THE UNIVERSITY OF MANITOBA

CYTOCHROMES OF *NITROBACTER*

BY

GHULAM RASUL CHAUDHRY

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GHULAM RASUL CHAUDHRY

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the University of Manitoba in partial fulfillment of the requirements
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To my parents and friends

ABSTRACT

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ABSTRACT

Optimal growth conditions were established for *Nitrobacter agilis* freshly isolated from soil. Oxygen tension below 10% or above 15% resulted in abnormal cell ultrastructure and reduced growth. High concentration of nitrite and nitrate also affected the growth and cell structure.

Optimally grown (2 g wet cell/ 10 l) cultures provided for the isolation and purification of three *c*-type cytochromes, cytochrome *a*-type oxidase, a copper-iron electron transfer protein, cytochrome *b*, ubiquinone, nitrite: cytochrome *c* reductase, NADH: cytochrome *c* oxido-reductase and a purple pigment.

Two of the three *c*-type cytochromes, cytochrome *c*-553 and *c*-550 had typical absorption spectra of cytochrome *c* and molecular weights of 11,500 and 12,500 respectively. Cytochrome *c*-553 was characteristically partially reduced when isolated and was not oxidized by ferricyanide. The completely reduced form displayed absorption peaks at 410, 523, and 553 nm. Cytochrome *c*-550 was similar to that purified previously by Ketchum *et al*. (1969) with an oxidized absorption peak at 411 nm and reduced peaks at 416, 521 and 550 nm. The amino acid compositions of the two cytochromes were different from each other. The N-terminal sequence of cytochrome *c*-550 showed a clear homology with the corresponding sequence portions of four *c*-type cytochromes from other sources, including horse heart.

The third cytochrome was an anomalous cytochrome of *c*′-type.

It had an oxidized absorption peak at 402 nm and bands at 525, 560 and 620 nm. The reduced form showed absorption maxima at 419, 523, 549 and 554 nm and a shoulder at 430 nm. The absorption maxima were markedly affected by pH. Sodium hydroxide at 0.1 N converted the anomalous spectra of cytochrome *c*-549, 554 to those of a typical cytochrome *c*. It had a molecular weight of 90,000 as estimated by gel filtration but appeared as a single polypeptide of 46,000 daltons by SDS polyacrylamide gel electrophoresis. Amino acid composition showed a high content of alanine. Cytochrome *c*-549, 554 was found to be highly autoxidizable. The ferrocytochrome reacted with CO and NO_2^- whereas both ferri- and ferrocytochrome combined with CN^-.

Cytochrome oxidase solubilized and purified from electron transport particles exhibited absorption spectra with maxima at 420 and 600 nm when oxidized and at 443 and 606 nm when reduced. The purified enzyme migrated as a single band during gel electrophoresis but appeared as three bands with molecular weights of 37,000, 25,000 and 13,000 in the presence of SDS and urea. Cytochrome *a*-type oxidase reacted rapidly with *Nitrobacter* cytochrome *c*-550 and horse heart cytochrome *c*. The enzyme activity was pH dependent and was inhibited by cyanide, azide and diethyl dithiocarbamate.

The copper-iron electron transfer protein similarly purified had a yellow-green color and absorbance maxima at 410 and 630 nm. The color disappeared upon reduction. It did not contain a methionine residue and had a molecular weight of 5,500 as judged by gel

filtration. The purified protein moved as a single band during electrophoresis. The copper-iron protein contained 0.34 g atom of copper and 0.60 g atom of iron per mole of protein and about 3% of phospholipid.

The isolated and partially purified cytochrome *b* was autoxidizable, reacted with CO and in the oxidized form had an absorption maximum at 405 nm; the reduced form maxima were at 432, 530 and 560 nm. Molecular weight estimated by gel filtration was 37,000. Fluorescence spectra indicated a flavin-like component present in cytochrome *b* preparations.

Absorption spectra, TLC, IR and PMR studies of the ubiquinone purified from whole cells suggest that it is probably coenzyme Q_{10}.

