Photoaffinity Labeling of the Antimycin Binding Site in *Rhodopseudomonas sphaeroides*

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PHOTOAFFINITY LABELING
OF THE ANTIMYCIN BINDING SITE
IN RHODOPSEUDOMONAS SPHAEROIDES

by
Emily Wilson

A thesis submitted in partial fulfillment
of the requirements for the degree

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in
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The purpose of this study was to identify the site of interaction of antimycin with the ubiquinone-cytochrome b-c1 oxidoreductase in the photosynthetic bacteria, *Rhodopseudomonas sphaeroides*. To accomplish this goal, three areas of research were undertaken: the synthesis of a radiolabeled, photoaffinity analog of antimycin, identification of the inhibitory characteristics of this analog, and the photoaffinity labeling of the antimycin binding site. All three areas were accomplished.

The major finding of this study was the identification of an 11,000 dalton polypeptide as the predominantly labeled protein. Although this polypeptide was not exclusively labeled, it was consistently labeled and showed competition with antimycin. These results are consistent with a similar study performed by Das Gupta and
Rieske (1973) with a mitochondrial preparation.

These results are not conclusive, but do show several interesting points. First, cytochrome \( b \) is not the only site of interaction of antimycin with the ubiquinone-cytochrome \( b-c_1 \) region of the electron transport chain. Secondly, an 11,000 dalton polypeptide is an important component of this protein complex. The function of this polypeptide is unknown, but should provide interesting research for future studies.
INTRODUCTION

Antimycin A is a potent and specific inhibitor of electron transport within the ubiquinone cytochrome b-c₁ oxidoreductase region of the photosynthetic bacteria (Dutton & Prince, 1978, Gabellini et al, 1982), the analogous Complex III region of mitochondria (Baum et al, 1967, Wikstrom & Berden, 1912), and the cytochrome b₆-f region of chloroplasts (Huber & Edwards, 1977). Because of the ubiquitous and highly specific nature of its inhibition, antimycin is a valuable tool for elucidating electron flow in the regions affected. Much information about the mechanism of the electron transfer through these regions is attributable to the use of antimycin. However, even though there is much knowledge about the effects of antimycin, the actual site of inhibition is unknown.

![Figure 1. General structure of antimycin A](image)

Antimycin A was first reported in 1948 as a fungicide produced by a species of *Streptomyces* (Leben & Keitt, 1948). The first evidence that antimycin toxicity was due to inhibition of mitochondrial respiration was reported by Ahmad et al in 1950. The site of action was further pinpointed by Chance and Williams (1956) who showed that
when respiring mitochondria were treated with antimycin, a redox crossover point was observed between cytochromes b and c. This evidence indicated that the site of inhibition was between these cytochromes. Hatefi and coworkers (1962) demonstrated that antimycin was a potent inhibitor of purified mitochondrial reduced ubiquinol-cytochrome c reductase (Complex III). Chance (1952) showed that antimycin caused oxidant-induced reduction of cytochrome b of the mitochondrial respiratory chain. This unusual property of antimycin has also been shown to occur in Rhodopseudomonas sphaeroides (Dutton & Prince, 1978). This one effect of antimycin has been a "test" for models proposed for electron transport through the ubiquinone-cytochrome b-c1 region. If a model cannot justify this event then it is not a viable model of electron transport through this region. Linear schemes of electron transport through this region have been abandoned because of failure to explain oxidant-induced reduction of cytochrome b (Bowyer & Trumpower, 1981).

The ubiquinone cytochrome b-c1 regions of the respiratory chain of mitochondria and the cyclic photosynthetic electron transport chain of R. sphaeroides show a high degree of similarity. Both systems contain similar redox components. There are two thermodynamically and spectrally distinct b cytochromes, a membrane-bound cytochrome c1, a membrane associated cytochrome c or c2, and an iron sulfur protein which is characterized by an EPR resonance at g=1.90. There is also a bound ubiquinone in both ubiquinone-cytochrome b-c1 regions. The similarity of electron transport components indicates that the
pathways of electron transport may also be similar if not identical in
the b-c$_1$ complexes of mitochondria and the photosynthetic bacteria
(Bowyer & Trumpower, 1981).

Past work on the mode of action of antimycin was done primarily
with mitochondria, submitochondrial particles, and purified Complex
III. However, in recent years the photosynthetic bacteria have been
used to study the kinetics of electron transport in this region.
Because short light flashes may be used as the oxidant, single
electron kinetics can be examined. These experiments have provided
information on the sequence and timing of the redox reactions.
Studies on both mitochondrial and bacterial systems may enable a
"Universal Model" of electron transport through the
ubiquinone-cytochrome b-c$_1$ oxidoreductase to be elaborated.

In spite of the large amounts of knowledge on the effects of
antimycin, the site of action is still controversial. A direct method
of identifying the site in purified Complex III from mitochondria was
attempted by das Gupta and Rieske in 1973. A photoactivatable azide
group was substituted for the formamido group on the aromatic ring of
antimycin (Fig.2.). Rieske's group showed that the analog inhibited
activity in a manner similar to antimycin. Using the tritiated
photoaffinity analog, a specifically labeled protein of molecular
weight of 11,500 was found. Rieske later reported variability in the
labeling pattern and the inhibitory characteristics of the
analog(Rieske, 1976).
Figure 2. Chemical structure of deformamidoazidoantimycin
A similar approach is used in the present work. Results of Dickie et al (1963) showed that replacing the dilactone ring of antimycin with a long chain alkyl amide still produced an inhibitor with similar properties to antimycin (Fig 3a). Furthermore, Neft and Farley (1971) showed that both 3- and 5-nitrosalicyl-N-(n-octadecyl)amide are active analogs of antimycin (Fig. 3b). Menick used the 3-nitrosalicyl-N-(n-octadecylamide) analog in flash studies with membrane vesicles from R. sphaeroides. (Menick, 1984) These studies corroborated that antimycin and this analog inhibited electron flow in a similar manner. With these results in mind, a photoactivatable analog, 3-azidosalicyl-N-(n-octadecyl)amide was synthesized (Fig 3c). Because the dilactone ring of antimycin is somewhat unstable, difficulties are encountered during the synthesis of the azido derivative. This instability may have contributed to the inconsistent results of das Gupta and Rieske (1973). In this regard, the 3-azidosalicyl-octadecylamide analog described in the present work offers experimental advantages.

![Chemical structures of several antimycin analogs](image)

**Figure 3.** Chemical structures of several antimycin analogs  
(a) 3-formamidosalicyl-N-(n-octadecyl)amide  
(b) 3-nitrosalicyl-N-(n-octadecyl)amide  
(c) 3-azidosalicyl-N-(n-octadecyl)amide
Rhodopseudomonas sphaeroides
and its Bioenergetics

*R. sphaeroides* is a facultative photosynthetic bacterium of the purple-nonsulfur type. When grown under anaerobic conditions in light, these bacteria perform light energy transfer and photosynthetic electron transport (Fig. 4). The major protein complexes involved in these reactions are the light harvesting pigment-proteins, the reaction center proteins, a soluble cytochrome \( c_2 \), and the ubiquinol cytochrome \( b-c_1 \) oxidoreductase.

