Relation of the Respiratory Chain-Linked Reduced Nicotinamide–Adenine Dinucleotide Dehydrogenase to Energy-Coupling Site 1*

M. Gutman,† T. P. Singer,‡ and H. Beinert§

ABSTRACT: Preparations of submitochondrial particles, upon treatment with reduced nicotinamide–adenine dinucleotide (NADH) in the aerobic state, undergo a cycle of absorbance changes, which may be monitored at the 470- minus 500-mμ wavelength pair. There is a rapid initial bleaching, followed by return of the color on exhaustion of the NADH. In the presence of rotenone or piericidin the cycle is prolonged, the return of color (reoxidation) is inhibited and the extent of reoxidation less than in uninhibited particles. On the basis of prior work the residual bleaching (and most or all of the initial bleaching by NADH) is thought to be due to chromophores associated with NADH dehydrogenase. ATP causes rapid reoxidation of the chromophore which remains permanently reduced in rotenone- and piericidin-blocked preparations. This effect is inhibited by oligomycin and 2,4-dinitrophenol. While ATP causes a complete reoxidation of the nonheme iron chromophores, it reduces cytochrome *b* in rotenone-inhibited preparations but this reduction is transient. Reoxidation of the chromophore monitored at 470 − 500 mμ is suggested to be an intraenzymic reversed electron flow, energized by ATP *via* phosphorylation site 1. Of the various iron–sulfur species associated with NADH dehydrogenase, whose reduction gives rise to characteristic electron paramagnetic resonance signals observed at temperatures between 4 and 20°K, the low potential iron–sulfur center 1 is reoxidized by the respiratory chain when NADH is exhausted, but the higher potential center 2 remains reduced. ATP causes reoxidation of the iron–sulfur of center 2. On the basis of these and of other observations it is tentatively proposed that coupling site 1 is directly associated with NADH dehydrogenase and is located on the O_2 side of iron–sulfur center 1 and the substrate side of both center 2 and the specific binding sites of rotenone and piericidin.

Bois and Estabrook (1969) recently described a cycle of absorbance changes observed on adding NADH to an aerobic sample of ETP,[1] which may be monitored with a dual-wavelength spectrophotometer at the wavelength pair 470 − 500 mμ. NADH causes rapid reduction (bleaching) of a chromophore, followed by reoxidation (recolorization) on exhaustion of the substrate. In the presence of rotenone, however, the slow reoxidation of the chromophore *via* the respiratory chain is incomplete and a fraction remains permanently bleached. This fraction has been tentatively identified as being due to iron–sulfur components of NADH dehydrogenase on the basis of its absorption spectrum and characteristic electron paramagnetic resonance (epr) signal at $g = 1.94$. This assignment has been recently confirmed by the authors (Gutman and Singer, 1970; Singer and Gutman, 1971).

The studies were extended in the previous paper of this series (Gutman and Singer, 1970), utilizing both specifically and unspecifically bound rotenone and piericidin (Horgan *et al.*, 1968). Analysis of the kinetic and thermodynamic parameters of the redox cycle indicate that most, perhaps all, of the bleaching at 470 − 500 mμ induced by NADH, in normal as well as inhibited preparations, reflects reduction of the flavin and nonheme iron moieties of the dehydrogenase, except for a small contribution by a *b*-type cytochrome. As is implicit in this assignment, the authors concurred with the conclusion (Bois and Estabrook, 1969) that the permanently bleached fraction of the chromophore also represents chromophores associated with the dehydrogenase.

In further studies with phosphorylating membrane preparations (ETP_H), in which the redox cycle occurs in the same manner as in nonphosphorylating preparations, it was discovered that ATP causes rapid reoxidation of this "permanently bleached chromophore" and that this reoxidation is inhibited by dinitrophenol and oligomycin (Gutman *et al.*, 1970a; Gutman and Singer, 1971). The present paper is a detailed account of the effect of ATP on the redox cycle, as monitored by spectrophotometric and epr techniques. Analysis of the data while confirming the assignment of coupling site 1 to the substrate side of the rotenone-sensitive site (Hinkle *et al.*, 1967), permits closer localization to a discrete region of NADH dehydrogenase.

Experimental Section

Materials and Methods. ETP_H from beef heart mitochondria was prepared according to Hansen and Smith (1964); Mn^{2+} was replaced by Mg^{2+} when epr studies were intended; oligomycin was purchased from the Sigma Chemical Co. and *Neurospora* NADase from Worthington. The redox cycle was measured in the Aminco-Chance dual-wavelength spec-

* From the Molecular Biology Division, Veterans Administration Hospital, San Francisco, California 94121, from the Department of Biochemistry and Biophysics, University of California Medical Center, San Francisco, California 94122, and from the Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53706. *Received July 29, 1971.* This work was supported by grants from the American Cancer Society (P-531), the National Science Foundation (GB8248), and the National Institutes of Health (HE 10027 and GM 12394). Paper XX in the series: Studies on the respiratory chain-linked reduced nicotinamide–adenine dinucleotide dehydrogenase.

† On leave of absence from Tel-Aviv University, Israel.

‡ To whom to address correspondence.

§ Recipient of a Research Career award (GM 18,442) from the Institute of General Medical Sciences.

[1] Abbreviations used are: BSA, bovine serum albumin; CoQ, coenzyme Q; ETP and ETP_H, nonphosphorylating and phosphorylating preparations of the inner membrane, respectively.

