ELECTRON PARAMAGNETIC RESONANCE STUDIES ON THE REDUCTION OF THE COMPONENTS OF COMPLEX I AND TRANSHYDROGENASE-INHIBITED COMPLEX I BY NADH AND NADPH*

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Summary: NADH treatment of complex I at pH 7-8 results in the appearance of electron paramagnetic resonance (epr) signals at x band due to reduced iron-sulfur centers 1, 2, 3 and 4, while NADPH treatment gives rise to the appearance of signals due to centers 2 and 3. Similar results are obtained with complex I preparations in which transhydrogenase activity from NADPH to NAD has been >95% inhibited by treatment of the complex with trypsin. At pH 6.5 and in the presence of rotenone, addition of NADPH to complex I or transhydrogenase-inhibited complex I results in partial reduction of iron-sulfur center 1 as well. These and other experiments with reduced 3-acetylpyridine adenine dinucleotide and NADPH + NAD as substrates have suggested that the differences in the reduction of complex I iron-sulfur centers by the above nucleotides are essentially quantitative and related to (a) the dehydrogenation rate of the nucleotides, and (b) autoxidation of complex I components under the epr experimental conditions.

Previous studies have shown that the respiratory chain of beef heart mitochondria can oxidize NADPH without the involvement of NAD and the NADPH → NAD transhydrogenase reaction (1-3). Treatment of submitochondrial particles with trypsin or butanedione in the presence of pH 9 borate buffer resulted in complete loss of NADPH → NAD transhydrogenase activity with little (∿10%) or no loss of NADH oxidase, NADPH oxidase, and NADH → AcPyAD transhydrogenase activities (3). NADPH oxidation by the respiratory chain is linked to ATP synthesis (P/O close to 3.0), and is inhibited by Amytal, rotenone, piericidin A, antimycin A, and cyanide (1,2). Both NADPH dehydrogenase and NADPH → NAD transhydrogenase activities fractionate mainly into complex I (NADH-ubiquinone reductase) (2).

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Earlier electron paramagnetic resonance (epr) studies had shown that complex I contains at least 4 major types of iron-sulfur centers which, together with flavin, are reduced by NADH (2,4). Under the same conditions, however, NADPH treatment of complex I resulted in the reduction of iron-sulfur center 2 and partially of centers 3 + 4, which exhibit overlapping epr signals (2). Iron-sulfur center 1 and flavin were not detectably reduced by NADPH, and subsequent examination of the partially reduced centers 3 + 4 indicated that the signal was due mainly or entirely to reduced center 3 (see below). Thus, it appeared that at pH 7-8 NADPH was capable of reducing those components of complex I (i.e., iron-sulfur centers 2 and 3) which exhibit reduction potentials close to zero, but not those (i.e., iron-sulfur centers 1 and 4) with reduction potentials 250-300 mV more negative (4-6).

Other results indicated, however, that the interaction of NADH and NADPH with complex I might be more complicated. (a) Rapid kinetic experiments of Orme-Johnson et al. (4) on NADH or AcPyADH reduced complex I showed that the sequence of appearance of the iron-sulfur center signals was not in accordance with their increasing reduction potentials. (b) Spectrophotometric studies on complex I and rotenone-treated ETP indicated that at 475 minus 510 nm NADPH results in greater reduction of flavin + iron-sulfur chromophores at pH values below neutrality (3). Thus further epr studies of NADPH-treated complex I were warranted, especially under conditions that possible complications due to transhydrogenation from NADPH to NAD (by inadvertant contamination) is completely excluded.

The availability of transhydrogenase-inhibited complex I provided the preparation needed for these studies. The present communication describes the results of epr studies with complex I and transhydrogenase-inhibited complex I (by trypsin treatment), using NADH and NADPH as substrates at appropriate pH values and in the presence and absence of rotenone.

METHODS AND MATERIALS

Complex I and ETP were prepared as described (7,8). Epr studies were

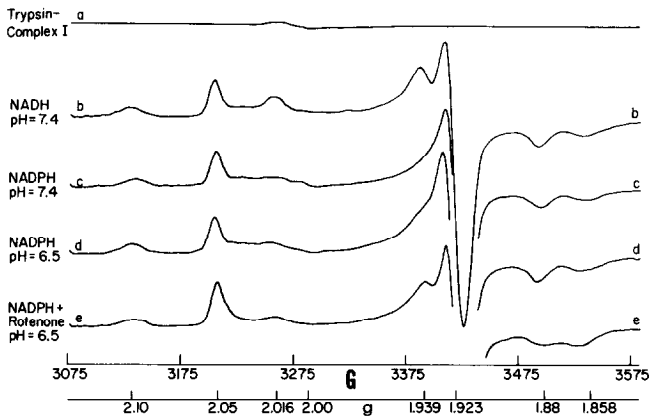

Fig. 1. First derivative epr spectra of trypsin-treated complex I reduced with NADH and NADPH at pH 7.4 and 6.5. Conditions: 0.35 ml of trypsin-treated complex I at 35 mg protein/ml of 0.25 M sucrose containing 100 mM potassium phosphate at the pH values shown. Where indicated 20 µl of 30 mM NADH or NADPH and 10 µl of 5 mM rotenone in ethanol were added.