Nitrite: cytochrome *c* reductase had a yellow color and catalyzed the reduction of cytochrome *c* with nitrite. It had a Km value of 30 mM for nitrite and its activity was inhibited by nitrate. The enzyme was unstable and lost its activity overnight at $-20^{o}C$. It contained adenine nucleotide, possibly FAD. The yellow pigment was bleached when reduced as was the color of enzyme preparations.

NADH: Cytochrome *c* oxido-reductase had a red color and catalyzed the reduction of cytochrome *c* in the presence of NADH. Its enzyme activity was inhibited by nitrate but not by nitrite. Both the enzyme preparation and the red pigment extracted from it exhibited similar absorption spectra with a 480 nm peak. The red color disappeared upon reduction.

The purple pigment had a weak activity of NH_2OH: cytochrome *c* reductase. The pigment was decolorized upon reduction and at a pH below 6.5.

The study reported in this thesis may be considered as a further positive step towards the understanding of the biochemistry of nitrite oxidation, an important process of the nitrogen cycle. Not only have some new redox components of the *Nitrobacter* respiratory chain been demonstrated but the basis for further exploration of the energetics of this organism has also been extended.

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ABBREVIATIONS

ATP	adenosine 5'-triphosphate
BIS	N,N'-methylene bisacrylamide
BSA	bovine serum albumin
^{13}CMR	carbon-13 magnetic resonance
CM	carboxymethyl
cyt.*c*	cytochrome *c*
DEAE	diethylaminoethyl
EDTA	ethylenediamine tetraacetic acid
ETP	electron transport particle(s)
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GSH	reduced glutathione
IR	infrared
NAD^+	oxidized nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
PMR	proton magnetic resonance
Q_9	coenzyme Q_9
Q_{10}	coenzyme Q_{10}
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet

INTRODUCTION

CHAPTER 1

INTRODUCTION

Inorganic nitrogen compounds exist in nature in several oxidation states from NH_3(-3) to HNO_3(+5). Whereas the conversion of its most oxidized form, nitrate, into its most reduced form, ammonia, is carried out by a wide variety of microorganisms and plants, the biological oxidation of reduced inorganic compounds is carried out predominantly by species of the nitrifying bacteria, *Nitrosomonas* and *Nitrobacter*. The former oxidizes ammonia to nitrite and the latter nitrite to nitrate.

Nitrobacter was first isolated and named by Winogradsky (1890) and is a chemoautotrophic bacterium that derives energy from the oxidation of nitrite (17.8 Kcal/mol) and assimilates CO_2 by using the Calvin cycle (Aleem 1965).

The era of biochemical studies of *Nitrobacter* essentially begins with the report that cytochromes are involved in nitrite oxidation (Lees and Simpson 1957). Aleem and Nason (1959) confirmed that nitrite oxidizing activity of *Nitrobacter agilis* resides in cytochrome-containing red particles. The subsequent work on the mechanism of nitrite oxidation by autotrophic bacteria over the years has formed the basis for several extensive reviews (Lees 1960, 1962, Nicholas 1963, Peck 1968, Wallace and Nicholas 1969, Aleem 1970, Kelly 1971, Suzuki 1974, Schlegel 1975, Aleem 1978).

Most of the studies to elucidate the mechanism of electron transfer (from nitrite to molecular O_2) and oxidative phosphorylation have been limited to those with whole cells, partially purified

fractions, and cell-free extracts or so-called electron transport particles (Straat and Nason 1965, Van Gool and Laudelout 1966, Kiesow 1967, Aleem 1968, Sewell and Aleem 1969, O'Kelley *et al.* 1972, Ingledew *et al.* 1974, Cobley 1976a,b). Exceptions include the report by Ketchum *et al* (1969) where they purified a *c*-type cytochrome and that by Yamanaka *et al.* (1979) where they isolated the terminal oxidase system (cytochrome *a*-type oxidase). More recently cytochrome *b* has also been isolated as a cytochrome *b* and *c* complex (Chaudhry *et al.* 1980). Very little is known about the rest of the components of the respiratory chain of *Nitrobacter.*

