involve F1 (ATPase). They are unaffected with respect to NPA binding by changes in the respiratory state or the energization state of mitochondria. Data regarding dissociation constants and the uncoupler potencies of NPA, 2,4-dinitrophenol, and azide have suggested that the uncoupler binding sites of mitochondria are functionally involved in the act of uncoupling.

METHODS AND MATERIALS

Heavy bovine heart mitochondria and various types of submitochondrial particles were prepared according to published procedures (9-11). Protein was estimated by the biuret method

1 The significance of the abbreviations used are: NPA, 2-azido-4-nitrophenol; DNP, 2,4-dinitrophenol; PCP, pentachlorophenol; Cl-CCP, carbonylcyanide m-chlorophenylhydrazone; S-13, 5-chloro-3-t-butyl-2′-chloro-4′-nitrosalicylanilide.
phase was saturated at low levels of NPA (see below), the low
mitochondrial protein suspended in 10 ml of 0.25
aration. concentrations, there is a relatively large uptake of NPA. This
occurs in two distinct phases (Fig. 1A).

RESULTS
Characteristics of 2-Azido-4-nitrophenol Binding to Mitochon-
dria—The binding of NPA to mitochondria is reversible and
occurs in two distinct phases (Fig. 1A). At low uncoupler
concentrations, there is a relatively large uptake of NPA. This
high affinity phase is followed by a weaker binding, which is a
linear function of NPA concentration. Whereas the high affinity
phase was saturated at low levels of NPA (see below), the low
affinity phase showed no such tendency even up to 600 μM NPA
(Fig. 2). Thus, it appeared to have the characteristics of either
very weak, but extensive, binding (> 10 nmol per mg of protein),
or a partition equilibrium between the medium and the mito-
chondrial phases (the results shown in Figs. 1 and 2 might be a
combination of both). By subtracting this low affinity binding
from the total binding of NPA to mitochondria, a hyperbolic
saturation curve is obtained (Fig. 1B), which is characteristic of
the specific binding of NPA to mitochondria. Maximal specific
binding (i.e. the concentration of NPA binding sites), as deter-
mined from double reciprocal plots of a number of experimental
curves similar to B of Fig. 1, is 0.56 ± 0.13 n mole of NPA per mg
of mitochondrial protein at 3° and pH 7.0. This value is of the
same order of magnitude as the concentration of F1 molecules,
estimated from electron microscopic (14) and aurovertin binding
data (15), or the concentration of electron carriers in the vicinity
of the three coupling sites (e.g., FMN + c1 + a0) (16, 17). A
Hill plot of the specific binding data is shown in Fig. 3. The
slope of the straight line is 1.03 in a set of data extending from
15% to 80% saturation of the binding sites. This indicates that
essentially all the specific binding sites have nearly equal affinity
for NPA, and bind NPA without appreciable cooperativity (18).
The dissociation constant (Kp) is 6 ± 3 μM at 3°, and is pH
independent between pH 7 and pH 9.4. The lack of an ap-
preciable pH effect on Kp together with our thermodynamic
studies (ΔH = -8 ± 1 Cal, Δ unitary entropy (ΔS°) = 2 ± 6
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tion.

The biphasic binding of NPA is not a general characteristic of
biomembranes. In the case of erythrocyte ghosts, which do not
contain the enzymic machinery for oxidative phosphorylation, the
binding of NPA was completely monophasic in the presence of
NPA up to 120 μM.
Fig. 3. Hill plot of the specific binding of NPA by mitochondria. [NPA]b,max, number of binding sites for NPA (nanomoles per milligram of protein); [NPA]f and [NPA]b, same as in Fig. 2.

Fig. 4. Effects of succinate and succinate plus ATP on NPA binding by mitochondria.

binding. Therefore, it may be concluded that NPA binding by mitochondria is an equilibrium process and not due to active accumulation driven by succinate oxidation or ATP hydrolysis.

Equilibrium binding studies such as shown above are not possible with water-insoluble uncouplers (e.g. Cl-CCP, S-13), because they are preferentially accumulated in mitochondria. Such studies are also very difficult with DNP, which is water-soluble and structurally very similar to NPA. The reason appears to be related to the fact that, as an uncoupler, DNP is 2 to 3 times less potent than NPA, and its dissociation constant is larger (see below). Thus, when a binding curve such as A of Fig. 1 was plotted for DNP, the slope of the initial, high affinity phase was not sufficiently different from the slope of the low-affinity phase to permit accurate separation and analysis of the two phases. In A of Fig. 1, the initial slope of the total NPA binding curve is nearly 5 times the slope at higher NPA concentrations. A similar experiment with radioactive DNP showed a slope difference of less than 2-fold.

