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Preliminary Characterization Studies on the *Neisseria catarrhalis* Respiratory Electron Transport Chain

PETER JURTSHUK AND T. W. MILLIGAN
Department of Biology, University of Houston, Houston, Texas 77004

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The *Neisseria catarrhalis* respiratory electron transport system was examined in a sonic type particulate membrane fraction and shown to have a moderately active succinate as well as nonpyridine nucleotide-dependent DL-lactate oxidoreductase and a very active tetramethyl-*p*-phenylenediamine oxidase. L-Malate and L-glutamate oxidation were found to be dependent on pyridine nucleotides and exclusively associated with a soluble (or nonmembranous) fraction. The primary cytochrome components in the electron transport system appear to be *c*-type in nature (555 nm and 550 nm) as well as cytochrome a_1 (600 nm) and cytochrome *o*.

Although Jyssum (7) has demonstrated the presence of the soluble tricarboxylic intermediate reactions in *Neisseria meningitidis*, and Tonhazy and Pelczar (10) have done the same for *N. gonorrhoeae*, very little is known about the characteristics of the soluble enzymes involved, and almost no information is available on the electron transport system of the genus *Neisseria*. This genus consists of obligately aerobic organisms having a very potent oxidase (Kovacs) reaction which is used diagnostically for primary isolations. The oxidase reaction involves the use of the electron donor tetramethyl-*p*-phenylenediamine (TMPD) and the oxidation of this dye is known to occur via the terminal oxidase reaction associated with most aerobic bacterial electron transport systems.

Jyssum and Joner (8), employing cell-free extracts of *N. meningitidis*, demonstrated that the reduction of mammalian ferricytochrome *c* was increased by the addition of hydroxylamine; and that the oxidation of ferrocytochrome *c* could be prevented by the addition of cyanide. Mammalian cytochrome *c*, however, is usually a very poor oxidant or reductant for bacterial electron transport systems (1, 6, 9). The purpose of our preliminary study was to examine enzymatically and spectrally the particulate electron transport particle of *Neisseria catarrhalis* (and *N. flava*) and characterize the nature of the electron transport system present. Our interest in *Neisseria* resides in its very active terminal cytochrome oxidase reaction that is probably related to its possessing *c*-type cytochromes and cytochrome *o* in its respiratory electron transport chain as is the case for *Azotobacter vinlandii* (Fed. Proc. **31**:888).

Resting cells were subsequently harvested, washed, and suspended in 0.02 M phosphate buffer, pH 7.5, standardized turbidimetrically, and disrupted by sonic oscillation (5). The resultant sonic homogenate was then separated into a soluble (S_3) fraction and a particulate (R_3) membranous fraction by a differential centrifugation procedure previously described (5). Manometric assays were employed to measure all oxidation rates used using both the soluble S_3 and particulate R_3 fractions. The TMPD assay was used to measure terminal cytochrome oxidase activity in the manner previously described (4), and the oxidation of other substrates (16.7 mM) was performed in a similar manner, but at pH 7.5. In assays employing oxidized nicotinamide adenine dinucleotide (NAD^+)(or oxidized nicotinamide adenine dinucleotide phosphate [$NADP^+$]), these compounds (1 mM) were present in the main compartment with the enzyme preparation. All assays were initiated (after temperature equilibration) by the addition of substrate (or TMPD and ascorbate) from the side arm.

Differential spectrophotometry studies were performed at room temperature (25 C) with a Beckman DK-2A recording spectrophotometer. Exact experimental conditions employed in performing spectral analyses are given in legends to the figures.

Table 1 shows the ability of the *N. catarrhalis* R_3 electron transport fraction to oxidize various substrates with or without the presence of pyridine nucleotides (NAD^+ or $NADP^+$). Of the

TABLE 1. *Manometric oxidation rates for various substrates by the N. catarrhalis R_3 electron transport particle*

Assay system (substrate-enzyme)	Membrane-bound oxidase activities[a]		
	No additions	+NAD^+	+$NADP^+$
Succinate			
R_3 fraction	0.09[b]	ND[c]	ND
DL-Lactate			
R_3 fraction	0.06[b]	ND	ND
R_3 fraction plus S_3 soluble fraction[d]	0.06	0.07	0.09
L-Malate			
R_3 fraction	0.001	0.001	0.001
R_3 fraction plus S_3 soluble fraction	0.014	0.021	0.051
L-Glutamate			
R_3 fraction	0.02	0.01	0.02
R_3 fraction plus S_3 soluble fraction	0.08	0.06	0.09
TMPD-ascorbate			
R_3 fraction	3.91[b]	ND	ND

[a] Expressed in microatoms of oxygen per minute per milligram of protein at 30 C.

