Hydrogen Transfer between Reduced Diphosphopyridine Nucleotide Dehydrogenase and the Respiratory Chain

II. AN INITIAL LAG IN THE OXIDATION OF REDUCED DIPHOSPHOPYRIDINE NUCLEOTIDE*

Shigeki Minakami,† F. J. Schindler,‡ and Ronald W. Estabrook§

From the Department of Biophysics and Physical Biochemistry, Johnson Foundation, University of Pennsylvania, Philadelphia 4, Pennsylvania

(Received for publication, December 11, 1963)

During studies of the reduced diphosphopyridine nucleotide oxidase activity of various heart muscle preparations, it was observed that some preparations showed a pronounced time lag before attaining the maximal rate of DPNH oxidation. Since this time lag was most apparent with Keilin-Hartree type of preparations from horse heart, a series of studies was carried out to determine the cause of this delay in the oxidation of DPNH. The present report summarizes the results of experiments on the reduction of alternative electron acceptors and the steady state kinetic changes of the endogenous cytochromes as well as the kinetics of reduction of coenzyme Q with DPNH as a substrate. The relation of this time lag to the sulfhydryl inhibitor-sensitive site described in the preceding paper (1) is discussed.

EXPERIMENTAL PROCEDURE

Materials and Methods

Heart Muscle Preparations—In general two types of heart muscle preparations were employed in the present studies. One type, here termed heart muscle particles, was prepared in a manner comparable to the method devised by Keilin and Hartree (2), but with the two following major modifications in the preparative procedure: (a) the water-washed heart mince was blended in the cold (4°) with 0.05 M phosphate buffer, pH 7.4, for 7 to 10 minutes in a Waring Blendor rather than ground with sand at room temperature for 1 hour in a mechanical mortar, and (b) the particulate material was concentrated by centrifugation for 30 minutes at 20,000 r.p.m. in a Spinco refrigerated centrifuge rather than precipitated by adjustment to pH 5.4. The pellet resulting from the high speed centrifugation was resuspended in 0.1 M phosphate buffer, pH 7.4, and stored at 4°. At pH 7.0 this type of preparation (heart muscle particles) contains both DPNH oxidase and succinic oxidase activity in the ratio of about 3:1, respectively; i.e. Qo, values at 22° averaged 300 for DPNH and 100 for succinate. The second type of preparation employed was the electron transport particle, isolated as described by Crane, Oishi, and Green (3). In this type of preparation, mitochondria were isolated from unwashed heart mince by blending for 3 minutes in a Waring Blendor in a 0.25 M sucrose and 0.01 M phosphate buffer medium. The resultant mitochondria were further subdivided into ETP by the alkaline treatment described by Crane et al. (3). At pH 7.0, this type of preparation (ETP) contains principally DPNH oxidase activity with relatively low succinoxidase activity; i.e. Qo, values at 22° averaged 1000 for DPNH and 100 for succinate.

Measurement of Oxygen Utilization and Reduction of Electron Acceptors—Oxygen uptake was measured polarographically with the oxygen electrode. The reduction of added electron acceptors (potassium ferricyanide, cytochrome c, and coenzyme Qo) was determined spectrophotometrically as described in the preceding paper (1). Reductions of the endogenous cytochromes and DPNH dehydrogenase flavoprotein were determined with a developmental model of an Aminco dual wave length monochromator at the wave lengths specified in the text. Changes in the extent of reduction of endogenous coenzyme Qo (ubiquinone) were determined by terminating the reaction at low temperature with a methanol petroleum ether mixture by a modification of the method described by Pumphrey and Redfearn (4). The change in absorbance at 275 μμμ upon addition of sodium borohydride to the extracts was then determined.