Specificity of Mitochondrial Uncoupler Binding Sites—The specificity of the NPA binding site for uncouplers is illustrated in Tables I and II and Fig. 5. Table I lists the effects of several uncouplers and inhibitors on the over-all binding of NPA to mitochondria. It is seen that uncouplers such as DNP, PCP, Cl-CCP, and azide inhibit the binding of NPA, whereas rutamycin and antimycin A, which are specific inhibitors of phosphorylation and electron transport respectively, have no effect on NPA binding. Table I also shows that the ionophore valinomycin, which is another modifier of membrane function, did not alter the extent of NPA binding to mitochondria. The finding that arsenate does not inhibit NPA binding (Table I) is particularly interesting in view of the fact that arsenate uncouples oxidative phosphorylation, but apparently between the sites of oligomycin inhibition and ATP synthesis (19-22) rather than between the oligomycin inhibition site and the respiratory chain. Therefore, the ineffectiveness of arsenate suggested that inhibition of NPA binding by other uncouplers might involve site specificity. Accordingly, it was found that DNP, azide, PCP, Cl-CCP, and S-13 inhibit NPA binding competitively. An example of such competitive inhibition is shown in Fig. 5. In these experiments, the uncoupler, S-13, which has been considered to act stoichiometrically (23-25), was deliberately used at a concentration (0.4 n mole per mg of protein) which is substoichiometric for total uncoupling. In addition, the considerable competitive inhibition of NPA binding by such small amounts of S-13 suggests that the mitochondrial site concerned with NPA binding is also functionally involved in the act of uncoupling. This point is further supported by the data of Table II, which allow a comparison of NPA with DNP in terms of their \( K_D \) and \( \phi_{1/2} \) values. The latter is the DNP dissociation constant determined from the competitive inhibition of NPA binding by DNP. Thus, it is seen that \( K_D \) and \( \phi_{1/2} \) values are essentially the same for each uncoupler, and that both \( K_D \) and \( \phi_{1/2} \) for DNP are larger than those for NPA by a factor of 2 to 3. In addition, the similarity of the \( K_D \) and \( \phi_{1/2} \) values for DNP further indicate that this compound competes with NPA for the same binding site, and

Table I

| Addition       | Inhibition % |
|----------------|-------------|
| NPA, 5.7 \( \mu \)M |             |
| + DNP, 33 \( \mu \)M | 28          |
| + PCP, 33 \( \mu \)M | 36          |
| + Cl-CCP, 33 \( \mu \)M | 59          |
| + S-13, 1 \( \mu \)M | 30          |
| + \( \text{Na}_2 \), 3 \( \text{mM} \) | 33          |
| + Antimycin A, 25 \( \mu \)M | 0           |
| + Rutamycin, 10 \( \mu \)M | 0           |
| + Valinomycin, 0.5 \( \mu \)M | 0           |
| + Arsenate, 22 \( \mu \)M | 0           |

Fig. 5. Double reciprocal (Benesi-Hildebrand) plot of the specific binding of NPA in the presence and absence of the uncoupler S-13 at 0.4 n mole per mg of protein.
TABLE II

| Uncoupler | $K_D$ | $K_{D,A}$ | $\phi_{C}$ |
|-----------|-------|-----------|------------|
| NPA       | 6 ± 3 | 15-25     | 5-10       |
| DNP       | 13-20 | 15-20     | 15-20      |

* Average of 10 determinations and standard deviation.

excludes the possibility of an allosteric effect of DNP on the affinity of mitochondria for NPA.

Location of Uncoupler Binding Sites—The NPA binding site is present in mitochondria, and in phosphorylating submitochondrial particles. It is also present to the same extent in submitochondrial particles, which are deficient in $F_1$ (4), incapable of phosphorylation or ATP utilization, but still competent, in the presence of oligomycin, for transhydrogenation driven by energy derived from substrate oxidation (11). These results indicate that the uncoupler binding site is located in the inner membrane but apparently not in $F_1$. This is indeed to be expected, because uncoupling is generally believed to occur between the electron transport system and the site of oligomycin inhibition (4).

