that this band, when reduced directly, also generated the 130-, 90-, and 40-kilodalton species. There was, however, no apparent interconversion between this and the 300-kilodalton species. The 130-kilodalton subunit is postulated here as the major binding or recognition subunit of the receptor. The other subunits may function by interacting with another insulin–receptor complex or with other membrane components. Such interactions could produce the required signals or messages for the appropriate biological responses.

Acknowledgments

We thank Helga Hsu for her excellent technical assistance.

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Interaction of Photosynthetic Electron Transport Inhibitors and the Rieske Iron–Sulfur Center in Chloroplasts and the Cytochrome b_6–f Complex†

Richard Malkin

ABSTRACT: The interaction of electron transport inhibitors with the Rieske iron–sulfur center in chloroplast membranes and in a purified chloroplast cytochrome complex (the b_6–f complex) has been studied by using electron paramagnetic resonance (EPR) spectroscopy. Several quinone inhibitors, all of which contain a halogen substituent and a bulky alkyl side chain, cause a shift in the EPR signal of the reduced Rieske iron–sulfur center from $g = 1.90$ to $g = 1.94$. This g-value shift occurs in untreated membranes as well as in the cytochrome complex, which contains the Rieske center and no other iron–sulfur centers. Other compounds known to inhibit electron transport in the region of the iron–sulfur center cause a smaller alteration in the EPR signal of the Rieske center and are able to interact with the quinone-binding site as evidenced by their displacement of quinones from the Rieske center. One substrate, plastoquinone 9, was also able to displace quinones from the Rieske center while others, such as plastoquinone 1 and duroquinone, did not show this effect. These results are considered in relation to the mode of interaction of quinones with the Rieske center in the photosynthetic membrane.

Electron transport between the two light reactions of chloroplast photosynthesis involves a series of carriers which include plastoquinone, the Rieske iron–sulfur center, cytochrome f, and plastocyanin (Trebst, 1974; Bendall, 1977; Crofts & Wood, 1978; Velthuys, 1980). Recent studies with the inhibitory plastoquinone analogue, DBMIB[1] (Trebst et al., 1970), have provided evidence for the interaction of quinones with the Rieske iron–sulfur center and have indicated that the iron–sulfur center can function to stabilize a semiquinone

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[1] Abbreviations: DBMIB, 2,5-dibromo-3-methyl-5-isopropylbenzoquinone; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; DNP-INT, 2,4-dinitrophenyl ether of iodonitrothymol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; BBB, 2-bromo-5-*tert*-butylbenzoquinone; DBBB, 2,3-dibromo-5-*tert*-butylbenzoquinone; DIBB, 2,3-diiodo-5-*tert*-butylbenzoquinone; DBDIB, 3,5-dibromo-2,6-diisopropylbenzoquinone; $NaDodSO_4$, sodium dodecyl sulfate.

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species of DBMIB (Malkin & Crowley, 1982; Malkin, 1981a,b). These results are of interest in relation to proposed Q-cycle mechanisms for proton translocation in energy-transducing membranes, in which the Rieske center has been proposed to have a key function (Velthuys, 1979; Bouges-Bocquet, 1980; Crowther & Hind, 1980; Trumpower, 1981).

For characterization of the nature of the relationship of quinones with the Rieske center in more detail, a study of the interaction of additional inhibitors with the Rieske center has been made by using EPR spectroscopy. In this work unfractionated chloroplast membranes and a highly purified cytochrome complex, whose preparation has recently been described, have been utilized (Hurt & Hauska, 1981). The latter material is practically free of chlorophyll, contains only four electron transport components (plastoquinone, cytochromes b_6 and f, and the Rieske center), and is functionally active in the transfer of electrons from reduced quinones to high-potential electron acceptors, such as plastocyanin and c-type cytochromes. With the cytochrome complex it has been possible to investigate the interaction of quinone analogues and other inhibitors with the Rieske center in greater detail because the preparation is devoid of interfering paramagnetic species. Evidence has been obtained for overlapping binding sites of various types of inhibitors at the Rieske iron–sulfur center.

