PARTIAL STRUCTURAL CHARACTERIZATION OF THE CYTOPLASMIC HEMOGLOBIN OF *NOSTOC COMMUNE* UTEX 584 EXPRESSED IN *ESCHERICHIA COLI*

by

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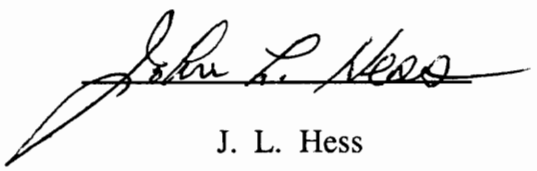

J. L. Hess

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ABSTRACT

Investigations into the nitrogen fixing apparatus in cyanobacterium *Nostoc commune* revealed a gene encoding for a hemoprotein, known as cyanoglobin. The cyanoglobin gene was isolated and subcloned into *Escherichia coli* previously.

The study presented here encompasses the optimization of growth conditions for the transformed *E. coli,* with subsequent induction of cyanoglobin synthesis. These conditions were applied to large-scale (24-l) fermentor culture, permitting purification of approximately 200 mg cyanoglobin. Structural analyses, including absorption spectroscopy and circular dichroism, are presented.

These studies indicate that cyanoglobin is a cytoplasmic hemoglobin with properties quite unlike those of leghemoglobin *a* and sperm whale myoglobin, which are used as references of comparison. For example, the optical spectral properties of oxycyanoglobin are different from those of leghemoglobin *a* and sperm whale myoglobin. In addition, the met-form of cyanoglobin has characteristics of a low-spin hemoglobin, in contrast to the high-spin met-forms of sperm whale myoglobin and leghemoglobin *a*. Unusually, the met-

form of cyanoglobin fails to coordinate the strong-field ligands, cyanide and azide, at pH 7 and pH 9. The Soret region circular dichroism (CD) spectrum of cyanoglobin is unlike that of sperm whale myoglobin, yet is very similar to leghemoglobin *a*, suggesting a similar heme environment in these two hemoproteins. Far-UV CD of cyanoglobin revealed alpha-helical character comparable to that of sperm whale myoglobin and leghemoglobin *a*. Cyanoglobin is the first monomeric hemoglobin detected in a prokaryote, raising questions concerning a possible role of cyanoglobin in early globin gene evolution.

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List Of Abbreviations

IPTG - isopropyl-β-D-thiogalactopyranoside

Tris.HCl - Tris(hydroxymethyl)aminomethane hydrochloride

EDTA - ethylenediaminetetraacetate

δ-ALA - delta-aminolevulinic acid

DTT - dithiothreitol

MWCO - molecular weight cut-off

DAB - diaminobenzidine

TBS - Tris-buffered saline

TBST - Tris-buffered saline with 0.05% Tween 20

PVDF - polyvinylidene difluoride

CAPS - (3-[cyclohexylamino]-1-propanesulfonic acid)

AUFS - absorbance units full-scale

SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis

PCR - polymerase chain reaction

ORF - open reading frame

nif - nitrogen fixing gene(s)

Fix L - hemoprotein of *Rhizobium meliloti*

Fix J - phosphokinase of *Rhizobium meliloti*

mRNA - messenger ribonucleic acid

vhb - *Vitreoscilla* hemoglobin gene

Hmp - hemoprotein of *E. coli* K-12

LB - Luria-Bertani media

MES - [2-(N-morpholino)ethanesulfonic acid

ESR - electron spin resonance

kD - kilodalton

ATP - adenosine triphosphate

glbN - cyanoglobin gene

CD - circular dichroism

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Introduction

The cyanobacteria, formerly classified as the blue-green algae, are a diverse group of cosmopolitan, photosynthetic prokaryotes that are believed to have been involved in the evolution of the Earth's oxygenic atmosphere (Schopf and Walter, 1982). A general feature of the cyanobacteria is their capacity to survive in extreme environments. Tolerance to such extremes is attributed to unique genetic responses that occur when water and nutrient availability is limiting. Cyanobacteria can be found in the Antarctic regions as well as the desert regions of Earth.

Nitrogen fixation and the cycling of global nitrogen by microorganisms are both ecologically and agriculturally important. The ammonia produced by nitrogenase is converted to glutamine and then to glutamate, via the glutamine synthetase-L-glutamine:2-oxoglutarate aminotransferase pathway, which is used as a precursor for further amino acid biosynthesis. The oxygen-labile nature of nitrogenase has long been established (Bothe, 1982). Inactivation of nitrogenase by oxygen arises from oxidation of the iron-sulphur center of the enzyme that is crucial for its activity. Certain filamentous cyanobacteria can fix nitrogen under both aerobic and anaerobic conditions. A common feature of these cyanobacteria is the formation of heterocysts in the absence of combined nitrogen. Approximately half of the filamentous, non-heterocystous cyanobacteria can fix nitrogen, but they do so only under anaerobic conditions (Stewart, 1978). The heterocyst, therefore, provides a spatial separation of the nitrogenase complex to avoid any deleterious effects of oxygen exposure during aerobic

growth (Rippka and Stanier, 1978). In effect, the "micro-anaerobic" environment that is sustained in the differentiated heterocyst permits nitrogen fixation to occur, concomitant with the oxygen-evolution that derives from the photosynthetic activities of adjacent vegetative cells.

In some strains of cyanobacteria, there also may be a temporal separation of photosynthetic respiration and nitrogen fixation activities. Such temporal separation of light (photosynthetic) and dark (nitrogen fixing) cycles has been postulated to occur in other non-heterocystous forms of cyanobacteria such as *Oscillatoria* sp. (Stal and Krumbein, 1985). Stal and Krumbein also presented evidence that would suggest both a high *de novo* synthesis of nitrogenase and an unknown mechanism of reversible modification of nitrogenase to an oxygen-stable form (Stal and Krumbein, 1985). Another mechanism of nitrogenase protection in cyanobacteria may involve oxygen scavenging by an oxygen-binding protein, hypothesized to occur in a particular species of *Nostoc* (Potts, et al. 1992).

Nostoc commune is a filamentous, heterocystous cyanobacterium that is particularly noted for its desiccation tolerance. Under nitrogen-limiting conditions, *N. commune* can fix nitrogen both aerobically and anaerobically. The nitrogen fixing (*nif*) gene cluster of *N. commune* UTEX 584 has been characterized (Defrancesco and Potts, 1988). The *nifUHD* cluster of this strain encodes the polypeptide products necessary for the functional activity of the nitrogenase complex. A *nifU*-like sequence is located approximately 1kb upstream from the *nifH* sequence (Defrancesco and Potts, 1988). Further investigations of this *nif* cluster revealed an open reading frame (ORF) between *nifU* and *nifH*. This ORF potentially encodes

a polypeptide of approximately 12 kilodaltons, with no similarity to any *nif* genes characterized thus far (Potts, et al. 1992). Restriction enzyme analysis permitted the isolation of the ORF fragment, and subsequent polymerase chain reaction (PCR) assays and subcloning of the insert into pT7-7 allowed transformation of *E. coli* BL21DE3.

Induction of recombinant protein synthesis in *E. coli* produced a 12.5 kilodalton hemoprotein, that has the capacity to bind carbon monoxide (Potts, et al. 1992). The recombinant hemoprotein isolated did not have significant amino acid sequence similarities to sperm whale myoglobin, the leghemoglobins, or the cytochromes. There was, however, significant amino acid sequence similarity to the protozoan cytoplasmic hemoglobins isolated from *Paramecium caudatum* and *Tetrahymena pyriformis* (Potts, et al. 1992). To reflect this similarity, the hemoprotein detected in *Nostoc commune* UTEX 584 was named cyanoglobin (Potts, et al. 1992). It was postulated initially that cyanoglobin may be involved in nitrogen fixation, specifically in the protection of the nitrogenase complex, because of its location within a *nif* cluster and the fact that the protein was synthesized in *Nostoc commune* under microaerobic, nitrogen-limiting conditions (Potts, et al. 1992).

The expression of cytoplasmic hemoglobins that function in the sequestering of free oxygen from the immediate vicinity of active nitrogenase occurs in nodulated legume roots (Appleby, 1984). The nitrogen fixing machinery in nodulated legume roots is provided in a symbiotic relationship with *Rhizobium* sp.. The requirement for high- oxygen flux to accommodate oxidative phosphorylation, concomitant with low oxygen tension for active nitrogenase synthesis in *Rhizobium* sp. is accomplished functionally by the leghemoglobins

(Appleby, 1984).

Oxygen equilibration experiments have shown that the leghemoglobins have an extremely high affinity for oxygen when in the ferrous form. The high oxygen affinity, which is critical for the biological function of the leghemoglobins, is the consequence of an extraordinarily fast oxygen combination rate constant and a moderately slow oxygen dissociation rate constant (Appleby, 1984). X-ray crystallography analyses of leghemoglobin *a* from soybean root nodules revealed that the oxygen binding pocket is more "open" than in other monomeric hemoglobins (Appleby, 1984). Furthermore, the "distal" histidine of leghemoglobin *a*, which is known to stabilize the bound dioxygen complex, was determined to be conformationally less restricted when compared to other monomeric hemoglobins that have a histidine in the distal position (Appleby, 1983). Circular dichroism studies suggest that the reorientations of amino acid side chains of leghemoglobin that occur in response to the binding of oxygen are significantly less than those experienced by sperm whale myoglobin (Nicola, 1975). Collectively, these data have been used to provide an explanation for the particularly high oxygen affinity of the leghemoglobins.

Plant cytoplasmic hemoglobins have also been detected in the actinorhizal nodules of *Casuarina* and *Myrica* sp. (Tjepkema and Asa, 1987). The heme content of the actinorhizal nodules is much lower than that of legume nodules. Although these hemoglobins can bind molecular oxygen, they are not considered as oxygen scavengers or as protectors of the nitrogenase complex because nitrogen fixation is maximal in actinorhizal root nodules at atmospheric oxygen tensions (Tjepkema and Asa, 1987). Current evidence suggests that the

actinorhizal hemoglobins may serve as an oxygen sensor(s), and may be involved in the shift between oxidative and fermentative plant metabolism (Silvester and Harris, 1990) The diazotrophic symbiont in actinorhizal root nodules is *Frankia* sp. Evidence suggests that a specialized, lignin-rich, thick cell-wall permits nitrogen fixation to occur in *Frankia* sp. *Frankia* sp., like heterocystous cyanobacteria, thus may provide a spatial barrier to the nitrogenase complex when growing at atmospheric oxygen tension (Silvester and Harris, 1990).

In addition to *Nostoc commune*, cytoplasmic hemoglobins have also been reported for other prokaryotic organisms. Free living *Rhizobium meliloti* contain a hemoprotein that is expressed under microaerobic growth conditions. Known as FixL, this hemoprotein was determined to bind molecular oxygen (Gilles-Gonzales, 1991). This protein is expressed from within the same operon as a protein known as FixJ. FixJ has been shown to possess kinase activity and is believed to act as an effector in the signal transduction pathway (Gilles-Gonzales, 1991). These two proteins are believed to act in concert to cause cellular changes via signal transduction when cells grow under microaerobic conditions. No evidence has been presented that would suggest a nitrogenase protection function for FixL. However, both of these proteins have been shown to regulate the expression of *nif* genes of *Rhizobium meliloti* in response to free oxygen (Gilles-Gonzales, 1991).