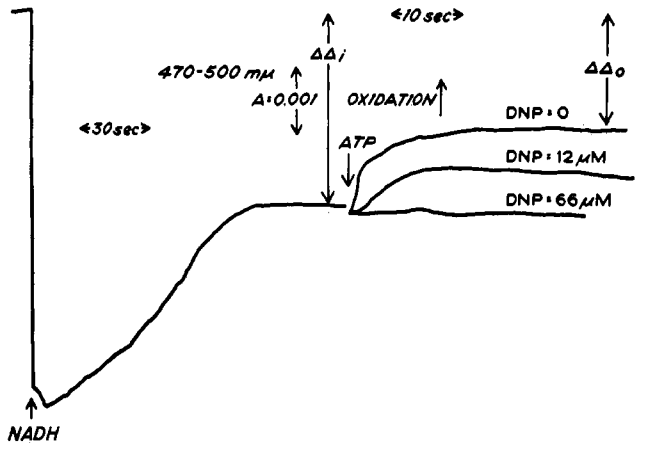

FIGURE 1: Kinetics of the absorbance changes observed at 470–500 mμ in rotenone-inhibited ETP_H and the effect of ATP. Beef heart ETP_H was suspended in 0.18 M sucrose–50 mM Tris-acetate–5 mM $MgSO_4$ (pH 7.4) at a protein concentration of 4 mg/ml with 0.5 nmole of rotenone present per mg of protein. The reaction was started by the addition of 0.25 mM NADH and absorbance was monitored at room temperature with the Aminco-Chance spectrophotometer. At the end of the redox cycle 3.6 mM ATP was added in the presence or absence of 2,4-dinitrophenol (DNP), as indicated. Note that the time scale changes after ATP addition.

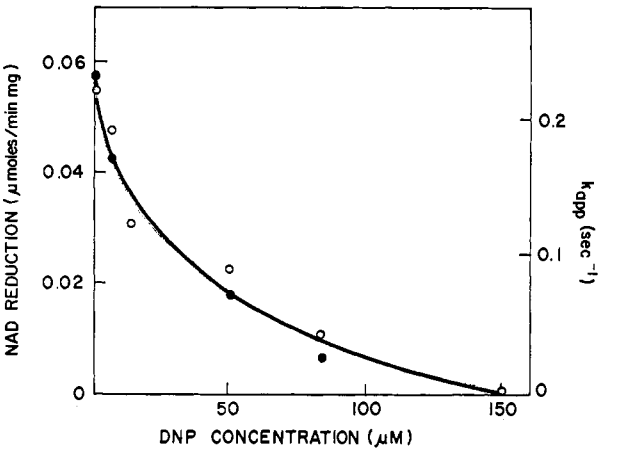

FIGURE 2: Effect of dinitrophenol on the ATP-linked reduction of NAD by succinate and on the rate of ATP-induced oxidation of the chromophore. ETP_H was washed with 0.25 M sucrose–50 mM Tris-acetate–5 mM $MgSO_4$ (pH 7.4) and resuspended in the same medium at a concentration of 4 mg of protein/ml. Aliquots were used to measure rates of NAD reduction at various concentrations of dinitrophenol (DNP). Another aliquot was treated with 0.5 nmole of piericidin A/mg of protein for 30 min at 0°, which gave complete inhibition of ATP-linked NAD reduction. The redox cycle was then measured at 470–500 mμ in the presence of varying concentrations of DNP. ATP was added, as in Figure 1, when the absorbance leveled off and the first-order rate constant for oxidation of the chromophore ($k_{app.}$) was determined.

trophotometer under the conditions previously specified (Gutman and Singer, 1970), except that the membrane preparation was suspended in 0.18 M sucrose–50 mM Tris-acetate–5 mM $MgSO_4$ (pH 7.4). Reverse electron transport was measured by the method of Ernster and Lee (1967).

Epr spectroscopy was carried out with a modified Varian V4500 instrument which permits operation at low power (Palmer, 1967). Cryogenic temperatures were produced with a helium boil-off system and monitored with a carbon resistor. The resistance values indicated by the resistor were calibrated with an iron-doped gold thermocouple, positioned in a tube in the cavity. The epr spectra of the particles were observed at X-band frequency at a variety of temperatures and microwave powers. A temperature of 13°K and a power of 0.3 mW at 6-G modulation amplitude were found to be optimal for resolution and signal-to-noise ratio with our instruments. Other methods were as in the preceding paper (Gutman and Singer, 1970).

Results

Effect of ATP on the Redox Cycle. As documented in previous publications (Gutman and Singer, 1970; Gutman *et al.*, 1970a; Singer and Gutman, 1971) on addition of NADH to an aerobic sample of ETP_H, there is an immediate bleaching, too fast to measure, followed by a rapid but measurable rate of reoxidation which starts when NADH is exhausted (Gutman and Singer, 1970). The extent of reoxidation, denoted as ΔA_{reox}, is incomplete and a residual bleaching ($\Delta\Delta_0$) remains. The chromophore responsible for the $\Delta\Delta_0$ value appears to be a reduced *b*-type cytochrome by its absorption spectrum and is the same in untreated and rotenone- or piericidin-inhibited samples (Bois and Estabrook, 1969; Gutman and Singer, 1970). This small absorbance, therefore, may be used to correct for the contribution of this component to the absorbance changes recorded at 470 – 500 mμ under a variety of experimental conditions (Gutman and Singer, 1970).

