Stimulation of NADH Oxidation During NADPH Dependent Microsomal Electron Transport Reactions

Jurgen Werringloer and Ronald W. Estabrook
Department of Biochemistry
The University of Texas Health Science Center
Southwestern Medical School
Dallas, Texas 75235

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Initiation of NADPH oxidation by rat liver microsomes results in a marked stimulation of the rate of NADH oxidation. The amount of NADH rapidly oxidized depends on the ratio of NADPH/NADH and the presence of various substrates for the function of cytochrome P-450. The present paper illustrates a simple direct method of quantitatively assessing these changes in NADH oxidation and the influence of the anaesthetic, halothane, or the drug, ethylmorphine, on the pattern of changes in NADH oxidation during microsomal electron transport reactions.

Liver microsomes are recognized as containing a very active NADH - cytochrome *c* reductase - a reaction in which cytochrome b_5 and the flavoprotein, NADH - cytochrome b_5 reductase, are known to function (1). In the absence of substrates for the fatty acid desaturase of microsomes, NADH oxidase activity is extremely low as determined from the rate of oxygen utilization or the spectrophotometric measurement of reduced pyridine nucleotide oxidation. Likewise, it has been known for many years (2-4) that NADH is not a very effective donor of reducing equivalents for the function of cytochrome P-450 dependent mixed function oxidation reactions. However, NADH oxidation is markedly stimulated when measured in the presence of NADPH (5). When a substrate capable of undergoing hydroxylation via cytochrome P-450 is present, this increase in the rate of NADH oxidation is associated with a "synergistic effect" observed as a marked increase in the rate as well as the extent of product formation (6-9). The purpose of the present communication is to demonstrate a direct spectrophotometric measurement to quantitatively evaluate the stimulation of NADH oxidation during NADPH oxidation by liver microsomes and to illustrate differences observed during cytochrome P-450 function, such as the formation of hydrogen peroxide, the operation of a mixed function oxidation reaction, *viz*, the

N-demethylation of ethylmorphine, or in the presence of a polyhalogenated compound, like halothane, which does not undergo a classic hydroxylation reaction (10).

Materials and Methods

Microsomes were prepared in 0.25 M sucrose as previously described (11) from the livers of 150-200 gm male Charles River CD (outbred albino) rats that had been pretreated by intraperitoneal injection of sodium phenobarbital (80 mg per kg.) for five days. After differential centrifugation the microsomal fraction was suspended to approximately 30 mg protein per ml in 0.25 M sucrose containing 50 mM tris-chloride buffer (pH 7.5) and used within 24 hours of preparation. For the spectrophotometric experiments described, microsomes were diluted in a buffer mixture consisting of 50 mM tris-chloride buffer, pH 7.5, 10 mM $MgCl_2$, 150 mM KCl, 0.5 μM rotenone and 2 mM 5'AMP to a protein concentration of 0.5 mg per ml. Halothane (Fluothane) was obtained from Ayerst Laboratories Inc., ethylmorphine (Dionin) from Merck Chemical Co., NADH (ChromatoPure) and NADPH from P-L Biochemicals Inc., and isocitrate dehydrogenase, type IV, (3.1 units per mg) and trisodium isocitrate from the Sigma Chemical Company.

Reduced pyridine nucleotide oxidation was measured at 340 nm using the split-beam mode of the Aminco DW 2 spectrophotometer with the cell compartment maintained at 25°. An extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ at 340 nm was used to determine the concentration of reduced pyridine nucleotides.

Results

The stimulation of NADH oxidation by rat liver microsomes, when measured spectrophotometrically in the presence of NADPH, is illustrated in Figure 1. As demonstrated by the experiment carried out in the presence of 2 mM halothane, the rate of NADH oxidation (Curve A) (in the absence of NADPH and in the presence of 0.5 μM rotenone to insure inhibition of NADH dehydrogenase associated with contaminating mitochondrial membrane fragments) is very slow, 1.2 nanomoles per minute per mg protein, compared to the initial rate of NADPH oxidation (Curve B), 27 nanomoles per minute per mg protein. However, when NADH is added (Curve C) to a system containing NADPH and an NADPH generating system (Curve D), the rate of NADH oxidation is stimulated nearly 20 fold to a rate of 23.5 nanomoles per minute per mg. A similar stimulation in the rate of NADH oxidation occurs if only NADPH is added in the absence of the NADPH generating system (Curve E). Of interest is the remarkably high affinity for NADH for this reaction, as indicated by the linearity of NADH oxidation (Figure 1, Curve C) until a concentration of less than 5 μM NADH is obtained. This high affinity presumably reflects the K_m for NADH for the flavo-