The most intensively studied prokaryotic cytoplasmic hemoglobin is that of the Gram-negative *Vitreoscilla* sp. Initially, this dimeric hemoprotein was thought to function as a terminal oxidase (Webster, 1988). Further studies demonstrated a reversible binding of

molecular oxygen by this protein, which is characteristic of a true cytoplasmic hemoglobin. *Vitreoscilla* hemoglobin shares significant amino acid sequence homology with all of the leghemoglobins and is thought to function as an oxygen storage protein because of the oxygen poor environments where *Vitreoscilla* spp. typically are found. Even though high amino acid sequence similarity exists with the leghemoglobins, *Vitreoscilla* hemoglobin possesses an extremely fast rate constant of oxygen dissociation (1000-fold higher than the leghemoglobins; Orri and Webster, 1986). Interestingly, the transcription from the promoter of the *Vitreoscilla* hemoglobin gene (*vhb*) is oxygen dependent (Khosla and Bailey, 1989). Increased levels of *vhb* mRNA transcripts are detected when *Vitreoscilla* spp. are grown under hypoxic conditions (Dikshit, 1989). In addition, *vhb* mRNA transcripts are virtually undetectable when *Vitreoscilla* spp. are grown under high oxygen tension (Dikshit, 1989). *E. coli* transformed with a plasmid that contains the *vhb* insert show enhanced growth characteristics under microaerobic conditions when compared to wild-type. Current studies suggest that the expression of *vhb* insert in *E. coli* increases the effective intracellular dissolved oxygen levels under microaerobic conditions, and therefore increases the relative activities of the terminal oxidases (Kallio et al., 1994). The result is a more efficient proton-pumping mechanism, leading to an increase in efficiency of ATP production.

A hemoglobin-like protein, Hmp, was detected in *E. coli* K-12 (Vasudevan, et al. 1991). Although it was not shown to be a true hemoglobin (defined as a protein that can reversibly bind oxygen), these workers demonstrated that Hmp could bind carbon monoxide. Hmp also has a dihydropteridine reductase activity. The reductase and putative oxygen

binding functions of Hmp are located on two different domains. The N-terminal domain of Hmp that putatively binds oxygen has significant amino acid sequence homology to the *Vitreoscilla* hemoglobin. At present, no function has been assigned to the *E. coli* K-12 Hmp.

Another hemoglobin with two domains, each with supposedly distinct functions, is the flavohemoglobin isolated from the lower eukaryote *Saccharomyces cerevisiae* (Zhu and Riggs, 1992). The protein reversibly binds dioxygen, and shows amino acid sequence similarities with *Vitreoscilla* hemoglobin and amino acid sequence similarities with other flavoproteins, such as ferredoxin reductase and cytochrome P-450 reductase (Zhu, 1992). Zhu and Riggs proposed a mechanism of oxygen binding and subsequent conformational changes within the protein that effect the activity of the reductase domain. These workers further postulated that the other bi-domain hemoproteins (of *E. coli* K-12 for example) may also function in a similar manner (Zhu, 1992).

Other cytoplasmic hemoglobins that have generated interest have been detected in two closely related protozoa *Paramecium caudatum* and *Tetrahymena pyrformis* (Tsubamoto et al., 1990 and Iwaasa et al., 1990). The protozoan hemoglobins share little amino acid sequence homology compared to the cytoplasmic hemoglobins isolated from other organisms, but share significant homology with each other and cyanoglobin. The protozoan hemoglobins are monomeric and are similar in size to cyanoglobin (Potts, et al. 1992). Although shown to reversibly bind oxygen, the spectral characteristics of the oxygenated protozoan hemoglobins, in the visible region, are unusual. The α/β peak ratio of the oxygenated forms of sperm whale myoglobin and the leghemoglobins are close to unity, whereas the α/β peak

ratio of the oxygenated forms of the protozoan hemoglobins is approximately 0.6. The met-form of the *Paramecium* hemoglobin is low-spin, similar to the met-form of cyanoglobin. Oxycyanoglobin has been observed qualitatively to have a relatively fast rate of autoxidation to the met-form (Potts, et al. 1992). Similarly, *Paramecium* hemoglobin has been shown to possess an autoxidation rate nearly 10-fold faster than sperm whale myoglobin (Tsubamoto, 1990).

With respect to the oxygen binding hemoproteins, the stabilization of the bound oxygen-heme complex is believed to occur at the distal side of heme iron. Interestingly, the protozoan hemoglobins and cyanoglobin have invariant glutamines at a position close to the putative distal heme binding site when aligned, and at least in the *Tetrahymena* hemoglobin, the glutamine has been postulated to act in place of the usual distal histidine found in sperm whale myoglobin and the leghemoglobins (Iwaasa, 1990). To date, no function has been assigned to the protozoan cytoplasmic hemoglobins or cyanoglobin.

The study presented in this paper involves the characterization of recombinant cyanoglobin. The optimization of the growth of *E. coli* BL21DE3 (pGlbN) and induction of cyanoglobin synthesis on the small-scale has allowed scale up to 24-l fermentor cultures. Approximately 200 mg of recombinant cyanoglobin was purified. A variety of conditions were used to attempt the crystallization of the recombinant cyanoglobin. The absorption and circular dichroism spectra of cyanoglobin, sperm whale myoglobin and leghemoglobin *a* are presented.

The structural characteristics of sperm whale myoglobin have been studied

exhaustively, which permits its use as a reference material in comparison to cyanoglobin. Leghemoglobin *a* is a plant hemoglobin that transports oxygen away from the site of nitrogenase activity. Nitrogenase is paramount to those organisms that rely upon its metabolic product, thus a protection mechanism must exist for nitrogen fixation activities during aerobic growth. Parallels between leghemoglobin *a* and cyanoglobin are emphasized and may reveal clues to the unknown relationship between cyanoglobin and nitrogenase in *Nostoc commune*.

The significant similarities between cyanoglobin and the protozoan hemoglobins embody a distinct group of hemoglobins including both prokaryotes and eukaryotes. Cyanoglobin is unique as a prokaryotic hemoglobin because it is monomeric and shows little sequence homology to the other prokaryotic hemoglobins studied thus far. Investigations into this unique group of oxygen-binding hemoproteins may help further unravel the enigma of the supposed eukaryotic link to the ancestral globin gene, from which all globin genes were derived.

Materials and Methods

Strains, Plasmids, and Growth Conditions

Escherichia coli strain BL21DE3 (pGlbN) was a gift from Dr. Malcolm Potts. The protocols used for *glbN* isolation and transformation of *E. coli* BL21DE3 have been described (Potts, et al. 1992). Actively growing cells were used as an inoculum for Luria-Bertani agar plates supplemented with 0.2 mg/ml ampicillin (sodium salt, Sigma; Sambrook et al., 1989) These plates were grown overnight at 37° C. Single colonies were subcultured to LB agar plates with ampicillin, and subcultured again to liquid LB medium (3 ml) with ampicillin. The liquid culture tubes were grown overnight, at 37° C, on a roller drum and then used as inocula for 250-ml liquid cultures using a 1% (v/v) inoculum. Conditions for growth were at 37° C with 270 rpm orbital shaking. Bacterial growth was monitored by turbidometry using the absorbance value at 550 nm versus time of incubation at 37° C. Logarithmic phase cells were then either processed for induction of cyanoglobin synthesis, or used as inocula for a 24-l preparative fermentor culture. The materials used for fermentor culture were scaled up proportionately. Growth conditions were identical to the small scale cultures except that the fermentor culture was sparged continually with sterile air, at 15 cc/min, and with stirring at 250 rpm.

Induction of Cyanoglobin Synthesis

Cells from the small scale growth were harvested by centrifugation in a chilled

GSA rotor, for 30 min, at 7000 x g. Cells from the fermentor growth were concentrated to 2 l using a Pellicon cell harvester with a 0.2-μm Millipore cassette. The cells in both cases were washed with prewarmed (37° C) M9 salts (Sambrook, 1989). The cells were then resuspended gently in prewarmed M9 salts with the following additions at their respective final concentrations: 0.2 mg/ml ampicillin, 0.1 mg/ml delta-aminolevulinic acid (sodium salt, Sigma), 1 μg/ml disodium magnesium EDTA, 12 μg/ml ferric ammonium citrate, and approximately 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The volume of the induction medium was one-half that of the growth medium. The culture was returned to orbital shaking or, in the case of the fermentor, was allowed to incubate with 250 rpm stirring and with sparging (5 cc/min) sterile air. The extent of induction of cyanoglobin synthesis was monitored by sonicating an aliquot (1 ml, aseptically withdrawn at timed intervals) of the induction culture five times for ten seconds each at 35% power, using a Fisher model 300 sonic dismembranator and allowing the material to cool on ice between each burst. The cell debris was removed by centrifugation for 5 minutes at 10,000 rpm in a Marathon microcentrifuge. The supernatant fraction was then diluted to 0.5 ml with 50 mM Tris.HCl pH 8.0. The ratio of A_{415nm}/A_{280nm} was used as a crude index of the presence of cyanoglobin and was measured on a Shimadzu spectrophotometer using 1-ml quartz cuvettes and diluent buffer as a reference. When this ratio no longer increased as a function of time, typically after six hours for the 125 ml induction culture, then induction was judged to be sufficient. The fermentor growth was allowed to induce for 15 hours under the conditions stated. This was sufficient time for cyanoglobin synthesis as judged by the deep scarlet red appearance of

the cells.

Cell Processing

Cells from both small scale and fermentor induction cultures were treated identically. The cells were pelleted by centrifugation, for 30 min, at 8000 x g in a chilled GSA rotor. The media were discarded, and the cells were washed with cold 50 mM Tris.HCl pH 7.5, and subsequently centrifuged again using the conditions stated previously. The cell paste was dispensed to a chilled mortar and then overlaid with a few ml of cold Tris.HCl pH 7.5 buffer that contained the following protease inhibitors: 5 mM EDTA, 0.5 mM DTT, 50 μM benzamidine, and 1 μg/ml leupeptin. Sterile alumina (Sigma) was added (approximately 2:1 ratio of alumina to cell paste) and the material was ground with a pestle for 5 min. Liquid nitrogen was added, allowed to evaporate, and then the material was ground until it thawed. More liquid nitrogen was added and the procedure was repeated to ensure adequate cell lysis. The liquid suspension was decanted to chilled Corex 30 centrifuge tubes and was centrifuged for 30 min, at 13,000 rpm, in a chilled SS-34 rotor. The scarlet red supernatant fraction was decanted and saved as the clarified cell lysate.

Purification of Cyanoglobin

Streptomycin sulfate (Sigma) was added to the clarified cell lysate to a final concentration of 0.2 mg/ml with stirring at 4° C for 20 minutes. The white precipitate that formed was removed by centrifugation. The scarlet red supernatant fraction was decanted

and saved. Enzyme grade ammonium sulfate (Fisher) was pulverized to a fine powder in a mortar and pestle and then added slowly to the supernatant fraction with stirring, overnight, at 4° C, to achieve 40% saturation (242 g/l). The straw colored material that precipitated was removed by centrifugation. The scarlet red supernatant was brought to 100% saturation with ammonium sulfate (457 g/l), with stirring, at 4° C for 3 hours. The deep scarlet red pellet was dissolved in a minimal volume of cold 50 mM Tris.HCl pH 8.0, 1 mM dithiothreitol (DDT), and was dialyzed against the same buffer twice, for 8 hours each, at 4° C. In analytical trials, ammonium sulfate removal from the pellets required 1 l of dialysis buffer, whereas pellets of the preparative material required 6 l of dialysis buffer. All dialyses used SpectroPor cellulose membranes with a molecular weight cutoff (MWCO) of 3.5 kilodalton (Spectrum, Houston, TX).