There exist two kinds of light-harvesting antenna pigment-protein complexes. The first is B875 with an absorption peak at 875 nm, and the second is B850 which has absorption peaks at 800 nm and 850 nm. These pigment complexes are composed of bacteriochlorophyll \( a \), carotenoids, and protein and function to absorb and transfer light energy to the reaction centers.

The reaction center is responsible for converting the light energy of the photon to a form usable by the cell. The reaction center is composed of three protein subunits with a total molecular weight of approximately 100,000 daltons, (Okamura et al, 1974) four molecules of bacteriochlorophyll \( a \), two molecules of bacteriopheophytin, a high spin ferrous iron and ubiquinone. Upon excitation by light a special bacteriochlorophyll \( a \) pair undergoes oxidation. The first electron acceptor is a short-lived
bacteriochlorophyll molecule which reduces bacteriopheophytin. Ubiquinone then accepts the electron from the bacteriopheophytin with ferrous iron serving as a modulator. Two types of quinone are present, a $Q_1$ which is more tightly bound and the first of the quinones to receive an electron, and $Q_{II}$ which is more loosely bound and receives the electron from $Q_1$.

$Q_{II}$ passes the electron to the ubiquinone-cytochrome $b$-$c_1$ oxidoreductase. The ubiquinone-cytochrome $b$-$c_1$ is composed of at least two types of $b$ cytochromes, a cytochrome $c_1$, a Rieske-type iron-sulfur protein (Rieske et al, 1964a) and a quinone ($Q_z$) which functions as a reductant for cytochrome $c$. The exact mechanism for electron flow through this region is still controversial. The linear schemes which were originally proposed have been abandoned because of failure to describe a mechanism for oxidant-induced reduction of cytochrome $b$ in the presence of antimycin A. Cyclic models are the likely candidates for the actual mechanism. The two pioneering cyclic models are the $Q$ cycle as proposed by Mitchell, 1975 (Fig.5a) and the $b$ cycle proposed by Wikstrom and Krab (1980) (Fig.5b). Many variations of these two models have been proposed (Fig 5c for example). None of the published models fit all of the known data.

Cytochrome $c_2$ is a mobile electron carrier. The function of cytochrome $c_2$ is to rereduce the oxidized reaction center, thereby completing the cycle. Fig. 4 shows a thermodynamic profile of the photosynthetic cycle.
Figure 4. Thermodynamic profile of light activation and cyclic photosynthesis in R. sphaeroides. (Taken from Prince et al., 1982)
$\Delta E_m$ contributes to $\Delta \psi$

$\Delta E_m$ drives $Q - c_2$ oxidoreductase

$\Delta E_m$ contributes to $\Delta \psi$
Figure 5. Cyclic models of electron flow through the ubiquinone-cytochrome b-c$_1$ oxidoreductase
(a) A modified Q-cycle proposed by Mitchell (1975) (taken from Bowyer & Trumpower, 1981)
(b) The b-cycle as proposed by Wikstrom & Krab (1980) (taken from Wikstrom & Krab, 1980)
(c) A modification of classical cycles as proposed by A. Crofts. (Taken from Wraight, 1982)
Chemical Properties of Antimycin

Fungal-produced antimycin is composed of four structurally related compounds, antimycin A₁, A₂, A₃ and A₄ (Lockwood et al., 1954) (Fig.1). The relative proportions of each vary among fungal strains with antimycin A₁ predominating. The common structure of A₁ and A₃ was determined by chemical degradation (van Tamelan et al., 1961, Dickie et al., 1963). Antimycin is composed of an acyl- and alkyl-substituted nine membered dilactone ring joined to 3-formamidosalicylic acid by an amide bond. Antimycin A₁ and A₃ differ by two methylene groups in the alkyl substituents.

In an attempt to identify the structurally-important functional groups a number of synthetic homologs and analogs of antimycin have been synthesized. Kinoshiota and Umezawa (1971) replaced the dilactone ring with a 15 membered dilactone ring, and Dickie and coworkers (1963) replaced the dilactone ring with an eighteen carbon alkyl group. Both of these analogs retained high inhibitory activity. These studies indicated that the dilactone ring functions in a supporting role, possibly providing the hydrophobicity necessary to penetrate the membrane. Structure-activity studies indicate that the other important functional groups necessary for inhibitory activity are the formamido and the phenolic hydroxyl groups of the aromatic ring. The formamido function is thought to be important as an electron withdrawing group because replacement by a nitro functional group in a
position either ortho or para to the hydroxyl, forming 3-nitro or 5-nitrosalicyl-N- (n-octadecyl)amide, also yields an active compound (Neft, 1971, Neft & Farley, 1971).

Effects of Antimycin on Mitochondrial Respiration

Many phenomena are observed in the properties of Complex III treated with antimycin. Electron transport between ubiquinol and ferricytochrome c is inhibited by stoichiometric concentrations of antimycin (Rieske et al, 1967, Rieske & Zaugg, 1962). Secondly antimycin strongly inhibits the cleavage of cytochromes b and c by dissociating reagents such as bile salts or guanidium salts (Rieske et al, 1967). Thirdly, the b cytochromes are altered in their redox and spectral properties. In the presence of antimycin the rate and extent of reduction of the b cytochromes by succinate or NADH and in purified Complex III by ubiquinol is greatly increased (Rieske, 1971, Pumphrey, 1962, Baum & Rieske, 1966). Other primary effects on cytochrome b are as follows:

1. Antimycin displaces the absorption peak of the ferro forms of both low and high potential cytochrome b-562 to longer wavelengths by 1.5-2.0 nm (red shift) (Chance, 1958, Pumphrey, 1962). It has no effect on the position of cytochrome b-566 or b-558 (Berden & Opperdoes, 1972).
2. Antimycin shifts the signal at $g=3.44$ of ferricytochrome b-562 in the EPR spectrum to $g=3.48$ (Dervartanian et al., 1973). There is no effect on $g=3.8$ due to ferricytochrome b=566 (Orme-Johnson et al., 1971).

3. Antimycin lowers the standard redox potential ($Em$) of high potential b-562 from 154 at pH 7.2 to 107 mV (Berden & Opperdoes, 1972).

4. Multiple splitting of the soret band (415 nm) of the oxidized complex and increased resolution of the absorption band in the visible region is seen with the addition of antimycin (Dervartanian et al., 1973).

These effects on cytochrome b indicate that antimycin affects the binding of one or both of the heme ligands sufficiently that it can be seen in the absorption spectrum of both ferro- and ferricytochrome b (Slater, 1973).

