CORRELATION OF ENZYMATIC ACTIVITY AND THE APPEARANCE OF THE EPR SIGNAL AT g = 1.94 IN NADH DEHYDROGENASE AND ITS THERMAL BREAKDOWN PRODUCTS[1]

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It has been shown by Sands and Beinert (1960) that the addition of NADH to mitochondria and submitochondrial particles elicits an asymmetric EPR signal at g = 1.94 (minor component at g = 2.00). This signal is distinguishable from a similar one produced by succinate. Since then it has become obvious that the type of material represented by asymmetric, strongly temperature-dependent signals at these g values occurs rather widely in organisms and in isolated enzyme systems which catalyze aerobic oxidations (Beinert et al., 1962a). Recent studies[3] on such enzyme systems have shown that the kinetic behavior of the component represented by this signal is compatible with its postulated function as a redox catalyst in electron transport. Although definitive proof is still lacking, the most plausible assumption at this time is that the signals at g = 1.94 and 2.00 indicate a species of reduced non-heme iron (Sands and Beinert, 1960; Beinert and Sands, 1960; Beinert and Lee,

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1961; Beinert et al., 1962a) and, for the sake of brevity, in this paper they will be referred to as "iron signal".

While the NADH-induced signal at g = 1.94 was seen in all preparations in which NADH dehydrogenase was still functionally and structurally linked to respiratory chain components, it was absent (Beinert and Sands, 1959) in acid-ethanol extracted preparations, such as the NADH-cytochrome reductases of Mahler et al. (1952) and of De Bernard (1957). In a collaborative study between these two laboratories, initiated in 1961, it was found that highly purified NADH dehydrogenase extracted by the phospholipase method (Ringler et al., 1963) on the other hand, gives qualitatively the same iron signal[4] on addition of NADH as do more complex preparations (Beinert and Sands, 1960). It will be shown in this paper that the kinetic behavior of the iron signal of the purified dehydrogenase is compatible with a catalytic role of this component[5] and further demonstrated that during the molecular transformation of NADH dehydrogenase to NADH-cytochrome reductase (Watari et al., 1962; Cremona et al., 1963) at 35° to 37° catalytic activity and iron signal are lost in a parallel manner.[6]

In order to ascertain whether the rates of appearance and disappearance of the iron signal in NADH dehydrogenase are compatible with the participation of the corresponding component in the catalytic cycle of this enzyme, the rapid freezing technique[3] (Bray, 1961) was utilized. On addition of NADH to the enzyme the iron signal is maximal within 8 msec, as would be expected in view of its high efficiency as a substrate. The problems posed by the high

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4. A typical EPR spectrum of this enzyme is shown in Fig. 7 of Beinert et al. (1962a). The signals obtained in the present work with lower enzyme concentrations showed a signal to noise ratio 1/2 to 1/3 of that in the quoted figure.

5. Similar results were obtained by two of us (H. B. and G. P.) in a study of NADH-coenzyme Q reductase preparations in collaboration with Drs. A. J. Merola and R. Coleman, Madison.

6. Dr. C. Veeger has kindly informed us that EPR studies on the conversion of NADH dehydrogenase into other forms have also been undertaken by Dr. Z. Kaniuga of the Laboratory for Physiological Chemistry, Amsterdam.