In the presence of rotenone (Figure 1) or piericidin A the redox cycle is greatly prolonged because the rate of reoxidation of the chromophore is inhibited, as anticipated from the fact that these inhibitors block the reoxidation of NADH dehydrogenase by the respiratory chain (Gutman *et al.*, 1970b). Further, for reasons discussed elsewhere (Gutman and Singer, 1970; Singer and Gutman, 1971), ΔA_{reox}, the extent of reoxidation, is also less than in uninhibited preparations. The extent of these inhibitions depends, of course, on the amount of rotenone or piericidin present. Under all conditions tested, however, the extent of bleaching by NADH is the same in control and inhibited preparations.

The amount of unreoxidized chromophore, denoted as $\Delta\Delta_i$ on the left side of Figure 1, remains stable at least for 1 hr under the experimental conditions. As mentioned earlier, $\Delta\Delta_i - \Delta\Delta_0$ is believed to represent an iron–sulfur species associated with NADH dehydrogenase. If ATP is added, rapid reoxidation of this chromophore occurs, so that the value of $\Delta\Delta_i - \Delta\Delta_0$ becomes zero or even negative. The reaction appears to be of first order, with a rate constant of approximately 0.35 sec^{-1}. Dinitrophenol inhibits this reoxidation at concentrations compatible with its function as an uncoupler (Figure 1). While the reaction remains a first-order one in the presence of dinitrophenol, both the rate and the extent of reoxidation are decreased. Figure 2 shows that at a series of concentrations this uncoupler affects the ATP-driven reoxidation of the chromophore and the energy-linked reduction of NAD identically. This correlation may be taken to suggest that the same dinitrophenol-sensitive component participates in both reactions although the possibility cannot be excluded that different components with identical affinities for the uncoupler are involved. ATP induced no reoxidation of the chromophore in nonphosphorylating preparations (ETP) or in aged ETP_H.

Effect of Oligomycin. As expected from its action as an inhibitor of oxidative phosphorylation and energy-linked reversal of electron transport, oligomycin inhibits the ATP-

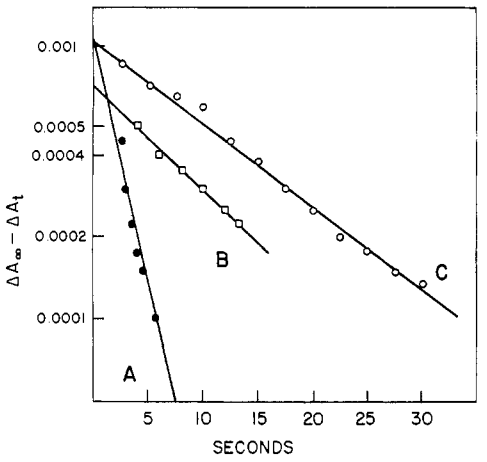

FIGURE 3: Effect of oligomycin and of *Neurospora* NADase on the ATP-induced oxidation of the chromophore. ETP_H was washed as in Figure 2 and resuspended in 0.18 M sucrose–50 mM Tris-acetate –5 mM $MgSO_4$–2% (w/v) BSA (pH 7.4) at a protein concentration of 4 mg/ml. Piericidin A (0.4 nmole/mg of protein) was added and the suspension was incubated for 30 min at 0°. Washing by centrifugation was omitted in order to avoid decline of the activity of the particles for reversed electron transport, but the presence of BSA assured that most of the piericidin was specifically bound. When all NADH was exhausted and the absorbance at 470–500 mμ became steady, 3.6 mM ATP was added. (A) No further addition; (B) oligomycin (0.4 μg/mg of protein) added before NADH; (C) NADase (0.05 IU) added after exhaustion of all NADH and the reaction mixture was incubated for 15 min at 25° prior to the addition of ATP in order to destroy all accumulated NAD. The first-order rate constants for reoxidation of the chromophore were A = 0.345 sec^{-1}, B = 0.086 sec^{-1}, C = 0.069 sec^{-1}. The ordinate denotes the difference between the maximal reoxidation of the chromophore monitored at 470–500 mμ (ΔA_∞) and the increment observed at any one time (ΔA_t)