Experimental Procedures

Washed thylakoid membranes from freshly harvested spinach were prepared as previously described (Malkin & Chain, 1980). The chloroplast cytochrome b_6–f complex was prepared as described by Hurt & Hauska (1981). In some preparations, a modification was made in the final sucrose gradient step in that the ammonium sulfate precipitate was redissolved in 30 mM Tris–succinate buffer (pH 6.5) containing 0.5% sodium cholate and applied directly to a 7–30% stepwise sucrose gradient which was prepared in 30 mM Tris–succinate (pH 6.5) containing 0.1% lecithin, 0.5% sodium cholate, and 30 mM octyl glucoside. After centrifugation for 15 h at 40000 rpm in a Beckman SW41 rotor, the brown band containing the cytochrome preparation was collected. Na-DodSO$_4$–polyacrylamide gel electrophoresis (14% acrylamide) of this material showed it to have the same polypeptide composition as the preparation made with 0.2% Triton [see Hurt & Hauska (1981)]. Concentrations of the cytochrome complex were based on the cytochrome f content; this carrier was analyzed from ferricyanide-oxidized minus ascorbate-reduced difference spectra. It has been shown by Hurt & Hauska (1981) that the complex contains one Rieske iron–sulfur center per cytochrome f.

EPR spectra were recorded with a modified JEOL X-band spectrometer at 10–15 K with 100 K Hz field modulation (Prince et al., 1980). Spectra were generally recorded with a nonsaturating power of 5 mW. The spin concentration of the Rieske iron–sulfur center was determined by double integration of the first-derivative signal and comparison with the integrated intensity of a standard chloroplast ferredoxin sample after the latter had been reduced with sodium dithionite.

The inhibitors used in this study were obtained from the following sources: DBMIB, BBB, DBBB, DIBB, DBDIB, and DNP-INT from Dr. A. Trebst [see Oettmeier et al. (1978) for a description of the preparation and properties of these compounds], UHDBT from Dr. B. L. Trumpower, and bathophenanthroline, o-phenanthroline, and antimycin A from the Sigma Chemical Co. Plastoquinone 1 and plastoquinone 9 were gifts from Dr. G. Hauska. All compounds, except UHDBT, were dissolved in Me_2SO at 10–50 mM concentrations and stored at 4 °C. UHDBT was used as an ethanol solution. Inhibitors were added in microliter amounts so that the final solvent concentration did not exceed 2%. Control experiments showed that the addition of Me_2SO or ethanol in these amounts had no effect on the observed EPR signals of the Rieske iron–sulfur center.

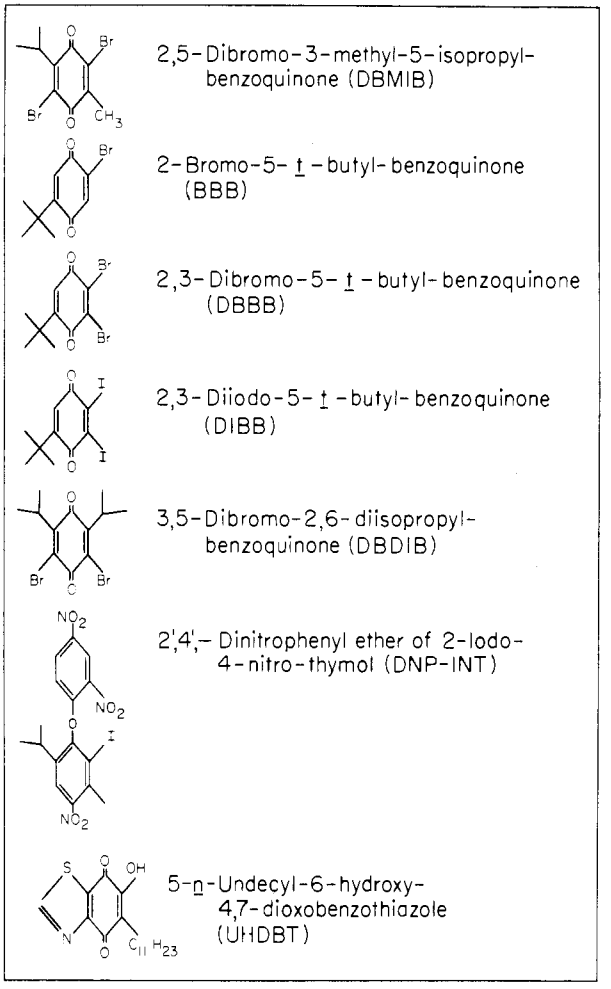

FIGURE 1: Formulas of inhibitors.