turnover number of the dehydrogenase (4.1×10^5 moles NADH/min./mole flavin at 30°, pH optimum (Ringler et al., 1963)) and the fact that the time resolution of the Bray apparatus[3] is 8 msec. were circumvented by the use of a slowly oxidized substrate, reduced acetylpyridine adenine dinucleotide (AcPyADH), low temperature (1°), and high pH (pH 9.0). We realize that a discrepancy of the turnover rate of the iron and that of the enzyme might not become apparent if we artificially limit reaction steps preceding iron reduction. Nevertheless, the present approach appeared to be the best choice in view of the limitations imposed by the technique. Under these conditions, with 1 mM ferricyanide as the oxidant, the turnover number is 3,240/min. at infinite AcPyADH concentration and 1,510/min. (42 msec./turnover) at 2.1 mM AcPyADH. (The measured velocity of the AcPyADH-ferricyanide reaction varies only slightly with ferricyanide concentration according to Minakami et al., (1963)). By means of a rapid-recording spectrophotometer the same turnover number was found in the range of 10 μg. to 11.5 mg. dehydrogenase per ml. Since the latter concentration sufficed for EPR studies, catalytic activity and iron signals were followed under identical conditions. Maximum development of the iron signal occurred in about 80 msec. and the half-time was 40 msec. at 2.1 mM AcPyADH under anaerobic conditions at 1°, pH 9.0. Hence, maximal development of the iron signal corresponds to two turnovers. This agreement between EPR data and catalytic assays is gratifying, particularly since two catalytic cycles would be required if both flavin and one or a pair of iron atoms were reduced under these conditions.

In further agreement with kinetic data (Minakami et al., 1963; Singer, 1963) the rate-limiting step in the NADH (or AcPyADH)-ferricyanide reaction was found to be the reduction of the enzyme by the substrate. Thus in enzyme samples previously reduced with excess NADH or AcPyADH (maximal iron signal), on rapid anaerobic mixing with low concentrations of ferricyanide at 1° no iron signal was detected at 8 msec. An oxidized substrate of suitable potential, such as AcPyAD, could replace ferricyanide as the oxidant.

The NADH-induced signal was diminished by about 70% upon the addition of a suitable excess of AcPyAD, but the kinetics of this reoxidation have not yet been measured.

Anaerobic titration of the enzyme with NADH suggested that about 5 moles of substrate/mole of dehydrogenase (based on total flavin) are required for full development of the signal. This is a maximum value since, for instance, no provisions were made to remove the NAD produced in the reaction, which is a competitive inhibitor (Minakami et al., 1963).

Since these studies have suggested that the kinetics of the appearance and decay of the iron signal given by highly purified NADH dehydrogenase are compatible with a possible role in the catalytic cycle, while acid-ethanol derivatives of the enzyme (Watari et al., 1962; Cremona et al., 1963) do not give this signal, it was of interest to follow the fate of the iron signal during the transformation of NADH dehydrogenase to NADH-cytochrome reductase. Studies on the kinetics and mechanism of this transformation under the influence of heat, organic solvents, acid pH, proteolytic enzymes, urea, and thiourea and the properties of the isolated products have been described[7] (Watari et al., 1962; Cremona et al. 1963; Singer and Kearney, 1962). In the present experiments thermal degradation of the dehydrogenase was followed, since the rate of this transformation is readily controlled by the selection of the temperature. It has been shown (Watari et al., 1962; Cremona et al., 1963; Minakami et al., 1963) that the incubation of NADH dehydrogenase at 37° and pH 7.4 results in gradual loss of its characteristic ferricyanide activity, accompanied by the emergence of cytochrome-and dichlorophenol-indophenol reductase activities. The product has been isolated (Cremona et al., 1963; Singer, 1963) and shown to be essentially indistinguishable from the Mahler enzyme (Mahler et al., 1952) and the preparation of King and Howard (King and Howard, 1962a). This thermal transformation has also been noted by others (Kaniuga and Veeger, 1962; King and Howard, 1962b).

7. H. Watari, E. B. Kearney and T. P. Singer, submitted to J. Biol. Chem.

Although ferricyanide activity and EPR signals were lost at the same rate at 35° as well as 37°, the process was too rapid at the higher temperature for convenient measurements. In view of the large energy of activation of the process (Cremona et al., 1963), lowering the temperature to 35° was sufficient to permit accurate sampling even during the initial, rapid phase of the transformation. As illustrated in Fig. 1, the disappearance of the NADH-ferricyanide activity on incubation of the enzyme at 35° is closely paralleled by the loss of substrate-induced iron signal. As previously re-

