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INHIBITION OF ERYTHROCYTE PLASMA MEMBRANE NADH DEHYDROGENASE BY NUCLEOTIDES AND UNCOUPLERS

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Erythrocyte ghost NADH dehydrogenase is inhibited in a competitive fashion by ATP and ADP whereas other nucleoside di- and triphosphates, cyclic nucleosides, as well as non-phosphorylating ATP analogs are relatively ineffective. In addition, this enzyme, measured with ferricyanide as electron acceptor, is inhibited by uncouplers of oxidative phosphorylation (proton-conducting reagents), the inhibition being competitive in character (i.e., the uncouplers were without influence upon maximum velocity). The effectiveness of the uncouplers was in the order of their hydrophobic character with the presence of the alkyl side chain rendering nonyl-dinitrophenol much more active than 2,6-dinitrophenol itself. Hydrophobic compounds that are not protonophores (e.g., eosin, proflavin or valinomycin) were not inhibitory. Whereas adenine nucleotides probably inhibit NADH oxidation competitively through structural similarity with the substrate, it appears unlikely that uncouplers compete at the NADH site directly. Rather, the apparently-competitive inhibition in the latter case may reflect competition for proton transfer to an acceptor residing in a hydrophobic region of the enzyme complex.

Introduction

There exists in plasma membranes from a number a cell types a collection of NADH dehydrogenases (NADH-acceptor reductases) [1] that differ from one another in their orientation within the membrane as well as their activity with various electron acceptors. These oxidoreductases are able, to varying extents, to donate electrons to ferricyanide, dichlorophenol indophenol, vanadate, cytochrome *c*, monodehydroascorbate, and methemoglobin [1–3]. However, the natural electron acceptor for these enzymes is not clear and the enzymes have been variously said to function as antioxidants, as regulators of the redox state of membrane-bound enzymes and carrier proteins, and as reducers of such plasma constituents as vanadate (which is less inhibitory to ATPases in its oxidized states) and dehydroascorbate [1,3]. One or more of these enzymes may be derived from NADH-cytochrome b_5 reductase of endoplasmic reticulum [4] reflecting, perhaps, in the case of erythrocytes, a residue of activity significant in precursor cells.

These NADH-acceptor reductases are subject to inhibition by sulfhydral-active agents such as *p*-chloromercuribenzoate and *N*-ethylmaleimide as well as flavin antagonists, such as atebrin [5]. A partially purified enzyme was shown to be inhibited by adenine nucleotides (with ADP being more effective than ATP or AMP); this inhibition was said to be competitive in nature, but no kinetic data were presented [4] and the action of other nucleotides was not investigated. Finally, erythro-

Abbreviations: p[NH]ppA, 5′-adenylyl imidodiphosphate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; pp[CH_2]pA, adenosine 5′-[α,β-methylene]triphosphate.

cyte NADH-ferricyanide reductase activity in open membranes has been shown to be increased by uncouplers of oxidative phosphorylation [6] and it has been suggested [1] that the stimulation reflects transmembrane proton translocation associated with activity of the enzyme, at least a portion of which spans the membrane [7]. Because there is little evidence for proton-driven oxidative phosphorylation associated with plasma membrane NADH oxidation, there is no a priori reason to expect an uncoupler (protonophore) to stimulate such electron transport. Therefore, we have studied erythrocyte NADH-acceptor reductase activity, examining its sensitivity to a number of lipophylic compounds (including uncouplers) as well as to a number of nucleotides. We report here that both uncouplers and adenine nucleotides inhibit membrane-bound enzyme in an apparently-competitive manner but suggest that the mechanism underlying such similar kinetic behavior is probably quite distinct in the two cases.

Methods

Human erythrocytes were drawn into heparinized tubes, centrifuged at $1350 \times g$ for 30 min, the buffy coat removed, and the cells then washed three times by centrifugation through a medium at 4°C containing 20 mM Hepes (pH 7.4), 11 mM glucose, 130 mM NaCl, and 4 mM KCl. Cells were finally diluted with the same medium to a final concentration of about $2 \cdot 10^9$ cells $\cdot$ ml^{-1}. Ghosts were prepared following a modification of the method of Buckley and Hawthorne [8]. Cells were lysed in nine volumes of cold Tris-EDTA (1.44 mM, pH 7.5), incubated for 5 min in an ice-water bath, and then centrifuged at $26000 \times g$ for 40 min at 4°C. The membrane pellet was then resuspended and washed by centrifugation in the same medium four times. Membranes were then reconstituted in 20 mM Tris-HCl at pH 7.4 and washed four times in the same medium as described above, after which the pellet was white. Finally, membranes were resuspended in a small volume of the same medium to a protein concentration of approximately 2.5 mg/ml.