Two EPR-detectable semiquione radicals are present in the mitochondrial complex. Both signals are affected by the presence of antimycin. Antimycin enhances the intensity of the $g=2.00$ signal that is observable at high microwave power, and also causes a shift in the potentiometric titration toward a more positive potential. The $g=2.00$ signal observable at low microwave power disappears upon addition of antimycin (Ohnishi & Trumpower, 1980). This site ($Q_c$) is thought to be closely linked to the antimycin site (Wraight, 1982).
Effects of Antimycin on R. sphaeroides

Antimycin affects the bacterial system in a similar manner to mitochondria. The rapid reduction of flash oxidized cytochrome c2 is abolished (Dutton & Jackson, 1972), and photoreduction of cytochrome b is stimulated. Oxidant-induced reduction of cytochrome b (Dutton & Prince, 1978) and the inhibition of the uptake of the second proton per electron after a short light flash have been shown (Petty & Dutton, 1976, Petty et al, 1977). Antimycin abolishes Phase III of the redshift of the absorption bands of endogenous carotenoid pigments. This shift registers electrogenic charge transfers across the membrane, and Phase III is closely related to ferricytochrome c2 reduction (Jackson & Dutton, 1973, Bashford et al, 1979).

Factors Affecting the Binding of Antimycin A

The Binding of antimycin to mitochondrial membranes and purified Complex III is specific and stoichiometric. The affinity of antimycin for its site is very high. The dissociation constant for antimycin in submitochondrial particles has been estimated to be 0.032 nM with the cytochromes b and c in their oxidized state (Berden & Slater, 1972). The affinity of antimycin for Complex III is related to the oxidation state of the complex. At low antimycin concentrations the oxidized complex binds antimycin better than the reduced complex (Berden &
Slater, 1972). Purified Complex III has a greater affinity for antimycin than membrane-bound complex. Dissociation constants for the oxidized complex have been measured at 0.1 pM (Rieske & das Gupta, 1972).

The binding of antimycin to intact Complex III was found to be essentially irreversible. No transfer of antimycin from treated complex to untreated complex was seen (Rieske et al, 1967). However, up to 70% of the bound antimycin could be extracted using taurocholate or acetone (Rieske et al, 1967). Conditions that gave cleavage of the complex also inhibited antimycin binding (Rieske et al, 1967).

Shape of the Antimycin Inhibition Titration Curve

The shape of the inhibition titration curve of the effects of antimycin in the mitochondria raised some discussion during the early seventies. Sigmoidal titration curves were found for the inhibition of respiration of submitochondrial particles with NADH or succinate as substrate (von Jagow & Boher, 1975) and for inhibition of the growth rate of yeast cells (Burger et al, 1976). The red shift of cytochrome b has been seen to follow curves which are hyperbolic or sigmoidal depending on the experimental conditions (Bryla et al, 1969a). The inhibition of cleavage of isolated Complex III is proportional to antimycin until saturation is reached. Bryla et al (1969b) proposed that the binding of antimycin was cooperative in nature. This
proposal was further supported by Berden and Slater (1972). An alternate proposal was put forth by Potter and Reif (1952) and supported experimentally by others (Kroger & Klinenburg, 1973a, Rieske & das Gupta, 1972). This proposal maintains that the sigmoidal curves of submitochondrial particle result from sequential electron transfer reactions. The electron transfer capacity of the ubiquinol cytochrome b-c region is higher than that of the preceding region from NADH or succinate to ubiquione. Only when the antimycin-sensitive region is sufficiently inhibited to limit the overall electron transport will respiration be significantly inhibited (Kroger & Klingenberg, 1973b).

van den Berg and coworkers (1979) used single turnover flash kinetics and controlled oxidation-reduction potential coditions to study the effects of antimycin on the cytochrome b-c\textsubscript{1} region of R. sphaeroides. The red shift of cytochrome b was found to be linearly proportional to the antimycin added to a titer of 0.7± 0.1 antimycin per reaction center. Other functions showing this linear relationship to antimycin added in R. sphaeroides are the reduction of ferricytochrome c, the oxidation of ferrocytochrome b, Phase III of the band shift of endogenous carotenoid pigments, and the uptake of 1 of the 2 protons per electron transferred. These results support the noncooperative model described for the mitochondrial system (Kroger & Klingenberg, 1973b).
Other Antimycin-Like Inhibitors

Several other antimycin-like inhibitors have been identified. Among these are 2-N-alkyl-4-hydroxyquinoline-N-oxides (HQNO), 3(3,4-dichlorophenyl 1,1-dimethyl urea (DCMU, Diuron), mucidin, and funiculosin. Little structural similarity is seen between these compounds and antimycin yet they show similar properties.

HQNO shows an inhibitory capability at least one order of magnitude less than antimycin. Also, the shape of the titration curve is different. Brandon et al (1972) compared inhibition curves for antimycin and HQNO using succinate oxidase, NADH oxidase, and ATP supported reverse electron transport in submitochondrial particles. Antimycin produced a concave or a sigmoidal curve whereas HQNO produced either a convex or hyperbolic curve. HQNO, like antimycin, induces extra reduction of cytochrome b-566 and a shoulder at 557-558 nm. The increase in absorbance at 557 nm is greater for HQNO than for antimycin. HQNO produces no red shift of cytochrome b$_{562}$ (Brandon et al, 1972). Results indicate that HQNO and antimycin compete for the same site (Van Ark & Berden, 1977).

Funiculosin is an antibiotic produced by *Penicillium funicolosin*. Moser et al (1977) proposed that its activity was much like that of antimycin. Nelson et al (1977) showed that funiculosin inhibits electron flow between cytochrome b and cytochrome c in submitochondrial particles, and also inhibits duroquinol-cytochrome c
reductase activity of isolated Complex III. Funicolosin also induces extra reduction of cytochrome $b$ in aerobic steady state conditions. The binding affinity of funiculosin is less than that of antimycin.

Mucidin, an efficient fungal antibiotic, has also been shown to induce a redox crossover point between cytochrome $b$ and cytochrome $c+c_1$ (Subik et al, 1974a). Mucidin inhibition displays sigmoidal relationships with activity (Subik et al, 1974b). In yeast submitochondrial particles, both antimycin and mucidin inhibit NADH oxidation 50% at concentrations of 0.1 nmole per mg of mitochondrial protein (Convent & Briquet, 1978).

Diuron was developed as a potent herbicide and is known to inhibit photosystem II electron transport in chloroplasts. It is also a respiratory electron transport inhibitor of yeast mitochondria. Inhibition of electron flow is between cytochromes $b$ and $c$ (Inoue et al, 1967). Diuron also causes the extra reduction of cytochrome $b$, but not the red shift of the band of cytochrome $b_{563}$ (Convert & Briquet, 1978).