conducted as before (2). Trypsin treatment of complex I (3) was carried out by adding trypsin in solution to complex I suspended in 0.25 M sucrose at a concentration of 0.1 mg trypsin per mg of complex I protein, followed by 75-90 min incubation at 0°. Assay of NADPH → NAD transhydrogenase activity (2,3) showed that after 60 min incubation more than 95% of transhydrogenase activity had been destroyed. Acrylamide gel electrophoresis in the presence of sodium dodecylsulfate was carried out essentially according to Weber and Osborn (9) as detailed elsewhere (10). In all the epr experiments, complex I samples ± rotenone (in 2.5% final volume of ethanol) and other additives were treated with substrate 75-90 seconds at room temperature before freezing in liquid nitrogen. Unless otherwise indicated, the epr experimental conditions were: microwave frequency, 9.206 GHz; 100 KHz modulation, 5G; power, 2 mW; gain, 10; and temperature, 13°K. Magnetic field was determined by proton magnetic resonance probe. The sources of the chemicals used were the same as before (2,3).

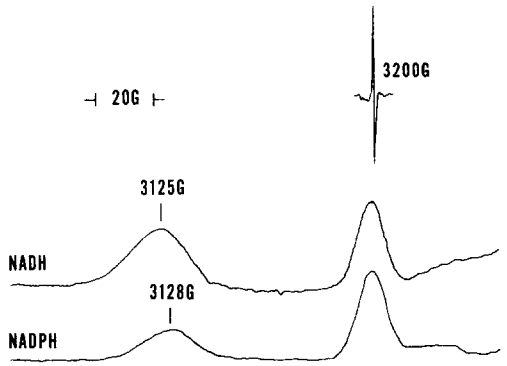

Fig. 2. First derivative epr spectra of NADH- and NADPH-treated complex I aligned against a marker at 3200 G to show the field positions of the g ≃ 2.1 signals of iron-sulfur centers 3 + 4. Conditions: 0.4 ml of complex I at 45 mg protein/ml of 0.25 M sucrose containing 50 mM Tris-HCl, pH 7.5. Where indicated 10 µl of 50 mM NADH or NADPH were added. Microwave frequency, 9.198 GHz; power, 2 mW; 100 KHz modulation, 4 G; temperature, 13°K.

RESULTS AND DISCUSSION

The four iron-sulfur centers of NADH-treated complex I show epr signals with the following approximate g values: center 1 at 2.02, 1.939, 1.923; center 2 at 2.05, 1.926, 1.922; and centers 3 + 4 at 2.1, 1.88, 1.86 (see also ref. 5). Under the same experimental conditions, NADPH-treated complex I exhibited epr signals in positions corresponding to center 2 and 3 + 4 only. Similar results were obtained (Fig. 1, traces b and c) when trypsin-treated complex I, in which NADPH → NAD transhydrogenase activity was >95% inhibited, was treated at pH 7.4 with NADH and NADPH, respectively. Alignment of the low-field signals (g ≃ 2.1) of centers 3 + 4 produced by NADH- and NADPH-treated complex I showed that the NADPH-produced signal was smaller and approximately 3 G upfield as compared to the NADH-produced signal (Fig. 2). These results indicated that, on the basis of the definition of Orme-Johnson

et al. (4,5), the NADPH-produced signal at g ≃ 2.1 was due mainly or entirely to center 3. Since NADPH oxidation is extremely slow at pH 7-8, but considerably faster at lower pH values (2), the effect of NADPH on the reduction of complex I iron-sulfur centers was examined at pH 6.5*. As seen in trace d of Fig. 1, addition of NADPH to trypsin-treated complex I at pH 6.5 resulted in a small degree of center 1 reduction, which was discernible in the region of g = 1.939. When the preparation was treated with rotenone before the addition of NADPH (trace e, Fig. 1), the reduction of center 1 was appreciably enhanced (note the signals at g ≃ 2.016 and 1.939).

