Analysis and modelling of respiratory metabolism in *Neisseria meningitidis*

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Abstract

Contents

List of Figures

List of Tables

Acknowledgements

Chapter 1

Introduction

1.1 Biology and pathology of *Neisseria meningitidis*

Neisseria meningitidis is a Gram-negative, bean-shaped diplococcal bacteria[1], surrounded by a lipid membrane containing outer membrane proteins and lipopolysaccharides[1]. When pathogenic, the bacteria also has a polysaccharide capsule attached to the membrane[1]. It is non-spore forming, non-motile but piliated, and lives as a parasite, with humans being its only host[2]. *N. meningitidis* inhabits the mucosal membranes primarily in the respiratory tract, and it is estimated that up to 20-25% of the population have this bacteria in their nasopharynx while being asymptomatic[2–4].

The *Neisseria* genus contains a number of non-pathogenic species which are part of the normal human flora including *N. subflava*, *N. flavescens* and *N. lactamica*. Two species of *Neisseria* are the causative agents of human diseases, *N. meningitidis*, which causes bacterial meningitis and *N. gonorrhoea* which causes gonorrhoea. Being β-proteobacteria[2], the *Neisseria* genus is also related to a number of other pathogenic bacteria including *Bordetella* and *Burkholderia*. This taxa also includes nitrogen-fixing bacteria such as *Nitrosomonas*[5].

N. meningitidis is classified into 13 different serogroups based on the differences in lipopolysaccharides, capsules, outer membrane proteins and adhesion molecules[1,2,6]. 3 of these 13 serogroups are the main cause of meningococcal meningitis, with serogroups B and C being the most prevalent[1]. Vaccines for Serogroup C are available, but serogroup B currently has no effective vaccine, as it mimics human antigens[2]. In addition to being the causative agent for meningococcal meningitis, *N. meningitidis* also causes septicaemia and the combination has a mortality rate of 10%[1,2].

Meningitis is caused by *N. meningitidis* entering the bloodstream and travelling to the meninges, a set of membranes that envelope the central nervous system, where the bacteria goes on to cause inflammation. Once it has entered the bloodstream, *N. meningitidis* is capable of switching its capsule by phase-variation to avoid host-immune detection[7,8]. After colonisation by the bacterium, in order to enter the bloodstream, it must first adhere to the mucosal tissue. This is facilitated by adhesion molecules on the outer membrane and by pili, with the latter being the primary source of adhesion[1,6]. Once the bacteria are adhered to the mucosal cells, additional contacts are made with the outer membrane

proteins. Interestingly, the presence of the polysaccharide capsule, which is required for survival in the bloodstream, interferes with these additional contacts[2]. *N. meningitidis* invades the bloodstream by being endocytosed by the mucosal epithelial cells, a process which is triggered by the pili and outer membrane proteins on the bacteria.

N. meningitidis is able to survive in the bloodstream (typically an antimicrobial environment) mainly by virtue of its polysaccharide capsule as this is able to protect the bacteria against various immune responses by the host including complement-mediated bacteriolysis and phagocytosis by neutrophils[1]. Despite these protective features, specific antibodies *do* provide full protection against the bacteria, but the time taken for these antibodies to be produced means that the host has a period of at least 1 week in which it must rely on innate immune response[1]. Evidence suggests that systemic infection by *N. meningitidis* can only occur in hosts which are immunocompromised in some way, specifically if they do not have the serum bactericidal antibodies against capsular or non-capsular antigens, or they are missing certain complement components[4]. A number of factors can increase the likelihood of contracting bacterial meningitis including smoking and travelling to epidemic regions[2]. In developed countries, the highest rates of invasive meningococcal meningitis are seen in infants and children less than 4 years-old, adolescents, military recruits and groups where crowding and new exposures occur such as college students living in dormitories, however the disease is capable of affecting all age groups[2].