In the present study attempts were made to isolate several redox systems of *Nitrobacter agilis* and to study some of their physicochemical properties. I have succeeded in isolating and identifying some of the so-far unknown or poorly understood redox components of *Nitrobacter*, for example *c*′-type cytochrome. It is hoped that these findings will provide a renewed interest and stimulus for workers to attempt a further elucidation of nitrite oxidation mechanism by *Nitrobacter.*

HISTORY

CHAPTER 2

HISTORY

2.1 INTRODUCTION

In the second half of the nineteenth century breakthroughs occurred in many areas of Science, particularly in the developing science of Microbiology. In the year 1862, for example, Pasteur first proposed that the oxidation of ammonia to nitrate might be a biological process performed by soil microorganisms (cited in Nelson 1931). It took another thirty years, however, to demonstrate that the biological oxidation of ammonia involved two steps, the oxidation of NH_4^+ to NO_2^-, and the oxidation of NO_2^- to NO_3^- (Schloesing and Muntz 1877, Warington 1891). At the same time, Winogradsky (1890) reported the isolation of bacteria responsible for the process of nitrification and called them *Nitrosomonas* and *Nitrobacter*, the former converting ammonia to nitrite and the latter oxidizing nitrite to nitrate (Lees 1955).

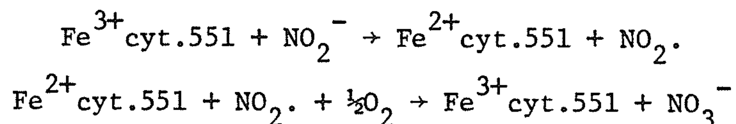
The classical studies of Winogradsky (1890) and Winogradsky and Omeliansky (1899) on the physiology of nitrifying bacteria led to the concept of obligate autotrophy. Members of the genus *Nitrobacter* fulfil their energy requirement for the fixation of carbon dioxide by the oxidation of nitrite, an inorganic substrate (Winogradsky 1922) and thus they are also called chemoautotrophs or chemolithotrophs (Lees 1955, Kelly 1971).

There have been three general approaches used in attempts to understand this mechanism of respiration or oxidation of substrate. First, classical respirometric studies with intact cells; secondly,

isolation and characterization of individual components of the respiratory and oxidative system, and finally the reconstitution and examination of the system after reassembling of the individual components. The purpose of this dissertation was to apply the second approach to further facilitate our understanding of nitrite oxidation by *Nitrobacter*. Since most of the studies so far were carried out with intact cells, crude extracts or partially purified preparations, my review of the literature begins with a general survey of these studies on the mechanism of nitrite oxidation by *Nitrobacter*, followed by a discussion of the individual components, particularly cytochromes of the respiratory chain of these bacteria.

2.2 MECHANISM OF NITRITE OXIDATION

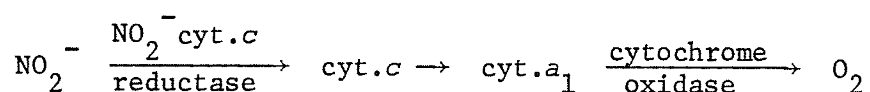
The classical studies by Meyerhof (1916, 1917a,b) on the respiratory process of nitrifying bacteria led to the belief that the substrate oxidation by chemoautotrophs was a process very similar to the cell respiration of heterotrophic organisms. An important discovery in this respect was made by Lees and Simpson (1957) when they observed that the addition of nitrite to a cell suspension of *Nitrobacter* resulted in appearance of three reduced cytochrome bands at 521-25, 551 and 589 nm. They implicated the role of this cytochrome in nitrite oxidation by *Nitrobacter* by postulating the following scheme:



$$Fe^{3+}cyt.551 + NO_2^- \rightarrow Fe^{2+}cyt.551 + NO_2.$$
$$Fe^{2+}cyt.551 + NO_2. + \tfrac{1}{2}O_2 \rightarrow Fe^{3+}cyt.551 + NO_3^-$$

The symbol $NO_2.$ in this scheme was proposed to represent some compound, free or bound to a carrier at the oxidation level of the nitrite radical.