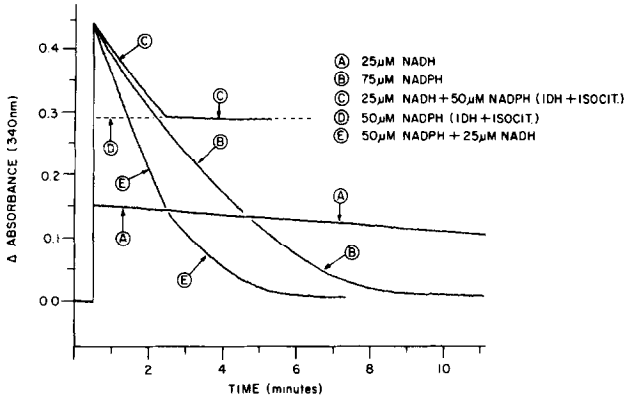

Figure 1. Stimulation of NADH oxidation by NADPH. Rat liver microsomes were diluted to 0.5 mg protein per ml in a reaction mixture containing 50 mM tris-chloride buffer (pH 7.5), 150 mM KCl, 10 mM $MgCl_2$, 0.5 μM rotenone, and 2 mM 5'AMP. Halothane was added to a final concentration of 2 mM and three ml aliquots placed in two cuvettes of 10 mm light path. The difference in absorbance at 340 nm was balanced and the reaction initiated by the addition of aliquots of reduced pyridine nucleotides as indicated on the figure. For experiments C and D the reaction cuvettes also contained 0.5 units per ml of isocitrate dehydrogenase and 5 mM sodium isocitrate. The results of each experiment are superimposed for clarity and comparison.

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protein, NADH-cytochrome b_5 reductase, since Strittmatter and Velick (1) have reported a value of 2.7 μM for the purified flavoprotein. Also the ability to observe a distinct break in the kinetic curve obtained when excess NADPH is present (Curve E) indicates the operation of the initial simultaneous oxidation of NADH and NADPH followed by the slower subsequent oxidation of only NADPH. When a similar series of experiments are carried out in the absence of halothane it can be shown than an inhibition of NADPH oxidation occurs in the presence of NADH; this can be directly demonstrated by carrying out the converse of the experiment shown in Figure 1, i.e. comparison of the rate of NADPH oxidation in the absence of NADH with that occurring in the presence of NADH plus an NADH generating system.

Spectrophotometric experiments, measuring reduced pyridine nucleotide oxidation in the absence of any generating system (for either NADPH or NADH), show that the amount of NADH rapidly oxidized is dependent on the concentration of NADPH added. As illustrated in Figure 2, addition of increasing concentrations of NADPH results in an increased extent of rapid oxidation of NADH. Failure to observe complete oxidation of NADH when using limiting concentrations of NADPH con-

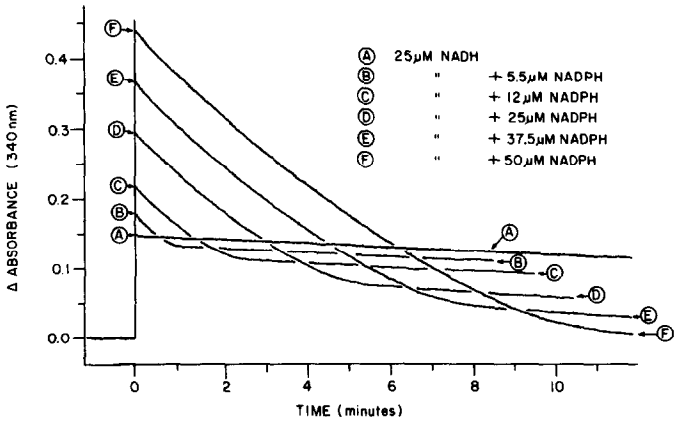

Figure 2. The effect of varying concentrations of NADPH on the extent of oxidation of NADH. A series of experiments similar to those described in Figure 1 (cf experiment E) were carried out in the absence of halothane where varying concentrations of NADPH were added to a reaction mixture containing 25 μM NADH. The concentration of NADPH added can be determined directly from the increase in absorbance above that contributed by curve A at zero time. The concentration of NADH oxidized can be calculated from the difference in the final absorbance attained and the time zero absorbance of Curve A.

firm the earlier conclusion (7) that and NADH-$NADP^+$ transhydrogenase is absent from liver microsomes. Likewise, evidence for the transfer of reducing equivalents from the flavoproteins, NADH-cytochrome b_5 reductase, to the flavoprotein, NADPH-cytochrome P-450 reductase or the direct interaction of NADH with the NADPH reactive flavoprotein can be excluded as possible means of NADH oxidation.