There is evidence to suggest that much of the damage done to the host during a meningococcal infection is actually caused by the host in an attempt to rid itself of the bacteria[9]. A systemic infection causes a massive inflammatory response and the resulting quantities of cytokines produced eventually lead to organ dysfunction and the proteases produced by neutrophil activation also lead to endothelial injury[9].

Once *N. meningitidis* has entered the bloodstream, it goes on to invade the cerebrospinal fluid (CSF), which serves as an excellent culture medium for the bacteria[4]. The host response to this infection is inflammation of the meninges, the membranes surrounding the central nervous system. This leads to a build-up of serous fluid in the brain causing cerebral swelling. Once the bacteria have entered the CSF, antimicrobial treatment is required otherwise the effects are almost invariably fatal[4].

Initially a meningococcal infection presents as a slight fever and chills, which may improve after 4-6 hours. Hemorrhagic skins lesions may appears between 8 and 18 hours, however roughly 20% of suffers never present with lesions. These skin lesions are possibly the most well known symptom of bacterial meningitis as they are characterised as a non-blanching (does not turn white under mild pressure) rash. The clearest evidence for meningococcal infection is a fever, stiff neck, aversion to bright light, vomiting, skin lesions and headaches. Unfortunately not all these symptoms may be present in all cases[4].

When meningococcal septicaemia occurs, renal function may be impaired as a direct consequence of cardiac impairment. Septicaemia causes "capillary leak" which reduces cardiac output and increases the effort required to breathe normally. Reduced cardiac output can also affect the gastrointestinal tract leading to reduced function. Once treated

these symptoms will usually subside as cardiac output improves[9].

In most cases the treatment for meningococcal meningitis is with antibiotics, where the primary aim is to achieve a rapid bactericidal effect in the CSF[1]. This treatment is suggested prior to positive identification of cultures of the bacteria obtained from the CSF as any delay is potentially life-threatening if the bacteria have indeed invaded the CSF[4].

1.2 Organisation of the respiratory chain of *N. meningitidis*

N. meningitidis is classified as an aerobe and as such has an oxidase pathway for reducing oxygen (O_2), but given that the environment in the nasopharynx is poor in oxygen, the bacteria must also be capable of respiring in a microaerobic environment. This is evidenced by the fact that bacterial isolates from the nasopharynx routinely contain both strict aerobes and strict anaerobes[10]. Genomic analysis of 2 strains of *N. meningitidis* shows that there are 3 terminal oxidases; 1 of each for reducing oxygen, nitrite (NO_2^-) and nitric oxide (NO)[11]. This analysis may be expanded as there are now many more genomes published. Experiments showed that under oxygen limiting conditions, *N. meningitidis* was capable of growth when nitrite was present in the media (Muller-Hinton Broth), and that nitrate (NO_3^-), the probable source for nitrite, had no effect on growth[11]. Additionally the bacteria require carbon dioxide, as shown by Tuttle and Scherp[12] and have 2 enzymes which catalyse the reduction of CO_2[4].

In vivo, nitrite is obtained as a product of digesting nitrate in food. There are a number of nitrate reducing enzymes present in the mouth and pharynx responsible for this[10]. Nitrite is also created by oxidation of nitric oxide, which is produced as a host signalling molecule and as a toxin as part of the host immune response[10,13].

The respiratory pathway for reducing nitrite in *N. meningitidis* involves 2 steps; nitrite is reduced to nitric oxide, which is then further reduced to nitrous oxide. This represents incomplete reduction, as a further reduction step would reduce nitrous oxide to dinitrogen gas[10,14].

Reduction of oxygen is favourable over nitrite reduction due to the redox potential differences. The redox potential of O_2/H_2O is $+820mV$, NO_2^-/NO is $+348mV$, thus O_2 has a higher tendency to acquire electrons resulting in a electrochemically favourable reaction[15]. The electron flow towards the oxidase is also preferred physiologically as it liberates more energy by virtue of the translocation of more protons than the reduction of nitrite. The translocated protons are ultimately used in the synthesis of ATP molecules for energy. This results in reduction of oxygen in preference to nitrite when both are present (in most cases).