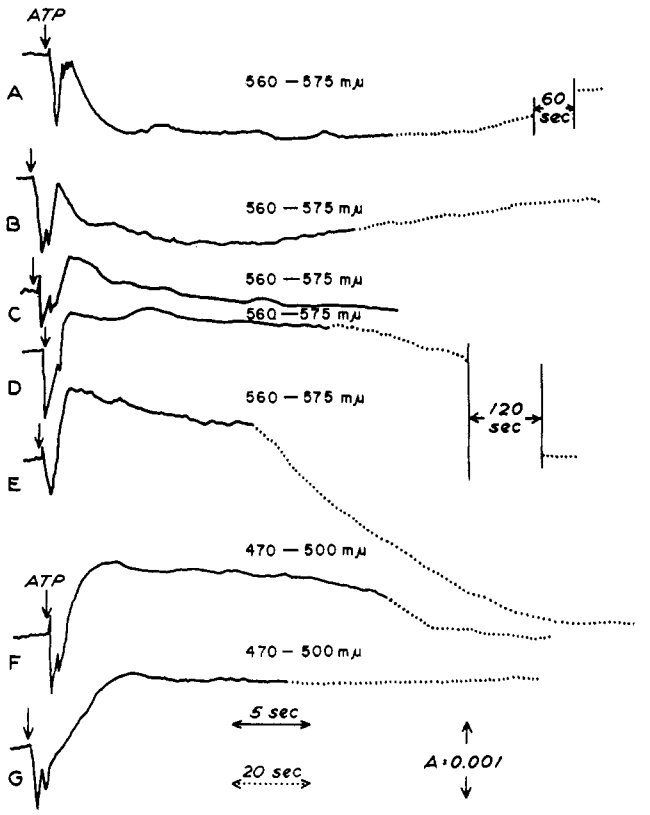

FIGURE 4: Effect of piericidin concentration on ATP-triggered absorbance changes. ETP_H was washed, resuspended, and treated with graded amounts of piericidin as described in the legend of Figure 3. Aliquots of 3 ml were placed in the cuvet of the spectrophotometer at 23°; 0.25 mM NADH was added to each sample, and the ensuing absorbance changes were monitored at 560–575 and 470–500 mμ. When the redox cycle was completed, as indicated by no further absorbance change at either wavelength pair, 4 mM ATP was added (vertical arrow) and the resulting absorbance changes were recorded. The piericidin concentrations (picomoles per milligram of protein) and remaining NADH oxidase activities (micromoles of NADH per minute) were (A) 0 and 0.9, (B) 20 and 0.8, (C) 100 and 0.6, (D) 200 and 0.55, and (E) 750 and 0.25. Tracings A–E were measured at 560 – 575 mμ, while F and G refer to 470 – 500 mμ and refer to samples containing 0 and 750 pmoles of piericidin per mg, respectively. The ΔΔ values for F and G were 0.9×10^{-3} and 1.9×10^{-3} *A*, respectively. Solid line denotes 5-sec/in. chart speed, dotted line 20 sec/in. Upward deflection denotes increased absorbance.

linked reoxidation of the chromophore. As seen in Figure 3, at a concentration of 0.4 μg/mg of protein, the rate constant for the oxidation of the chromophore is only one-fourth that of the control, while in other experiments at 0.8 μg/mg of protein inhibition was complete. The decrease in the extent of reoxidation in Figure 3 by oligomycin is relatively small. The energy-linked reduction of NAD by succinate was found to be nearly completely inhibited at a lower concentration of oligomycin (0.24 μg/mg of protein). The reason for the discrepancy might be that while the latter process measures substrate utilization in the micromole range, the former measures a single turnover, so that even a slight, residual activity might finally lead to oxidation of the reduced chromophore.

NAD Requirement for the ATP-Induced Reoxidation of the Chromophore. One of the questions which arises in connection with the ATP-induced reoxidation of the chromophore monitored at 470 – 500 mμ is the nature of the electron acceptor. An obvious possibility is that the NAD accumulated during the first part of the redox cycle fulfills this role. In order to test this hypothesis the preparation was incubated at the end of the cycle for 15 min with a large excess of *Neurospora* NADase and then ATP was added. The half time for the hydrolysis of the estimated amount of NAD present was 0.35 min. Therefore complete hydrolysis of the accumulated NAD could be reasonably expected in 15 min. Under these conditions the rate of the ATP-triggered reoxidation of the chromophore was 80% inhibited but eventually reached completion (Figure 3). This experiment suggests that in piericidin-inhibited preparations NAD is the primary electron sink for the reoxidation of the chromophore. The possible mechanism of the ATP-dependent reoxidation in the absence of NAD is dealt with in the Discussion.

Effect of ATP on Cytochrome b. Hinkle *et al.* (1967) reported that in submitochondrial particles, in the presence of succinate, sulfide, malonate, and antimycin A, the addition of ATP causes oxidation of cytochrome *b* and that this reaction is inhibited by rotenone and uncoupling agents. In ETP_H, at the end of the redox cycle initiated by NADH, ATP similarly induces a rapid but transient oxidation of cytochrome *b* in the absence of succinate or of the inhibitors mentioned. This transient oxidation may be observed either at 560 – 575 mμ (Figure 4, curve A) or at 470 – 500 mμ (Figure 4, curve F), where cytochrome *b* still shows appreciable absorbance. The characteristics of this oxidation observed at the two wavelength pairs are quite similar and the same rate constant (1.06 sec^{-1}) may be derived for the initial oxidation rate from tracing A or F. The slight difference in the kinetics of the decay phase is not due to the different wavelength pairs used but to variations in the length of the ATP cycle between

experiments. The ratio of absorbance changes at the two wavelength pairs, $\Delta A_{560-575\ m\mu}:\Delta A_{470-500\ m\mu}$ ranged from 1.5 to 3.3 in different experiments, with most values near 3. This may be compared with 3.2, the value reported by Goldberger *et al.* (1961) for purified cytochrome *b* and with 3.4, the figure for the cytochrome *b* component of ETP which is permanently reduced by NADH (Gutman and Singer, 1970). This ATP-induced oxidation of cytochrome *b* is regarded as quite analogous to the observations of Hinkle *et al.* (1967) but is transient in the present experimental conditions owing to the absence of a pool of reducing equivalents, such as succinate in the studies of Hinkle *et al.*

When the experiment presented is repeated with ETP_H samples inhibited with increasing amounts of piericidin, there is a gradual shift from an ATP-induced oxidation to a *reduction* of cytochrome *b* (Figure 4, curves A–E). Reduction of cytochrome *b* under the influence of ATP would not occur under the experimental conditions of Hinkle *et al.* (1967) because of the presence of succinate. The fact that the component reduced in piericidin-inhibited samples is cytochrome *b* was verified by its absorption spectrum. The difference spectrum of the sample corresponding to curve E in Figure 4 at the time of maximal reduction minus the same immediately before ATP addition showed typical maxima at 563 and 534–536 mμ. (There is an uncertainty in the exact position of the maximum of about 2 mμ owing to the very high-scale expansion).