The dialyzed material was then concentrated under nitrogen pressure employing a 50-ml Amicon concentrating cell and a Diaflo 3,500 MWCO nylon membrane. One milliliter of the concentrated material from the analytical studies was then applied to a 4-cm x 0.5-cm column of Q-sepharose ion-exchange resin (FastFlow, Pharmacia LKB) preequilibrated with 50 mM Tris.HCl pH 8.0. Subjective observation indicated that cyanoglobin appeared in the flow-through fractions. After 10-1 ml fractions were collected, a 1 M sodium chloride step gradient was applied and bound proteins were eluted. In preparative experimants, material obtained from the dialyzed ammonium sulfate fraction was batch adsorbed to 100 ml of Q-sepharose Fast Flow resin, equilibrated with 50 mM Tris.HCl pH 8.0 with stirring, for 20 min, at 4° C. The material that did not adsorb to the resin was scarlet red in color and was

removed by filtering the slurry through a Whatman 3 filter, in a Buchnner funnel apparatus, with suction. The post Q-sepharose fraction was concentrated as described above and then passed through a G-25 gel filtration resin (medium grade, Pharmacia) that had been preequilibrated with 50 mM Tris.HCl pH 8.0 and 1 mM DDT, at 4° C. The scarlet red effluent was collected and concentrated under nitrogen pressure as described above. The concentrated material was then passed through a Superose 12 gel filtration resin (Pharmacia), preequilibrated with 50 mM Tris.HCl pH 8.0 and 1 mM DDT, at room temperature. All reagents were filtered with Millipore 0.2-μm nylon membranes and were degassed under vacuum. The flow rate was 0.5 ml/min and the chart speed was set at 1 cm/ml. Multiple injections of 0.2 ml were applied and the $A_{280\,nm}$ and $A_{415\,nm}$ were monitored simultaneously using a Pharmacia LKB dual wavelength in-line spectrophotometer. Fractions that contained the relevant peaks in absorbance were pooled and then concentrated using Centricon 3000 concentrating cartridges, following the manufacturer's recommendations (Amicon). Protein concentrations of the various fractions obtained in the study were determined using the Coomassie G-250 microassay system, using bovine serum albumin as a standard and following the manufacturer's recommendations for use (Pierce).

SDS-PAGE

SDS-PAGE was performed using 15-18% (w/v) 1.5-mm slab gels and the procedure of Laemmli (Laemmli, 1970). The protein bands were visualized by staining in 1% (w/v) Coomassie R-250 in 10% (v/v) acetic acid:45% (v/v) methanol solution and then destaining

with a 10% acetic acid:10% methanol solution. Some SDS-PAGE gels were stained for hemoproteins, utilizing the intrinsic pseudo-peroxidase activity of the heme iron and diaminobenzidine (DAB; Francis, 1984). Briefly, the SDS-PAGE protocol was modified to exclude any reducing agents in the sample buffer. The samples were also not subjected to boiling and were electrophoresed in the dark, at 4° C. After electrophoresis, the gels were incubated in 100 ml 20 mM Tris·HCl pH 7.3 with 50% methanol, for 20 minutes. This incubation step was repeated using fresh buffer. DAB (Sigma) was added to 120 ml of 50 mM sodium citrate pH 4.4, with vigorous stirring, to give a final concentration of 2.5 mg/ml DAB. Immediately prior to use, 3 ml of a 30% (v/v) stock solution of hydrogen peroxide (Sigma) was added to give a final concentration of 0.75% (v/v) hydrogen peroxide. The solution was added immediately to the gel and the mixture was incubated with shaking, for approximately 30 min. The stained hemoproteins were fixed by washing the gel with approximately 100 ml of 12.5% (w/v) trichloroacetic acid, for 30 minutes. The cloudy white background was eliminated by washing the gel in distilled deionized water for approximately 30 min. The gels were then photographed immediately. Other protein bands on the gel were visualized by further staining with Coomassie R-250. Prestained markers were used as protein molecular mass standards (BRL, Bethesda Research Laboratories).

Immunoblotting

Immunoblotting was performed using rabbit polyclonal anti-cyanoglobin antibodies (Potts, 1992). The antibodies were diluted 1:10 in Tris buffered saline (TBS) and were then

incubated overnight together with polyvinyl difluoridene (PVDF) membranes saturated with cell extracts from *E. coli* BL21DE3 (pT7-7). This procedure ensured that any antibodies, not specific for cyanoglobin, were eliminated from the preparation. The anti-cyanoglobin antibodies in this final form were obtained from Dr. Malcolm Potts. The immunoblotting procedure used was a modification of the protocol used by Matsudaira (1987). PVDF membranes (Immobilon P Millipore) were used as the transfer matrix and were pretreated by washing in methanol for a few seconds, followed by washing in distilled deionized water for approximately 10 minutes. The PVDF membranes were finally washed in 10 mM CAPS pH 10.3 with 10% (v/v) methanol until ready to use. Proteins were resolved in SDS-PAGE gels that were placed on two pieces of blotting paper (Millipore), that were saturated with 10 mM CAPS pH 10.3 with 10% methanol. The PVDF membrane was placed on top of the gel, and two more pieces of blotting paper were added. The "sandwich" was then placed in a transfer apparatus (Hoeffer) filled with 10 mM CAPS pH 10.3 with 10% methanol. The "sandwich" was situated so that the resolved proteins migrated towards the anode, and thus to the membrane. The transfer was allowed to proceed for 30 minutes at 500 mA constant current. Cool water circulated through the transfer apparatus throughout the procedure. The membrane was removed from the sandwich and was washed twice, for ten minutes each, in 50 ml of Tris buffered saline with 0.05% Tween 20 (Sigma) (TBST). Any sites on the PVDF membrane not occupied by protein were blocked by washing in 50 ml of 0.5% (w/v) bovine serum albumin (Sigma) in TBST, for 30 minutes, with shaking. The membrane was washed three times, for five minutes each with 50 ml of TBST. The primary anti-cyanoglobin

antibody was added at a final dilution of 1:7000 (71 µl in 50 ml of TBST) to the membrane with shaking overnight. The membrane was washed three times in 50 ml TBST for five minutes each. The secondary antibody, goat anti-rabbit IgG horseradish peroxidase conjugate (Biorad), was then added at a final dilution of 1:2500 (20 µl in 50 ml TBST) and incubated with shaking for three hours. The membrane was washed twice in 50 ml of TBST for ten minutes each, followed by one wash for ten minutes in 50 ml TBS (no Tween). The membranes were processed as follows: 30 mg of 4-chloro-1-napthol (Biorad) was dissolved in 10 ml of ice cold methanol and 30 µl of 30% hydrogen peroxide (Sigma) was added to 50 ml of TBS. The two solutions were mixed and immediately added to the membrane with shaking. An intense blue precipitate formed at the location of the antigenic proteins. The reaction was stopped by washing the blot in distilled deionized water for a few minutes. The membrane was dried with a hand-held hair dryer and then photographed immediately. All incubations and washings during immunoblotting were performed at room temperature.

Crystallization Trials

Crystallization of the purified, recombinant cyanoglobin was attempted using the hanging drop vapor diffusion method (McPherson, 1990). A 24-well cell culture plate (Linbro) was used to hold the contents of the reservoir. The droplets containing the protein samples were pipetted onto 22-mm glass coverslips that were silconized with Sigmacote (Sigma) and then washed with methanol before use. The coverslip was inverted and then sealed onto the culture plate well using a small amount of mineral oil. Both drop and reservoir contained

varying amounts of ammonium sulfate as the precipitant over the pH range 7.2-7.8 using 20 mM Tris.HCl. Separate trials were conducted at room temperature and at 4° C. Crystallization preparations were also performed at pH 8.9 and 9.3 using 50 mM CAPS, although these preparations were allowed to equilibrate at 4° C only. The cyanoglobin sample used was a concentrated pool from the Superose 12 fraction as described on p. 14. Protein concentrations in the droplets ranged from 1 mg/ml to 3 mg/ml. All solutions used for crystallization trials were filtered with Millipore 0.2-μm nylon membranes and then degassed under vacuum. The preparations were examined by light microscopy immediately after preparation, and then intermittently thereafter for up to one week. Controls were prepared with no protein sample to ensure that the precipitated material in the drop, at equilibrium, was not ammonium sulfate.

Absorption Spectroscopy

Spectra were obtained on a Cary 209 scanning spectrophotometer that was zeroed at all wavelengths using diluent buffer as a reference. Some samples were scanned using a Hewlett-Packard diode array spectrophotometer that was also zeroed at all wavelengths with diluent buffer. The scans using the Cary 209 were obtained from 300 nm to 500 nm at an AUFS of 1.0, and from 500 nm to 700 nm using an AUFS of either 0.1 or 0.2. The chart speed for all scans using the Cary 209 was 1 cm/20 nm scanned. All scans using the Cary 209 employed 3-ml fused silica quartz cuvettes fitted with teflon stoppers. Data analysis required manual reading of absorbance values every 4 nm, and then inputing these values into a computer

program capable of graphical display. Spectra from the Hewlett-Packard instrument were saved as a "text" file using the integrated computer program, and then imported into an appropriate computer graphing program (DeltaGraph).

Hemoproteins of interest in this study were sperm whale myoglobin (Sigma), leghemoglobin A (a generous gift from Dr. Robert Klucas, University of Nebraska), and purified cyanoglobin obtained from pooled and concentrated Superose 12 fractions. Met-forms of the hemoproteins were produced by adding a ten-fold molar excess of potassium ferricyanide to a concentrated sample of the hemoprotein. Excess potassium ferricyanide was removed by passing the material through a G-25 (Pharmacia) column equilibrated with 20 mM potassium phosphate pH 7.0. The buffer was filtered with a Millipore 0.2-μm nylon membrane and then degassed under vacuum before use. The colored effluent was collected in its entirety and then diluted to 3 ml with 20 mM potassium phosphate buffer pH 7.0. Solutions of sodium azide and potassium cyanide were produced as 500 mM stock solutions in 20 mM potassium phosphate pH 7.0. Small aliquots of these ligand solutions were added so as to achieve minimal dilution of the 3 ml sample and near 1000-fold molar excess of ligand to hemoprotein. Some studies involving the synthesis of the ferric derivatives were performed at pH 9.0 using 20 mM CAPS.

Deoxy derivatives were produced by adding a few crystals of solid sodium dithionite (Baxter) to a diluted protein sample, inverting the cuvette, and then immediately collecting the spectra. Carbonmonoxy derivatives were produced by gently perfusing carbon monoxide gas for about 30 seconds through a dithionite-reduced protein solution.

The oxygenated derivatives were produced by first forming the deoxy-forms under completely anaerobic conditions in a nitrogen-flushed glove box. All materials used were placed in the glove box and kept overnight before use. Potassium phosphate buffer pH 7.0 (20mM) was perfused vigorously with nitrogen with stirring, for about two hours, and then placed in the glove box overnight before use. A column of Bio-Gel P-6DG gel filtration resin (Biorad) was poured in a Pasteur pipette stoppered with glass wool (approximately 1 ml packed resin). The column was then equilibrated with the degassed 20 mM potassium phosphate pH 7.0. Sodium dithionite, (5 µl of a 250 mM stock), was passed through the column and collected to ensure complete removal of any residual oxygen from the column. Methylviologen-impregnated filter papers were used to follow the presence of sodium dithionite. If the effluent did not cause a deep blue color to form, then all dithionite was assumed to be removed. Stock sodium dithionite (5 µl as described above) was added to 18 µl of concentrated protein solution and then this material was passed through the P-6DG column and the colored effluent was collected in its entirety (typically 150 µl). To ensure that the effluent was devoid of sodium dithionite, a small amount was placed on methylviologen-impregnated filter paper, with no observed color change. The sodium dithionite was then eluted from the column and collected in a separate container. The presence of sodium dithionite was determined by the deep blue color that formed when a small amount of effluent was placed on methylviologen impregnated filter paper. The deoxygenated hemoprotein was diluted to 3 ml with 20 mM potassium phosphate buffer, and was dispensed anaerobically into a quartz cuvette fitted with a rubber stopper and a septum. The cuvette was tightly

stoppered, removed from the glove box, and then spectra were taken immediately on the Hewlett-Packard diode array spectrophotometer. A reference sample of buffer was handled identically. Data collection was performed via an integrated computer program. Compressed air was gently perfused through the solution for about 30 seconds using a syringe. The cuvette was inverted and the spectra of the oxy-form was collected. The oxycyanoglobin was allowed to stand at room temperature and spectra were collected at two, four, and then finally twenty-four hours to follow any autooxidation events.