Results and Discussion

Previous work has shown that the addition of DBMIB to spinach chloroplasts as well as membranes from other photosynthetic systems results in an interaction between the inhibitor and the Rieske iron–sulfur center (Malkin, 1981a,b). This electron carrier has an EPR g value of 1.89 in the reduced state in thylakoid membranes, and this g value shifts to 1.94 after the addition of DBMIB. Other inhibitory compounds, such as DNP-INT, UHDBT, and bathophenanthroline, which inhibit electron transport in the same region of the electron transport chain (Bering et al., 1977; Trebst & Reimer, 1977; Trebst et al., 1978; Malkin et al., 1981), do not produce this pronounced shift. It has been possible to identify several additional inhibitors, the structures of which are shown in Figure 1, which have an effect similar to that of DBMIB in that a g-value shift to 1.94 is observed in their presence in untreated chloroplast membranes (Figure 2). In the case of one inhibitor (BBB), the shift is not complete even after the addition of 10 equiv of inhibitor per Rieske center, but this may be related to the relatively poor inhibitory effectiveness of this compound as compared with the other compounds (Oettmeier et al., 1978). Other quinone inhibitors which are nonhalogenated, such as 2,5-dibutyl- and 2,6-dibutylbenzo-

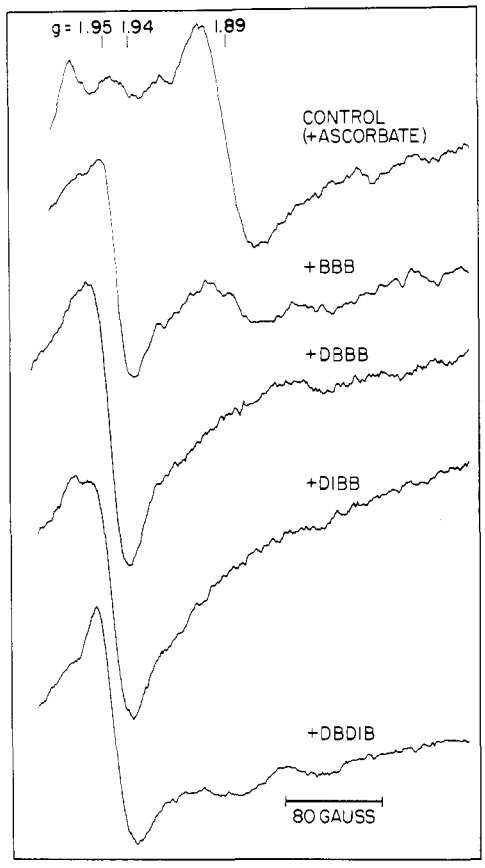

FIGURE 2: Effect of quinone inhibitors on the Rieske iron–sulfur center in chloroplast membranes. Washed chloroplast membranes (3.3 mg of chlorophyll/mL) in the presence of 2 mM ascorbate were treated with respective inhibitors at the indicated concentrations for 2 min prior to freezing to 77 K: BBB, 500 nmol; DBBB, DIBB, and DBDIB, 100 nmol.

quinone, as well as UHDBT (see Figure 1), did not produce a large shift, while a halogenated nonquinone (DNP-INT) was also ineffective, although both UHDBT and DNP-INT are compounds whose inhibitory potency are at least as great as that of DBMIB (Trebst et al., 1978; Malkin et al., 1981).

Although our previous results were strongly suggestive that quinone inhibitors interact directly with the Rieske iron–sulfur center, this result could not be tested with a preparation which contained only the Rieske center (Malkin, 1981b). The availability of a purified chloroplast cytochrome b_6–f complex which contained the Rieske center allowed for a more rigorous testing of this idea. Quantitative EPR analysis of the EPR signal of the Rieske center observed after the addition of ascorbate, hydroquinone, or dithionite confirmed the findings of Hurt & Hauska (1981) that the preparation contains one iron–sulfur center, containing two non-heme iron atoms, per cytochrome f. On the basis of the spectra observed in the presence of dithionite, it is also possible to conclude that no other iron–sulfur centers are present in the complex.

