Studies on the Respiratory Chain-linked Reduced Nicotinamide Adenine Dinucleotide Dehydrogenase

XIV. LOCATION OF THE SITES OF INHIBITION OF ROTENONE, BARBITURATES, AND PIERICIDIN BY MEANS OF ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY*

(Received for publication, October 20, 1967)

GRAHAM PALMER†
From the Biophysics Research Division, Institute of Science and Technology, University of Michigan, Ann Arbor, Michigan 48107

DOUGLAS J. HORGAN,§ HOWARD TISDALE, AND THOMAS P. SINGER
From the Division of Molecular Biology, Veterans Administration Hospital, San Francisco, California 94121, and the Department of Biochemistry, University of California School of Medicine, San Francisco, California 94122

HELmut BEINERT¶
From the Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53706

SUMMARY

On the addition of NADH to submitochondrial particles in which NADH oxidase is blocked by rotenone, piericidin A, or Amytal, the g = 1.94 signal of NADH dehydrogenase appears in essentially the same manner as in untreated preparations. However, the appearance of the NADH-induced iron signal of succinate dehydrogenase and of cytochromes b and c₁ is inhibited by these agents. It is concluded that Amytal, rotenone, and piericidin block NADH oxidation on the O₂ side of the non-heme iron of NADH dehydrogenase.

There has been considerable discussion in the literature concerning the reaction site of rotenone and barbiturates in the NADH oxidase chain. On the basis of spectrophotometric studies on mitochondria, it has been suggested that Amytal (2) and rotenone (3) block the reduction of NADH dehydrogenase by NADH in phosphorylating preparations but inhibit reoxidation of the flavoprotein in nonphosphorylating ones (4, 5). Since neither Amytal nor rotenone inhibits the NADH-ferricyanide reaction or transhydrogenation in soluble or particulate preparations, it has been proposed that these inhibitors act on the O₂ side of NADH dehydrogenase regardless of the state of phosphorylation (6-8) and that contrary results with mitochondria may be due to shortcomings of the spectrophotometric method when applied to NADH dehydrogenase flavin in complex preparations (1, 7). A similar localization has been suggested by Burgos and Redfearn (9).

It has been recently reported (1, 10) that, on complete extraction of NADH dehydrogenase from mitochondria or from the nonphosphorylating ETP fully inhibited with rotenone-₁³C, the radioactivity remained firmly bound to the insoluble residue. Furthermore Amytal and piericidin competitively inhibited the binding of rotenone-₁³C in the NADH dehydrogenase segment of the chain. It appears quite probable, therefore, that all these inhibitors act between NADH dehydrogenase and coenzyme Q. The present experiments provide additional, independent evidence for this localization from studies of the effect of these inhibitors on the appearance of the NADH-induced g = 1.94 signals in submitochondrial particles.

EXPERIMENTAL PROCEDURE

ETP was prepared by the method of Ringler, Minsakami, and Singer (11). The particles were titrated to greater than 99% inhibition with either rotenone or piericidin according to the

* Supported by grants from the American Heart Association, the Life Insurance Medical Research Fund, the National Science Foundation (GB 5392), and the United States Public Health Service (HE 10027, GM 12176, and GM 12294). The preceding paper in this series is Reference 1.
† Career Development Awardee (GM-K2-21, 213).
§ Fellow of the Bay Area Heart Association; Fulbright Scholar.
¶ Research Career Awardee (GM-K6-18,442).

1 The abbreviations used are: ETP, electron transport particle; EPR, electron paramagnetic resonance.
Fig. 1. Effect of inhibitors on the EPR spectra of ETP reduced with NADH. A, normal untreated ETP; B, as A plus 10 µl of 0.5 mM NADH; C, as A plus 5 µl of 1% (w/v) antimycin A and subsequently 10 µl of 0.5 mM NADH; D, as A plus 10 µl of 0.2 mM Amytal and subsequently 10 µl of 0.5 mM NADH; E, rotenone-inhibited ETP plus 10 µl of 0.5 mM NADH; F, piericidin-inhibited ETP plus 10 µl of 0.5 mM NADH. The samples were frozen 30 sec after mixing began. Spectra were recorded at 85°C with the following instrumental conditions: modulation amplitude, 12 gauss; microwave power, 27 milliwatts; magnetic field sweep, 400 gauss per min; time constant, 1 sec. The rotenone-inhibited ETP was prepared by adding 200 µl of 1.35 mM rotenone to 100 mg of ETP (protein basis) suspended in 26 ml of 0.25 M sucrose-0.025 M phosphate (pH 7.4)-2% (w/v) bovine serum albumin (99% inhibition of NADH oxidase). After standing for a few minutes at 0°C, the mixture was centrifuged for 30 min at 30,000 rpm in the No. 30 rotor of the Spinco model L ultracentrifuge. The piericidin-treated sample was prepared in the same manner except that 200 µl of 3.6 mM inhibitor were added per 100 mg of ETP (99% inhibition).

procedure of Horgan and Singer (10). Samples inhibited with either Amytal or antimycin were incubated for 5 min at 0°C with these inhibitors, prior to addition of substrates.