Circular Dichroism

All circular dichroism (CD) spectra were collected on a Jasco J-700 (Japan Spectroscopic Company) spectropolarimeter that was standardized on a daily basis with a 0.06% (w/v) solution of ammonium d-10-camphor sulfonate. The optical rotation of this solution was calibrated to be 1.904×10^2 millidegrees $\pm$ 1 millidegree at 290.5 nm using a 1-cm quartz cell. Scan rates were 1 nm/minute with a response time of four seconds and a step resolution of 0.5 nm. Data collection was performed by the integrated computer program. On occasion, scans were performed on hemoprotein samples immediately after the absorption spectra were collected. The absorbance of these samples was assured to be less than 1.00 over all wavelengths measured. All other scans were performed on samples that were not subjected to treatment with any reduction or oxidation reagents. CD spectra were collected on all samples in quartz cuvettes, ranging from 0.2 to 0.5-cm for scans from 600 nm to 250 nm, and in a 0.01-cm quartz cuvette from 250 nm to 180 nm. The instrument was flushed

continuously with "unscrubbed" tank nitrogen throughout all scans. Nitrogen tanks were replaced when 4/5 of the contents were depleted according to readings on the pressure gauge. All reagents used for CD analysis were filtered with a 0.2-μm nylon filter and thoroughly degassed under vacuum.

Results

Growth

The growth characteristics of *E. coli* strain BL21DE3 (pGlbN) were comparable to that of the untransformed wild-type. Luria-Bertani (LB) agar plates containing ampicillin revealed large colonies when grown overnight at 37° C. The results were typical of a bacterial growth curve (Figure 1). In repetitive trials under the experimental conditions used, lag-phase growth occurred until approximately 2 hours after inoculation, and then logarithmic phase growth ensued. Mid-logarithmic phase growth occurred at approximately 2.5 hours, and after 3 hours, the cells entered stationary phase growth.

When growth conditions were scaled up to 1-l, a longer incubation time (3 hours) was required to achieve mid-logarithmic phase growth. However, the 24-l fermentor growth culture also required only 3 hours of incubation to achieve mid-logarithmic phase growth, which is attributed to the increased efficiency of culture aeration that is obtainable when the culture was sparged continually with sterile air. Cyanoglobin was purified from only one fermentor culture in this study. The wet cell weight yield after induction was 50 g. A second fermentor culture and induction was performed and the dry cell weight yield was 27 g, though cyanoglobin has not yet been purified from these cells.

Induction of Cyanoglobin Synthesis

Recombinant cyanoglobin synthesis was induced after the addition of IPTG to the

M9 induction medium. The cells became increasingly scarlet-red in color as the induction time increased. The optimal time for induction of the 125 ml culture was approximately 7 hours at 37° C (Figure 2). After this time, the amount of cyanoglobin synthesis did not increase significantly, most likely due to nutrient deprivation in the culture medium. The amount of cyanoglobin synthesized was determined not to be limited by the delta-aminolevulinic acid (δ-ALA) concentration used in this study because tripling the δ-ALA concentration in the induction medium did not produce higher yields of cyanoglobin nor was the time course of cyanoglobin synthesis increased. During induction of cyanoglobin synthesis, no significant amount of *E. coli* BL21DE3 (pGlbN) growth occurred based the nearly constant A_{550nm} as a function of induction time.

Scale-up of conditions for induction of cyanoglobin synthesis required longer induction times. Specifically, a 500-ml induction culture typically required overnight induction (12-15 hours) before the A_{415nm}/A_{280nm} ratio no longer increased significantly . The appearance of the culture after overnight induction could be described qualitatively as "cream of tomato soup". Such qualitative observation was used as an index of cyanoglobin synthesis in the 24-l fermentor culture in that the intense scarlet-red color of the cells was used as an indication of sufficient cyanoglobin synthesis (Figure 3). The time required for the cells to attain such an appearance was only 15 hours under the conditions stated in the text, again probably attributable to the increased efficiency of culture aeration obtainable when using a fermentor apparatus.

The efficacy of induction of cyanoglobin synthesis was apparently dependent on how

the *E. coli* BL21DE3 (pGlbN) cells were grown. Colonies from LB agar plates (subcultured from the glycerol stocks) stored at 4° C longer than one week gave decreased cyanoglobin yields when compared to colonies used from LB agar plates less than 24 hours old. Furthermore, qualitative observations indicated that induction of cyanoglobin synthesis was apparently dependent on the growth phase of the cells to be processed for induction. Stationary phase *E. coli* BL21DE3 (pGlbN) cells seemed to give inconsistent yields of recombinant cyanoglobin. Experience dictated that mid-logarithmic phase cells processed for induction gave consistent, high yields of the recombinant cyanoglobin.

Cell Processing

After the induction of cyanoglobin synthesis was judged to be sufficient, the cells were lysed. Centrifugation of the cell debris revealed a scarlet-red, viscous supernatant fraction, indicating that cyanoglobin is present in the soluble phase when expressed in *E. coli* BL21DE3 (pGlbN). The resultant cell free lysate was diluted in 50 mM Tris.HCl pH 8.0 and subsequent spectral analysis revealed a strong absorbance between 400-430 nm, which is characteristic of the Soret region of heme-containing proteins. There were also two small peaks at 580 nm and 540 nm, which are characteristic of an oxygenated hemoprotein. Such a spectral profile confirms that cyanoglobin is overexpressed in *E. coli* BL21DE3 (pGlbN), at least partly, as the oxy-form. Subsequent dialysis of the diluted cell free lysate revealed no significant decrease in the A_{415nm}/A_{280nm} ratio. Therefore, all heme present in the cell free lysate was associated with cyanoglobin or with other native *E. coli* BL21DE3

heme-containing proteins.

Purification of Cyanoglobin

The scarlet-red cell-free lysate was then subjected to nucleic acid precipitation using streptomycin sulfate. The white precipitate that formed was removed by centrifugation and the resultant scarlet-red supernatant fraction had a markedly reduced viscosity. The supernatant fraction was then subjected to ammonium sulfate precipitation. Cyanoglobin began to precipitate from solution at 40% saturation of ammonium sulfate. At 80% saturation, there was still a significant amount of red color in the supernatant fraction. At 100% saturation, the supernatant was colorless and the pellets were scarlet-red. Removal of ammonium sulfate from the pellets by dialysis against 50 mM Tris.HCl pH 8.0 with 1 mM DTT caused a slight, straw-colored precipitate to form. The precipitate was removed by centrifugation and subsequent SDS-PAGE analysis revealed that the precipitated material did not contain cyanoglobin.

Preliminary analytical scale column chromatography of cyanoglobin included a variety of separation techniques (Table 1). The elution profile of cyanoglobin using Q-sepharose ion-exchange resin at pH 8.0 using 50 mM Tris.HCl consisted of a major peak of A_{415nm} that appeared in the flow-through fractions (Figure 4). Therefore, cyanoglobin did not adsorb to the resin under these conditions. A 1 M NaCl step gradient eluted proteins from the resin that had an associated A_{415nm}, which were believed to be native *E. coli* BL21DE3 heme-containing proteins. The efficacy of this step was deemed acceptable

because of the relatively large amounts of non-cyanoglobin proteins that adsorbed to the resin under these conditions.

Cyanoglobin did adsorb to Q-sepharose ion-exchange resin at pH 11.0 using 50 mM ethanolamine. No proteins that absorb at 280 nm appeared in the flow through fractions and the resultant proteins, eluted from the resin with a 1 M NaCl step gradient, revealed a 10-fold decrease in A_{415nm} when compared to pH 8.0 (Figure 5). The decrease in heme content was attributed to the spontaneous release of the non-covalently bound heme moiety from cyanoglobin under alkaline conditions. The loss of heme from cyanoglobin under these conditions precluded further use of this step.

Cyanoglobin did not adsorb to strong cationic exchange resins, such as sulfopropyl and Mono S at pH 6.0 using 50 mM MES (Figure 6). Substantially less contaminating proteins adsorbed to the resin under these conditions when compared to the Q-sepharose step at pH 8.0 using 50 mM Tris.HCl. Therefore, the latter was judged to provide a more sufficient enrichment of cyanoglobin.

Hydrophobic interaction chromatography using phenyl sepharose did show promise because cyanoglobin adsorbed to the resin under high salt conditions. However, the high salt concentrations (> 1 M ammonium sulfate) required for cyanoglobin to adsorb to phenyl sepharose also caused most all other proteins in the sample to adsorb to the resin as well. The descending salt gradient used did not provide good resolution of cyanoglobin from the other proteins in the sample. Therefore, use of this step for preparative purposes would be too laborious and expensive for the little enrichment of cyanoglobin achieved.

The gel filtration chromatography trials proved to be highly effective. However, directly applying the concentrated post Q-sepharose fraction to the Superose 12 column caused high pressure problems at below normal flow rates. A yellowish discoloration soon appeared at the top of the Superose 12 gel bed after repeated injections. However, passing the post Q-sepharose fraction through a G-25 resin prior to Superose 12 chromatography effectively eliminated the problems with column contamination. The Superose 12 elution profile of cyanoglobin under the conditions described in the text and subsequent SDS-PAGE analysis of the designated fractions reveal that essentially pure protein was obtained with this step (Figure 7).

The final preparative purification scheme used in this study and the SDS-PAGE analysis of the pertinent fractions revealed that both the overexpression of the recombinant cyanoglobin and its purification to apparent homogeneity was accomplished (Figure 8). The Q-sepharose step (Lane C) has a band pattern that clearly shows that an enrichment was effected by using this step. Lane E shows the effectiveness of the Superose 12 step, demonstrating a homogeneous protein preparation migrating at approximately 12 kD.

SDS-PAGE and Immunoanalysis

The hemoproteins used in this study were subjected to SDS-PAGE analysis. Two identical gels were run and one was developed using Coomassie R-250 and the other was subjected to immunoanalysis as described in the text. The Coomassie stained gel shows that sperm whale myoglobin migrates to 18 kD, leghemoglobin *a* to 15 kD, and cyanoglobin to

12 kD (Figure 9). Also of note is the heterogeneity of the sperm whale myoglobin sample. The upper migrating band in the sperm whale myoglobin sample is approximately twice the molecular mass as the lower migrating band. Therefore, non-specific aggregation into dimeric forms was suspected.

Immunoanalysis of the concomitantly run SDS-PAGE gel revealed that both leghemoglobin *a* and sperm whale myoglobin are not immunologically reactive with the anti-cyanoglobin antibodies (Figure 9). Both apo-cyanoglobin and holo-cyanoglobin reacted with the anti-cyanoglobin antibodies used in the procedure, demonstrating that the cognate epitopes that are recognized by the rabbit anti-cyanoglobin antibodies are protein, and not heme, in nature. The holo-cyanoglobin did reveal a very slight cross reaction to an upper migrating band at approximately 25 kD, which was undetectable from the Coomassie stained SDS-PAGE gel.