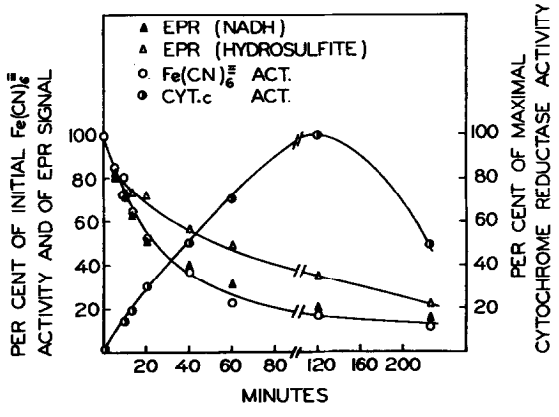

Figure 1. Time course of inactivation of NADH dehydrogenase, emergence of cytochrome reductase, and decay of substrate-induced EPR signal at g = 1.94. The enzyme (27.3 mg./ml., specific activity = 324) in 0.03 M phosphate, pH 7.8, was incubated aerobically at 34.9° in the dark. Samples removed at the times indicated were rapidly cooled in ice. Aliquots were assayed after appropriate dilution with 0.06 M triethanolamine-1% serum albumin (w/v), pH 7.8, for NADH-ferricyanide activity by the modified method (triethanolamine, pH 7.8, Ringler et al., 1963) and for cytochrome reductase activity (Minakami et al., 1963). Results were calculated for infinite concentration of electron acceptor. Ferricyanide activity is expressed as % of initial activity and cytochrome reductase as % of maximal activity emerging. Aliquots of 0.20 ml. were placed in anaerobic quartz tubes (Beinert and Sands, 1961), 0.02 ml. 1 M glycine, pH 9, was added and the tubes were repeatedly evacuated and filled with purified N_2. 0.01 ml. of 0.1 M NADH was added under a flow of N_2; the samples were frozen 1 minute after mixing and the EPR was measured at -176° (Beinert et al., 1962b) at 25 mwatt microwave power and 19 gauss modulation amplitude. The samples were then thawed, a trace of solid hydrosulfite was added under N_2, the samples were refrozen again after 15 minutes at 22° and tested for EPR as above. The % of the initial peak-to-peak amplitude of the g = 1.94 signal (first derivative) is plotted.

ported (Cremona et al., 1963; Singer and Kearney, 1962) the appearance of cytochrome reductase activity, being a secondary manifestation of structural breakdown, lags behind the loss of ferricyanide activity. Examination of the kinetics

of the appearance of the iron signal in a sample removed at the half-way point during the transformation indicated no material difference from the kinetic properties of the untreated enzyme. Fig. 1 further shows that the decay of the hydrosulfite-reducible iron signal initially also follows the loss of catalytic activity but that a deviation between the two processes occurs in the later phase.

In connection with the close agreement shown between the loss of NADH-ferricyanide activity and of substrate-induced iron signal during thermal breakdown of the dehydrogenase, it may be noted that under these same conditions considerable labilization of non-heme iron and loss of spectral characteristics attributed to iron are known to occur (Cremona et al., 1963; Singer and Kearney, 1962), although the iron atoms involved in these phenomena are not necessarily the same.

It was further noted that the presence of NADH in anaerobiosis protected the dehydrogenase from thermal breakdown. Thus while in one hour at 37°, pH 7.8, 85% of the substrate-reduced iron signal and 90% of the ferricyanide activity were lost, with 5 mM NADH under N_2 only 16% of the iron signal and some 30% of the ferricyanide activity disappeared under these conditions and the formation of cytochrome reductase activity was correspondingly prevented. Aerobic incubation with NADH even at room temperature inactivates, rather than protects, the enzyme.

In contrast to the clean-cut $g = 1.94$ signals observed with untreated NADH dehydrogenase on reduction with substrate, NADH elicits no marked changes in free radical signal intensity in the enzyme. Considerable changes, however, were induced by addition of substrate at the end of the thermal degradation and also in the acid-ethanol product (Beinert and Sands, 1959).

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