

**STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF  
CYANOglobin: A PERIPHERAL MEMBRANE HEMOGLOBIN IN *NOSTOC  
COMMUNE* UTEX 584 (CYANOBACTERIA)**

Marc V. Thorsteinsson

Dissertation submitted to the Faculty of the  
Virginia Polytechnic Institute and State University  
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY  
in  
Biochemistry

D. R. Bevan, Co-Chairman  
M. Potts, Co-Chairman  
J. L. Hess  
R. E. Ebel  
R. F. Helm

Keywords: Cyanobacteria, hemoglobin, oxygen, nitrogen fixation

December 3, 1997  
Blacksburg, Virginia

Copyright 1997, Marc V. Thorsteinsson

**STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF  
CYANOGLLOBIN: A PERIPHERAL MEMBRANE HEMOGLOBIN IN *NOSTOC  
COMMUNE* UTEX 584 (CYANOBACTERIA)**

by

Marc Victor Thorsteinsson

David R. Bevan, Chairman

Department of Biochemistry

**ABSTRACT**

Investigations of the nitrogen fixing (*nif*) genes in the cyanobacterium *Nostoc commune* UTEX 584 revealed a gene encoding a hemoprotein, named cyanoglobin. The cyanoglobin gene was isolated and subcloned into *Escherichia coli* previously. Cyanoglobin possesses a high oxygen affinity. The study presented here investigated the functional role of cyanoglobin, and encompassed the determination of the kinetic basis for the high oxygen affinity of cyanoglobin through kinetic studies utilizing stopped-flow spectrophotometry and flash photolysis. In addition, studies of cyanoglobin, in the presence of a variety of ligands, employed as structural probes of the distal pocket architecture, are presented. These data are interpreted in terms of structural models of cyanoglobin produced by homology modelling and hemoglobins with known crystal structures. Cyanoglobin coordinated oxygen and a variety of ligands with high rates of association, which explained the high oxygen affinity of cyanoglobin. Cyanoglobin possessed high rates of autoxidation and heme loss. The ligand binding behavior of cyanoglobin was more similar to leghemoglobin than to sperm whale myoglobin. The ligand binding behavior of cyanoglobin is explained in terms of a highly reactive, and solvent exposed, heme-iron. The 5' region of *glbN* interacted with NtcA, the global regulator of nitrogen metabolism in cyanobacteria, which may provide an indication of the nitrogen deprivation signal required for cyanoglobin expression *in vivo*. Finally, the isolation and N-terminal sequencing of a potential cyanoglobin homolog in *Anabaena* sp. strain PCC 7120 is presented. Collectively, the data obtained in this study may support the model of cyanoglobin function described by Hill, et al., that cyanoglobin sequesters oxygen, and presents it to, or is a part of, a terminal cytochrome oxidase complex in

*Nostoc commune* UTEX 584 under microaerobic conditions, when nitrogen fixation, and thus ATP demand, is maximal.



## Acknowledgements

The individuals personally involved with the success of my career are too many to mention here, which underscores the fact that science cannot be accomplished alone. Therefore I thank each and every person in the department of biochemistry at Virginia Tech. Specific mention of appreciation go all of my committee members: Dr. David Bevan, Dr Malcolm Potts, Dr. John Hess, Dr. Richard Ebel, and Dr. Richard Helm. I would also like to thank the members of Dr. Newton's lab for allowing me use of their diode array spectrophotometer.

I would like to personally thank Dr. Dennis Dean, who availed to me free reign of his laboratory facilities, which were instrumental to the success of many of my experiments. I would also like to personally thank the entire Dean lab group, who made me feel at home in their lab, and simply, were friends. I always enjoyed talking science and experiments of the day with Limin, Jason, Valerie, PJ, Len, Dee, and the many other students who have worked in the Dean lab.