A series of controls was performed identically in the immunoanalysis procedure to ensure that the upper migrating band in holo-cyanoglobin sample was of cyanoglobin origin. Sera from the rabbit prior to immunization with cyanoglobin was analyzed and no reaction was observed. Therefore, the rabbit anti-cyanoglobin antibodies were devoid of native-rabbit antibodies. Cell free lysates of *E. coli* BL21DE3 and *E. coli* BL21DE3 (pT7-7) were also analyzed and showed no reaction. Therefore, no rabbit antibodies to proteins of *E. coli* BL21DE3 or pT7-7 origin were present in the rabbit anti-cyanoglobin antibodies preparation.

The notion that there may be slight aggregation of cyanoglobin into dimeric forms

when subjected to SDS-PAGE was further investigated by staining with diaminobenzidine. This stain is heme specific and revealed that the upper migrating bands in both the sperm whale myoglobin and holo-cyanoglobin samples are heme-containing proteins (Figure 10). Therefore, the upper migrating band detected in the immunoanalysis procedure was not caused by an immunologically related, non-cyanoglobin, protein present in the sample.

Absorption Spectroscopy

A variety of derivatives of sperm whale myoglobin, leghemoglobin *a*, and cyanoglobin were produced and their absorption spectral properties characterized in this study (Table 2). Heme-containing proteins have a strong absorbance in the 400-430 nm range, commonly referred to as the Soret (γ) peak. Also typical of heme-containing proteins is the appearance of peaks at approximately 540 nm and 580 nm, referred to as the β and α peaks respectively. The location and intensity of the above mentioned peaks are diagnostic of the environment of the heme moiety.

The deoxy-forms of the hemoproteins were produced by anaerobic reduction with sodium dithionite. The spectral properties in the Soret region of the deoxy-cyanoglobin were similar to the other hemoproteins (Figure 11). The extinction coefficient of the Soret peak of the deoxy-forms are typically lower when compared to the oxy-forms (described later). The Soret peak is also much broader when compared to the other derivatives of cyanoglobin produced in this study. The visible region of the deoxy-forms have only a β peak at approximately 550 nm.

When the deoxy-hemoproteins coordinate molecular dioxygen, the α peak appears at approximately 580 nm and the β peak shifts to approximately 540 nm (Figure 12). Both oxysperm whale myoglobin and oxyleghemoglobin *a* have similar visible region spectral profiles. In particular, the α/β peak ratios are close to unity. Such is not the case in oxycyanoglobin which exhibits an atypically low α/β peak ratio. Interestingly, these unique spectral properties of oxycyanoglobin in the visible region are also observed in the oxyprotozoan cytoplasmic hemoglobins. After four hours at room temperature, there was no noticeable change in the spectral characteristics of oxycyanoglobin. However, after twenty-four hours at room temperature, oxycyanoglobin was completely autoxidized, revealing a spectrum identical to the met-cyanoglobin form (data not shown).

When gaseous carbon monoxide was perfused through a solution of the deoxy-hemoproteins, drastic spectral changes occurred (Figure 13). Of note is the dramatic increase and sharpening of the Soret peak, as well as the β peak splitting into β and α peaks. The resultant β and α peaks are located approximately in the same position as in the oxy-forms but are less sharp. Comparatively, the carbonmonoxy-forms of the hemoproteins studied here showed no unique differences to each other.

Large differences were seen, however, in the ferric (met) forms of the hemoproteins. Both met-sperm whale myoglobin and met-leghemoglobin *a* have α peaks at approximately 630 nm (Figure 14). Met-cyanoglobin, however, had markedly different visible region spectral characteristics by having a β peak at approximately 540 nm and an α shoulder at approximately 570 nm, which is not observed in either met-sperm whale myoglobin or met-

leghemoglobin *a*. The spectral profiles of met-sperm whale myoglobin and met-leghemoglobin *a* suggest that the heme iron is in a high-spin state, which has been confirmed by other workers using ESR. In contrast, the heme iron in met-cyanoglobin appears to be in the low-spin state, which would suggest that the sixth coordinating ligand in met-cyanoglobin is a strong-field ligand. Interestingly, the spectral properties of met-cyanoglobin were identical at both pH 7 and at pH 9 (data not shown). In contrast, both met-sperm whale myoglobin and met-leghemoglobin *a* exhibit pH dependent spectral differences (Antonini and Brunori, 1971). Another remarkable feature of met-cyanoglobin was its inability to coordinate the exogenous, strong-field ligands azide and cyanide under the conditions used, in contrast to the marked spectral changes that occurred when met-sperm whale myoglobin coordinated these ligands (figure 15). Upon binding the strong-field ligands of cyanide or azide, there was a decrease in the extinction coefficient and a shifting to longer wavelengths of the Soret peak. In addition, the visible region α peak in met-sperm whale myoglobin was shifted to an intense β peak at 540 nm. The spectral profiles of the known low-spin cyanmet and azidomet-sperm whale myoglobin derivatives were very similar to the spectral profile of met-cyanoglobin in the visible region.

Circular Dichroism

Circular dichroism (CD) spectra of hemoproteins reported here were obtained on samples that were untreated with ferricyanide or dithionite. The raw CD data (in millidegrees) are expressed as molar ellipticity ($[\theta]$) in degree $\cdot$ cm$^2\cdot$ decimol^{-1}. The CD data

in the far-UV (190-250 nm) are expressed as mean residue molar ellipticities by assuming a mean residue weight of 116 for all hemoproteins (Nicola, 1975).

The CD of the hemoproteins in the Soret region revealed large differences (Figure 16). Specifically, sperm whale myoglobin had a positive ellipticity at θ_{409nm}. Cyanoglobin and leghemoglobin *a*, on the other hand, had strongly negative ellipticities at θ_{412nm} and θ_{404nm} respectively, with the ellipticity maximum of cyanoglobin, being less intense when compared to leghemoglobin *a*. Further analysis of the CD spectrum of leghemoglobin *a* suggested that there was possibly a mixture of oxyferrous and met-forms in the untreated sample because the intensity of the CD band was intermediate to the published intensities of these two forms (Nicola, 1975). Compared to sperm whale myoglobin, the Soret region CD of cyanoglobin is opposite in sign of ellipticity and not nearly as intense.

The far-UV CD spectra of the hemoproteins revealed that all have relatively high alpha-helical contents based on the two negative θ_{209nm} and θ_{220nm} bands (Figure 17). The intensities of these bands was strongest in sperm whale myoglobin. Comparatively, the intensities of the bands of cyanoglobin and leghemoglobin *a* are similar. However, in both sperm whale myoglobin and leghemoglobin *a* the band at θ_{209nm} was always more intense than the corresponding band at θ_{220nm}. In cyanoglobin, however, the band at θ_{209nm} was less intense than the corresponding band at θ_{220nm}.

Crystallization

A variety of conditions of buffer pH and precipitant concentrations were examined

in attempts to crystallize cyanoglobin. Although no crystals were formed under the conditions used, there was progress in defining the most appropriate range for further trials. Ammonium sulfate was used as the precipitant, and it was noted that low saturations of this precipitant (4-14%) in the drop at pH 7.2 did not cause precipitation of cyanoglobin (Table 3). Increasing the precipitant concentration in the drop did cause precipitates to form, but only after a few days of incubation at 4° C. Increasing the reservoir concentration of precipitant caused the precipitates in the drop to become more "clumpy", compared to the finely dispersed precipitates that occurred when lower concentrations of reservoir precipitant were used. Drop concentrations of precipitant that appeared to be most promising were in the 30-35% saturation of ammonium sulfate range. Varying the pH of the equilibration buffer was also investigated (Table 4). There did not appear to be any significant difference between crystallization trials performed at pH 7.2 and those performed in the pH range 7.4 - 7.8. Increasing the cyanoglobin concentration in the drop to 3 mg/ml also appeared to have no effect on the amount of precipitate observed (Table 5). Large differences, however, were observed in samples in the pH range 8.9 - 9.3. These set-ups also used a higher concentration of cyanoglobin (3 mg/ml) in the drop than used previously. Trials performed at pH 9.3, a value close to the calculated isoelectric point of cyanoglobin (9.7), precipitated almost immediately, whereas those performed at pH 8.9 precipitated after a few hours of incubation at 4 ° C.

Figure 1. Growth of *E. coli* strain BL21DE3 (pGlbN) in 250 ml Luria-Bertani liquid media containing 0.2 mg/ml ampicillin.

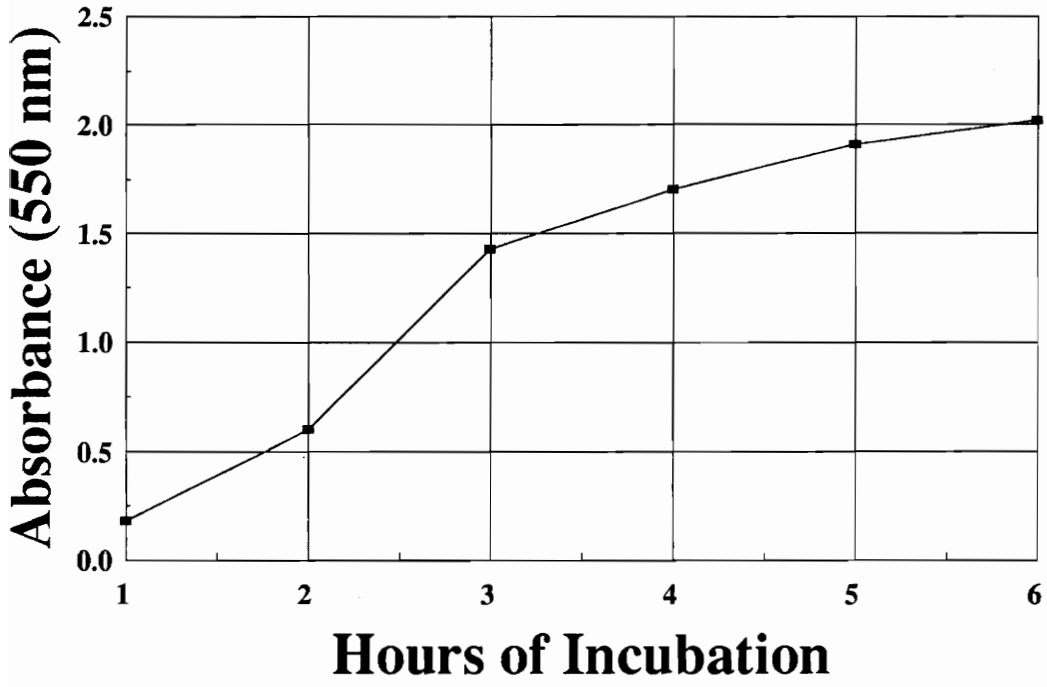
Absorbance (550 nm)
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
6
Hours of Incubation

Figure 2. Induction of cyanoglobin synthesis in 125 ml modified M9 salts.