As shown in Figure 3, a preparation of the cytochrome complex purified through the ammonium sulfate fractionation step contains the Rieske center as evidenced by the EPR g values at 2.03 and 1.90 after ascorbate reduction: the large free-radical signal at $g = 2.00$ originates from an additional unidentified paramagnetic center in the preparation. The addition of 1 equiv of DBMIB per iron–sulfur center results in a marked change in the signal with decrease in signal amplitude at $g = 2.03$ and 1.90 and the appearance of a signal with g values of 2.01 and 1.95. The total spin concentration determined by double integration of these EPR signals remains constant, indicating a quantitative conversion of the Rieske

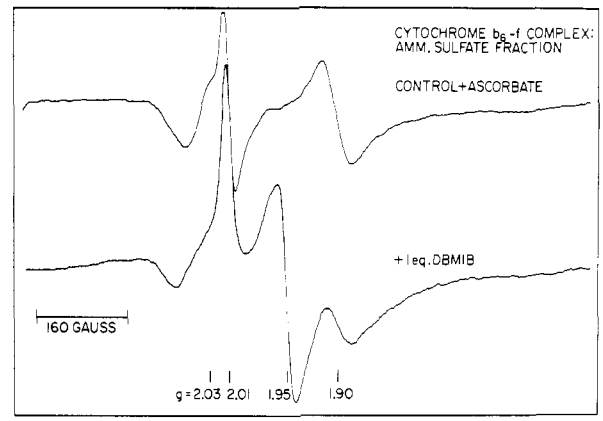

FIGURE 3: Effect of DBMIB on the Rieske iron–sulfur center in the chloroplast cytochrome b_6–f complex. The 45–55% ammonium sulfate fraction of the chloroplast cytochrome b_6–f complex (concentration = 13 nmol of cytochrome f/mL) was treated with the indicated number of equivalents of DBMIB in the presence of 10 mM ascorbate for 2 min prior to freezing to 77 K. EPR conditions: field setting, 3400 ± 500 G; modulation amplitude, 12.5 G; microwave power, 5 mW; temperature, 12 K.

center to the altered center. Except for a slight difference in g value, this result is similar to that reported for untreated membranes using higher amounts of DBMIB (Malkin, 1981b).

When a more purified preparation of the cytochrome complex is used, slightly different results are obtained. The ammonium sulfate fraction has been purified on a sucrose gradient containing either 0.2% Triton or 0.5% sodium cholate plus 30 mM octyl glucoside to yield the five polypeptide preparation described by Hurt & Hauska (1981). These gradient preparations still contain the Rieske center (Figure 4), but the reaction with DBMIB is decreased in both preparations. In the Triton preparation (Figure 4A), 1 equiv of DBMIB has no significant effect on the Rieske center, while the addition of 5 equiv of inhibitor produces only a 25% shift in g value. The cholate–octyl glucoside preparation (Figure 4B) showed a more sensitive reaction to DBMIB in that 1 equiv produced a 25% shift while 5 equiv yielded a 75% shifted Rieske signal. The poor response to DBMIB in the Triton preparation appears to be related to the presence of this specific detergent, since the addition of 0.2% Triton to the ammonium sulfate fraction markedly decreased the effectiveness of 1 equiv of DBMIB to produce the g-value shift of the Rieske center.

The quinone analogues which have been shown to shift the g value of the Rieske center in chloroplasts (Figure 2) are also able to produce a similar shift in the cytochrome b_6–f complex. As shown in Figure 5, all the quinone analogues produce shifted Rieske center signals, and the g values of the shifted signals are similar, but the line shapes are not identical with all of the inhibitors.

It has also been possible to test other compounds which are known to be efficient inhibitors of electron transport in the cytochrome b_6–f complex for their possible interaction with the Rieske center. Three compounds (UHDBT, DNP-INT, and bathophenanthroline; see Figure 1) have been found to inhibit electron transport from plastohydroquinone to plastocyanin in the cytochrome b_6–f complex (Hurt & Hauska, 1981). As shown in Figure 6, these inhibitors do not produce the large g-value shift observed with the quinone analogues previously studied, but more subtle changes in the EPR spectrum of the Rieske center are observed. UHDBT does not affect the g value at $g = 1.90$, but a significant narrowing of line shape occurs. A similar result has been observed after the addition of UHDBT to untreated chloroplast membranes (Malkin & Crowley, 1982). A more significant shift of the