The NPA binding site appears not to involve the bulk of the mitochondrial lipids, as prior removal of more than 80% of mitochondrial lipids with aqueous acetone (26) did not change the extent of NPA binding. Preparations of the electron transfer complexes (27) did not bind NPA. However, these negative results might be misleading because preparations of the complexes contain deoxycholate, which (a) uncouples oxidative phosphorylation, and (b) might inhibit NPA binding as a result of its unspecific interaction with the mitochondrial hydrophobic regions.

Covalent Labeling of Uncoupler Binding Sites with 2-Azido-4-nitrophenol—Three important questions with regard to covalent labeling of the mitochondrial uncoupler binding sites with NPA are as follows. (a) Does NPA bind to mitochondria covalently when irradiated? (b) Is the labeling of mitochondrial components specific or diffuse? (c) Does covalent labeling occur at the uncoupler binding sites?

With regard to $a$ and $b$, our preliminary results have shown that under appropriate conditions, mitochondria could be labeled with radioactive NPA to the extent of 0.5 nmole per mg of protein. Furthermore, acrylamide gel electrophoresis of NPA-labeled mitochondria in the presence of sodium dodecyl sulfate and mercaptoethanol showed that more than 40% of radioactivity was associated with a protein band in the region of molecular weight 20,000 to 30,000. These results are shown in Fig. 6 (see the region of slices 10 to 15). Further work on the isolation and characterization of the NPA-labeled material is currently in progress.

With regard to $c$, covalent binding experiments were carried out in the presence of several concentrations of DNP, which was shown to be a competitive inhibitor of NPA binding under nonphotolytic conditions. Thus, NPA, mitochondria, and increasing concentrations of DNP were irradiated for 3 min and the particles were analyzed for covalently bound uncoupler. Duplicate experiments were carried out in the dark, and the extent of equilibrium binding of NPA was determined. The results are presented in Fig. 7 as a normalized Dixon-type plot. It is seen that DNP inhibits competitively both the equilibrium and the covalent binding of NPA, thus indicating that the same un-
above. A similar control experiment was carried out with a portion of the same preparation of mitochondria involving all the treatments and washing steps, except that the cold NPA was added after irradiation of mitochondria. The control mitochondria, which did not involve covalently bound, cold NPA, showed a specific uptake of radioactive NPA of 0.34 nmole per mg of protein, which is in agreement with previous results (29) obtained at the pH 7.8 used for this experiment. By comparison, the mitochondria, which were prelabeled with cold NPA, showed only 50% as much capacity for the specific binding of tritiated NPA. These results further indicate that the same finite number of binding sites appear to be involved in equilibrium and covalent binding of NPA to mitochondria.

**DISCUSSION**

**Stoichiometry of Uncoupler Binding**—The chemical hypothesis of oxidative phosphorylation assumes the presence of a high energy intermediate with which uncouplers interact. Therefore, the stoichiometry of uncoupling under various conditions has been a subject of interest in several laboratories (23–25, 31, 32). Potent, water insoluble uncouplers, such as carbonylcyanide phenylhydrazones and S-13, have been used for this purpose, and functional parameters, such as loss of phosphorylation activity, loss of respiratory control, stimulation of ATPase activity, and release of azide-inhibited respiration, have been studied as a function of uncoupler concentration. Margolis et al. (31) showed that the \( \phi_{1/2} \) of carbonylcyanide m-chlorophenylhydrazone for uncoupling oxidative phosphorylation was about $5 \times 10^{-11}$ moles per mg of protein of bovine heart mitochondria, and calculated that for 100% uncoupling it was sufficient to add 1 uncoupler molecule per 9 respiratory chains, or 27 coupling sites. The latter group calculated that in rat liver mitochondria, at comparable oxidation rates, the number of moles of S-13 per mole of cytochrome \( a \) needed for complete uncoupling were 0.30, 0.81, and 1.1 when the substrates were, respectively, ascorbate plus tetramethyl-p-phenylenediamine, succinate, and glutamate-malate. Using functional parameters other than P:O values, Wilson (23) showed that in rat liver mitochondria the amounts of S-13 needed per respiratory chain for complete effect were 0.6, 1.0, and 1.35 molecules when the assays were, respectively, the loss of respiratory control, the stimulation of ATPase, and the release of azide-inhibited respiration. He has concluded, in contrast to the above authors, that the S-13 titer is necessary. This is probably related to the kinetics of the different reactions measured by Wilson.