The enzyme was assayed using a Perkin-Elmer Model 350 dual wavelength spectrophotometer with either ferricyanide, cytochrome *c*, or vanadate as electron acceptor. NADH-ferricyanide reductase activity was measured at 340 nm at 20°C with a reaction mixture containing 16 mM Tris-HCl buffer (pH 7.4), 0.5 mM EDTA, 0.1 mM NADH, 200 μM potassium ferricyanide and approx. 20 μg of membrane. NADH-cytochrome *c* reductase was measured at 550 nm and at 20°C with a reaction mixture containing 16 mM Tris-HCl buffer (pH 7.4), 0.5 mM EDTA, 100 μM NADH, 7.6 μM cytochrome *c* (Sigma type III) and approx. 30 μg of membrane. NADH-vanadate reductase was measured at 340 nm with a reaction medium identical to that used in the ferricyanide assay but containing 0.5 mM sodium vanadate instead of ferricyanide. Protein was measured by the method of Lowry et al. [9]. All chemicals were of at least reagent grade, all biochemicals being purchased from Sigma Chemical Company.

Results and Discussion

Activity of human erythrocytes ghost NADH reductase is illustrated in Table I in which it is seen that treatment with the detergent, Triton X-100, significantly enhances activity when ferricyanide, or vanadate is employed as electron acceptor. Because stimulation of ferricyanide reductase activity by detergent is characteristic of vesicles in their native (right-side-out) state, [10] it appears that these membranes are largely in the latter configuration. This conclusion is supported

TABLE I

NADH-(ACCEPTOR) REDUCTASE: COMPARISON OF ELECTRON ACCEPTORS

Conditions are as described in Methods with 0.05% Triton X-100 or 0.02% saponin where present. Rates are the mean of three values.

Conditions	Rate (nmol/min per mg)
Ferricyanide	38
Ferricyanide + Triton X-100	172
Cytochrome *c*	18
Cytochrome *c* + Triton X-100	0
Cytochrome *c* + saponin	27
Vanadate	8
Vanadate + Triton X-100	33

by experiments, not shown in the table, where Triton X-100 failed to increase acetylcholinesterase activity. This enzyme, which is located on the external surface of the plasma membrane was measured as described in Ref. 11. Because Triton X-100 is strongly inhibitory to NADH-cytochrome *c* reductase, the detergent saponin was employed, instead. The relatively modest increase in activity observed may reflect partial inhibition of cytochrome *c* reductase activity by the detergent.

The influence of detergent on activity is also evident in values for the apparent K_m, for NADH obtained by a double reciprocal plot, where the electron acceptor was ferricyanide. In the presence of 0.05% Triton X-100, the apparent K_m was 8.5 μM, whereas in its absence, the K_m rose to 60. Kitajima et al. [4] studying a purified reductase, reported a value of 6.4 μM.

Table II summarizes inhibition of NADH-ferricyanide reductase activity by a number of nucleotides. Of these, adenine nucleotides (including NAD^+) are the most potent inhibitors, their effectiveness being in the order ADP, ATP, NAD^+, AMP. The non-phosphorylating ATP analogs, p[NH]ppA and pp[CH_2]pA were only moderately inhibitory as were the other nucleoside di- and triphosphates tested. In the presence of detergent, the nucleoside diphosphate was more inhibitory than the corresponding triphosphate, whereas, in its absence, this distinction was largely abolished. In general, the detergent increased sensitivity to nucleotides and, of course, led to greatly-enhanced reductase activity (Table I). The sensitivity to nucleotides was reflected in an inhibitor constant (K_i, obtained from a Dixon plot) of 190 μM in the presence of detergent and about 900 μM in its absence. These values should be compared with 80 μM obtained by Kitajima et al. [4], who studied purified enzyme.