Reduction of oxygen in *N. meningitidis* is carried out by the oxygen reductase (oxidase) cytochrome cbb_3, a membrane-bound heme-copper oxidase[16]. cbb_3 is capable of binding oxygen and nitric oxide, which means that during nitrite reduction (denitrification), the oxidase can be competitively inhibited (chemically) by the intermediate product of denitrification. cbb_3 can be permanently damaged at high concentrations of NO and O_2, as they can both bind at the cbb_3 active site and react together to form peroxyni-

trite[17–19].

Nitrite is reduced by the nitrite reductase AniA, which is a copper containing reductase. This reduction does not involve translocation of protons, and thus does not produce any useable energy. Nitrite is reduced to nitric oxide which can then be further reduced by a nitric oxide reductase NorB. Since *N. meningitidis* is capable of reducing nitric oxide, a host toxin, directly, this may help it defend itself against part of the host immune response[10,20] as has been shown in tissue culture by Anjum et al.[19].

The reduction processes carried out by these enzymes are shown in the table in Table 1.2.

Reduction			**Enzyme**
NO_2^-	$\rightarrow$	NO	AniA
NO	$\rightarrow$	N_2O	NorB
O_2	$\rightarrow$	H_2O	*cbb*$_3$

Table 1.1: The reductions catalysed by the respiratory enzymes in *N. meningitidis*

The major source for electrons in both respiratory pathways is NADH, although electrons can also be obtained from pyruvate and lactate amongst others. These reduced substrates lead to reduction of ubiquinone to ubiquinol in the ubiquinone pool that exists within the bacteria. Ubiquinol is oxidised either by the cytochrome *bc*$_1$ complex or directly by the NorB enzyme whilst reducing NO to N_2O. Cytochrome *bc*$_1$ is oxidised by a number of intermediate cytochromes which act to transport electrons to the terminal oxidases; AniA and *cbb*$_3$. The c_5 cytochrome transports electrons from the *bc*$_1$ complex to AniA, and two cytochromes, $c_{2/x}$ and c_4, transport electrons to *cbb*$_3$. It is not understood why *cbb*$_3$ has 2 alternate cytochromes, and there is evidence to suggest that it can also be supplied, in a limited capacity, by the c_5 cytochrome as well[21]. The electron transport chain is shown graphically in Figure1.1.

In addition to the difference in favourability between the two respiratory pathways, there is also a great deal of regulation, both at the enzymatic and transcriptional level. Chemical inhibition also plays a part in regulation as briefly mentioned previously. Expression of AniA is regulated by two processes, the reduction of oxygen and the presence of nitrite. The presence of oxygen down-regulates the expression of an activator of AniA expression. This activator is FNR (fumarate and nitrate reduction regulator), and the presence of oxygen effectively means that AniA expression is repressed by the reduced expression of FNR. In *N. meningitidis*, FNR appears to work slightly differently than in facultative anaerobes such as *E. coli*, in that FNR is still expressed at quite high concentrations of oxygen, and is itself down-regulated by a separate co-factor[22].

The presence of nitrite triggers the two component NarP/NarQ system which activates expression of AniA in response to increasing levels of nitrite[10]. The activity of AniA is also controlled by the competition for electrons by the other reductase enzymes in the respiratory chain. Both NorB and *cbb*$_3$ have a higher affinity for electrons than AniA, and as a result the presence of these enzymes (when active) has an inhibitory effect on AniA. The regulation of AniA is further complicated by the production of nitric oxide, and the