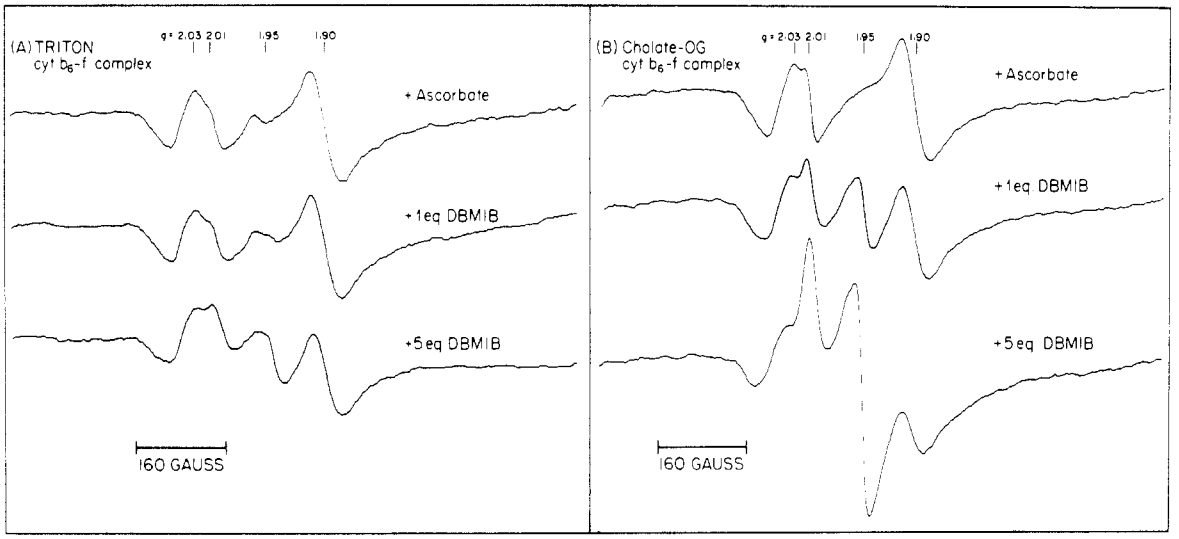

FIGURE 4: Effect of DBMIB on the Rieske iron–sulfur center in the Triton and cholate containing octyl glucoside chloroplast cytochrome b_6–f complex. The sucrose gradient cytochrome b_6–f complexes prepared with either Triton (A) or cholate containing octyl glucoside (OG) (B) (see Experimental Procedures) (concentration = 12 nmol of cytochrome f/mL) were treated with the indicated number of equivalents of DBMIB in the presence of 10 mM ascorbate for 2 min prior to freezing to 77 K. EPR conditions were as in Figure 3.

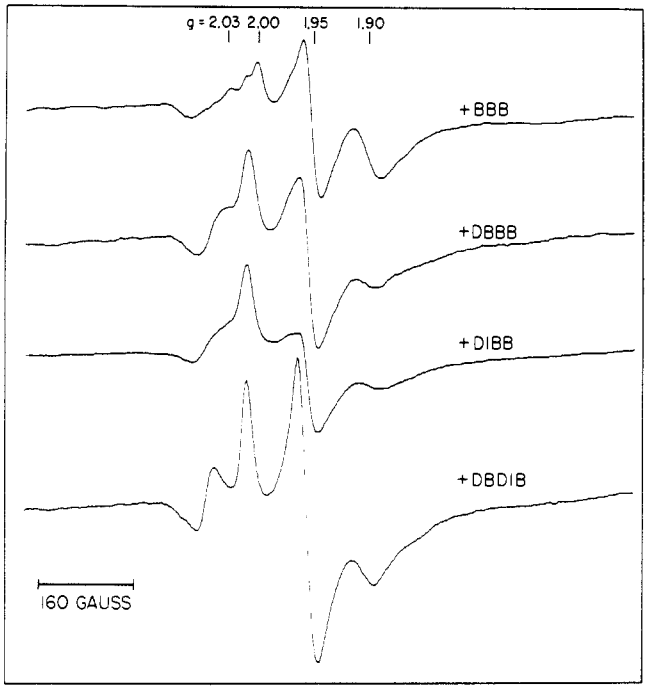

FIGURE 5: Effect of quinone inhibitors on the Rieske iron–sulfur center in the chloroplast cytochrome b_6–f complex. The sucrose gradient chloroplast cytochrome b_6–f complex prepared with cholate containing octyl glucoside (concentration = 12 nmol of cytochrome f/mL) was treated with 10 equiv of the respective inhibitor for 2 min prior to freezing to 77 K. EPR conditions were as in Figure 3.

g value at 2.02 occurs in the presence of UHDBT as the inhibitor produces a new g value at 2.03. In the case of the Rieske center in bacterial chromatophores, UHDBT has been reported to narrow the $g = 1.89$ signal and to shift the g_z signal to 2.03. In addition to these g-value shifts, UHDBT induces a positive shift of ~70 mV in the E_m of the bacterial Rieske center (Bowyer et al., 1980). DNP-INT also affects the Rieske center in that g values of 2.02 and 1.91 are observed with this inhibitor. The EPR spectrum in the presence of bathophenanthroline is indistinguishable from that of the control sample.