X-band (9 GHz) EPR spectra were obtained with a Varian V-4500 spectrometer equipped with a microwave bridge and a circulator (12). The low temperature accessory resembled that described by Hansen, Kalal, and Beinert (13) except that the facility for automatic temperature control was omitted. Samples (0.25-ml) of normal or inhibited ETP (approximately 100 mg per ml) were loaded into the 3-mm-inner diameter quartz EPR tubes. Other reagents as shown were layered on the ETP, with the use of calibrated narrow bore polyethylene tubing. Efficient stirring was accomplished with a motor-driven Nichrome wire which terminated in a small loop. Samples were subsequently centrifuged briefly for 5 sec in a bench top centrifuge to remove included air, incubated for the requisite amount of time, and then frozen in liquid nitrogen. The operations of stirring, centrifuging, incubation, and freezing were standardized as much as possible to facilitate comparison of samples.

Optical difference spectra were obtained with an Aminco-Chance double beam spectrophotometer operating in the split beam mode.

RESULTS

The effects of rotenone, piericidin, and Amytal on the EPR spectrum of ETP are illustrated in Figs. 1 and 2. Fig. 1A shows the EPR spectrum of untreated ETP. The observed absorption is due to the oxidized copper component of cytochrome oxidase. This spectrum was not changed by rotenone, piericidin, Amytal, Seconal, antimycin A, or ethanol in the quantities used for the experiments. On addition of NADH (Fig. 1B), the EPR spectrum changed profoundly. Two major effects may be discerned: a substantial decrease in the copper signal, and the appearance of new resonances at high field. These have been shown to be due to the non-heme iron of NADH dehydrogenase and succinate dehydrogenase, and those located in the cytochrome b-c₁ portion of the respiratory chain (14). As an aid in identification, these are labeled D, S, and R (cf. Fig. 2G), respectively. Because these resonances overlap, quantitative estimates of the contributions of each species to the total resonance is difficult (see Refer-

Fig. 2. Spectra A to F are the same as in Fig. 1, except that all of the samples had an additional 2-min incubation at room temperature. G, as A, subsequently reduced with a few grains of dithionite. In this spectrum D, S, and R are used to indicate the positions of the non-heme iron associated with NADH dehydrogenase, succinate dehydrogenase, and cytochromes b and c₁, respectively.
once 15 for a comparison of the EPR spectra of the isolated species). In particular, the principal slope immediately to the left of the \( g = 1.94 \) signal may be a misleading measure of the intensity of the iron signal, because of a negative contribution of the copper component. We prefer to measure intensities by reference to the high field base-line.

When NADH is added to ETP which has been blocked with rotenone or piericidin, the EPR spectra obtained (Figs. 1 and 2, E and F) show qualitative differences from that obtained in the absence of the inhibitor; the copper signal retains much of its original intensity, and the signal due to succinate dehydrogenase iron is now absent. The resonances due to NADH dehydrogenase iron and the \( b_{-}c \) non-heme iron component are still present. Quantitatively, the intensity of the NADH dehydrogenase iron signal is about 70% of that found in the absence of the inhibitor. Much, if not all, of this small decrease can be attributed to the absence of the succinate dehydrogenase iron signal, which makes a substantial contribution to the intensity at position \( D \) (Fig. 2G). Thus, in the presence of these inhibitors, the intensity of the NADH dehydrogenase iron signal, and quite possibly that of the \( b_{-}c \) iron, is not substantially affected, although the overall oxidase activity is decreased by 99%. It might be argued that in the length of time necessary to perform the experiment enough substrate has been oxidized to consume all of the available oxygen and that the system has become anaerobic. However, comparison of the intensity of the copper resonance in the uninhibited and inhibited samples shows that the terminal oxidase is clearly more oxidized in the latter, indicating the continued availability of oxygen to the inhibited particles.

With the inhibitors mentioned, essentially similar spectra were obtained after 30 sec (Fig. 1) and 150 sec (Fig. 2), except that at the later time the \( b_{-}c \) non-heme iron signal was more pronounced. With Amytal the inhibition appears to be less effective, for although the 30-sec spectrum (Fig. 1D) was quite similar to that obtained with rotenone (Fig. 1E), substantial reduction of the succinate dehydrogenase iron had occurred by 150 sec (Fig. 2D, ooe signal at position \( S \)). The experiments with antimycin A (Fig. 1C and 2C) show a similar effect, with succinate dehydrogenase iron even more reduced at 30 sec.

The observation that under the conditions of our experiments the \( b_{-}c \) iron is substantially reduced in the inhibited particles is difficult to reconcile with the widely held belief that rotenone and related inhibitors act specifically in the flavoprotein segment of the NADH oxidase chain. In order to confirm the finding that a second reaction site for rotenone and piericidin may exist in the cytochrome \( b_{-}c \) region, optical spectra were recorded with a dual wavelength spectrophotometer operating in the split beam mode. The results are shown in Fig. 3 for rotenone. There is a slow but real reduction of cytochrome \( b \) as judged from the time-dependent appearance of a peak at 432 \( \mu m \) and trough at 418 \( \mu m \). These extremes correspond to those found with antimycin-treated liver mitochondria reduced with NADH (16).