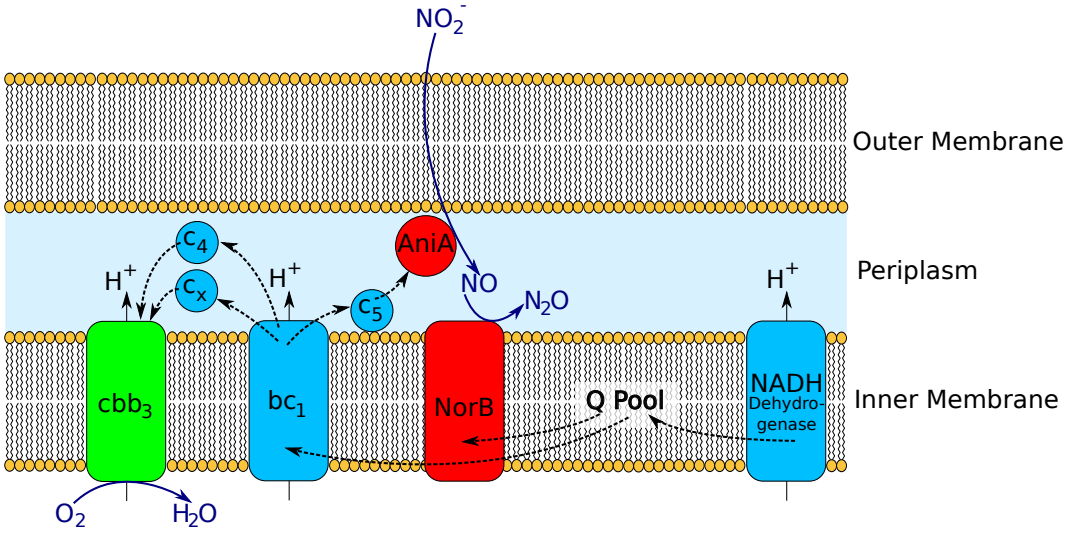

Figure 1.1: Layout of the components of the respiratory system in *Neisseria meningitidis*. Oxygen reducing components are shown in green, nitrogen reducing components in red. Components transporting electrons are coloured light blue, and their transport is indicated by dashed arrows. Respiratory substrates are shown in dark blue, with corresponding arrows linking them to their reducing enzymes. Components which produce membrane potential are also indicated.

presence of a protein, NsrR.

Nitric oxide has a direct inhibitory effect on the expression of AniA, as does the NsrR protein. Nitric oxide also inhibits the NsrR protein, leading to a de-represssion of AniA[20]. In the absence of nitric oxide, AniA is almost fully repressed by active NsrR. As NO concentrations increase, NsrR is inactivated allowing full activation of AniA. Once NO reaches a sufficiently high level it will begin to inhibit AniA[10,22].

NorB is less tightly regulated by respiratory components, as it is only acted upon by NsrR, however it is regulated by FNR and ArsR outside the respiratory chain[23]. This regulation by NsrR works in a similar way to how NsrR acts upon AniA. When there is no nitric oxide present, the NsrR acts to inhibit NorB since there is no substrate for it to reduce. In the presence of nitric oxide, NsrR is inhibited, leading to the activation of NorB which is now able to reduce NO to N_2O. In this case nitric oxide is acting as a de-repressor of NorB.

This complicated set of regulatory relationships between the different components of the respiratory pathways is shown in Figure 1.2.

1.3 Modelling

A limited amount of modelling has been carried out on bacterial respiratory chains, these focused on the denitrification pathway and treated the pathway as a simple electrical circuit[24]. An alternative approach involved modeling respiration using "P systems" which are probabilistic models of events. This assigned a probability of each reaction happening, dependant on the state of the system and then iterated through a given set of steps evaluating probabilities and altering values based on the outcome[25]. This approach to modelling was limited in that it was only predicting the quantities of 1 component in