Soon after, nitrite oxidation was demonstrated in a cell-free particulate fraction from *Nitrobacter* (Aleem and Alexander 1958). Aleem and Nason (1960) subsequently reported that the oxidation of nitrite was catalyzed by the cytochrome-electron transport particle termed "nitrite oxidase" and the process involved an enzymatic transfer of electrons from nitrite to molecular oxygen mediated by *c* and a_1-type cytochrome as shown below:



$$NO_2^- \xrightarrow[\text{reductase}]{NO_2^-\ \text{cyt.}c} \text{cyt.}c \rightarrow \text{cyt.}a_1 \xrightarrow[\text{oxidase}]{\text{cytochrome}} O_2$$

They further found that nitrite oxidation was coupled to the synthesis of ATP, with a P/O ratio 0.2. A similar value for the P/O ratio was also observed by other workers (Fischer and Laudelout 1965) who considered that these low values were consistent with the molar growth yield of the organism.

While discussing the problem of nitrite oxidation, Lees (1962) pointed out that the redox potential of nitrite/nitrate couple ($E_{m,7}$ + 0.42V) was appreciably higher than that of the cytochrome *c* of *Nitrobacter*, which had an $E_{m,7.5}$ of +0.25V (Butt and Lees 1958). He therefore, suggested that to oxidize nitrite, *Nitrobacter* might have to modify it in some way, perhaps by synthesizing some such compound as adenyl nitrite, so as to lower the redox potential of nitrite to one more compatible with reduction of cytochrome *c*. Although this idea did not agree with the kinetic studies of Kiesow discussed below (Kiesow 1963, 1964, 1967), it nevertheless changed the pattern of future thinking on the mechanism of nitrite oxidation.

Spectrophotometric studies conducted with *Nitrobacter* particles showed that the reduction of cytochrome a_1 by nitrite could be

achieved both aerobically and anaerobically, whereas cytochrome *c* was reduced only aerobically. Addition of ATP however, resulted in the reduction of cytochrome *c* under anaerobic conditions as well (Kiesow 1967). These observations led to a conclusion that the reduction of cytochrome *c* requires energy but that of cytochrome a_1 does not. According to Aleem (1968), 8.3 kcal of energy is required to bridge the gap between cytochrome *c* and NO_3^-/NO_2^- system. Van Gool and Laudelout (1967) reported that the activation energy for the reduction of cytochrome *c* by nitrite is 15 kcal. These findings were further substantiated by uncoupler and inhibitor studies. It was proposed that cytochrome a_1 was the site of entry of nitrite in the *Nitrobacter* electron transport chain and the reduction of cytochrome *c* involved an energy-dependent reversal of electron transfer from cytochrome a_1 (Aleem 1968).

On the other hand a direct reduction of cytochrome *c* by nitrite in the presence of a nitrite-cytochrome *c* reductase was reported (O'Kelley *et al.* 1970) and the experiments supporting the energy-dependent reduction of cytochrome *c* and reversal of electron transfer flow from a_1 (Aleem 1968) were criticized on the basis of lack of sufficient control experiments (O'Kelley *et al.* 1970). They suggested that the entry site of nitrite is not at cytochrome a_1 level but at or prior to cytochrome *c*. The same workers also reported that nitrite showed higher affinity for enzyme (nitrite-cytochrome *c*-reductase) at a lower pH, suggesting HNO_2 rather than NO_2^- as the substrate for the nitrite oxidase system. In *Nitrosomonas*, ammonia also showed greater affinity at higher pH, suggesting NH_3 rather than

NH_4^+ as the substrate for oxidation (Suzuki 1974). Aleem (1970), however, postulated a hydrated form of nitrite ($NO_2^-.H_2O$) as the substrate of nitrite oxidation.

Redox potential studies at various pH values (Ingledew *et al.* 1974) showed that pH had no effect on the E_m of *Nitrobacter* cytochrome *c*, *a* and a_3, but the redox potentials of cytochrome a_1 components (two types of cytochrome a_1 had been identified by Straat and Nason 1965) became more positive with decreasing pH. Aleem (1978) has not only reported a similar pH dependent redox potential change for cytochrome a_1, but he further observed an analogous change in the midpoint potential of nitrite/nitrate in the presence of *Nitrobacter* cell-free extracts. The E_m of nitrite became more positive from pH 9.0 (E_m= 328 mV) to pH 6.8 (E_m = 414 mV). He therefore concluded that the reduction of cytochrome a_1 by nitrite posed no thermodynamic barrier, whereas that of cytochrome *c* did contrary to the findings of O'Kelley *et al.* (1970).