The inset in Fig. 3 shows a similar experiment with higher scale expansion (0.05-A full scale on recorder) in order to permit following the time course of the appearance of the \( \alpha \) and \( \beta \) bands of cytochrome \( b \). Under the experimental conditions, reduction of cytochrome \( b \) was complete in 5 to 6 min. The ratio of the peak to trough amplitudes of the Soret and \( \alpha \)-bands remained essentially constant in the course of the experiment.

In identical experiments with piericidin A as inhibitor, the results were quite similar to those illustrated for rotenone.

**Discussion**

The data presented here are consistent with the proposition that the primary site of action of the inhibitors rotenone, piericidin, and Amytal is on the oxygen side of the non-heme iron of the NADH dehydrogenase. This conclusion is based on the observation that in particles inhibited by more than 90% with respect to their NADH oxidase activity the \( g = 1.94 \) signal associated with this part of the chain has 65 to 75% of the intensity found in uninhibited particles, and the observed loss in intensity is obviously due to the lack of reduction of the iron component of succinate dehydrogenase. It has been recently reported (17) that dicyclohexylcarbodiimide also blocks electron transport in the NADH oxidase chain between the non-heme iron of NADH dehydrogenase and cytochrome \( b \), as judged from both optical and EPR spectroscopy.

Our data also support the contention (10) that the action of piericidin and of rotenone on electron transport particles is not necessarily specific, i.e. that more than one inhibition site appears to be operative. The appearance of the \( b_{-}c \) non-heme iron signal and the observation that cytochrome \( b \) is slowly reduced both argue for a second inhibitor site between the \( b_{-}c \) non-heme iron and cytochrome \( c_{1} \), i.e. in the same general region as the antimycin A site. The relatively slow appearance of the substrate-induced non-heme iron signal in the \( b_{-}c \) region and of the spectrum of reduced cytochrome \( b \) (unaccompanied by any reduction of cytochromes \( c \) and \( c_{1} \)) in rotenone and piericidin treated

![Fig. 3. Effect of NADH on the optical spectrum of rotenone-inhibited ETP. The inhibited particles, prepared as in Fig. 1, suspended in 0.25 m sucrose-0.025 m phosphate buffer, pH 7.4, at a protein concentration of 2.5 mg per ml, were placed in the reference and sample cuvettes of the Aminco-Chance spectrophotometer (3.2-ml total volume, 10-mm light path). The reference cell received at zero time 30 \( \mu l \) of sucrose-phosphate; the sample cell, 30 \( \mu l \) of 0.1 m NADH. The temperature was 24°. Scanning was started immediately (5 \( \mu m \) per sec) at 0.1 absorbance (full scale) and was repeated at the times indicated. At the end of the experiment, solid Na\( _{2} \)S\( \text{O}_{4} \) was added and the spectrum was plotted on 0.5 absorbance (full scale). The inset shows the 490 to 560 \( \mu m \) region for a similar experiment performed at a higher scale expansion, in order to illustrate the appearance of the \( \alpha \) and \( \beta \)-bands.](http://www.jbc.org/issue/243/4/article_386_Fig3.png)
particles may be explained as a result of a slow leak in the primary inhibition site (between flavoprotein and coenzyme Q) which permits a slow flux of reducing equivalent to cytochrome b, coupled with a complete block in the b-c₁ region, which prevents reoxidation of cytochrome b.

The results presented, together with the studies on the binding of rotenone-¹⁴C (1, 10) indicate that rotenone, Amytal, and piericidin A inhibit NADH oxidation on the O₁ site of the non-heme iron moieties of NADH dehydrogenase, regardless of the state of phosphorylation, in accord with Scheme 1. It would appear that NADH dehydrogenase preparations which have lost the iron and labile sulfur components responsible for the g = 1.94 signal have also lost the physiological reaction site (between dehydrogenase and coenzyme Q), since the inhibitors mentioned act between the dehydrogenase and coenzyme Q.

REFERENCES

15. BEINERT, H., in A. SAN PIETRO (Editor), Non-heme iron proteins-role in energy conversion, Antioch Press, Yellow Springs, Ohio, 1965, p. 23.
Studies on the Respiratory Chain-linked Reduced Nicotinamide Adenine Dinucleotide Dehydrogenase: XIV. LOCATION OF THE SITES OF INHIBITION OF RÖTENONE, BARBITURATES, AND PIERICIDIN BY MEANS OF ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY

Graham Palmer, Douglas J. Horgan, Howard Tisdale, Thomas P. Singer and Helmut Beinert


Access the most updated version of this article at http://www.jbc.org/content/243/4/844

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/243/4/844.full.html#ref-list-1