TABLE II

INHIBITION OF NADH-FERRICYANIDE REDUCTASE BY NUCLEOTIDES

Conditions are as described in Methods. The detergent, Triton X-100, where included, was at a concentration of 0.05%. Values for nucleotide concentration producing 50% inhibition were obtained in absence of detergent.

Compound	Inhibition at 2.0 mM (%)		Concn. (mM) producing 50% inhibition
	+ detergent	− detergent	
ATP	67	52	1.9
ADP	78	67	1.5
AMP	23	23	5.3
CTP	16	28	4.2
CDP	32	27	4.0
UTP	20	18	5.4
UDP	31	10	> 8.0
GTP	43	13	> 8.0
GDP	44	15	> 8.0
ITP	37	13	> 8.0
NAD^+	42	37	3.0
cyclic AMP	31	31	5.2
cyclic AMP	10	4	> 8.0
p[NH]ppA	23	20	6.4
pp[CH_2]pA	15	15	> 8.0

Fig. 1 shows a double reciprocal (Lineweaver-Burk) plot of ADP inhibition at different NADH concentrations; convergence at a single value for the reciprocal of V_{max} is consistent with a competitive form of inhibition. As ADP comprises a portion of the NADH molecule, such competition is not unexpected nor is it unreasonable that ADP should be the most effective of the nucleotides examined.

Inhibition of NADH-ferricyanide reductase by

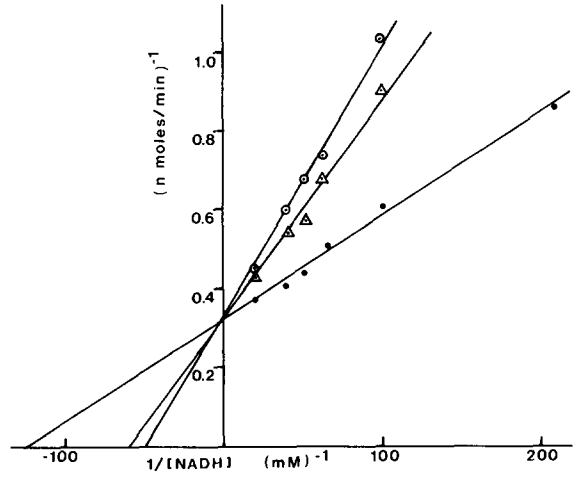

Fig. 1. Inhibition of NADH-ferricyanide reductase by ADP. Conditions are as in the legend to Table II. Closed circles denote control; triangles, 300 μM ADP; open circles, 600 μM ADP.

uncouplers of oxidative phosphorylation and other hydrophobic compounds is illustrated in Table III. It is evident that the uncouplers, dinitrophenol, nonyl-dinitrophenol and FCCP, produce substantial inhibition in micromolar concentrations and that the other compounds tested were ineffective. The inhibitor constant for FCCP was found to be 19 μM. Just as 6-nonyl-2,4-dinitrophenol is a more potent mitochondrial uncoupler than its parent 2,4-dinitrophenol [12], the nonyl derivative is a much more effective inhibitor of the reductase, presumably owing to its increased hydrophobic character.

The failure of valinomycin to inhibit suggests that the inhibition requires the conduction of protons and not merely positive charge to (or across) a hydrophobic region. The failure of 2-hydroxyl-1,4-naphthoquinone to inhibit is consistent with the previous observation [13,14] that this compound, unlike some other hydroxynaphthoquinones, is relatively ineffective as a mitochondrial uncoupler. As this compound probably acts as an inhibitor of the mitochondrial respiratory chain by serving as a ubiquinone analog, the lack of inhibition argues against involvement of quinones as electron carriers in the NADH reductase system. Finally, 2,4,5,7-tetrabromofluorescein (eosin) and 3,6-diaminoacridine (proflavin) have both been employed as conformation-sensitive probes of the hydrophobic regions of enzymes and interact strongly with such regions at low concentrations [15,16]. Their failure to inhibit the reductase, again, indicates that proton conduction is a necessary requirement for the inhibition and that hydrophobicity alone is insufficient. Fig. 2 is a double-reciprocal plot showing substrate concentration dependency in the presence and absence of nonyldinitrophenyl. Again, convergence to a single maximum velocity is observed suggesting a competitive form of inhibition.