RESULTS

DPNH Oxidase Activity

During polarographic measurements of the DPNH oxidase activity of horse heart muscle preparations, it was frequently observed that there was a pronounced time lag before the establishment of the maximum rate of DPNH oxidation. A typical experimental showing the time course of DPNH oxidation by this heart muscle particle type of preparation is illustrated in Fig. 1. A lag period of about 30 seconds in duration occurs after the first addition of DPNH, as shown in Fig. 1 by the extrapolation from the maximal rate of oxygen uptake (dashed line). A similar lag time in the rate of DPNH oxidation is observed when the decrease of absorbance at 340 μμμ is used as a measure of DPNH oxidase activity of heart muscle particles. If succinate is employed as substrate with the same preparation, the initiation of the rate of oxygen uptake shows no comparable lag before the onset of the maximal rate of succinate oxidation.

* Supported in part by United States Public Health Service Research Grant RG 9950.
† Recipient of a Fulbright Travel Grant. Permanent address, Department of Physiological Chemistry and Nutrition, Faculty of Medicine, University of Tokyo, Japan.
‡ Predoctoral Fellow of the United States Public Health Service.
§ This work was carried out during the tenure of a Research Career Development Award (GM-K3-4111).

† The abbreviation used is: ETP, electron transport particle.
‡ B. Chance, personal communication.
participates in the oxidation of DPNH but not succinate is.

It appears that some component in heart muscle particles, the rate of reduction of which is equal to that of DPNH, was added prior to DPNH.

The first and second additions of DPNH, experiments comparable with the DPNH dehydrogenase of heart muscle particles, and the rate of oxygen uptake, it was observed that the rate of reduction of cytochrome c was not apparent and the optimal rate of DPNH oxidation was immediately established. A similar type of experiment, in which a limiting amount of succinate was first incubated with heart muscle particles before the addition of DPNH, did not alter the initial lag in the kinetics of DPNH oxidation.

In those preparations in which the addition of cytochrome c stimulated the rate of oxygen uptake, it was observed that the duration of the delay in DPNH oxidation was further accentuated. Another method of increasing the duration of the initial time lag in DPNH oxidation was to age the heart muscle preparation for prolonged periods at 4°C (1 week) or by continuous aeration by stirring at room temperature for more than 1 hour.

Partial inhibition (about 50%) of DPNH oxidation with cyanide was made as indicated to establish anaerobiosis. Oxygen uptake was measured polarographically in a closed vessel with the membrane-covered Clark oxygen electrode.

Studies with External Electron Acceptors

In an attempt to establish the locus of the component associated with the time lag observed during the oxygen uptake studies with heart muscle particles, the rate of reduction of external electron acceptors such as cytochrome c, coenzyme Q1, and potassium ferricyanide (K3Fe(CN)6) were measured. Typical results of these experiments are illustrated in Fig. 2. As shown in Fig. 2 (left), a time lag similar to that observed during the oxygen uptake studies is apparent when the DPNH-initiated reduction of cytochrome c is measured at 550 μM. This result is similar to that apparent in the data of Slater (Fig. 4 in Reference 5), in which a delay in the attainment of the optimal rate of cytochrome c reduction was observed when DPNH was employed as substrate with the Keilin and Hartree type of heart muscle preparation. Measurements with coenzyme Q1 as acceptor were determined by measuring the decrease of DPNH absorbance at 340 μM. In this case, antimycin A was added to the reaction mixture to inhibit electron transport to oxygen. Under these conditions, a similar time lag was also apparent. Potassium ferricyanide (K3Fe(CN)6) has been used routinely (6) in the assay of the DPNH dehydrogenase activity of heart muscle mitochondria. As illustrated in Fig. 2 (right), no initial time lag in the rate of the K3Fe(CN)6 reduction was observed; i.e. the maximal rate of ferricyanide reduction was achieved immediately. In each instance described above, parallel experiments were carried out with an ETP preparation. No time lag was detectable in measurements of the rate of the reaction of any of the external electron acceptors.
the acceptors with the respiratory chain of the FTP type of preparation. Thus, the locus of the component associated with the lag period observed during DPNH oxidation with various heart muscle preparations appears to reside between the site of ferricyanide intervention and coenzyme Q\textsubscript{1} interaction with the respiratory chain.