[b] Reaction completely inhibited by KCN (10^{-4} M).

[c] Not determined.

[d] The S_3 soluble fraction that was added to the reaction mixture contained 8.32 mg of total protein. All specific activity calculations were based solely on the protein content of the R_3 fraction even when the S_3 soluble fraction was added to the assay system.

substrates tested, only succinate, DL-lactate, and TMPD were oxidized to any appreciable extent (specific activity greater than 0.05) when oxygen was used as a terminal electron acceptor. L-Malate and L-glutamate oxidation rates by the *N. catarrhalis* R_3 fraction were negligible even in the presence of pyridine nucleotides, until the supplemental factors in the S_3 fraction were added to stimulate the oxidation rates. The S_3 fraction contained both the soluble L-malate and L-glutamate dehydrogenases as well as other soluble cofactors such as pyridine nucleotides. Of the two substrates tested (L-malate and L-glutamate), $NADP^+$ rather than NAD^+ gave higher activity rates in assay reaction mixtures in which the combined R_3 and S_3 fractions were used. Interestingly, TMPD was oxidized at a rate 40 to 50 times higher than any of the other substrates tested with the *N. catarrhalis* R_3 fraction, which shows the unusual nature of this interesting terminal oxidase reaction. KCN (1×10^{-4} M) was found to completely inhibit succinate, DL-lactate, and TMPD oxidation by the *N. catarrhalis* R_3 electron transport fraction (Table 1, footnote *c*). The data obtained for L-glutamate oxidation were consistent with the findings reported by Holten and Jyssum (2) for *N. meningitidis*. These workers observed that this meningococcus contained both an NAD^+- and $NADP^+$-linked L-glutamate dehydrogenase and that the $NADP^+$-linked dehydrogenase was stimulated when the organism was grown with glucose as the main carbohydrate source. In our studies on the *N. catarrhalis* R_3 electron transport fraction, it was surprising to find an inactive (nonpyridine nucleotide-dependent) L-malate oxidase since Jones and King (3) found that all strict aerobic organisms possessed a flavoprotein-type particulate L-malate oxidase that does not require NAD^+ nor $NADP^+$ for activity, and that facultative anaerobes oxidized L-malate primarily through a soluble NAD^+-linked dehydrogenase. However, Jones and King utilized the phenazine methosulfate-dichloroindophenol (PMS-DCIP) reductase assay in the presence of KCN, whereas we employed only oxygen as the electron acceptor in our preliminary study. Preliminary experiments utilizing a PMS-DCIP spectrophotometric assay have revealed that *N. flava* contains an active L-malate PMS-DCIP reductase activity (specific activity of 0.06 μmol per min per mg of protein), but as yet we have not determined whether this activity is present in *N. catarrhalis*. The data shown in Table 1 for *N. catarrhalis* could be duplicated, with minor variations, for *N. flava* to show that the two organisms possess a similar electron transport system.

Figure 1 shows a difference spectrum (dithionite-reduced minus oxidized) of the *N. catarrhalis* R_3 fraction at a protein concentration of 8.4 mg/ml. The presence of *c*-type cytochromes is demonstrated by the well-defined α-peak at 550 nm and the shoulder of the curve at 555 nm as well as the beta absorption characteristics at 524 and 530 nm. A strong Soret absorption peak is noted at 423 nm. The shoulder absorption at 555 nm and 530 nm may indicate the presence of a *b*-type cytochrome, and the broad absorption shoulder at 600 nm in Fig. 1 suggests the presence of cytochrome a_1. The spectrum in Fig. 1 also shows the presence of flavoprotein (and possibly nonheme iron) by the marked "bleaching" observed in the 450 nm region. The absorption characteristics shown in Fig. 1 for the *N. catarrhalis* R_3 fraction are typical and nothing of an unusual nature can be noted. A similar type difference spectrum was obtained for *N. flava*.