Proposed Binding Sites for Antimycin

As discussed, antimycin has profound affects on the cytochrome $b$-$c_1$ region of the mitochondrial respiratory chain, the bacterial photosynthetic electron transport chain, and the photosynthetic electron transport chain of chloroplasts. Because no function of this
region is immune to perturbation by antimycin, the site of interaction can be considered an important site in this region. The actual nature of the antimycin site is still obscure.

Two redox components which have been implicated in the past are the Rieske-type iron sulfur protein (Rieske et al, 1964a) and the cytochrome b. The other proposed sites are the binding site for $Q_c$ and the hypothetical component X which may function as a control point within the electron transfer chain. The iron sulfur protein was thought to be important because antimycin has metal chelating properties (Tappel, 1960). However, Rieske et al (1964b) were able to split the iron-sulfur protein away from the mitochondrial complex III. This iron-sulfurless complex was still sensitive to antimycin. Therefore, the iron-sulfur protein was dismissed as a potential site for antimycin interaction.

Cytochrome b has long been considered the prime site of interaction of antimycin. As seen with both mitochondrial systems and the photosynthetic systems of R. sphaeroides, the b cytochromes show pronounced spectral changes when antimycin is added. Storey (1972) has proposed a direct binding of antimycin to mitochondrial cytochrome $b_{562}$. This would be analogous to cytochrome $a_3/CO$ binding. Berden and Slater (1972) have provided contradicting data. Calculations involving the energy transfer of antimycin to cytochrome $b$ (fluorescence quenching) seem to suggest that the distance between the two groups is 20 A which would be too far for a direct binding of the phenolic hydroxyl of antimycin to the heme of cytochrome b. Von Jagow
and Boher (1975) also support direct binding of antimycin to b_{566} (mitochondrial) on the basis of titration data. However, this is in contrast to the spectral studies which show that b_{562} is the only b cytochrome affected (Berden & Opperdoes, 1972).

A third component which has been proposed is a component X which may serve as a redox switching point and be involved in antimycin stimulated oxidant-induced reduction of cytochrome b. The central role of X with regard to structure and function would offer an explanation of the diverse effects of antimycin on cytocrome b-c_1 region (Baum et al, 1967).

Recent work indicates that antimycin may function as an inhibitor by displacing either a quinone or a quinol from the Q_c site (Cramer & Crofts, 1982). The Q_c site is proposed to function in the stabilization of the semiquione observable by EPR at g=2.00 (low microwave power) (Ohnishi & Trumpower, 1980). It is speculated that the redox changes observable upon addition of antimycin may be caused by the loss or gain of electrons as a semiquinone species is converted to either a quinol or a quinone, by electron transfer to or from neighboring redox centers before the inhibitor displaces the group (Velthuys, 1982).
Multivalent Model of Inhibitor Binding

Rieske (1980) has proposed that the primary inhibitor site for all antimycin-like inhibitors is a cavity within the ubiquinone-cytochrome b-c\textsubscript{1} region. This would explain why an inhibitor could perturb several different subunit proteins. This cavity model can explain many of the effects of antimycin such as protection of the complex against cleavage by bile salts, quenching of fluorescence in complex-bound antimycin, and that an intact protein complex is needed for binding of antimycin. A multivalent model of inhibitor binding could be used to explain the inconsistencies in effects of different antimycin-like inhibitors in different organisms. Different molecular shapes and functional moieties of the inhibitors will perturb to different degrees the subunits that are exposed to the inhibitor on the wall of the cavity (Fig. 6).

Figure 6. A diagramatic representation of the Multivalent Binding Model of Inhibitor Binding to Complex III
The components shown are cytochrome b (b), cytochrome c\textsubscript{1} (c\textsubscript{1}) iron-sulfur protein (Fe), core protein (CP), and inhibitor (I). (Taken from Rieske, 1980)
Photoaffinity Theory

Photoaffinity labeling represents a special type of affinity labeling in which the reactive chemical group is replaced by an inert, but photoactivatable substituent. The biological applications of such molecules were first studied by Singh et al in 1966. He initiated the use of carbene precursors, and in 1969 Fleet and coworkers used an arylazide nitrene-precursor for investigations of an antibody recognition site. Since these pioneering studies many applications of photoaffinity ligands have been successfully used.

Two types of photoaffinity probes in use today are those which yield either a carbene or a nitrene upon irradiation. Carbenes contain a carbon with a nonbonding electron pair and are generally very reactive. Carbenes can be generated by photolysis of diazoalkanes, ketones, diazirines and \( \alpha \)-ketodiazocompounds with concurrent loss of molecular nitrogen. Carbene precursors have the disadvantage that the major activation absorbance bands are in the short UV region. Irradiation at these wavelengths can damage protein and other macromolecules (Gilchrist & Rees, 1969). Also, carbenes have a tendency to undergo Wolff rearrangements to form ketenes (Charmovich et al, 1968).

The nitrogen analog of a carbene is the nitrene. Nitrenes can be generated from alkyl- and arylazido groups, isocyanates, and carbonylazides (Gilchrist & Rees, 1969). Nitrenes offer the advantage
of being photoactivatable at longer wavelengths (Chowdry et al, 1976). However, they are longer lived than carbenes (10^-4 sec), and they are more selective. In many cases they act as electrophiles and react with many of the nucleophilic groups that are attacked by ordinary protein modifiers.

Several of the criteria that should be met before using a nitrene and its azide precursor as a photoaffinity precursor are as follows. The nitrene precursor should contain functional groups which allows it to bind with the desired receptor site. The nitrene should be generated at wavelengths that will not damage the receptor site. The precursor should be chemically stable at the temperature used, and it should not react with the solvent (water) (Singer, 1967).
MATERIALS AND METHODS

Chemicals

Octadecylamine and 3-nitrosalicylic acid were obtained from Aldrich Chemical Co. Horse heart cytochrome c (Type III), N,N'-dicyclohexylcarbodiimide (DCCD), Tris(hydroxymethyl)aminomethane (tris base), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), phosphatidylglycerol, phosphatidylethanolamine, succinic acid, and 2,5-diphenyloxazole (PPO), were obtained from Sigma. Sodium dodecylsulfate (NaDodSO₄), glycine, and N,N'-methylenebisacrylamide were obtained from Serva. Acrylamide was obtained from Eastman Kodak Chemicals. Fluorographic solutions were obtained from either New England Nuclear (Enhance) or from Research Products International (Fluorohance). All other chemicals were reagent grade.

Tritiation of 3-nitrosalicyl-N-(n-octadecyl)amide was performed by the Wilzbach method at New England Nuclear.