**Steady State Measurements**

**Cytochrome c**—The high affinity for DPNH of the DPNH dehydrogenase associated with the respiratory chain permits the observation of repetitive cycles of reduction and oxidation of the endogenous cytochrome pigments by limiting quantities of DPNH (cf. Fig. 1). When the change in absorbance of cytochromes c + c\textsubscript{1} of horse heart muscle particles was determined, reduction cycles of the type illustrated in Fig. 3A were obtained. As shown in this figure, the first addition of DPNH causes a rapid partial reduction of the cytochromes followed by a slower kinetic phase without establishing a steady state of reduction of the pigment. Upon utilization of the DPNH added, the cytochromes are reoxidized to a level nearly comparable to that observed before the addition of DPNH. When a second addition of DPNH is made, the extent of cytochrome c + c\textsubscript{1} reduction is greater and the appearance of the slower kinetic phase of reduction is less pronounced. This is most apparent by comparing the third cycle with the first cycle of cytochrome reduction. The associated increase in oxidative activity as illustrated in Fig. 1 is also reflected in the duration of the reductive cycles; i.e., the time for the second cycle is shorter than that of the first cycle, even though equal amounts of DPNH have been added to the preparation. Determinations of the duration of the reductive cycles illustrated in Fig. 3A show times of 45, 29, and 25 seconds for the respective additions of DPNH. A comparable study employing ETP is shown in Fig. 3B. In this instance, little or no difference in the kinetics of cytochrome reduction or in the duration of the reductive cycles was apparent. This is in conformity with previous observations (7) describing the reduction of the cytochromes of the DPNH oxidase preparation of Mackler and Green (8).

**Flavoprotein**—A similar series of studies were performed in which the change in absorbance at 465 \( \mu \)m relative to 500 \( \mu \)m was measured. Although the wave length pair measures the contributions of both the nonheme iron and flavin components of DPNH dehydrogenase, it can serve as a qualitative indicator for determining the relative degree of flavoprotein participation in the reaction. In addition, interference from changes in the extent of oxidation and reduction of the cytochromes further complicates (9) the interpretation of the spectral changes measured at 465 \( \mu \)m. In spite of these difficulties, this wave length pair remains as the only optical means of assessing flavoprotein reduction.

As shown in Fig. 4, the addition of DPNH causes a rapid decrease in absorbance at 465 \( \mu \)m, indicative of the reduction of flavoprotein. There is only a small contribution of a slower kinetic phase to the initial absorbance changes recorded at 465 \( \mu \)m. Second and third additions of DPNH, after the DPNH previously added was exhausted, showed comparable degrees of absorbance change. Thus, the changes generally attributed to flavoprotein (see above) appear to differ from those observed with cytochrome c + c\textsubscript{1} in the less pronounced slower kinetic phase of pigment reduction as well as any alteration in the magnitude of absorbance change during the repetitive cyclic reduction of the pigments.

**Coenzyme Q**—During studies designed to determine the kinetics of reduction of endogenous coenzyme Q\textsubscript{10}, it was observed that the rate of coenzyme Q\textsubscript{10} reduction was often slower with DPNH than with succinate. Fig. 5 shows the extent of coenzyme Q\textsubscript{10} reduction determined at short time intervals after addition of substrate. These experiments illustrate how pretreatment of the heart muscle preparation with DPNH markedly alters the kinetics of coenzyme Q\textsubscript{10} reduction. This is apparent in de-
Fig. 5. Kinetics of coenzyme Q₁₀ (ubiquinone) reduction following addition of DPNH to a heart muscle preparation. Samples of horse heart muscle preparation (6 mg of protein, 0.034 μmole of endogenous quinone) were diluted to 1.0 ml with 0.1 M phosphate buffer, pH 7.4. At zero time, 1 ml of a 1.2 mM solution of DPNH was added to the diluted heart muscle preparation. The reaction was terminated at the times indicated by the simultaneous addition of 5 ml of petroleum ether and 4 ml of methanol cooled to the temperature of Dry-Ice. The coenzyme Q₁₀ was extracted and assayed as indicated under "Materials and Methods." •, results of studies of the untreated preparation during the aerobic steady state of DPNH oxidation; □, results with the untreated preparation in which potassium cyanide was added to give a final concentration of 1 mM; ○, comparable experiments in the absence and presence of cyanide, respectively, but with a heart muscle preparation which was pretreated by addition of 2.3 μmoles of DPNH followed by shaking in air until examination with the hand spectroscope showed the cytochromes fully oxidized. In all those experiments in which cyanide was present, it was added 30 seconds before the final addition of DPNH. Parallel experiments of oxygen uptake indicated a V₉₀₀₀ for DPNH oxidation equivalent to 35 μg O₂ sec⁻¹. The temperature of the reaction medium was 21°C.