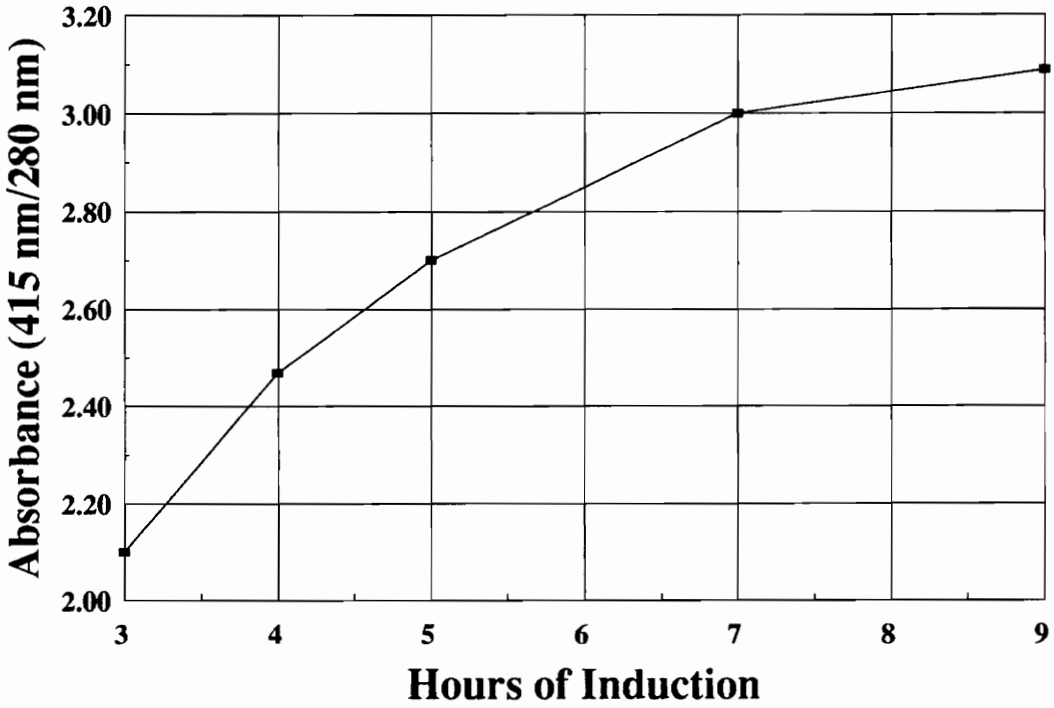
Absorbance (415 nm/280 nm)
3.20
3.00
2.80
2.60
2.40
2.20
2.00
3
4
5
6
7
8
9
Hours of Induction

Figure 3. Harvesting of *E. coli* Bl21DE3 (pGlbN) after overnight induction.

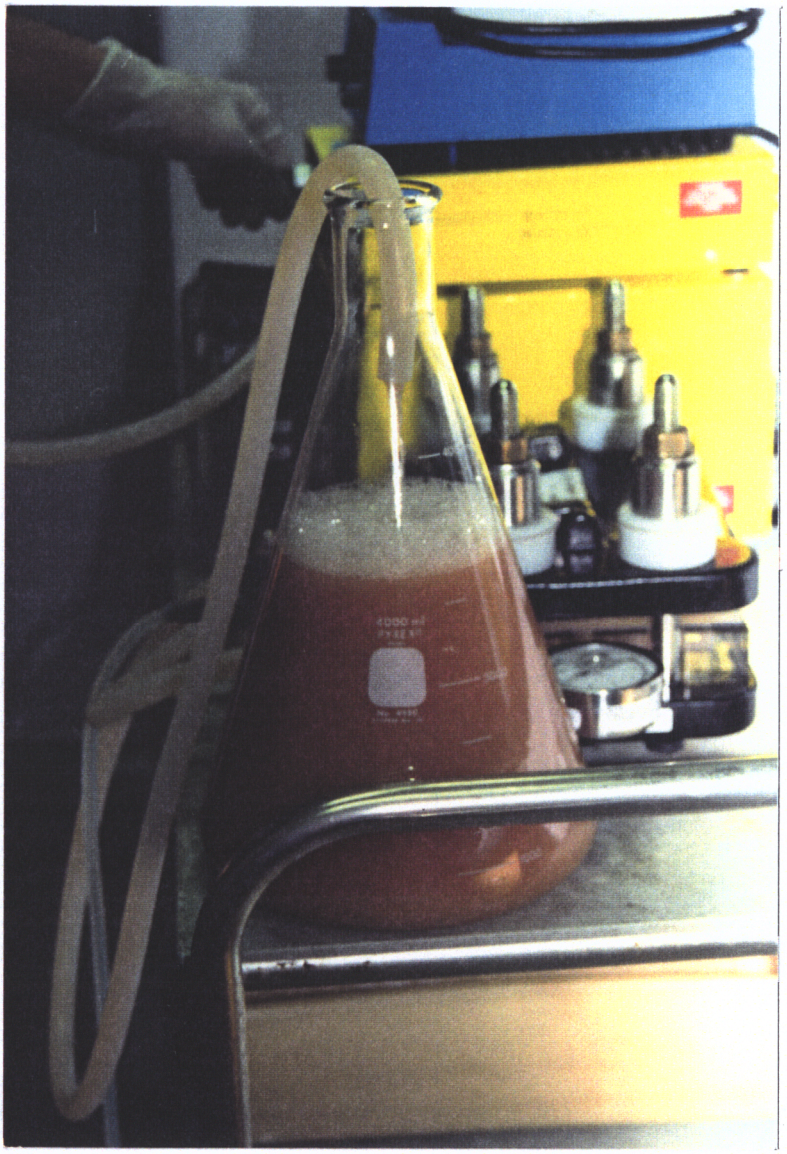

Figure 4. Q-sepharose chromatography of GlbN using 50 mM Tris.HCl pH 8.0. (●) A_{280nm}; (■) A_{415nm}; (▲) 1 M NaCl.

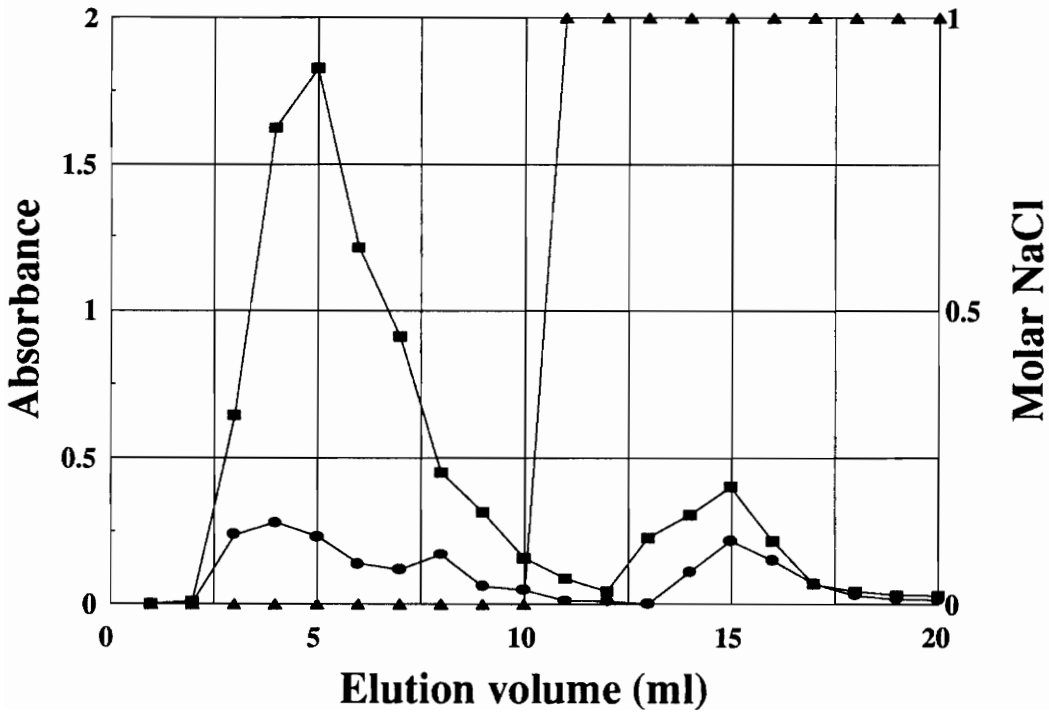
Absorbance
Molar NaCl
Elution volume (ml)
2
1.5
1
0.5
0
1
0.5
0
0
5
10
15
20

Figure 5. Q-sepharose chromatography of GlbN using 50 mM ethanolamine pH 11. (●) A_{280nm}; (■) A_{415nm}; (▲) 1 M NaCl.

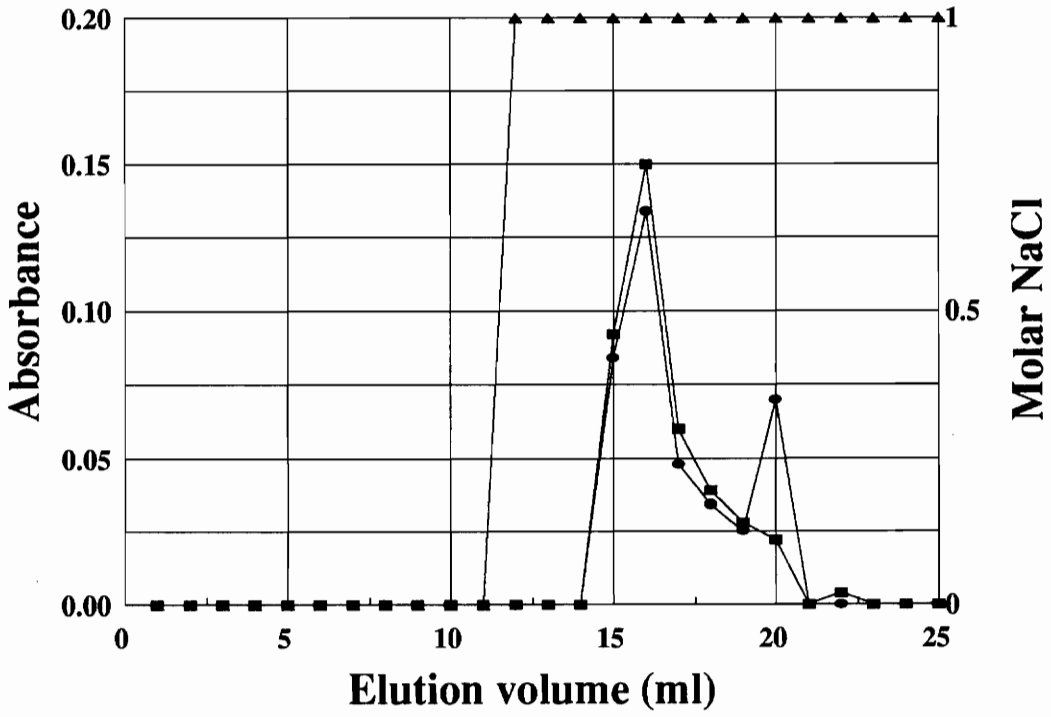
0.20
0.15
0.10
0.05
0.00
Absorbance
1
0.5
0
Molar NaCl
0
5
10
15
20
25
Elution volume (ml)

Figure 6. Sulfopropyl chromatography of GlbN using 50 mM MES pH 6.0. (●) A_{280nm}; (■) A_{415nm}; (▲) 1 M NaCl.