The above data clearly show that each particular assay condition yields a different uncoupling titer. This is to be expected, because in general kinetic studies yield a functional titer, not a binding site titer. Consider, for example, the enzyme-catalyzed sequence \( A \rightarrow B \rightarrow C \) in which the rate of C production is being measured, but the first reaction is rate limiting and can be stimulated by uncouplers. In such a system, study of the kinetics of C production as a function of increasing uncoupler concentration does not necessarily yield information regarding the titer of the uncoupler with respect to the first reaction. This is because long before saturation of the first reaction with respect to uncoupler is achieved, the second reaction might become rate limiting. Consequently, the result will not be a thermodynamic saturation titer, but a kinetic effectiveness titer, which could vary with the enzymatic reactions under consideration. Because of these complications, it is important to distinguish clearly between the stoichiometry of uncoupling as studied by kinetic methods and the stoichiometry of uncoupler molecules bound per respiratory chain or coupling site.

In our studies, the concentration of uncoupler binding sites was determined from equilibrium binding experiments. Thus the results showed a constant saturation level and an invariable KD regardless of whether the mitochondria were in the oxidized or reduced state, energized by addition of ATP (Fig. 4), de-energized by addition of arsenate (Table I), inhibited at the level of the respiratory chain with antimycin A, or inhibited at the level of high energy transfer reactions with rutamycin. The latter two conclusions also arise from the data of Table I in which antimycin A and rutamycin neither decreased nor increased the extent of NPA binding.

The concentration of uncoupler binding sites found in bovine heart mitochondria by the equilibrium binding method is $0.56 \pm 0.13$ nmole per mg of protein. In these mitochondria, the concentration of electron carriers ranges from 0.15 to 0.7 nmole per mg of protein (except for coenzyme Q, which may be a mobile carrier), and the concentration of F1 molecules is approximately 0.2 nmole per mg of protein (calculated from the data of Reference 4). These comparable stoichiometries between electron carriers, phosphorylation enzymes, and the components at or near the coupling sites suggest that energy conservation, energy coupling and dissipation by uncouplers, and high energy transfer are all related molecular interactions rather than electrochemical or conformational effects transferred from one bulk phase to another. For the latter hypotheses, a stoichiometric relationship of the component of these three systems would not be particularly necessary.

**Effect of Uncoupler Binding on Membrane Structure**—It has been shown that uncouplers change the gross structure of mitochondrial cristae in the energized state (35–37), and reverse the ATP-induced fluorescence enhancement of bound 8-anilino-1-naphthalenesulfonic acid (38). These results have suggested
that uncoupling of energized mitochondria is associated with the conformation changes of cristae detectable both by electron microscopy and by a probe for membrane hydrophobic-hydrophilic interphases. Our data (small ΔS°; invariable K\textsubscript{D} in the absence and presence of succinate plus ATP, antimycin A, rutamycin) indicate, however, that uncoupler binding (as distinct from uncoupling) causes neither a significant net conformation change (i.e. in terms of ordering and disordering) of the membranes, nor a specific conformation change of the uncoupler binding sites. These conclusions are in agreement with the results of Zimmer et al. (39) obtained with a spin label.

Specificity of Uncoupler Binding Sites—The diversity of structure and size of the uncouplers used in this work (S-13, Cl-CCP, PCP, DNP, and azide) is difficult to reconcile with the fact that all these uncouplers are competitive inhibitors of NPA binding, and therefore appear to bind to the same site. Wilson et al. (24) have suggested a general acid-base catalysis for the mechanism of action of uncouplers, in which certain uncouplers are considered to be active in protonated (acidic) form and others in anionic (basic) form. These differences also appeared to have no effect on the recognition of uncouplers by the binding sites described above, since both the "acidic" uncoupler, Cl-CCP, and the "basic" uncoupler, S-13, were competitive inhibitors of NPA binding. The one common feature in the above uncouplers is that in all of them the negative charge is delocalized in a π electron system. This common characteristic may have functional significance, but can hardly account for a conventional site specificity. Therefore, we feel that our competitive binding data suggest a unique structure for the uncoupler binding sites of mitochondria. The isolation and characterization of the mitochondrial component covalently labeled with NPA should provide considerable insight into this puzzle.