Methods

Synthesis of a Photoactivatable Antimycin Analog

3-nitrosalicyl-N-(n-octadecyl)amide(I). Octadecylamine (2.7 g, 10 mMol) was dissolved in 50 ml of tetrahydrofuran (THF), and 15 ml of THF in which 1.83 g (10 mmol) of 3-nitrosalicylic acid had been dissolved was added. To this solution was added 4 g (20 mmol) of DCCD dissolved in 5 ml of THF. The solution was stirred at room
temperature for 4 h. Glacial acetic acid (0.5 ml) was added to decompose excess DCCD. The dicyclohexylurea precipitate was removed by filtration. The solvent was removed by flash evaporation at 25°C. The product was suspended in ethanol:water (3:1 v/v) and 2 drops of 10 N sodium hydroxide were added. The product was recrystallized from ethanol:water. Purity was confirmed using silica gel thin layer chromatography (TLC) with a solvent system of chloroform:methanol (99:1 v/v); an Rf of 0.75 was found. The yellow crystalline material was characterized by a m.p. of 88°C. The percent yield for the reaction was 50%.

3-aminosalicyl-N-(n-octadecyl)amide (II). Compound I was reduced to the amine derivative and recovered as the amine hydrochloride as described previously (Neft, 1971, Neft & Farley, 1971). The product was recovered with 70% yield, the m.p. was 155°C, and an Rf value of 0.45 was found using silica gel TLC with a solvent system of chloroform: methanol:ammonia (99:1:0.1).

Alternately, 0.5 g (1.5 mmol) was dissolved in 20 mls of chloroform, and 0.2 g of 5% palladium on carbon was added. The mixture was sealed in a 130 ml serum bottle with a butyl rubber stopper. The bottle was evacuated and then filled to approximately 5 psi with H2 gas. The bottle was refilled with H2 gas as necessary, and the mixture was stirred at room temperature for 48 hrs. The amine derivative (II) is insoluble in chloroform, and precipitates upon reduction forming an amorphous solid with the catalyst. The carbon-amine precipitate was filtered and taken up in absolute ethanol
in which II is soluble. The carbon was removed by filtration or centrifugation. II was not purified further.

3-azidosalicyl-N-(n-octadecyl)amide (III). The synthesis of III was performed in the dark and on ice. Compound II (0.26 g, 0.6 mMol) was dissolved in 50 ml of absolute ethanol to which 0.11 g (1mmol) of fluoroboric acid was added. Sodium nitrite (NaNO₂) (0.076 g, 1mMol) dissolved in a minimal amount of water was added dropwise to the ethanolic mixture. The solution was stirred for 30 minutes, or until the formation of 3-diazosalicyl-N-(n-octadecyl)amide was complete. Completeness was judged by using silica gel thin layer chromatography with a chloroform:methanol (99:1v/v) solvent system (Rₖ of 0.75). Sodium azide (NaN₃) (0.065 g, 1mMol) was dissolved in a minimal amount of water and added dropwise to the reaction mixture. The solution was stirred for 1 h. The reaction mixture was treated with decolorizing charcoal for 30 min at room temperature with stirring. The charcoal was removed by filtration. Compound III was precipitated with water, collected by filtration, and crystallized from ethanol:water (9:1). Silica gel TLC with chloroform:methanol (99:1v/v) solvent showed an Rₖ of 0.95. The mp was 75°C, and the percent yield was 90%. C, H, N analysis: calculated C, 69.76; H, 9.76; N, 13.05 found C, 69.73; H, 9.55; N, 13.09 (The analysis was performed by M-H-W Laboratories, Phoenix, Az.)

For the three step reaction, an overall yield of 30% was obtained.
$[^3\text{H}]$-3-nitrosalicyl-N-(octadecyl)amide $(^3\text{H}]$-I). The $[^3\text{H}]$-I was prepared by the Wilzbach method by New England Nuclear.

The crude material was purified using preparative thin layer chromatography with 20 X 20 cm, 2mm thick, silica gel F-254 plates as support. The plates were previously activated at 100 °C for 1 h. The solvent system was chloroform: methanol (10:1 v/v). A yield of 16 mCi of purified $^3\text{H}$-I was obtained from 1,115 mCi of crude material.

$[^3\text{H}]$-3-azidosalicyl-N-(n-octadecyl)amide $(^3\text{H}]$-III). Synthesis of $[^3\text{H}]$-III was as described for III. However, the amount of $[^3\text{H}]$-I used per synthesis was approximately 2 mCi, and the other components were altered proportionately. Final concentrations of $[^3\text{H}]$-III were calculated using a molar extinction coefficient of 3250 at 310 nm. The radiospecific activity of the $[^3\text{H}]$-III was 9.7 mCi/umol.

**Organism**

*R. sphaeroides* strain Ga (Crounse et al., 1963) was used for all membrane labeling work, and in all inhibition studies. Cultures were maintained on YEMG agar stabs (Lascelles, 1969).

**Growth Medium and Conditions**

*R. sphaeroides* strain Ga was grown in MG media (Lascelles, 1969). Cells were grown anaerobically in either one liter Roux bottles or in
a 4 liter fermenter flask. Light was provided by 100 watt incandescent light bulbs. The light intensity was approximately 4000 lux. Cells were harvested at mid-log phase (optical density at 680 nm of 1-1.5) by centrifugation at 3,000 X g for 10 minutes. The pellet was suspended and washed once with 25 mM phosphate buffer, pH 7.8 and recentrifuged. Cells were suspended in 25mM phosphate buffer, pH 7.8 to an O.D. of 40 at 680 nm.

Preparation of Chromatophores

Chromatophores were prepared by the method of Takemoto and Bachmann (1979) with slight modification. The suspended cells were ruptured by one passage through a French pressure cell at 1281 Kg/cm². The crude extract was treated with DNAase and centrifuged at 10,000 X g for 20 min. The supernatant was then centrifuged at 150,000 X g for 1 h. The pellet was resuspended in 25 mM phosphate buffer, pH 7.8 and homogenized in a Dounce homogenizer. The membranes were purified on a column of Biogel A 150 (100-200 mesh). The sample was eluted with 25 mM phosphate buffer, pH 7.8. The peak pigmented fractions were collected and pooled. Chromatophores were pelleted by centrifugation at 150,000 X g for 1 h. The chromatophores were resuspended in 25 mM Hepes buffer, pH 7.8. All manipulations were performed at 5°C.
Protein Determinations

Membrane protein determinations were performed by the method of Markwell et al (1978). A standard curve was prepared using bovine serum albumin.