Other photosynthetic electron transport inhibitors have been tested with the cytochrome complex for their ability to induce g-value shifts in the Rieske center. Antimycin A, o-phenanthroline, and DCMU had no effect on the Rieske center EPR signal when added at 5–10-fold excess over the concentration of center present.

Although the g-value shifts produced by UHDBT and DNP-INT are small, evidence that these inhibitors interact near the DBMIB binding site on the Rieske center has been obtained in displacement studies. It has been observed in chloroplast membranes that excess UHDBT displaces DBMIB from the Rieske center, producing a g value at 1.89 (Malkin, 1981b). As shown in Figure 7, similar effects can be observed with the chloroplast cytochrome complex. The complex is first treated with 5 equiv of DBMIB, and then increasing amounts of either UHDBT or DNP-INT are added. When approximately 20 equiv of either inhibitor is present (the ratio of UHDBT or DNP-INT to DBMIB is 4), the $g = 1.90$ signal has reappeared and the signal at $g = 1.95$ has decreased. Increasing amounts of UHDBT and DNP-INT produce a larger signal at $g = 1.90$. In contrast to the displacement of DBMIB by these two compounds, bathophenanthroline, antimycin A, o-phenanthroline, and DCMU at comparable concentrations (approximately 5-fold excess over DBMIB) were not able to displace DBMIB from its Rieske center binding site.

In addition to the inhibitors shown in Figure 7, one quinone substrate of the cytochrome b_6–f complex has been found which is able to displace DBMIB from its binding site. As has been shown by Hurt & Hauska (1981), both plastohydroquinone 1 and plastohydroquinone 9 serve as electron donors for the cytochrome complex oxidoreductase activity with plastocyanin or c-type cytochromes as electron acceptors. Durohydroquinone is also an electron donor with the complex. Some of these substrates have been tested for their ability to displace DBMIB from the Rieske center, and the results are shown in Figure 8. Of the compounds tested (durohydroquinone, duroquinone, plastoquinones 1 and 9), only plastoquinone 9 is able to displace DBMIB from its binding site on the Rieske center.

On the basis of these findings, the inhibitory binding sites at the Rieske center can be described in the following manner. One site is present on the protein which is linked closely to the functional iron–sulfur center. The binding of quinone analogues, and presumably plastohydroquinone, would occur at this site, and this binding has pronounced effects on the EPR

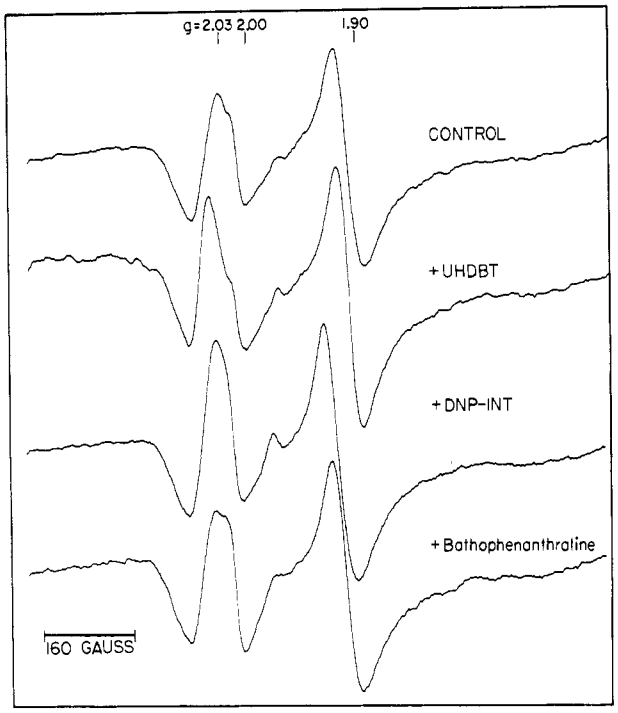

FIGURE 6: Effect of photosynthetic inhibitors on the Rieske iron–sulfur center in the chloroplast cytochrome b_6–f complex. Experimental and EPR conditions were as in Figure 5. 10 equiv of the respective inhibitor was added to each sample.