Observation of inhibition of reductase by low concentrations of uncouplers is in sharp contrast with the report of Löw et al. [6] which describes significant stimulation by FCCP. The reason for this contrast is not clear, but it is important to note that the present study was carried out with sealed ghosts in their native sidedness, whereas the stimulation of activity reported in Ref. 6 was observed with open ghosts. Moreover, FCCP and gramicidin were presumably added as an ethanolic solution, and ethanol, itself, at very low concentra-

TABLE III

INHIBITION OF NADH-FERRICYANIDE REDUCTASE ACTIVITY BY UNCOUPLERS AND IONOPHORES

Conditions are as described in the Methods section with 0.05% Triton X-100 included in the reaction mixture. All compounds except 2,4-dinitrophenol and eosin were added as an ethanolic solution, in which case the final ethanol concentration did not exceed 4 % and a comparable volume of ethanol was included in the control.

Compound	Concn. (μM)	% inhibition
2,4-Dinitrophenol	50	12
	100	44
6-Nonyl-2,4-dinitrophenol	10	31
	50	75
FCCP	15	18
	30	26
	60	81
Valinomycin (+KCl)	80	0
	120	6
Antimycin	20	0
2-Hydroxyl-3-cyclohexyl-1,4-naphthoquinone	20	0
	60	0
2,4,5,7-Tetrabromofluorescein	1000	0
	1000	4

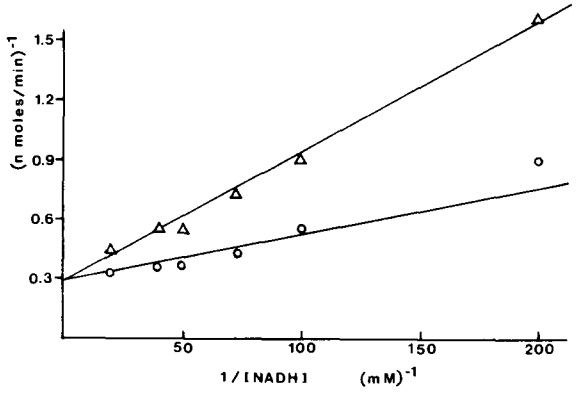

Fig. 2. Inhibition of NADH-ferricyanide reductase by 6-nonyl-2,4-dinitrophenol. Conditions are as in the legend for Table II. Circles denote control; triangles, activity in presence of 50 μM nonyl-dinitrophenol.

tions, stimulates activity of NADH-ferricyanide reductase in open vesicles [3].

It should be added that NADH-cytochrome *c* reductase exhibits similar nucleotide sensitivity when compared with NADH-ferricyanide reductase, but, on the other hand, is much less sensitivity to uncouplers. For example, 3.0 mM ADP produces 90% inhibition of NADH-cytochrome *c* reductase (exactly the inhibition observed with ferricyanide reductase in the presence of detergent) and the other nucleoside di- and triphosphates were similarly less effective. In contrast, dinitrophenol, nonyl-dinitrophenol and FCCP, all at a concentration of 100 μM, produced less than 15% inhibition of cytochrome *c* reductase activity. The cause of this insensitivity (when compared to the high sensitivity of NADH-ferricyanide reductase, see Table III) is unknown but may reflect the orientation of functional sites in the two instances, with NADH-ferricyanide reductase, but not cytochrome c reductase, spanning the membrane (see Ref. 1).

Whereas it is likely that the close structural relationship between ADP and NADH underlies site-competition between the two compounds, no such structural similarity exists between NADH and the uncouplers (or, indeed, between the uncoupling phenols and FCCP). Because uncoupler inhibition requires proton conduction and is enhanced by increased hydrophobicity, we imagine that inhibition is a consequence of proton transfer to (or through) a hydrophobic region. If the membrane-spanning NADH-ferricyanide reductase were electrogenic and the membrane vesicles sufficiently impermeable, then the expected influence of uncouplers would be stimulation of activity by collapse of an opposing potential. As we do not observe this, we suggest instead that the uncouplers may function by conducting protons to a hydrophobic, internal region of the enzyme, there, perhaps to compete with an internal proton donor for a common acceptor.

Acknowledgments

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