The ratio of NADPH to NADH required to oxidize NADH varies depending on the presence or absence of substrates for cytochrome P-450 catalyzed reactions. As shown in Figure 3, this ratio may vary from approximately 1 to 2, i.e. in the presence of the anaesthetic, halothane, one equivalent of NADPH results in the rapid oxidation of one equivalent of NADH while in the absence of any exogenous substrate (control conditions) approximately two equivalents of NADPH are required for the oxidation of one equivalent of NADH. With a substrate such as ethylmorphine, which undergoes N-demethylation to form formaldehyde in a cytochrome P-450 dependent reaction, intermediate results are obtained. Although the basis for these differences remains to be more fully defined, experiments currently in progress, relating the amount of reduced

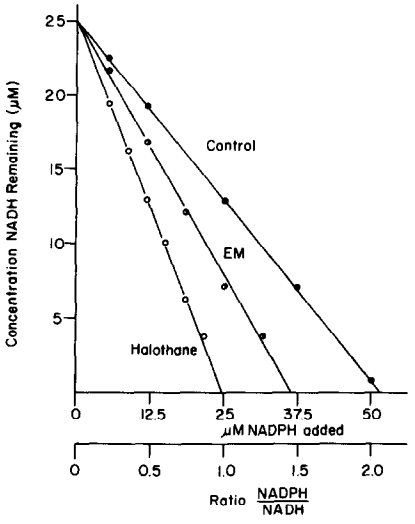

Figure 3. The influence of varying concentrations of NADPH on the extent of oxidation of NADH in the presence of halothane or ethylmorphine. A series of experiments similar to those illustrated in Figure 2 were carried out in the presence of 2 mM halothane, 5 mM ethylmorphine (EM) or no substrate addition (control). Rat liver microsomes were incubated with 25 μM NADH and varying concentrations of NADPH added. The amount of NADPH required to oxidize NADH is indicated by the ratio scale.

pyridine nucleotide oxidized to the amount of oxygen utilized and products formed, suggest the operation of at least two pathways of electron transport in liver microsomes involving cytochrome P-450, viz, a system leading to the formation of hydrogen peroxide which does not respond in a synergistic manner to NADH and a cytochrome P-450 dependent system normally functional in mixed function oxidation reactions where NADH can donate electrons (possibly via cytochrome b_5) as described previously (4). The reaction sequence responsible for the formation of hydrogen peroxide during NADPH oxidation by liver microsomes and a fuller evaluation of the contribution of "endogenous substrates" on the balance of utilization of reducing equivalents derived from NADPH and NADH, will be required prior to a more detailed determination of the mechanism of stimulation of NADH oxidation during NADPH oxidation by rat liver microsomes. The direct demonstration of an ordered reaction where one observes a marked stimulation of NADH oxidation associated with the NADPH dependent function of cytochrome P-450 during the mixed function oxidation of ethylmorphine or

the uncoupler like reaction observed in the presence of halothane lends further credence to the mechanism of distinct electron donating steps during the cyclic function of cytochrome P-450.

The described experimental method also may prove to be a valuable tool to quantitatively assess the contribution of the "synergistic" and "non-synergistic" pathways of reduced pyridine nucleotide oxidation and the analysis of the reaction sequences associated with microsomal electron transport.

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References

1. Strittmatter, P. and Velick, S.F. (1957) J. Biol. Chem., 227, 785-799.
2. Axelrod, J. (1955) J. Pharmacol. Exp. Ther. 115, 259-267.
3. Conney, A. H., Brown, R. R., Miller, J. A. and Miller E. C. (1957) Cancer Res. 17, 628-633.
4. Hildebrandt, A. G. and Estabrook, R. W. (1971) Arch. Biochem. Biophys. 143, 66-79.
5. Cohen, B. S. and Estabrook, R. W. (1971) Arch. Biochem. Biophys. 143, 54-65.
6. Nilsson, A. and Johnson, B. C. (1963) Arch. Biochem. Biophys. 101, 494-498.
7. Cohen, B. S. and Estabrook, R. W. (1971) Arch. Biochem. Biophys. 143, 46-53.
8. Correia, M. A. and Mannering, G. J. (1973) Mol. Pharmacol. 9, 455-469.
9. Correia, M. A. and Mannering, G. J. (1973) Mol. Pharmacol. 9, 470-485.
10. Staudt, H., Lichtenberger, F. and Ullrich, V. (1974) Eur. J. Biochem. 46, 99-106.
11. Franklin, M. R. and Estabrook, R. W. (1971) Arch. Biochem. Biophys. 143, 318-329.