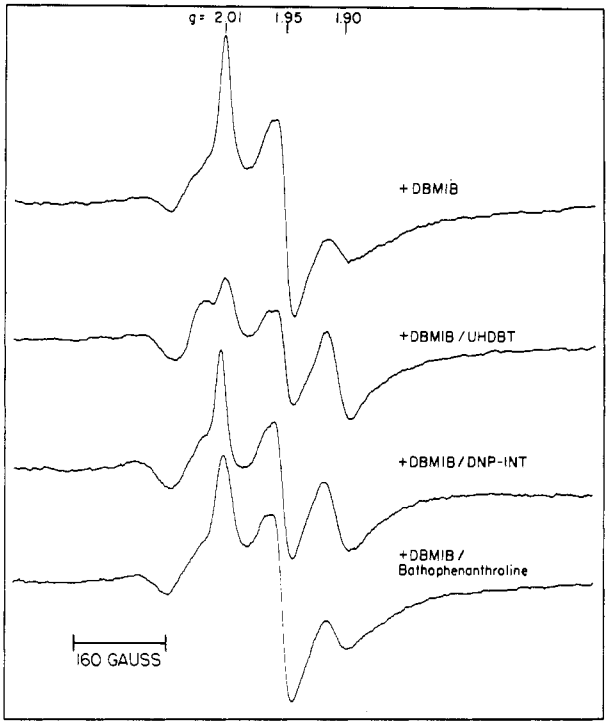

FIGURE 7: Displacement of DBMIB from the Rieske iron–sulfur center in the chloroplast cytochrome b_6–f complex by inhibitors. The cholate containing octyl glucoside cytochrome b_6–f complex (concentration = 13 nmol of cytochrome f/mL) was treated with 5 equiv of DBMIB in the presence of 10 mM sodium ascorbate. 20 equiv of either UHDBT, DNP-INT, or bathophenanthroline was then added and the samples were incubated for 2 min prior to freezing to 77 K. EPR conditions were as in Figure 3.

signal of the iron–sulfur center and its redox properties (Malkin, 1981a). A second binding site is also present but is not as closely associated with the iron–sulfur center since only a small g-value shift is noted upon inhibitor binding. This site binds rather diverse compounds such as a dinitrophenyl

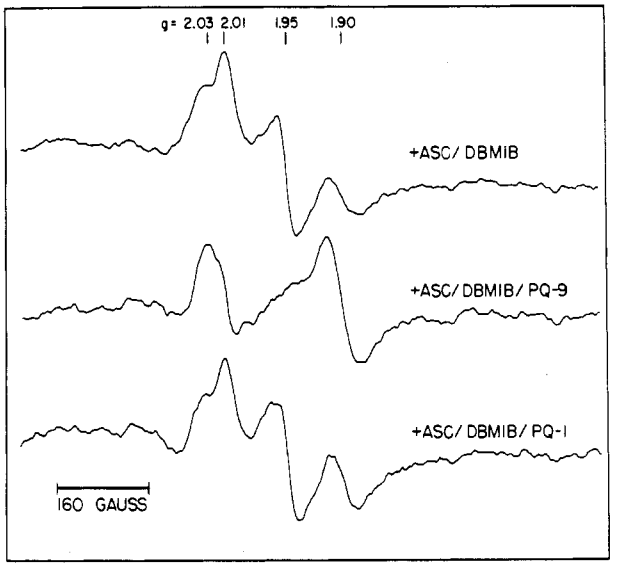

FIGURE 8: Displacement of DBMIB from the Rieske iron–sulfur center in the chloroplast cytochrome b_6–f complex by quinone substrates. Conditions were as in Figure 7 except that plastoquinone 1 (PQ-1) or plastoquinone 9 (PQ-9) was added to a 20-fold excess over UHDBT as indicated.

ether (DNP-INT) and a long-chain quinone analogue (UHDBT). Because of the displacement of quinone analogues by compounds at the second site, there must be an overlap of these sites on the Rieske protein.