While in uninhibited samples oxidation of cytochrome *b* with identical kinetics is observed at both 560 − 575 mμ and 470 − 500 mμ, in piericidin-inhibited samples this is not the case (*cf.* Figure 4, curves E and G). This is because in the presence of piericidin ATP triggers not only a reduction of cytochrome *b* but also an oxidation of the residual chromophore absorbing at 470 − 500 mμ. While the former is expected to cause bleaching at this wavelength, the latter results in increased absorbance; the net result is that seen in tracing G.

The ATP-induced reduction of cytochrome *b*, calculated from tracings such as curve E in Figure 4 amounts to only 1.7×10^{-11} mole of cytochrome *b* per mg of protein or some 2% of the total cytochrome *b* content of the particles. This reduction may be regarded as a reversed electron flow from coupling site 2, with electrons accumulating at the cytochrome *b* level because of the piericidin block, instead of reaching NAD. This hypothesis was verified by adding antimycin A at the end of the NADH redox cycle but before ATP (Figure 5, curve B). Comparison to the control sample (curve A), identical except for the absence of antimycin A, shows that the latter inhibitor prevents the ATP-induced reduction of cytochrome *b* but facilitates oxidation of the cytochrome. This is in accord with expectations, since piericidin inhibition was partial (53% of oxidase activity) while antimycin inhibition was complete. Curve C shows that oligomycin causes nearly complete inhibition of the reduction of cytochrome *b* by ATP in piericidin-blocked particles.

Thus the response of cytochrome *b* to ATP appears to depend on the difference in electron flux between the flavoprotein–cytochrome *b* and cytochrome b–c_1 junctions. If only the former is blocked, as occurs with piericidin, reduction of cytochrome *b* is observed; if inhibition at the latter site exceeds that at the flavoprotein junction, oxidation ensues. The reason for the slow oxidation of cytochrome *b* which follows the ATP-induced reduction (Figure 4, curve E) is not clear.

Epr Studies. In recent studies at 4–20°K of the epr signals of particle fractions of different complexity derived from beef

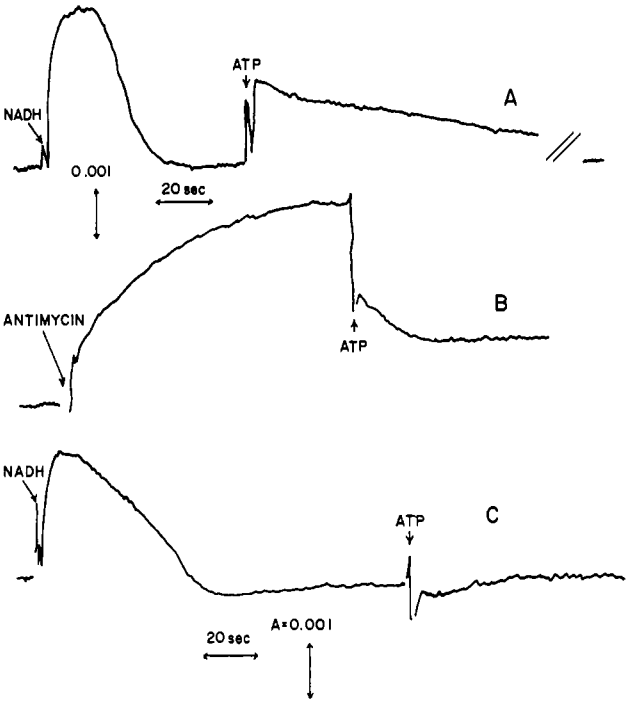

FIGURE 5: Effect of ATP on the redox state of cytochrome *b* in piericidin-inhibited ETP_H at 560–575 mμ. The particles were washed, resuspended, and treated with piericidin A as in Figure 3. ATP (3.6 mM) was added when all NADH was exhausted and absorbance at 470–500 mμ became steady. (A) Control. (B) At the end of the redox cycle initiated by NADH (not shown) the particles were incubated for 30 min at room temperature in order to allow oxidation of all NADH, then 0.5 mM thenoyltrifluoroacetone was added to inhibit succinate dehydrogenase, followed by 0.49 μg of antimycin A/mg of protein at the point indicated. When reduction of cytochrome *b* was complete, 3.6 mM ATP added. (C) Same as part A except that 0.8 μg of oligomycin was added per mg of protein prior to NADH. Upward deflection denotes increased absorbance.

heart mitochondria, four distinct iron–sulfur centers associated with the NADH dehydrogenase region of the electron-transfer system have been recognized (Orme-Johnson *et al.*, 1971, 1972). According to their average *g* values, they are of the ferredoxin type. The peak positions on the *g*-value scale are [center 1 (2.022, 1.938, 1.923), 2 (2.054, 1.922), 3 (2.101, 1.886, 1.864), 4 (2.103, 1.861)] measured only for NADH–ubiquinone reductase. These values are not to be considered as *g* values, but they are likely to be close to them. In ETP the four centers are present in approximately equimolar amounts and were also found to be equimolar with the flavin in NADH ubiquinone reductase. The soluble NADH dehydrogenase of Cremona and Kearney (1964) also shows four components. Centers 2–4 are not well resolved at temperatures exceeding 25°K. Resonances of center 4 overlap with resonances of center 3, so that center 4 is not always resolved even at very low temperatures. Center 1 has two resonances in the $g = 1.94$ region which are, however, not resolved at temperatures exceeding 25°K. The resonance of center 1 is the only resonance of NADH dehydrogenase which is detectable at liquid nitrogen temperature. This component was therefore the only one accessible to study in our previous investigations of the dehydrogenase (Beinert *et al.*, 1965). It was found to be readily reoxidized by ferricyanide. According to reductive titrations the order of oxidation–reduction potentials of the centers is $2 > 3 > 4 > 1$ (Orme-Johnson *et al.*, 1971).