Lastly, I would like to thank my collaborators: Dr. Richard Weber at the University of Aarhus, Denmark, Dr. Brian Hales at Louisiana State University, Dr. John Olson at Rice University, and Dr. Gerd LaMar at the University of California at Davis. My gratitude for their interest in cyanoglobin, and their efforts, cannot be overstated.

The work presented here is dedicated to my father and his model of integrity and industry; to believe that work is good, labor is better. My father was the philosopher, who encouraged me to be curious about life and nature. For this, I owe him a great debt.

## List of Abbreviations

<b>EDTA</b> - ethylenediaminetetraacetate	<b>O<sub>2</sub></b> - oxygen
<b>DTT</b> - dithiothreitol	<b>CO</b> - carbon monoxide
<b>MWCO</b> - molecular weight cut-off	<b>NO</b> - nitric oxide
<b>PVDF</b> - polyvinylidene difluoride	<b>R-NC</b> - alkylisonitrile
<b>PCR</b> - polymerase chain reaction	<b>ORF</b> - open reading frame
<b>nif</b> - nitrogen fixing gene(s)	<b>WT</b> - wild type
<b>FixL</b> - hemoprotein of <i>Rhizobium meliloti</i>	<b>LB</b> - Luria-Bertani media
<b>FixJ</b> - response regulator of <i>Rhizobium meliloti</i>	<b>ATP</b> - adenosine triphosphate
<b>DNA</b> - deoxyribonucleic acid	<b>kD</b> - kilodalton
<b>mRNA</b> - messenger ribonucleic acid	<b>glbN</b> - cyanoglobin gene
<b>vhb</b> - <i>Vitreoscilla</i> hemoglobin gene	<b>CD</b> - circular dichroism
<b>Hmp</b> - hemoprotein of <i>E. coli</i> K-12	<b>PITC</b> - phenylisothiocyanate
<b>NMR</b> - Nuclear magnetic resonance	<b>k<sub>obs</sub></b> - observed rate constant
<b>EPR</b> - electron paramagnetic resonance	<b>k'<sub>x</sub></b> - association rate constant of X
<b>TAE</b> - Tris/acetate/EDTA buffer	<b>k<sub>x</sub></b> - dissociation rate constant of X
<b>TE</b> - Tris/EDTA buffer	<b>K<sub>x</sub></b> - calculated affinity of X ( $k'_x/k_x$ )
<b>TBST</b> - Tris-buffered saline with 0.05% Tween 20	<b>TBS</b> - Tris-buffered saline
<b>IPTG</b> - isopropylthio-B-D-galactopyranoside	
<b>CAPS</b> - (3-[cyclohexylamino]-1-propanesulfonic acid)	
<b>MES</b> - [2-(N-morpholino)]ethanesulfonic acid	
<b>Tris.HCl</b> - Tris(hydroxymethyl)aminomethane hydrochloride	
<b>SDS-PAGE</b> - sodium dodecyl sulfate-polyacrylamide gel electrophoresis	
<b>FPLC</b> - Fast protein liquid chromatography	
<b>HPLC</b> - High pressure liquid chromatography	
<b>NtcA</b> - <u>N</u> itrogen <u>c</u> ontrol protein in cyanobacteria	
<b>FNR</b> - <u>F</u> umarate and <u>n</u> itrate reductase <u>r</u> egulator protein	
<b>PDB</b> - Protein Data Bank	

## Table of Contents

Abstract . . . . .	. ii
Acknowledgements . . . . .	.iv
List of Abbreviations . . . . .	.v
Table of Contents . . . . .	.vi
List of Figures . . . . .	viii
List of Tables . . . . .	.xi
Introduction . . . . .	. 1
Part I. Cyanobacteria and cyanoglobin. . . . .	. 1
Part II. Bacterial hemoglobins . . . . .	. 7
Part III. The electronic and structural basis for ligand binding to hemoglobins . . . . .	. 9
Part IV. Nitrogen control in bacteria . . . . .	. 19
Part V. Specific aims and significance . . . . .	. 21
Materials and Methods . . . . .	. 24
Bacterial strains and plasmids. . . . .	. 24
Growth conditions for bacteria . . . . .	. 24
Large scale growth of <i>E. coli</i> (pGlbN) . . . . .	. 25
Isolation and purification of cyanoglobin . . . . .	. 26
Quantitation of heme in cyanoglobin . . . . .	. 28
Exogenous ligand binding to cyanoglobin . . . . .	. 29
Kinetics of Ligand Binding. . . . .	. 30