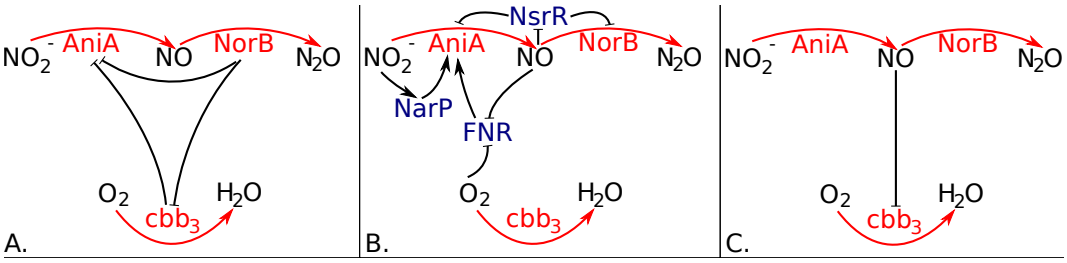

Figure 1.2: Regulation of respiratory components in *Neisseria meningitidis*. Enzymes and enzymatic reactions are shown in red. *A.* describes the regulation caused by competetion for electrons between the respiratory enzymes. *B.* shows the genetic regulation, which also involves a number of additional components in dark blue. *C.* shows chemical inhibition of the respiratory components.

each of 2 "compartments"; oxygen in the cell membrane and carbon dioxide in the thylakoid membrane (the model was developed using cyanobacteria).

Since when modelling respiration in a cell, the most important factor is the change in concentration of components over time without any particular spatial constraints, ordinary differential equations (ODEs) are an appropriate technique. In these systems the model does not change with regard to the spatial arrangement of any of the components. If the system requires changes in time *and* space, then partial differential equations (PDEs) would be necessary (and more complicated)[26].

Ordinary differential equations only depend on one variable; the time (t). In this case, the change in concentration over time for each component can be modelled as a single differential equation. For multiple components this leads to multiple differential equations with some that rely on the result of another (if the rate of one reaction is directly related to the concentration of another component). These ODEs must then be solved in parallel at a suitable timescale.

Complications arise when using differential equations if the processes are considered to be stochastic, as a differential equation model assumes that every component can have a continuous value, which is not the case as molecules are discrete. However if the system being modeled is sufficiently large, this effect can be ignored. If the reaction component size is small ($<$ 100s of molecules) stochastic simulation algorithms have to be used as described by Gillespie[27]. This method requires far more computation than solving ODEs, as the model will spend most of its time calculating values for reactions involving large molecules even though this is not necessary as the reaction is not stochastic. Additionally, the time interval used between reaction steps is usually very small, meaning the simulation progresses slowly[26].

A number of software packages exist that are capable of this type of modeling such as the Systems Biology Workbench[28] and COPASI[29]. These allow you to enter biochemical reactions in a format familiar to biologists, and have pre-defined libraries for types of reactions such as mass-action, or one with Michaelas-Menton kinetics etc. The mathematical equations are then derived automatically from the reactions and can be modified by hand if necessary. Parameters for the mathematical equations must be entered, and these will usually be derived from experimental data, or in some cases educated guesses (at

least initially). Once a parameter set has been created, the modelling software can run a time-course using a relevant solver-algorithm. COPASI includes 4 solvers, LSODA (Livermore Solver for Ordinary Differential Equations)[30] for deterministic systems (such as ODEs), Gibson-Bruck[31] for stochastic systems and Runge-Kutta and LSODA for hybrid systems (where portions are not considered to be stochastic).

Chapter 2

Materials and Methods

Chapter 3

Parameter Estimation Methodologies

Chapter 4

Model - Construction and Parameters

4.1 Construction

4.1.1 Converting Biological Reactions into Differential Equations

4.1.2 Assumptions and their Justifications

4.2 Parameters

Chapter 5

Oxygen reduction in *N. meningitidis*

5.1 Aerobic reduction of Oxygen

5.1.1 Introduction

5.1.2 Results

5.1.3 Discussion

Chapter 6

Nitric Oxide Reduction in *N. meningitidis*

Chapter 7

Nitrite Reduction in *N. meningitidis*

Chapter 8

AniA and NorB expression in *N. meningitidis*

Chapter 9

The Completed Model

Appendix

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