determining the kinetics of coenzyme Q₁₀ reduction, either during the attainment of the aerobic steady state or in a cyanide-inhibited system. The measurements of the rate of coenzyme Q₁₀ reduction, therefore, reflect the spectrophotometric measurements of cytochrome c₁ + c₁ reduction and the associated time lag of oxygen utilization determined polarographically. This assigns the locus of the slowly reacting component before the reduction of coenzyme Q.

Cytochrome b—Measurements of the cyclic reduction of cytochrome b showed a kinetic pattern comparable to that described above for cytochromes c₁ + c₁ (cf. Fig. 3). One difference was apparent, however, as illustrated in Fig. 6A. Upon utilization of the first aliquot of DPNH added to the reaction mixture, only a small absorbance decrease associated with cytochrome b oxidation was observed. This indicated an irreversible reduction of a pigment by DPNH. A second addition of DPNH caused a cyclic reduction of cytochrome b with the absorbance change returning to the level observed before the second addition of DPNH. Studies with the wavelength-scanning recording spectrophotometer showed that the atypical
response (i.e. the first cycle of reduction and oxidation) observed with cytochrome b reduction was associated with the reduction of a pigment with absorption band maxima at 563 and 431 nm. Such a spectrum is illustrated in Fig. 7. Although these spectra indicate the relatively small contribution of this type of pigment when compared to the concentration of cytochrome b reducible on anaerobiosis, the concentration of this b type of pigment is relatively large when compared to the amount of cytochrome b reduced during the aerobic steady state. The presence of this cytochrome b-type pigment was not exclusively associated with preparations which demonstrated the time lag in the rate of DPNH oxidation, for a similar pigment has been observed with ETP. In addition, experiments carried out in triethanolamine buffer (Fig. 6B) did not show the presence of this additional cytochrome b-type pigment even though the time lag in DPNH oxidation remained apparent (cf. the duration of the steady state cycles of reduction). The nature of this pigment and its failure to be oxidized upon depletion of DPNH remain obscure and require further study.

**DISCUSSION**

The recognition and characterization of components participating in the transfer of reducing equivalents between DPNH dehydrogenase and the cytochrome chain or coenzyme Q are unknown. Electron spin resonance studies by Beinert et al. (10, 11) have demonstrated the rapid reduction of non-heme iron in DPNH dehydrogenase preparations. The change in oxidation and reduction state of coenzyme Q has also been determined during DPNH oxidation, although definitive kinetic evidence for its direct participation in transfer reactions is still a matter of dispute. More recently Ziegler (12) has suggested that an inorganic iron sulfhydryl complex may function in the parallel portion of the respiratory chain, mediating the reaction between succinic dehydrogenase and coenzyme Q10.

The participation of sulfhydryl groups in the reaction between primary DPNH dehydrogenase and coenzyme Q was implicated from studies presented in the preceding paper (1). This component either could be an integral part of the DPNH dehydrogenase flavoprotein without functioning in the DPNH ferricyanide reaction or could be associated with an independent molecule between DPNH dehydrogenase flavoprotein and coenzyme Q10.