Prior to the availability of transhydrogenase-inhibited complex I, partial reduction of center 1 with NADPH at pH 6.5 could not have been confidently ascribed to the direct oxidation of NADPH. This is because the NADPH → NAD transhydrogenase reaction is severalfold faster than direct NADPH oxidation, and both reaction rates are similarly augmented by lowering of pH (2). Therefore, the presence of contaminating traces of NAD could have resulted in sufficient NADH production by the facilitated transhydrogenase reaction to cause the partial reduction of center 1 observed in traces d and e of Fig. 1. This possible complication is excluded, however, from the results obtained with trypsin-treated complex I. Hence, it can be concluded that NADPH is capable of reducing center 1 when its oxidation rate is made more favorable at a lower pH**. The increase in the extent of center 1 reduction in the presence of rotenone (trace e, Fig. 1) suggested that this compound prevents electron leak from complex I. The rates of NADH and NADPH oxidation by aerobic solutions of complex I at pH 7.4 and 22° were estimated to be roughly about 20 and 5 nmoles·min^{-1}·mg^{-1} protein in the absence, and 7 and 1 nmoles·min^{-1}·mg^{-1} protein in the presence of 5 μM rotenone, respectively.

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* Lower pH values could not be used, because the bile salt-solubilized complex I became too viscous at or below pH 6.0.

** In submitochondrial particles NADPH oxidation at pH 6.5 is about 4 times faster than at pH 7.5 (2).

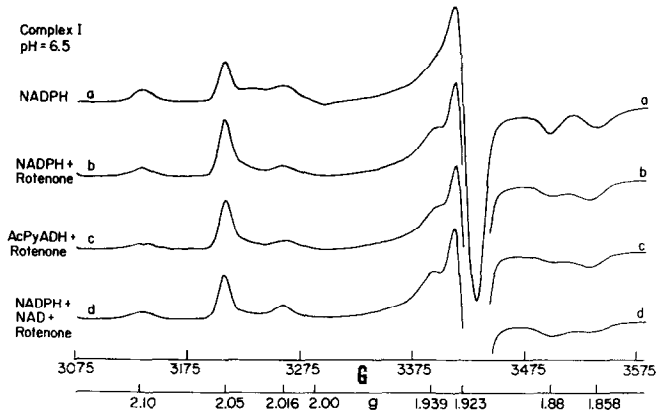

Fig. 3. First derivative epr spectra of complex I at pH 6.5 reduced with NADPH, AcPyADH, and NADPH + NAD. Conditions were the same as in Fig. 1. Where indicated 5 µl of 100 mM NAD and 20 µl of 30 mM AcPyADH were added.

Albracht and Dooijewaard (11) feel that the signals in the regions of g = 2.1 and 1.88 are due to center 3, while the corresponding center 4 signals (g_z and g_x) are at g = 2.037 and 1.863. That the g_z signal at g ≃ 2.05 ascribed to center 2 may not be due to a single entity is in agreement with our earlier studies where subtraction of the epr spectrum of NADPH-treated complex I from that of NADH-treated complex I did not just reduce or eradicate the g ≃ 2.05 signal, but left behind in the difference spectrum a derivative-type signal in this region (cf. signal 0 of Fig. 5, ref. 2). However, if according to Albracht and Dooijewaard the g ≃ 1.86 signal is due to center 4, then we would have to conclude (a) that under conditions that center 1 is not detectably reduced (i.e., trace c of Fig. 1), center 4 is still reduced by substrate, and (b) that under these conditions the 3 G upfield change of the g ≃ 2.1 signal is not associated with the subtraction of center 4 contribution to this signal.

Figure 3 shows data at pH 6.5 for complex I not treated with trypsin. It is seen that traces a and b of Fig. 3 are very similar to traces d and e of Fig. 1 insofar as the degree of center 1 reduction is concerned. Thus the

partial reduction of center 1 by NADPH appears to be related to the greater activity of NADPH dehydrogenase at the lower pH values plus the inhibition by rotenone of electron leak from complex I rather than to modification of complex I by trypsin***. In Fig. 3, trace c shows that, at pH 6.5 and in the presence of rotenone, the degree of center 1 reduction by AcPyADH is comparable to that by NADPH (trace b), and trace d shows that the presence of NAD together with NADPH increases the extent of center 1 reduction. At pH 6.5, the oxidation rates by ETP of NADPH and AcPyADH are comparable, and addition of NAD together with NADPH increases the oxidation rate of NADPH by 3-4 fold. Therefore, it seems that the differences in the reduction of complex I components by NADH, AcPyADH, NADPH, and NADPH + NAD are essentially quantitative and related mainly to the balance between the dehydrogenation rates of these nucleotides by complex I and electron leak from complex I by autoxidation. It is seen in Fig. 1 and 3 that wherever rotenone was added the signal at g ≃ 1.88 was considerably diminished. This appears to be due, at least in part, to the added ethanol (2.5% v/v) in which rotenone was dissolved.

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*** Polyacrylamide gel electrophoresis of trypsin-treated complex I in the presence of sodium dodecyl sulfate and mercaptoethanol has shown that at least four polypeptides of complex I have been modified by trypsin. However, as shown elsewhere (3) the rates of NADH and NADPH oxidation by submitochondrial particles are scarcely altered even after prolonged treatment of the particles with trypsin at 0°.