Succinate-Cytochrome c Reductase Inhibition Assay

Inhibition studies using chromatophores were performed by measuring the percent inhibition of the reduction of cytochrome c when different concentrations of compound III were present as compared to the uninhibited chromatophores. The assay mixture added to both the reaction and the reference cuvette, consisted of 0.40 mM KCN, 20 uM cytochrome c, 25 mM KH₂PO₄, pH 7.5, and 5 to 20 ul of chromatophores in a total volume of 1 ml. The assay was started by the addition of 20 uM succinate to the reaction cuvette. Reduction of exogenous cytochrome c was measured by monitoring the absorbance change at 550 nm on a Beckman Model 35 spectrophotometer. Inhibitor (antimycin or III) was added to the reaction cuvette and the change in slope was measured. Alternately, compound III was incubated with the reaction mixture for 10 minutes, and subsequently the reaction was started by the addition of succinate.
Labeling of the Antimycin Site

Aliquots (50 ul) of purified ubiquinol-cytochrome b-c\textsubscript{1} complex from strain R-26 of *R. sphaeroides* (a gift from Don Menick) containing 0.4 nmol of cytochrome b, 0.5% Triton X-100, 10mM sodium phosphate buffer, pH 6.8 were either incubated with 10 ul of ethanol or 5 ul of antimycin A (10mM) for 10 minutes. Aliquots of [\textsuperscript{3}H]-III (0.4uCi, 0.0045umol) were added to each of the tubes. The reaction mixtures were irradiated in quartz tubes by either a Spectraline model scl UV penlight for 10 minutes or for 30 seconds in a Rayonet UV photoreactor using 12 lamps at 300 nm. The irradiation was performed at room temperature.

Aliquots (100 ul) of chromatophores were preincubated with ethanol or antimycin as described previously. To each sample was added 5 ul of [\textsuperscript{3}H]-III and irradiated either immediately or after 10 minutes of incubation. Light systems were as described for the purified complex.

Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis

Labeled samples were diluted 1:1 with a solubilization buffer containing 0.139 M Tris, 0.5% NadodSO\textsubscript{4}, 20% sucrose adjusted to pH 7.8 with glacial acetic acid and heated at 70°C for 5 minutes. Gels were cast and run as described by Anderson et al (1983). This method incorporates the use of 8% acrylamide and 8M urea in the separating
gel and was useful in separating the bulk lipid molecules and detergent from low molecular weight polypeptides. Approximately 25-50 ug of protein was applied to each lane of the gel. Gels were run in a Hoefer SE 600 dual slab gel apparatus at 12°C with a constant current of 50 mamps. The gels were stained using Coomassie Blue stain (Fairbanks et al, 1971), and destained using a 10% isopropyl alcohol, 10% acetic acid solution.

**Fluorography**

The gels were prepared for fluorography by either using a commercially available fluorographic solution (Enhance or Fluorohance) or acetic acid and PPO as described by Skinner and Griswold (1983). Gels were dried onto Whatman I filter paper using a Biorad slab gel dryer model 224. The dried gels were exposed to Kodak X-Omat R x-ray film at -70°C for 2 weeks. The fluorograms were developed using Cronex developer for 5 minutes at 25 C and Kodak fixer.

**Phospholipid Extraction and Quantitation**

To determine the amount of protein bound [³H]-III as compared to lipid bound label, chromatophores were labeled with the photoreactive species and lipids were extracted (Lascelles & Szilagyi, 1965). Chromatophores (200 ul) were incubated with 0.32 uCi of [³H]-III. The sample was irradiated for 20 minutes with the UV penlight. The sample
was washed twice with 25 mM tris buffer, pH 7.8. The chromatophores were pelleted by centrifugation at 150,000 X g for 1 h. The samples were resuspended and extracted with a 2:1 solution of chloroform and methanol. The protein was pelleted by low speed centrifugation and resuspended. An aliquot was counted on a Beckmann LS-100c scintillation counter. The lipids were chromatographed as previously described (Skipski & Barclay, 1969). Standards of phosphatidylglycerol and phosphatidylethanolamine which had been treated with $^3$H-III were also chromatographed. The TLC plates were sprayed with molybdenum blue reagent, a general stain for phospholipids (Dittmer & Lester, 1964). Sections of the Silica gel plate were scraped and counted in order to quantitate the radioactivity.
RESULTS AND DISCUSSION

Synthesis of a Photoactivatable Antimycin Analog

A photoaffinity analog of antimycin, 3-azidosalicyl-N-(n-octadecyl)amide was synthesized as diagrammed in Fig. 7. Each step in the synthesis was accomplished with yields exceeding 50%.

The published method for synthesis of 3-nitrosalicyl-N-(n-octadecyl)amide (Neft & Farley, 1971, Neft, 1971) used 2-phenylenephosphorochloridite (Anshutz et al, 1943) as the coupling agent for amide bond formation. The overall reaction for this step in the synthesis is as follows:

Attempts to duplicate this work yielded a yellow compound with a melting point of over 200°C. This material may have been a phenolic salt as described by Neft (1971). Also, the published yields for the formation of the product were low. Therefore, the use of DCCD as the coupling agent for amide bond formation was investigated. Using DCCD as the coupling agent, a yellow crystalline product with a melting point of 87-88°C was obtained. This melting point compares favorably with the published values of 85-86°C (Neft & Farley, 1971). The yield of this reaction was greater than or equal to 50% using DCCD as
compared with the published yield of 38% using o-phenylene phosphorochloridite. The mechanism for the formation of an amide bond using DCCD is as follows:

\[
\begin{align*}
\text{RCOH} + \text{R'N=C=NR'} & \rightarrow \text{RCO-C=NR'} \\
\text{RCOC}=\text{NR'} & \rightarrow \text{RCO-C=NR'} \rightarrow \text{RNHR'} + \text{RNHCNHR'} \\
\text{R'} &= \bigcirc
\end{align*}
\]

The catalytic reduction (Pd/C, H₂ gas) of Compound I to 3-aminosalicyl-N-(n-octadecyl)amide was performed as published (Neft, 1971) with good yield. Adaptations were made in the technique in order to accommodate smaller reactant quantities and to better contain the radioactive materials. The use of sealed septum bottles as described in the METHODS section alleviated both of these concerns.

The synthesis of 3-azidosalicyl-N-(n-octadecyl)amide (III) was performed in the dark because the product is sensitive to light. Compound III is also thermally sensitive, and was concentrated by precipitation with water instead of flash evaporation in order to avoid decomposition.
Figure 7. Outline of the synthesis of 3-azidosalicyl-N-(n-octadecyl)amide
\[
\text{NO}_2 \text{COOH} + \text{CH}_3(\text{CH}_2)_{17}\text{NH}_2 \\
\xrightarrow{\text{OCCD}} \\
\text{NO}_2 \overset{\text{OH}}{\text{C}}\overset{\text{N}}{\text{ICH}}(\text{CH}_2)_{17}\text{CH}_3 \\
\xrightarrow{\text{Pt/C} \ H_2} \\
\text{NO}_2 \overset{\text{NH}_2}{\text{OH}} \overset{\text{C}}{\text{NiCH}}(\text{CH}_2)_{17}\text{CH}_3 \\
\xrightarrow{1. \text{NaNO}_2 \ 2. \text{NaN}_3} \\
\text{NO}_2 \overset{\text{N}_3}{\text{OH}} \overset{\text{C}}{\text{NiCH}}(\text{CH}_2)_{17}\text{CH}_3
\]
Demonstration of the photoactivation of 3-azidosalicyl-N-(n-octadecyl)amide was accomplished by taking a UV-visible spectrum of the compound and subsequently irradiating with UV light for a given time. A spectrum was taken after each period of irradiation, and disappearance of signal intensity observed. Ji (1977) has published similar spectra as evidence of photoactivation of arylazides (Fig. 8).