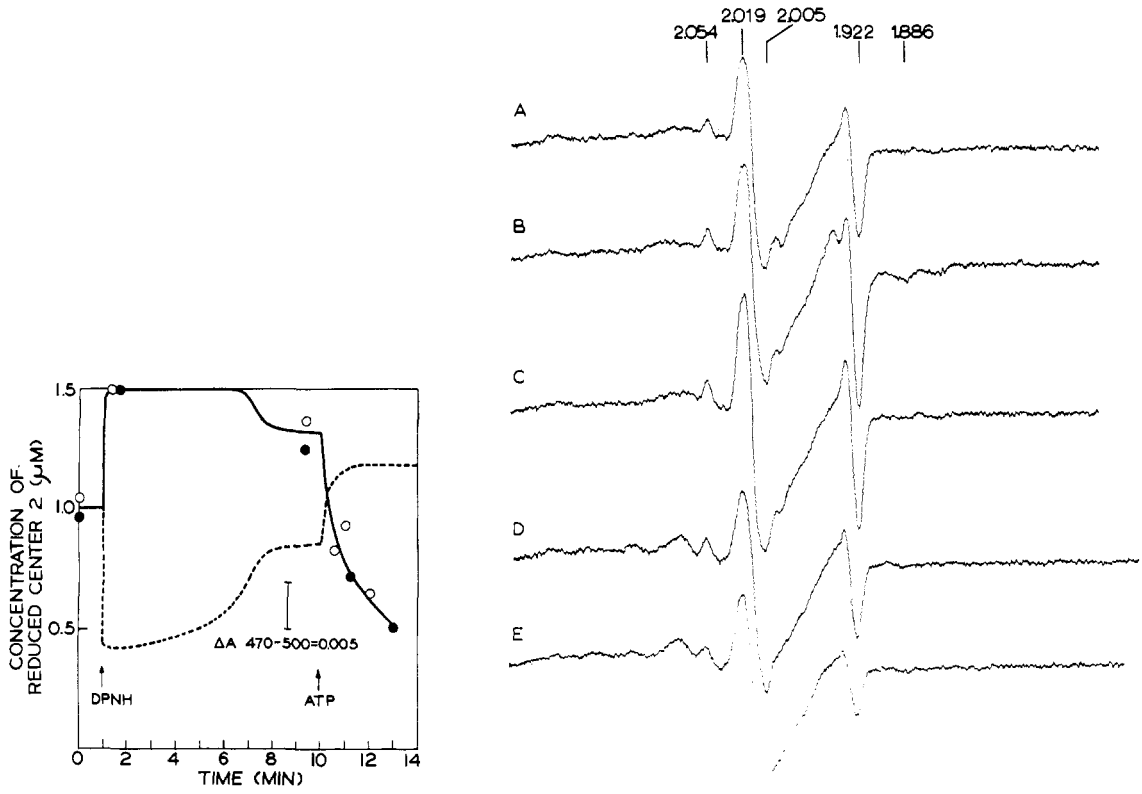

FIGURE 6: Correlation between the redox state of iron–sulfur center 2 and absorbance changes at 470 − 500 mμ and the effect of ATP on these parameters. Left side: an ETP_H sample (NADH dehydrogenase activity = 41 μmoles of NADH/min per mg at 30°), inhibited with 0.525 nmole of piericidin A/mg of protein, was suspended in 0.18 M sucrose–50 mM Tris–5 mM $MgCl_2$–2% (w/v) bovine serum albumin to a concentration of 10 mg of protein/ml. The redox cycle was carried out as previously described (Gutman and Singer, 1970). NADH was added at 0 time, causing immediate reduction, the extent of which is shown by samples taken at 1 min. Reoxidation ensuing on exhaustion of NADH is shown by the samples at 9 min. ATP (6 mM) was added at 10 min and samples were withdrawn at the times shown. The sampling time was less than 10 sec; samples were frozen in isopentane. The dashed line represents absorbance changes at 470–500 mμ, the solid line the micromolar concentration of reduced iron–sulfur center 2, calculated by comparison to CuEDTA standards. The solid and open circles represent samples from separate, parallel experiments. Note that the scale on the ordinate refers to the solid line only, the scale for absorbance is given separately in the figure. Right side: epr spectra recorded on samples of piericidin-treated ETP_H before and after the oxidation–reduction cycle initiated by NADH and after addition of ATP. Epr spectroscopy was carried out as indicated under Materials and Methods. (A) Particles as prepared and piericidin treated; (B) after aerobic addition of 220 μM NADH; (C) after reoxidation; (D) 1 min after addition of 6 mM ATP; (E) 5 min after ATP. The concentrations given are final concentrations. Note that after addition of ATP extra lines appear, particularly visible at low field. These lines are due to Mn^{2+}, which is a contamination of ATP, although the ATP used was purified by chromatography on Dowex 50 resin. There is also a large, strongly temperature-dependent signal with derivative peaks at $g = 2.019$ and 2.005 (*cf.* Orme-Johnson *et al.*, 1971, 1972), which stems from the oxidized form of an unknown electron acceptor. However, neither this nor the Mn^{2+} resonances interfere with the evaluation of the signals from centers 1 and 2.