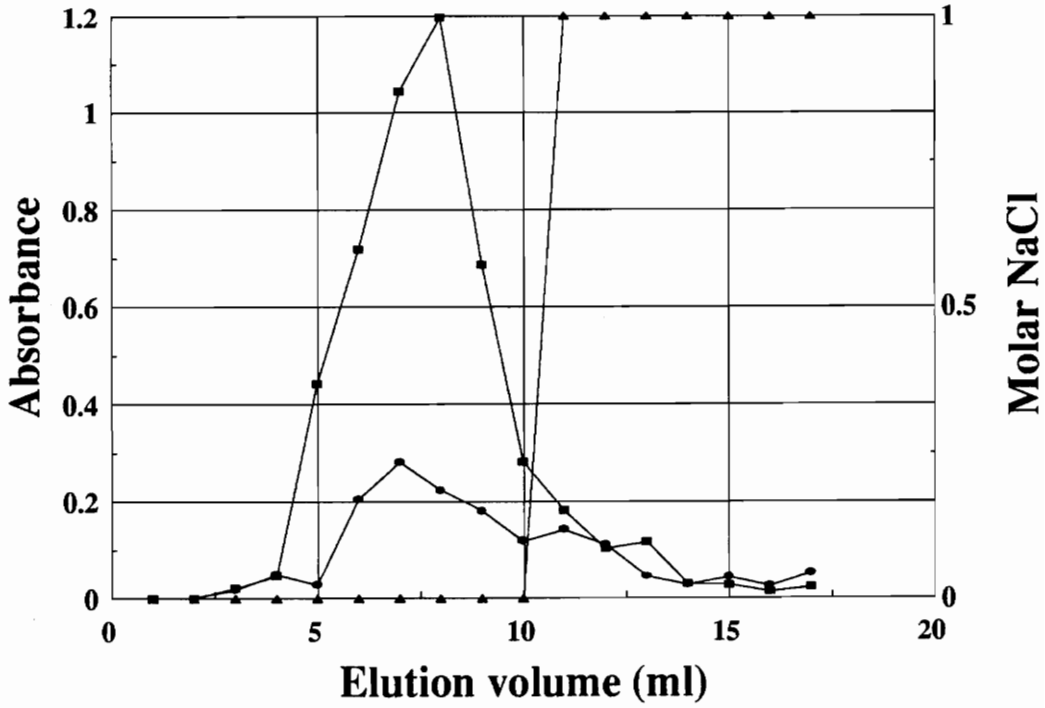
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance
1
0.5
0
Molar NaCl
0
5
10
15
20
Elution volume (ml)

Figure 7. Superose 12 gel filtration of GlbN using 50 mM Tris.HCl pH 8.0 with 1 mM DTT. (inset) Coomassie stained SDS-PAGE gel of designated fractions.

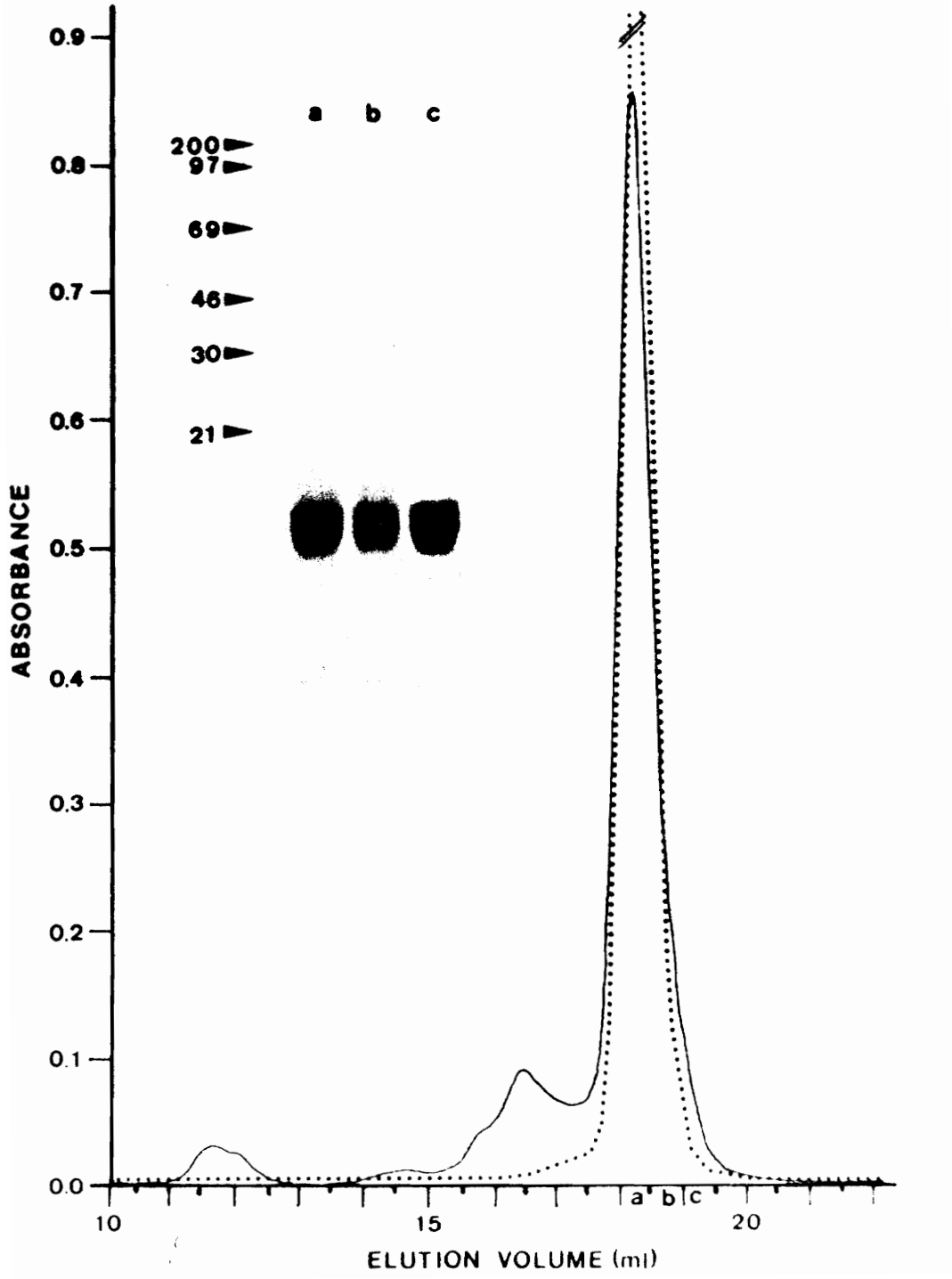
a b c
200
97
69
46
30
21
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ABSORBANCE
10
15
20
a b c
ELUTION VOLUME (ml)

Figure 8. (a) Preparative purification scheme used in this study. (b) SDS-PAGE gel of fractions obtained. Lane A - Cell free lysate; Lane B - 40-100% ammonium sulfate; Lane C - Q-sepharose; Lane D - G-25; Lane E -Superose 12. Molecular weight markers are displayed in kilodaltons.

(a)

Clarified Cell Lysate

↓

Streptomycin Sulfate

↓

40-100% Ammonium Sulfate

↓

Q-sepharose Batch Adsorption

↓

G-25 Gel Filtration

↓

Superose 12 Gel Filtration

(b)

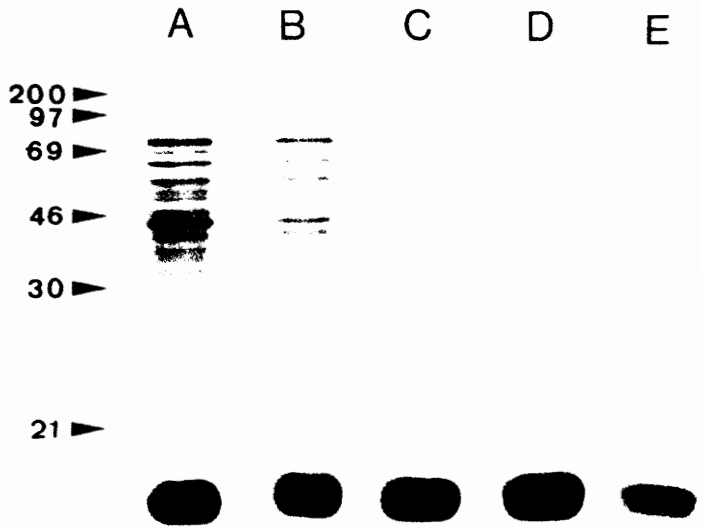

Figure 9. SDS-PAGE and immunoanalysis of hemoproteins used in this study.

SDS-PAGE
Lane A - Purified GlbN (this study)
Lane C - Leghemoglobin *a*
Lane E - Sperm whale myoglobin
Lane G - Purified apo-GlbN

Immunoanalysis
Lane B - Purified GlbN (this study)
Lane D - Leghemoglobin *a*
Lane F - Sperm whale myoglobin
Lane H - Purified apo-GlbN

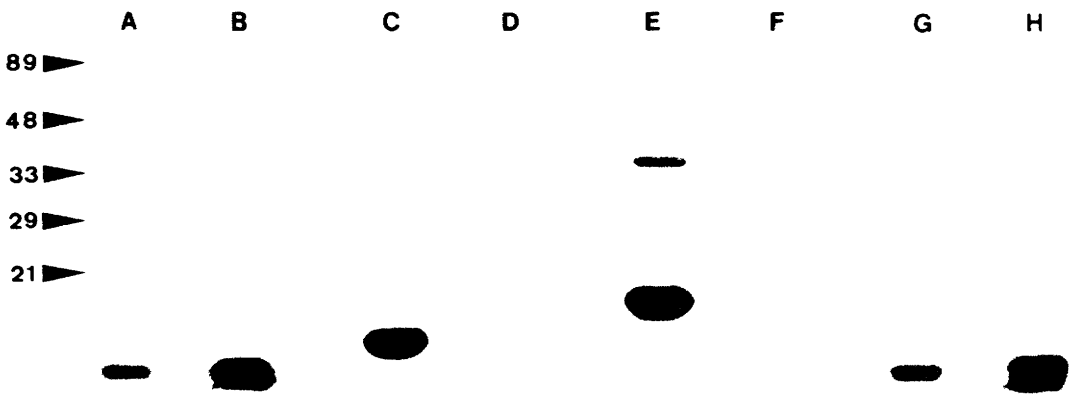
A
B
C
D
E
F
G
H
89
48
33
29
21

Figure 10. SDS-PAGE gel developed with diaminobenzidine stain.

Lane A - Superose 12 pool
Lane B - Sperm whale myoglobin
DF = Dye Front

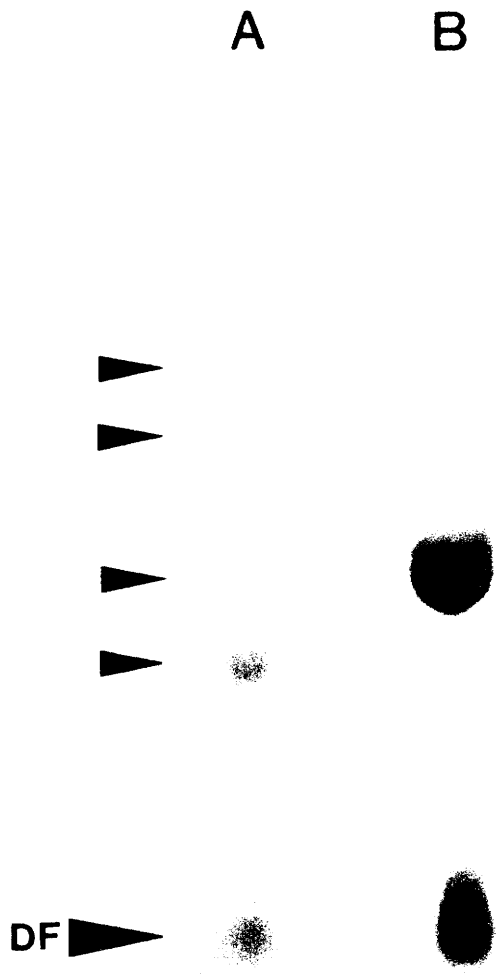
A
B
DF

Figure 11. Absorption spectra of deoxy-hemoproteins in 20 mM potassium phosphate buffer pH 7. Concentrations 4 - 6 μM. (----) sperm whale myoglobin; (—) leghemoglobin *a*; (--•--) cyanoglobin.