Inhibition of Succinate-Cytochrome c Reductase Activity

The 3-azidosalicyl-N-(n-octadecyl)amide inhibited succinate-cytochrome c reductase activity by 50% at a level of 58 umoles of analog/mg protein. The inhibition titration curve was slightly sigmoidal (Fig. 9). By comparison, the amount of 3-nitrosalicyl-N-(n-octadecyl)amide needed to inhibit succinate-cytochrome c reductase in mitochondria by 50% is 30 umoles/mg of protein and is also sigmoidal (Neft, 1971). This is as would be predicted on the basis of the electron withdrawing capabilities of a nitro-function as compared to an azido-function. Neft and Farley (1971) have postulated that the role of the formamido-function in antimycin is as an electron withdrawing moiety. The azido-substituent of the photoaffinity analog used in this study does not function as an electron withdrawing group to the same extent as the nitro- or the formamido-functions of 3-nitrosalicyl-N-(n-octadecyl)amide or antimycin. This could account for the approximate doubling of the concentration of
Figure 8. Photoactivation of 3-azidosalicyl-N-(n-octadecyl) amide.
3-azidosalicyl-N-(n-octadecyl)amide in absolute ethanol was irradiated with a Spectraline UV penlight for the times indicated (min). Following irradiation, the spectra were recorded.
Figure 9. Inhibition of succinate-cytochrome c reductase activity in chromatophores of *R. sphaeroides* by 3-azidosalicyl-N-(n-octadecyl)amide.
3-azidosalicyl-N-(n-octadecyl)amide needed for 50% inhibition of succinate-cytochrome c reductase activity. However, the difference in activity may also be due to differing amounts of ubiquinone-cytochrome b-c₁ in the two systems. Antimycin A inhibits the same reaction in mitochondria by 50% at a concentration of 0.05 umoles/mg protein (Neft, 1971).

Photoaffinity Labeling of the Antimycin Binding Site

The strategy for labeling the antimycin site is based on two fundamental premises. These conditions are that the 3-azidosalicyl-N-(n-octadecyl)amide has a high affinity for a specific polypeptide in the membrane, and that the reactive arylnitrene, which is generated upon irradiation, will covalently bind to this same polypeptide. Incorporating a radiolabel into the analog allowed identification of a specific antimycin binding polypeptide by NaDodSO₄ PAGE analysis.

In both purified ubiquinone-cytochrome b-c₁ oxidoreductase (Fig. 10) and chromatophores from R. sphaeroides, (Fig. 13) a polypeptide with a molecular weight of approximately 11,000 was the predominantly labeled species (Figs. 12). Figs. 11 and 13 show comassie blue stained NaDodSO₄ PAGEs of purified ubiquinone-cytochrome b-c₁ oxidoreductase and chromatophores respectively. This labeling was competitively inhibited by preincubation of the reaction mixture with
antimycin before adding the radioactive analog and irradiating. Inhibition of analog binding by antimycin is interpreted to mean that the analog used in this study and antimycin occupy the same site. Antimycin has a very high affinity for the site relative to 3-azidosalicyl-N-(n-octadecyl)amide (Rieske & das Gupta, 1972). Therefore, little labeling by the radioactive analog would be expected when precircubation with antimycin precedes addition of the analog (Figs. 10, 13 lanes D, E, F).

Several other proteins were labeled in the chromatophore preparation (Figs. 13, 14). These proteins were not consistently labeled by the analog, and were labeled to a lesser extent than the 11,000 dalton polypeptide. These proteins seem to correspond to bands within the purified protein complex (ubiquione-cytochrome b-c1 oxidoreductase) (Fig. 11), and show competition with antimycin under some conditions.

The large band of radioactivity present in the bottom of the NaDdSO₄-PAGE fluorogram is most likely unreacted [³H]-azidosalicyl-N-(n-octadecyl)amide. In order to test this hypothesis, chromatophores were treated with the radioactive photoaffinity label and irradiated as previously described. The lipids were extracted and an analysis of the distribution of the radioactivity performed. Table 1 shows the distribution of counts in the protein and lipid fractions. The organic fraction was dried, redissolved, and TLC performed on the residue. The TLC plates were sprayed with molybdenum reagent, a general stain for phospholipids.
Figure 10. NaDodSO₄-PAGE fluorogram of isolated ubiquinone-cytochrome b-c₁ oxidoreductase which has been treated with [³H]-azidosalicyl-N-(n-octadecyl)amide and subsequently irradiated (Lanes A, B, and C). Lanes D, E, and F were preincubated with antimycin before addition of the radioactive analog and irradiation. Six aliquots of purified cytochrome b-c₁ oxidoreductase containing 0.4 nmols of cytochrome b, 0.5% Triton X-100, 10 mM sodium phosphate buffer, pH 6.8 were preincubated with either 10 ul of ethanol (Lanes A, B, and C) or with 5 ul of antimycin A (10 mM) (Lanes D, E, and F) for 10 minutes. Each aliquot was then treated with 0.4 uCi (0.0045 umoles) of [³H]-III. Lanes A and D were incubated one minute prior to irradiation. Lanes B and E were incubated five minutes prior to irradiation and lanes C and F were irradiated immediately. Gels were run by the method of Anderson et al (1983) and prepared for fluorography as described in the text.
Figure 11. NaDodSO₄-PAGE of purified ubiquinone-cytochrome $b-c_{1}$ oxidoreductase (lanes C, D, and E). Lanes A and B contain molecular weight standards. Lane A contains Pharmacia Polypeptide Molecular Weight Standards: Myoglobin (17,201), Myoglobin fragment I and II (14,632) and Myoglobin fragment I (8,235). Lane B contains Pharmacia Low Molecular Weight Protein Standards: Phosphorylase b (94,000), Albumin (67,000), Ovalbumin (43,000), Carbonic Anhydrase (30,000) Trypsin Inhibitor (20,100) and $\alpha$-Lactalbumin (14,400).
Figure 12. Molecular weight estimation of the major antimycin binding polypeptide from NaDodSO₄-PAGE (Anderson et al 1983). Standard proteins and their molecular weights are: soybean trypsin inhibitor, 20100; α-lactalbumin, 14,400; Myoglobin, 17,200; and Myoglobin I, 8240. The estimated molecular weight of the antimycin binding polypeptide is 11,000.
Figure 13. NaDodSO₄-PAGE fluorogram of chromatophores treated with the radioactive photoaffinity analog. Lanes A, B, and C were treated with the analog and subsequently irradiated. Lanes D, E, and F were preincubated with antimycin before addition of the photoaffinity analog and irradiation. Aliquots of chromatophores containing 100 µl (100-150 µg of protein) were either incubated for 10 minutes with 10 µl of ethanol (lanes A, B, and C) or with 10 µl of (10 mM) antimycin A (lanes D, E, and F). Subsequently, 10 µl of [³H]-III (0.8 uCi) were added to each sample. Each sample was then irradiated immediately as described in the text. Gels were run by the method of Anderson et al (1983) and prepared for fluorography as described in the text.
Figure 14. NaDodSO₄-PAGE of Chromatophores from R. sphaeroides.