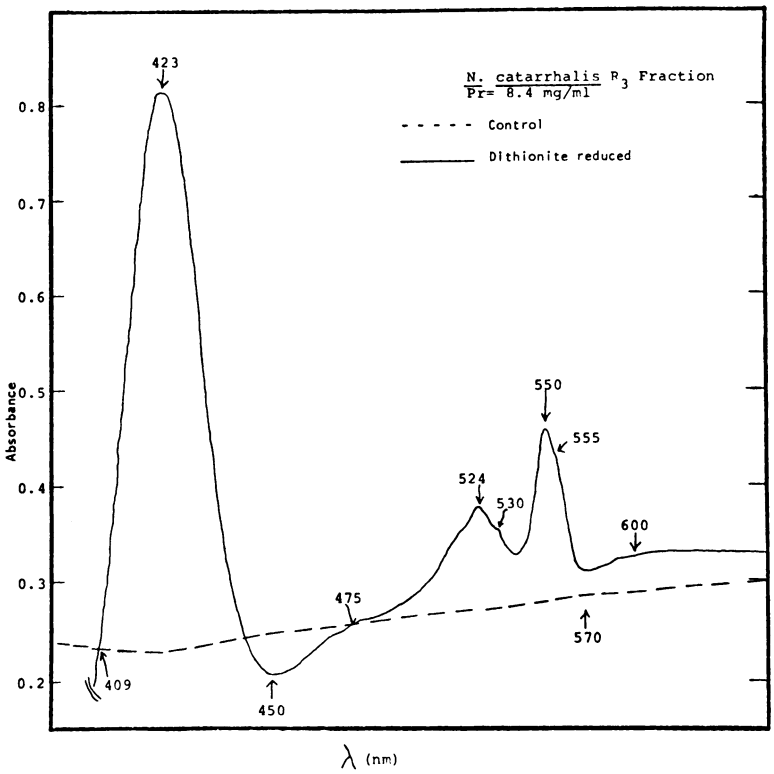

FIG. 1. *Room temperature (25 C) difference spectra (dithionite reduced minus oxidized) exhibiting the steady state reduction of the N. catarrhalis R_3 electron transport components. The control shows the difference spectra between the reference and sample mixtures before any additions. The solid line illustrates those components in the sample mixture that were reduced by the addition of a small amount of $Na_2S_2O_4$ crystals.*

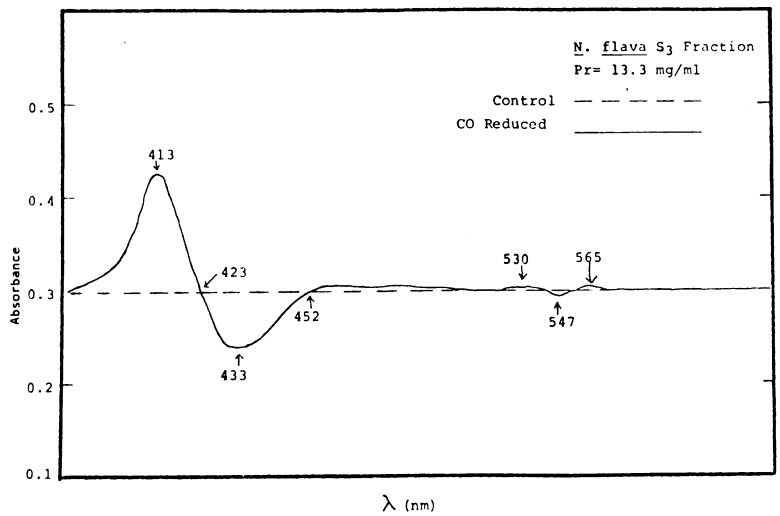

FIG. 2. *Room temperature difference spectra of membrane fragments present in N. flava S_3 fraction (carbon monoxide-dithionite reduced minus dithionite reduced). A small amount of dithionite crystals were added to a test tube containing 5.0 ml of S_3 fraction, and after adequate mixing samples from this tube were added to the reference and sample cuvettes. The control shows the difference between the reference and sample cuvettes after dithionite addition. The sample cuvette was then removed and carbon monoxide was gently bubbled through the enzyme solution for 90 s. The N. flava S_3 fraction was present in both cuvettes at a concentration of 13.3 mg of protein suspended in 1.0 ml of 0.02 M phosphate buffer, pH 7.5.*

Figure 2 shows a difference spectrum (carbon monoxide-dithionite reduced minus dithionite reduced) of membrane components in *N. flava* S_3 fraction. This represents a typical difference spectrum of cytochrome *o*, which has broad, ill-defined peaks in the 565 to 568 and the 530 to 537 nm regions and a sharp, well-defined peak in the 413 to 418 nm region (8a). A similar difference spectrum could also be obtained for *N. catarrhalis*, which would suggest that cytochrome *o* is a major component in both these *Neisseria* species. The presence of a *c*-type cytochrome in conjunction with cytochrome *o* would account for the high TMPD oxidase activity as is the case with the *Azotobacter vinelandii* electron transport system (Fed. Proc. **31**:888). It is probable that the two major terminal oxidase components in *Neisseria* species are cytochrome *o* and a_1.

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