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Characteristics of the Isolated Purine Nucleotide Binding Protein from Brown Fat Mitochondria†

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ABSTRACT: The isolation of a purine nucleotide binding protein (NbP), the putative uncoupling protein, from hamster brown adipose tissue mitochondria and some of its functional characteristics are described. (1) Among various detergents tested, Triton is the most suitable; the total GDP binding capacity can be recovered after solubilization by Triton and is rather stable in this extract. (2) For separation of NbP from the ADP/ATP carrier, differences in the solubilizing conditions and the stability at room temperature between both proteins are exploited. The preparation is substantially free of ADP/ATP carrier. (3) The purified NbP has a binding capacity for 16 μmol of GDP/g of protein, corresponding to a 16-fold purification from mitochondria. (4) In sodium dodecyl sulfate–polyacrylamide gel electrophoresis a single band of M_r 32000 is found. A dimer structure is suggested from chemical cross-linking, from the binding capacity for GDP, and from the previously reported centrifugation equilibrium. (5) The isolated NbP preparation consists of Triton–protein–phospholipid mixed micelles with a Stokes radius of 60.5 Å as determined by gel filtration. The Triton binding is 1.9 g/g of protein, and the phospholipid binding is 0.2 g/g of protein. (6) The amino acid composition has a polarity index of 43.5%. The N-terminal peptide has the sequence Val-Asp-Pro-Thr-Thr-Ser-Glu-Val. (7) The affinity of NbP for different purine nucleotides decreases in the order GTP > GDP > ATP > ITP > ADP > IDP. The affinity for the monophosphates is 100 times lower. (8) Photooxidation and the lysine reagent 2,4,6-trinitrobenzenesulfonic acid decrease the binding capacity without influencing the affinity of the unaffected sites. GDP protects against photooxidation.

Mitochondria isolated from brown adipose tissue are largely uncoupled (Nicholls & Lindberg, 1973). Coupling can be restored by adding purine nucleotides (Hohorst & Rafael, 1968; Pedersen, 1970). These mitochondria possess an abnormally high permeability to protons as well as to chloride, bromide, and nitrate, which is decreased by purine nucleotides (Nicholls et al., 1974). A specific binding site for purine nucleotide was determined on the outer surface of the inner membrane of brown adipose tissue mitochondria (Rafael & Heldt, 1976; Nicholls, 1976). The number of these binding sites was found to increase during cold adaptation, corresponding to the thermogenic activity of brown adipose tissue (Rafael & Heldt, 1976; Sudin & Cannon, 1980).

In a study of polypeptide composition of rat brown adipose tissue mitochondria, Ricquier & Kader (1976) reported a prominent M_r 32000 component. Desautels et al. (1978) correlated the increase in binding of purine nucleotides during cold acclimation with the M_r 32000 component. By using 8-azido-[^{32}P]ATP,[1] Heaton et al. (1978) showed that the M_r 32000 component becomes the major labeled protein and that the labeling was prevented by a 10-fold excess of GDP (Heaton et al., 1978) but not by CAT. They claimed that the M_r 32000 component is the regulatory site of the energy-dissipating ion channel. Isolation of this protein was first reported by Ricquier et al. (1979) with GDP–agarose affinity chromatography. However, the purification was partial and with low yield.

The M_r 32000 protein has been tentatively referred to by us (Lin & Klingenberg, 1980a) as the "uncoupling protein" to emphasize its putative functional role in brown adipose tissue mitochondria. Recently the name "thermogenin" has been suggested (Lindberg et al., 1981). On the basis of the concept that NbP has certain similarities to the ADP/ATP carrier, the isolation method developed for the ADP/ATP carrier has been applied to NbP with some modifications, which are necessary to ensure the separation between the two proteins. This method was first briefly outlined for the isolation from cold-adapted hamster (Lin & Klingenberg, 1980a,b) and was then also applied for that purpose to cold-adapted rat (D. Ricquier, C. S. Lin, and M. Klingenberg, unpublished results). In view of the importance and growing interest in this protein a full account of the isolation procedures is given here. Furthermore, some characteristics of the isolated uncoupling protein are reported, in order to provide a basis for understanding its function.

Materials and Methods

Emulphogen BC 720 was obtained from GAF Co., New York, NY, [^{3}H]GDP, [^{14}C]ADP, and [^{14}C]ATP were from New England Nuclear, dimethylsuberimidate was from Pierce

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[1] Abbreviations: NbP, nucleotide binding protein; CAT, carboxyatractylate; DMS, dimethylsuberimidate; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Mops, 3-(*N*-morpholino)propanesulfonic acid; NEM, *N*-ethylmaleimide; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-phosphate; GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; GMP, guanosine 5′-phosphate; ITP, inosine 5′-triphosphate; IDP, inosine 5′-diphosphate; IMP, inosine 5′-phosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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