It was of interest to compare the epr behavior of these iron–sulfur centers during the redox cycle with the spectrophotometric observations described above. Figure 6 illustrates an experiment conducted at room temperature, in which optical changes were monitored at 470 − 500 mμ and at various times aliquots were removed, quickly frozen in liquid N_2, and the epr signals were subsequently recorded at 13°K. The left side of the figure compares the changes in absorbance to those of epr signals corresponding to centers 1 and 2. To illustrate the resolution obtained, signals recorded at various states of the system are shown on the right side. Prior to the addition of NADH the signals, corresponding to the reduced forms of centers 1 and 2, were 5 and 50%, respectively, taking the integrated area of the signal of center 2 after NADH treatment, and an equimolar quantity of center 1 as representing 100% for each signal type. We have documented elsewhere (Gutman *et al.*, 1971b) that when the color begins to return, indicating depletion of the added NADH, reoxidation of center 1 also begins and reaches its maximal value by 6 min, at which time no significant reoxidation of center 2 has occured. Thus the residual absorbance at 470 − 500 mμ, which has been attributed to iron–sulfur components, is indeed accompanied by a residual epr signal, namely that of center 2. After the addition of ATP a large portion of center 2 which was still reduced, is now apparently oxidized. The small decline in the concentration of reduced center 2 between 6 and 9 min is either experimental error or may represent a contribution of other iron–sulfur centers. In the absence of ATP no significant reoxidation occurs in the same time span.

A detailed study of the kinetics of the disappearance of the signal of center 2 as compared to the increase in absorbance at 470–500 mμ would involve a large number of additional experiments and has, therefore, not been carried out yet. The data available, however, from a number of experiments indicate that the initial portion of the absorbance increase following the addition of ATP is more rapid than the changes of the epr signal. This indication, namely that part of the absorbance changes brought about by ATP are due to events other than the reoxidation of center 2, is supported by a consideration of the quantities of material represented by the epr and optical absorption changes. If, as it appears to be, center 2 of NADH dehydrogenase is an iron–sulfur center of the ferre-

doxin type, we can derive an estimate of the expected absorbance changes at 470–500 mμ from the corresponding values of known compounds of this class. Thus we find that Δ_{ox-red} 470–500 mμ for the ferredoxins of spinach, parsley, *Clostridium acidi urici*, and *Clostridium pasteurianum*, and for the iron–sulfur proteins of beef and pig adrenals falls into the range of 0.8–1.5 mM^{-1} cm^{-1}. On this basis the absorbance changes observed in our experiments exceed those expected from the changes of the oxidation state of center 2 severalfold. Since approximately at least 18 nonheme iron atoms and some 25 labile sulfur groups are associated with the NADH dehydrogenase region, of which probably only 8, and at most 16, are represented in the recognized iron–sulfur centers, it is possible that the unaccounted portion of the absorbance change is also due to iron–sulfur groups, which we have not yet learned to measure by epr spectroscopy.

While this remains an open question, our experiments thus far have clearly shown that an iron–sulfur component of NADH dehydrogenase is indeed reoxidized by ATP under the conditions described and that this explains at least part of the previously observed absorbance changes following ATP addition. These results suggest that energy coupling site 1 is located in the span between iron-sulfur centers 1 and 2 of NADH dehydrogenase, although this is not meant to exclude that a more detailed analysis may eventually allow an even closer localization.

Discussion

It has been known for many years that energy coupling site 1 is in the vicinity of NADH dehydrogenase (Lehninger, 1964). Ferricyanide is an excellent oxidant for NADH dehydrogenase and causes immediate reoxidation of the iron–sulfur components responsible for the $g = 1.94$ signal of the enzyme seen at liquid N_2 temperature (Beinert *et al.*, 1965) but does not permit energy conservation, although it is not an uncoupler (Schatz and Racker, 1966), while ATP formation is readily coupled to NADH oxidation with CoQ_1 as an oxidant (Schatz and Racker, 1966). Thus energy conservation site 1 would seem to be on the substrate side of CoQ_1, but on the O_2 side of iron–sulfur center 1 of NADH dehydrogenase.

One reason why a closer localization hitherto has not been achieved has been the absence of a reliable method for monitoring the redox state of the iron–sulfur and flavin components of NADH dehydrogenase. As discussed in recent reviews (Singer and Gutman, 1971; Gutman and Singer, 1971), all spectrophotometric methods available suffered from the shortcoming that the reduction of the dehydrogenase by substrate was examined in the anerobic state or in the absence of cytochrome oxidase activity, so that a variety of components of the respiratory chain became reduced and thus obscured the absorbance changes of the enzyme itself. Recently, however, a spectrophotometric method was described (Bois and Estabrook, 1969) which appears to be free from these objections. Analysis of the absorbance changes at the 470- minus 500-mμ wavelength pair initiated by NADH in uninhibited and rotenone-or piericidin-blocked particles in the aerobic state suggested that, after correction for the minor contribution of a *b*-type cytochrome, most or all of it may be ascribable to chromophores associated with NADH dehydrogenase (Gutman and Singer, 1970; Singer and Gutman, 1971). An extension of the method to phosphorylating particles and to an examination of the effect of ATP on the absorbance changes at 470 − 500 mμ appeared therefore logical in attempting to delineate further the locus of site 1.