Lanes A and B are molecular weight standards. Lanes C and D the chromatophore preparation. Gels were run by the method of Anderson et al (1983).

Lane A contains Pharmacia Low Molecular Weight Protein Standards: Phosphorylase b (94,000), Albumin (67,000), Ovalbumin (43,000), Carbonic Anhydrase (30,000), Trypsin Inhibitor (20,100), and α-Lactalbumin (14,400).

Lane B contains Pharmacia Polypeptide Molecular Weight Standards: Myoglobin (17,201), Myoglobin fragments I and II (14,632), and Myoglobin fragment I (8,235).
The spots corresponding to phosphatidylglycerol and phosphatidylethanolamine, the major lipid components of *R. sphaeroides* (Al-Bayatti & Takemoto, 1981), were scraped and counted. There was no significant amount of radiation found in the phosphatidylglycerol, and only a small amount of the applied radiation was present in the phosphatidylethanolamine (Table 2). The largest amount of radiation present in the organic extract comigrated with the unreacted 3-azidosalicyl-N-(n-octadecyl)amide. The hydrophobic nature of the antimycin analog used in this work could explain why this large amount of radioactive material did not wash out of the gel during the staining and destaining procedures.

The use of the NaDodSO₄-PAGE system described by Anderson et al (1983) enhanced the separation of the labeled protein from the large radioactive band previously described. Numerous attempts to identify the antimycin binding site using a conventional Laemmli (1970) gel system with total acrylamide concentrations ranging from 12-16% did not resolve the 11,000 dalton polypeptide from the detergent-lipid-unreacted analog band.

The identification of a protein with a molecular weight of 11,000 daltons as the antimycin binding protein is the major finding of this work. This finding supports the work of das Gupta and Rieske (1973), who reported the binding of deformamidoazidoantimycin to an 11,500 dalton protein in beef heart mitochondria.
Table 1. Quantitation of Applied Radiation

| Fraction | Radioactivity | % |
|----------|---------------|---|
| Total    | 0.3 uCi       | 100 |
| Wash     | 0.1 uCi       | 34  |
| Organic  | 0.17 uCi      | 56  |
| Protein  | 0.03 uCi      | 10  |

Table 2. Quantitation of Radiation in the Organic Extract

| Fraction | Radioactivity | % |
|----------|---------------|---|
| PE       | 0.003 uCi     | 1  |
| PG       | 0 uCi         | 0  |
| $[^3]H$-III | 0.165 uCi   | 55 |

*Total of 56% taken from table I
The function of this protein is unknown. However, it seems to be of importance to the ubiquinone-cytochrome b-c\textsubscript{1} oxidoreductase because small molecular weight proteins are present in most of the purified functional ubiquinone-cytochrome b-c\textsubscript{1} complexes from both mitochondria and \textit{R. sphaeroides}. Purified preparations of mitochondrial ubiquinone-cytochrome b-c\textsubscript{1} oxidoreductases of das Gupta and Rieske (1973), Capaldi (1974), Hare and Crane (1974), and Gellerfors and Nelson (1975) all contain components with molecular weights in the range of 10,000 to 12,000 daltons. Recent purifications of this protein complex in \textit{R. sphaeroides} by Yu and Yu (1982), Takamiya et al (1982), and Menick (1984) all contain proteins of this general size.

Of the possible antimycin binding sites proposed earlier in the LITERATURE REVIEW of this thesis, only the proposal that antimycin binds directly to cytochrome b would be affected by the results of this work. The published molecular weight of cytochrome b in mitochondrial systems (beef heart) is approximately 31,000 daltons (von Jagow et al, 1978) and in \textit{R. sphaeroides} around 40,000 daltons (Gabellini et al, 1982, Menick, 1984) Both of the photoaffinity labeling studies performed thus far seem to be in agreement as to the approximate size of the protein which most strongly interacts with these analogs. Since the weight of this protein does not correspond to the published molecular weights of cytochrome b in either system, it can be concluded that the mode of action of antimycin is not solely by direct binding to cytochrome b.
The multivalent binding model described by Rieske (1980) and the hypothesis that antimycin interacts with one of the quinone binding sites seem to be the most plausible models of antimycin interaction with the ubiquinone-cytochrome b-c₁ oxidoreductase. Yu and Yu (1980) have reported using a photoaffinity quinone analog to label the quinone binding site of mitochondrial ubiquinone-cytochrome b-c₁ oxidoreductase. The protein labeled has a molecular weight of approximately 15,000 daltons. Attempts by these authors to show competition of antimycin for this quinone binding site were negative. This evidence does not rule out the possibility that antimycin interacts with a quinone binding protein because more recent work indicates that there are two semi-quinone species which interact at two different sites (Ohnishi & Trumpower, 1980).

The multivalent binding model proposed by Rieske (1980) also seems to be a viable model for antimycin binding. The binding studies using chromatophores showed several proteins labeled by the radioactive photoaffinity analog with the 11,000 dalton polypeptide being predominantly labeled (Fig. 10,13). The interpretation of this finding is not readily apparent, but these results could be used to support the multivalent binding model.

Although the specificity of 3-azidosalcy1-N-(n-octadecyl)amide for the has not been rigorously tested, several fundamental aspects of the analog would offer credence to this supposition. These properties are as follow: 1) Preincubation of either purified ubiquinone-cytochrome b-c₁ complex or chromatophores with antimycin
prior to exposure to the photoaffinity analog inhibited analog binding. 2) \(^{3}\text{H}\)-III inhibits electron transport in a manner similar to antimycin. 3) Both antimycin and this analog show sigmoidal titration curves under certain conditions. 4) The concentration of this analog necessary for inhibition is low enough to indicate a specific site of interaction. 5) The photoactivatable azido is located adjacent to the phenolic hydroxyl group which is considered to be the functional group of primary importance in inhibition. 6) A study using deformamidoazidoantimycin as a photoaffinity label in a mitochondrial system yielded similar results. 7) Similar results were obtained in this study with both a purified system and chromatophores.

In summary, this study has shown that direct binding to cytochrome b is not the only site of interaction action of antimycin. The primary site of binding for the analog used in this study was a polypeptide with a molecular weight of 11,000. This polypeptide was labeled both predominantly and consistently, but not exclusively. The other proteins labeled showed variability both in the amount of label bound and consistency in binding the analog. Therefore, this study does not offer unequivocal evidence for the exclusive role of the 11,000 dalton polypeptide as the antimycin binding site. More research is necessary to distinguish between a single protein as the binding site and the multivalent binding theory.
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