Nature of Uncoupler Binding Component—It has been shown that the mitochondrial uncoupler binding sites are located in the inner membrane and do not appear to involve F\textsubscript{1} and the bulk of mitochondrial lipids. The preliminary results of the covovalent binding studies suggest protein involvement in the range of molecular weight 20,000 to 30,000. This provisional molecular weight range excludes a number of electron carriers (e.g. DPNH dehydrogenase, cytochrome c\textsubscript{1}, and cytochrome c\textsubscript{3}) as possible candidates for uncoupler binding. The ineffectiveness of rutamycin and antimycin A on NPA binding also excludes oligomycin sensitivity-conferring protein (40) and the antimycin-binding site of complex III. Since S-13 releases the inhibition of respiration by azide, Wilson (23) has suggested that the site of action of S-13 is associated with cytochrome oxidase. The subunits of bovine heart cytochrome oxidase (41) do not fit well into the molecular weight range of 20,000 to 30,000. However, the suggestion of Wilson is now amenable to direct test, since Hagan and Racker (42) have reconstituted site I oxidative phosphorylation from complex I and coupling factors, one of which contains cytochrome oxidase. This reconstituted system was shown to be sensitive to the uncoupler, dihexafluoracetone-acetone.

An important question with regard to the uncoupler binding sites described above is whether these sites are also involved in the act of uncoupling. We have no rigorous proof for this, but three pieces of information favor this possibility: (a) K\textsubscript{D} values for NPA, DNP (Table II), and azide (not shown) are very close to their respective φ\textsubscript{1/2} values, (b) the specific binding data as analyzed by a Hill plot (Fig. 3) indicate only one type of binding (i.e. in terms of affinity) from 15% to 89% saturation, and (c) all the uncouplers tested to date were competitive inhibitors of NPA binding.

Mechanism of Uncoupling—As stated earlier, the chemical hypothesis of oxidative phosphorylation assumes the presence of a high energy intermediate (X\textsuperscript{*}) with which uncoupler \( \Phi \) interacts and dissipates its energy. Thus the interaction of the uncoupler with X\textsuperscript{*} may be written as

\[
X^* + \Phi \rightleftharpoons X^* - \Phi \rightarrow X - \Phi \rightleftharpoons X + \Phi
\]  

(3)

Our results on NPA binding and competitive inhibition of binding by other uncouplers indicate that uncouplers must also be capable of reversible interaction with the de-energized form of X as shown in Equation 4

\[
X^* + \Phi \rightleftharpoons X^* - \Phi \rightarrow X - \Phi \rightleftharpoons X + \Phi
\]  

(4)

This is because the concentration of X\textsuperscript{*}, and therefore of X\textsuperscript{*} - \( \Phi \), would be expected to vary considerably in the presence and absence of ATP plus succinate, antimycin A, oligomycin, or azide, whereas the concentration of X can be independent of these conditions. In these considerations, we do not imply that \( \Phi \) interacts with the high energy intermediate site on X. Uncouplers might interact with an allosteric site on X or X\textsuperscript{*}, but have a destabilizing effect on X\textsuperscript{*}.

An important consideration with regard to the mechanism of uncoupling is the properties of mitochondria containing covalently bound NPA. This problem is currently under investigation. Our preliminary results seem to suggest that mitochondria partially labeled with NPA show essentially no change in P:O values and state 4 rates with succinate or 3-hydroxybutyrate as substrate. However, state 3 rate and oligomycin-sensitive ATPase activity were both decreased to approximately the same extent. The decreased state 3 rate could not be increased by the addition of uncouplers to the reaction mixture. These effects could be due either to damage or specific labeling of mitochondria. The latter possibility is compatible with a mechanism in which a component required for oxidative phosphorylation is prevented from turning over by photoaffinity labeling.

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1 The hypothetical high energy intermediate (X\textsuperscript{*}) which reacts with uncouplers has been designated A \rightleftharpoons C by Harker (43, 44) and X \rightleftharpoons I by Chance and Williams (2). For the differences between A \rightleftharpoons C and X \rightleftharpoons I, see References 2, 43 and 44.
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