Homology modelling of cyanoglobin . . . . .	. 34
Analysis of the <i>nifU-glbN</i> intergenic region . . . . .	. 34
Characterization of the 18-kD immuno-reactive protein . . . . .	. 39
Results . . . . .	. 44
Isolation, purification, and quantitation of cyanoglobin . . . . .	. 44
Exogenous ligand binding to cyanoglobin . . . . .	. 46
Kinetics of ligand binding to cyanoglobin . . . . .	. 50
Homology modelling of cyanoglobin . . . . .	. 55
Analysis of the <i>nifU-glbN</i> intergenic region . . . . .	. 57
Characterization of the 18-kD immuno-reactive protein . . . . .	. 59
Discussion . . . . .	115
Isolation and purification of cyanoglobin . . . . .	115
Exogenous ligand binding to cyanoglobin . . . . .	115
Kinetics of ligand binding to cyanoglobin . . . . .	118
Homology modelling of cyanoglobin . . . . .	123
Analysis of the <i>nifU-glbN</i> intergenic region . . . . .	. 123
Characterization of the 18-kD immuno-reactive protein . . . . .	. 124
The structure and function of cyanoglobin . . . . .	. 125
References . . . . .	126
Vita. . . . .	135



## List of Figures

Fig. 1	Published crystal structure of met-sperm whale myoglobin and residues of the distal pocket. . . . .	. 64
Fig. 2	Cyanoglobin is expressed in <i>E. coli</i> (pGlbN) as the oxygenated form. . . . .	. 65
Fig. 3	Phenyl sepharose hydrophobic interaction chromatography discriminates between oxygenated and autoxidized cyanoglobin. . . . .	. 66
Fig. 4	Phenyl sepharose hydrophobic interaction chromatography discriminates between oxygenated and autoxidized cyanoglobin. . . . .	. 67
Fig. 5	Cyanoglobin can be purified in the oxygenated form. . . . .	. 68
Fig. 6	Cyanoglobin is expressed in <i>E. coli</i> (pGlbN) as a <i>b</i> type heme-containing protein. . . . .	. 69
Fig. 7	The Soret extinction coefficient of oxycyanoglobin, when compared to that of met-cyanoglobin, is unusual. . . . .	. 70
Fig. 8	Cyanoglobin coordinates cyanide at pH 7. . . . .	. 71
Fig. 9	Cyanoglobin coordinates azide at pH 7. . . . .	. 72
Fig. 10	Cyanoglobin coordinates fluoride at pH 7. . . . .	. 73
Fig. 11	Cyanoglobin coordinates the bulky ligand imidazole at pH 7. . . . .	. 74
Fig. 12	Cyanoglobin coordinates the bulky ligand nictotinate at pH 7. . . . .	. 75
Fig. 13	Cyanoglobin coordinates acetate at pH 7. . . . .	. 76
Fig. 14	Cyanoglobin coordinates acetate at pH 5.5. . . . .	. 77
Fig. 15	Cyanoglobin coordinates the bulky ligands methylisonitrile and ethylisonitrile at pH 7. . . . .	. 78