The results presented in this paper indicate a modified time course in the rate of DPNH oxidation, presumably associated with the alteration of a component functional between DPNH dehydrogenase and coenzyme Q. The locus of the component associated with the time lag in DPNH oxidation is supported by three types of evidence. The first is the time course of the reaction when various electron acceptors are employed with heart muscle preparations. The time lag observed when oxygen is the terminal electron acceptor is also apparent when cytochrome c or coenzyme Q10 is utilized but not when potassium ferricyanide reduction is measured. The second concerns observations on the steady state reduction of the cytochromes; the measurement of reduction of cytochromes c and c1 as well as cytochrome b shows an initial slow phase in the kinetics of reduction as well as an increased extent of reduction of these pigments in the aerobic steady state. This is in contrast to the changes observed at 465 nm, where the slower kinetic phase is less pronounced and the extent of pigment reduction remains relatively constant. Third is the measurement of the rate of coenzyme Q10 reduction upon addition of DPNH or succinate. The much slower kinetics of coenzyme Q10 reduction observed with the initial addition of DPNH supports the assignment of the component responsible for the initial time lag between DPNH dehydrogenase and coenzyme Q10.

Attempts to identify the component further by the addition of a variety of reagents and assessing their influence on the time lag observed during DPNH oxidation have been unsuccessful. Compounds such as Versene, cysteine, oxidized and reduced glutathione, dithionite, mercaptoethanol, ferric or ferrous ions, succinate, and DPN have no influence on the time course of the reaction. Of interest, and perhaps a clue to the nature of the compound, is the observation that the addition of a low concentration of DPNH and the associated transfer of reducing equivalents does modify the rate-limiting component. This, together with the finding that the Keilin-Hartree preparation (which is exhaustively washed with water) has a tendency to show the initial time lag, whereas ETP failed to show a similar lag, suggests the possibility that the component related to this time lag could be associated with the sulfhydryl inhibitor-sensitive site discussed in the previous paper. If it was a sulfhydryl component which was modified during the preparation of heart muscle particles to form an inactive disulfide complex, one can envision the concomitant reduction of this disulfide during the early phase of DPNH oxidation. This presumes that the initial concentration of active sulfhydryl is rate-limiting for DPNH oxidation and that during DPNH oxidation the concentration of the active sulfhydryl form would increase with an associated increase in the rate of oxygen utilization.

When spectrophotometric studies were carried out to determine whether any spectral change might have occurred during the oxidation of limited quantities of DPNH, it was observed that a cytochrome b-type pigment remained reduced even after DPNH depletion. The failure to show a consistent correlation between the appearance of the time lag in DPNH oxidation and the presence of this additional cytochrome b-type pigment precludes the assumption that these two are related. Further studies may reveal that this cytochrome b pigment is merely a reflection of the reorganization of the respiratory chain associated with disruption of (the structural integrity of the system (13).

**SUMMARY**

1. The present study has demonstrated the presence of a pronounced time lag in the initiation of oxidation of reduced diphosphopyridine nucleotide by some heart muscle preparations.

2. Studies with alternative electron acceptors such as cytochrome c, coenzyme Q10, and potassium ferricyanide indicate that the time lag observed in DPNH oxidation is related to a component functional between the sites of ferricyanide and coenzyme Q10 interaction with the respiratory chain.

3. Spectrophotometric measurements of the steady state reduction of cytochrome c + c1 as well as flavoprotein by DPNH support the hypothesis that the reaction between DPNH dehydrogenase and the cytochrome system is responsible for the observed time lag.

4. Studies of the kinetics of reduction of endogenous coenzyme Q10 by DPNH indicate that the observed time lag is related to a component operative between DPNH dehydrogenase flavoprotein and coenzyme Q10.

5. The possible role of sulfhydryl groups is discussed in terms
of the time course for the attainment of the maximal rate of DPNH oxidation as well as the influence of preliminary incubation of the preparation with DPNH.

Acknowledgments—The authors are indebted to Dr. B. Chance for his interest and advice during the course of experimentation and preparation of the manuscript. The technical assistance of Mr. S. Wolfgang and Mr. S. Cherim is gratefully acknowledged.

REFERENCES

Hydrogen Transfer between Reduced Diphosphopyridine Nucleotide Dehydrogenase and the Respiratory Chain: II. AN INITIAL LAG IN THE OXIDATION OF REDUCED DIPHOSPHOPYRIDINE NUCLEOTIDE
Shigeki Minakami, F. J. Schindler and Ronald W. Estabrook


Access the most updated version of this article at http://www.jbc.org/content/239/6/2049.citation

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/239/6/2049.citation.full.html#ref-list-1