In previous studies (Gutman and Singer, 1970) the observation that the "permanently bleached chromophore" ($\Delta\Delta_i - \Delta\Delta_0$) is always less than predicted from the inhibition of NADH oxidase activity was proposed to be due to heterogeneity of the NADH dehydrogenase population and the existence of lateral electron transport between NADH dehydrogenase molecules. While this suggestion may have some merit in explaining the observations quoted, it has become clear in the present study that the heterogeneity of the iron–sulfur centers in NADH dehydrogenase must also be considered.

The data presented in this paper and observations on the behavior of center 1 iron–sulfur (Gutman *et al.*, 1971b) show that while center 1 is reoxidized without ATP in piericidin-blocked submitochondrial particles, center 2 remains reduced until ATP is added. Thus the $\Delta\Delta_i - \Delta\Delta_0$ value observed in spectrophotometric experiments represents center 2 iron–sulfur, with possible contributions from other iron–sulfur centers which could not be monitored by epr because of the concentration limits imposed by the spectrophotometric equipment. The reason why center 2 remains reduced may be that lateral electron transport to the center 2 components of other NADH dehydrogenase molecules (which remain active at the piericidin concentrations used) is either too slow or may not occur, while electron flux *via* center 1 to NAD is energetically unfeasible because of the differences in redox potentials of centers 1 and 2.

Reoxidation of the "permanently" bleached chromophore on the addition of ATP may now be visualized as a lowering of the redox potential of center 2 as the system is energized, so that electrons can flow back to center 1 and thence to NAD. The conclusion that ATP acts as an energizer is based on the inhibition of the reoxidation by oligomycin and DNP and on the absence of any ATP effect in nonphosphorylating preparations or in aged ETP_H. Energy coupling is visualized as occurring at coupling site 1. In this sense the phenomenon is analogous to the ATP-induced oxidation of reduced cytochrome *b* (Hinkle *et al.*, 1967), but in our instance the reduced carrier being oxidized is an iron–sulfur component associated with NADH dehydrogenase. The reason why NAD is considered to be the electron acceptor is that if NAD is removed with NADase prior to the addition of ATP, the ATP-induced reoxidation of the chromophore occurs at a very much slower rate. The nature of the electron acceptor under these conditions is not known. The possibility that a slight autooxidation of the flavin occurs, as suggested by Hinkle *et al.* (1967) cannot be eliminated but alternative possibilities exist. For example, ATP might facilitate lateral (interchain) electron transport (Lee *et al.*, 1969) to uninhibited NADH dehydrogenase molecules and thence to the respiratory chain.

The response of cytochrome *b* to ATP is more complicated than that of the iron–sulfur chromophore. It should be emphasized that the fraction of cytochrome *b* undergoing oxidation or reduction under the influence of ATP in the present experiments is less than 2% of the total present. An additional complication is that, unlike the "permanently reduced" iron–sulfur components which are present in all preparations tested, the permanently reduced cytochrome *b* is variable and sometimes appears to be absent. In general, two qualitatively distinct responses to ATP have been observed. ATP induces oxidation of cytochrome *b* under conditions resembling those of Hinkle *et al.* (1967) in the sense that electron flux is minimal or absent, such as in uninhibited particles after all NADH is oxidized or in the piericidin-

blocked samples when a long time is allowed for exhaustion of the NADH and antimycin is subsequently added. ATP causes a reduction of cytochrome *b* in piericidin-inhibited particles if the cytochrome b–c_1 junction is operative and this reduction is not affected by complete inhibition of succinate dehydrogenase with thenoyltrifluoroacetone (Gutman and Singer, 1970). It appears that the energy-dependent oxidation–reduction of this fraction of cytochrome *b* may utilize coupling sites either on the substrate or on the O_2 side of the cytochrome and that the direction of electron flux is a function of the relative extents of the inhibition in the alternative directions.

The present experiments permit a closer localization of coupling site 1 than has been possible until now. Hinkle *et al.* (1967) suggested that coupling site 1 is located between cytochrome *b* and an unidentified, autooxidizable component which is located on the substrate side of the rotenone block. The experiments presented are in accord with this assignment but permit a closer localization.

Evidence has been presented that the type V SH group of NADH dehydrogenase is involved in the binding of rotenone and piericidin (Gutman *et al.*, 1970b, 1971a; Singer and Gutman, 1971). Since this type of SH appears to be an integral component of NADH dehydrogenase, coupling site 1 is not only on the substrate side of the rotenone block but also on the substrate side of the type V SH group of the dehydrogenase. Since the permanently reduced chromophore associated with NADH dehydrogenase is also on the substrate side of type V SH (Gutman and Singer, 1970), its reoxidation by ATP is in accord with this assignment. As discussed above, the iron–sulfur component responsible for the epr signal of center 1 of the enzyme is on the substrate side of coupling site 1. It is suggested, therefore, that, assuming a linear arrangement of components, the coupling site is associated with NADH dehydrogenase itself as an enzyme unit, and is located between iron–sulfur center 1 and both type V SH group and iron–sulfur center 2.

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