Fig. 16 Sperm whale myoglobin coordinates the bulky ligands methylisonitrile and ethylisonitrile at pH 7.	. . . . .	. 79
Fig. 17 The high-spin to low-spin transition in met-cyanoglobin occurs between pH 9 and pH 10.	. . . . .	. 80
Fig. 18 Cyanoglobin possesses an extremely fast rate of oxygen association.	. . . . .	. 81
Fig. 19 Cyanoglobin possesses an extremely fast rate of oxygen dissociation.	. . . . .	. 82
Fig. 20 Cyanoglobin possesses an extremely fast rate of CO association.	. . . . .	. 83
Fig. 21. Cyanoglobin possesses an extremely fast rate of CO association.	. . . . .	. 84
Fig. 22 Cyanoglobin possesses an extremely fast rate of alkylisonitrile association.	. . . . .	. 85
Fig. 23 Cyanoglobin possesses an extremely fast rate of alkylisonitrile association.	. . . . .	. 86
Fig. 24 Cyanoglobin possesses a relatively slow rate of alkylisonitrile dissociation.	. . . . .	. 87
Fig. 25 Cyanoglobin possesses an extremely fast rate of azide association.	. . . . .	. 88
Fig. 26 Oxycyanoglobin possesses a relatively fast rate of autoxidation to the met-form.	. . . . .	. 89
Fig. 27 The fast rate of autoxidation of oxycyanoglobin is pH dependent.	. . . . .	. 90
Fig. 28 Cyanoglobin possesses an extremely fast rate of hemin loss.	. . . . .	. 91
Fig. 29 Cyanoglobin possesses a three-on-three helical fold (globin fold).	. . . . .	. 92

Fig. 30. The MODELLER program provides meaningful homology models. . . . .	93
Fig. 31. Cyanoglobin possesses a three-on-three helical fold (globin fold) . . . . .	94
Fig. 32. Cyanoglobin possesses a relatively solvent-exposed heme moiety. . . . .	95
Fig. 33. Ramachandran analysis of the modelled structures of <i>Aplysia</i> myoglobin and cyanoglobin. . . . .	96
Fig. 34 The <i>nifU-glbN</i> intergenic region contains extensive sequence similarity with the promoter region of <i>glnA</i> (glutamine synthase).___ . . . . .	97
Fig. 35 Isolation and cloning of DNA fragments containing the <i>nifU-glbN</i> intergenic region. . . . .	98
Fig. 36 The overexpression of NtcA in <i>E. coli</i> (pCSI26). . . . .	99
Fig. 37 The <i>nifU-glbN</i> intergenic region interacts with NtcA. . . . .	100
Fig. 38 The 18 kD immuno-reactive protein is localized in the cell membrane in <i>Anabaena</i> 7120.. . . . .	101
Fig. 39 The 18 kD immuno-reactive protein is a peripheral membrane protein. . . . .	102
Fig. 40 The 18 kD immuno-reactive protein is not a phycobiliprotein. . . . .	103
Fig. 41 The 18 kD immuno-reactive protein migrates as a triplet. . . . .	104
Fig. 42 The 18 kD immuno-reactive protein exists as only one isoform. . . . .	105
Fig. 43 The 18 kD immuno-reactive protein is the large (50S) ribosomal subunit L12 protein homolog in <i>Anabaena</i> 7120. . . . .	106
Fig. 44 The 18 kD immuno-reactive protein is specifically degraded to 14kD as a result of cathepsin C treatment. . . . .	107
Fig. 45 Models of genetic regulation of <i>glbN</i> expression by NtcA. . . . .	108

## List of Tables

Table 1	Listing of bacterial strains and plasmids used in this study. . . . .	109
Table 2	Cyanoglobin exists as a monomer in solution even at high concentrations. . . . .	110
Table 3	The absorbance maxima and extinction coefficients of cyanoglobin derivatives. . . . .	111
Table 4	Kinetic parameters of gaseous ligand binding to cyanoglobin and selected hemoglobins and myoglobins.. . . .	112
Table 5	Kinetic parameters of alkylisonitrile ligand binding to cyanoglobin and selected hemoglobins and myoglobins. . . . .	113
Table 6	Kinetic parameters of azide ligand binding, autoxidation, and hemin loss in cyanoglobin and selected hemoglobins and myoglobins. . . . .	114