Recent studies suggested that the rate of nitrite oxidation by electron transport particles was directly related to the value of electrical potential of the membrane, $\Delta\psi$, but the NADH oxidation could be stimulated by the conditions which reduced both $\Delta\psi$ and ΔpH components of the proton motive force (Cobley 1976a).

A satisfactory mechanism of nitrite oxidation has to explain the following observations:

1. ATP induced changes in the mid-point potential of cytochrome *c* (Ingledew and Chappell 1975). 2. pH dependent redox potential of cytochrome a_1 (Ingledew *et al.* 1974) and NO_2^- (Aleem 1978), 3. the effect of

temperature on the reduction of cytochromes *c* and *a* (Van Gool and Landelout 1967) and 4. the energy independent reduction of cytochrome *c* (O'Kelley 1970).

Nitrite oxidation supplies both energy as ATP and reducing power as NADH for the chemosynthetic bacterium, *Nitrobacter*. Because of the redox potentials of NO_3^-/NO_2^- couple and NAD^+/NADH couple ($E_{m,7}$ - 0.32 V), the direct reduction of NAD^+ by nitrite is thermodynamically impossible unless energy is provided.

The first experimental evidence for NAD^+ reduction coupled to nitrite oxidation was offered by Kiesow (1963). He found that the NAD^+ reduction by nitrite in *Nitrobacter* cell-free preparations was inhibited by an uncoupler, 2,4 dinitrophenol (Kiesow 1964). Similar observations were reported independently by Aleem *et al.* (1963) and it was proposed that the reduction process of NAD^+ in *Nitrobacter* was energy-dependent, involving the reversed electron flow from reduced cytochrome *c*. The amount of energy required for the reduction of NAD^+ by nitrite was 2 ATP according to Kiesow (1967) and 4-6 ATP according to Sewell and Aleem (1969).

NADH oxidation by cell-free extracts of *Nitrobacter* was coupled to ATP synthesis aerobically, or anaerobically in the presence of nitrate (Kiesow 1964). A membrane fraction from *Nitrobacter*, so-called nitrite oxidase particles, oxidized NADH with a P/O ratio of 2.0 (Aleem 1968). On the basis of inhibitor effects on the oxidation of NADH, it was concluded that the electron transport chain of the chemosynthetic bacteria, *Nitrobacter* was analogous to the mitochondrial system (Aleem 1968). Justification for such a proposal requires the

isolation and identification of individual components involved in NADH oxidation.

2.3 CYTOCHROMES

Cytochromes are defined as haemoproteins whose principal biological function is electron and/or hydrogen transport by virtue of a reversible valency change of their haem iron (Commission Enzyme on the International Union of Biochemistry 1961). Our knowledge about cytochromes developed from the preliminary observations of MacMunn (1886) during his spectroscopic studies of mammalian tissue. His discovery of a colored pigment different from haemoglobin or myoglobin was criticized by the fellow workers (Hoppe-Seyler 1890, Levy 1889) and was overlooked until 1925, when the skillful observations of Keilin (1925) confirmed MacMunn's original findings. He found that the new compound was intimately related to cellular respiration and thus named it cytochrome. He also revealed that the four banded absorption spectrum of the "cytochrome" was in fact due to different compounds which he called cytochromes *a*, *b* and *c*. Initially, cytochromes were believed to be present only in aerobic organisms. Their later discovery in anaerobic organisms (Ishimoto and Koyama 1954, Kamen and Vernon 1954, Vernon and Kamen 1954) made it clear that cytochromes function in almost all types of organisms.

Cytochromes of *Nitrobacter* were first reported by Lees and Simpson (1957).

2.3.1 *a*-type cytochromes: Cytochromes of *a*-type as well as *c*-type were first implicated in nitrite oxidation of *Nitrobacter* (Lees and Simpson 1957). Later observations confirmed these results (Zavarzin