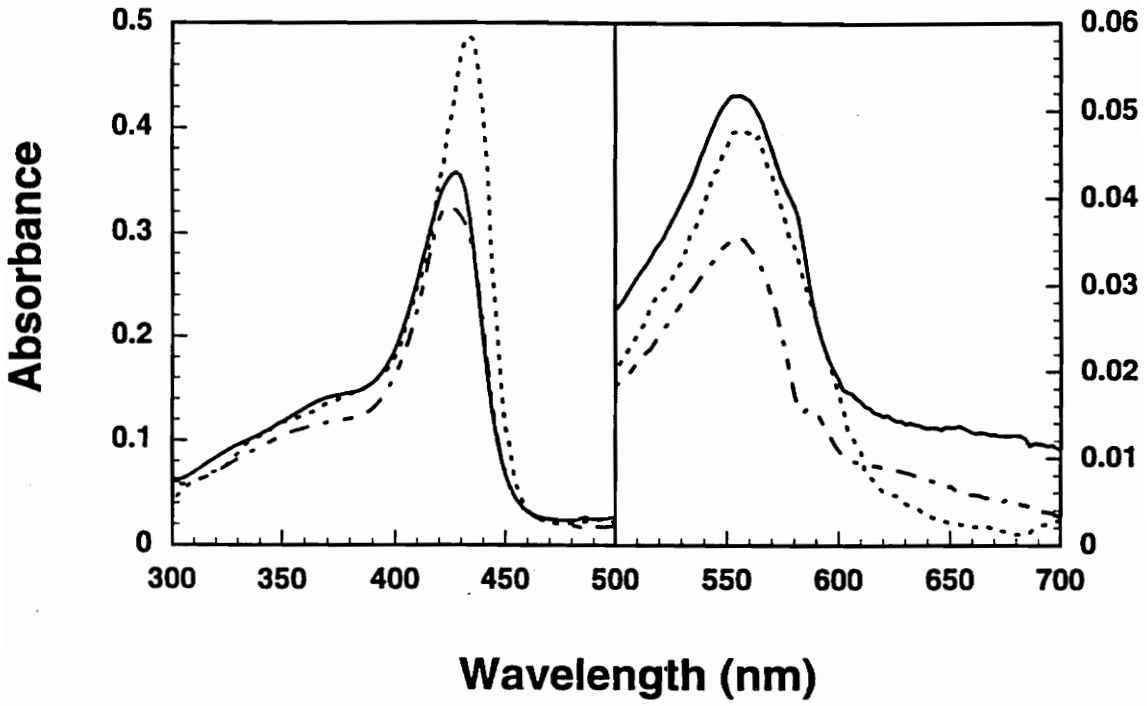
Absorbance
0.5
0.4
0.3
0.2
0.1
0
0.06
0.05
0.04
0.03
0.02
0.01
0
300
350
400
450
500
550
600
650
700
Wavelength (nm)

Figure 12. Absorption spectra of oxy-hemoproteins in 20 mM potassium phosphate buffer pH 7. Concentrations 4 - 6 µM. (-----) sperm whale myoglobin; (——) leghemoglobin *a*; (--•--) cyanoglobin.

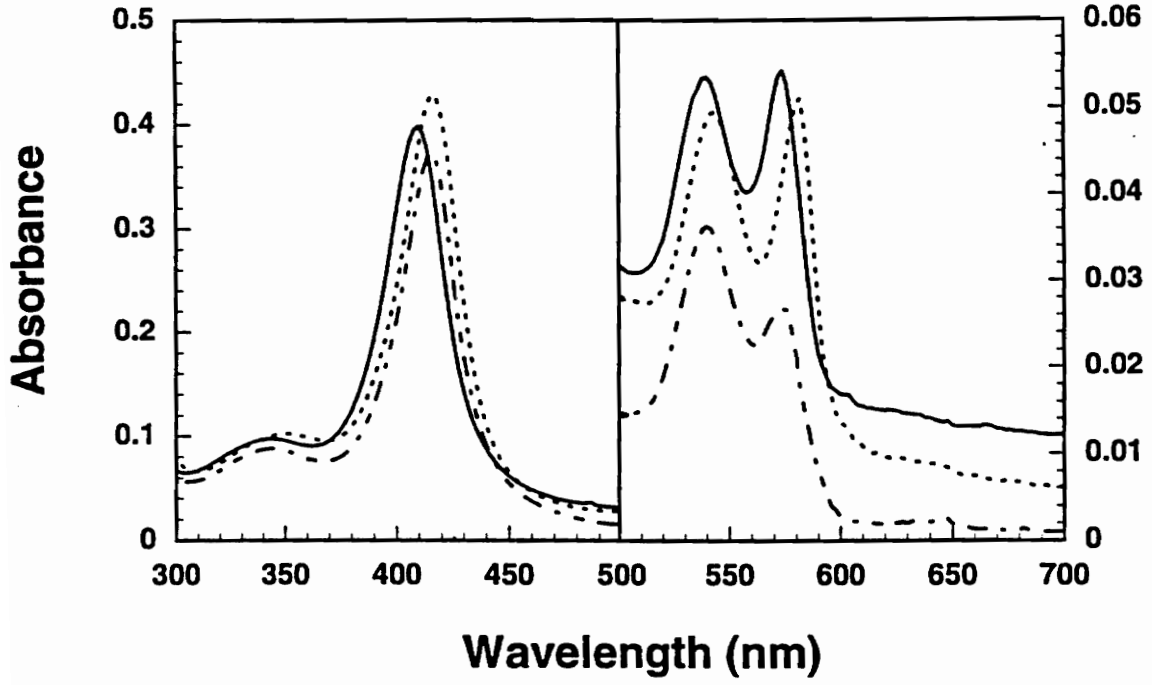
Absorbance
0.5
0.4
0.3
0.2
0.1
0
0.06
0.05
0.04
0.03
0.02
0.01
0
300
350
400
450
500
550
600
650
700
Wavelength (nm)

Figure 13. Absorption spectra of carbonmonoxy hemoproteins in 20 potassium phosphate buffer pH 7. Concentrations 5 - 8 μM. (-----) leghemoglobin *a*; (——); sperm whale myoglobin; (--•--) cyanoglobin.

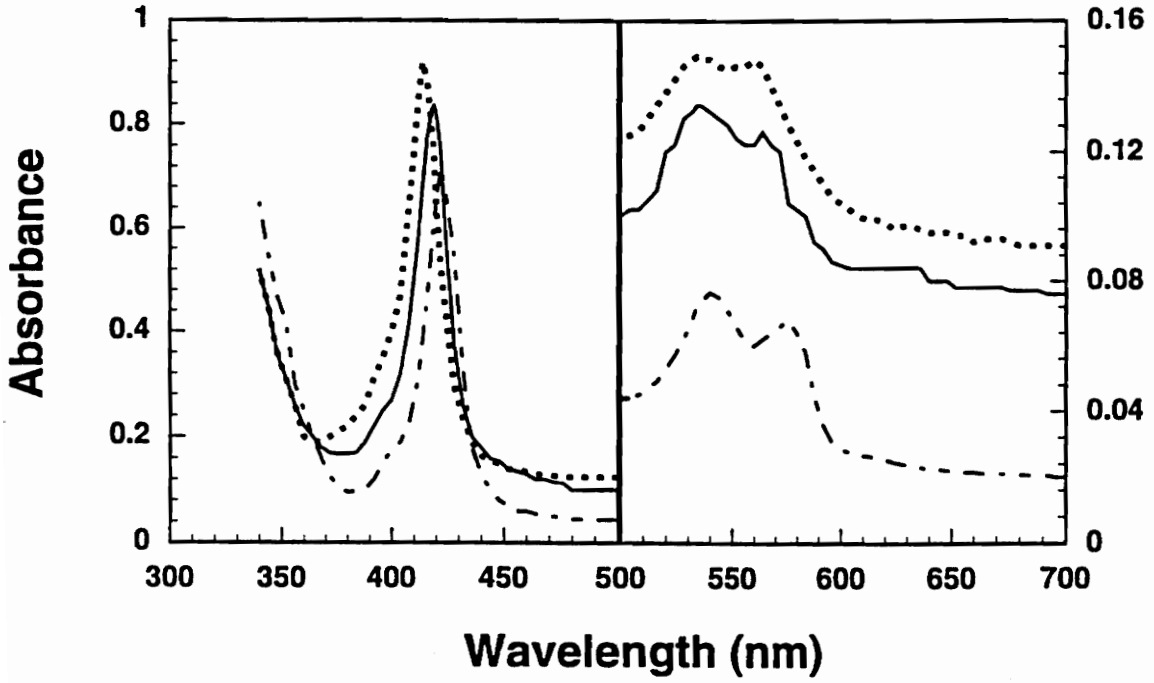
Absorbance
1
0.8
0.6
0.4
0.2
0
0.16
0.12
0.08
0.04
0
300
350
400
450
500
550
600
650
700
Wavelength (nm)

Figure 14. Absorption spectra of ferric (met) hemoproteins in 20 mM potassium phosphate buffer pH 7. Concentrations 6 - 8 μM. (----) leghemoglobin *a*; (——) cyanoglobin; (--•--) sperm whale myoglobin.

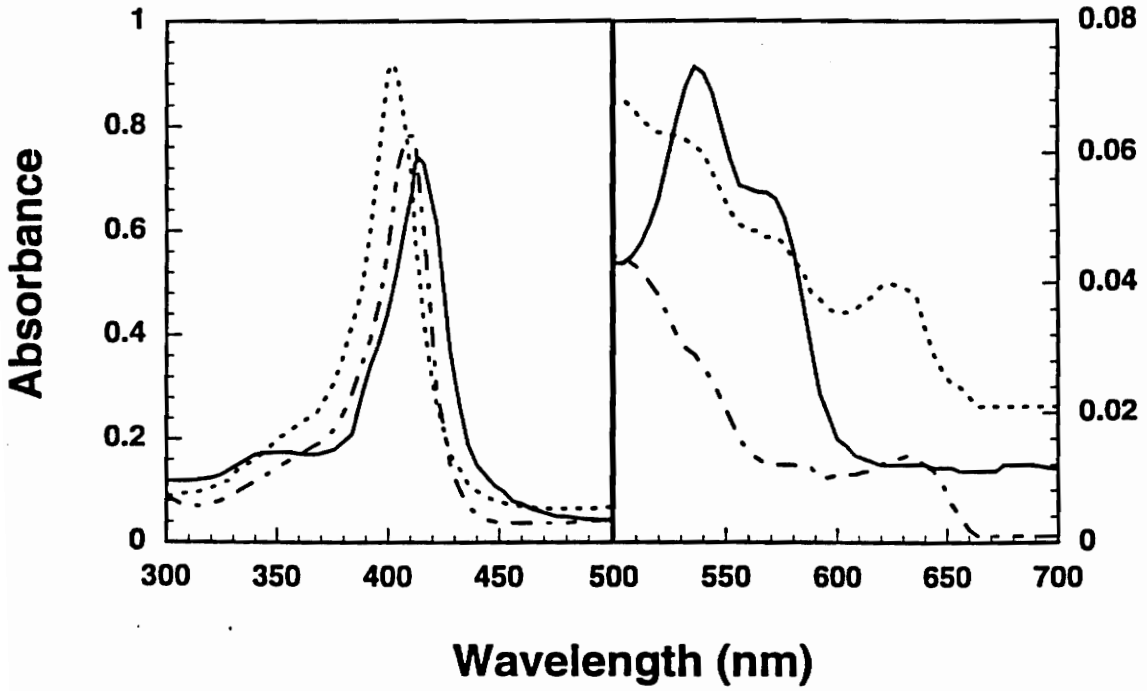
Absorbance
1
0.8
0.6
0.4
0.2
0
0.08
0.06
0.04
0.02
0
300
350
400
450
500
550
600
650
700
Wavelength (nm)

Figure 15. Absorption spectra of ferric (met) derivatives of sperm whale myoglobin in 20 mM potassium phosphate buffer pH 7. Concentration 8 μM. (-----) met-SWMb; (——) azidomet-SWMb; (--•--) cyanmet-SWMb.

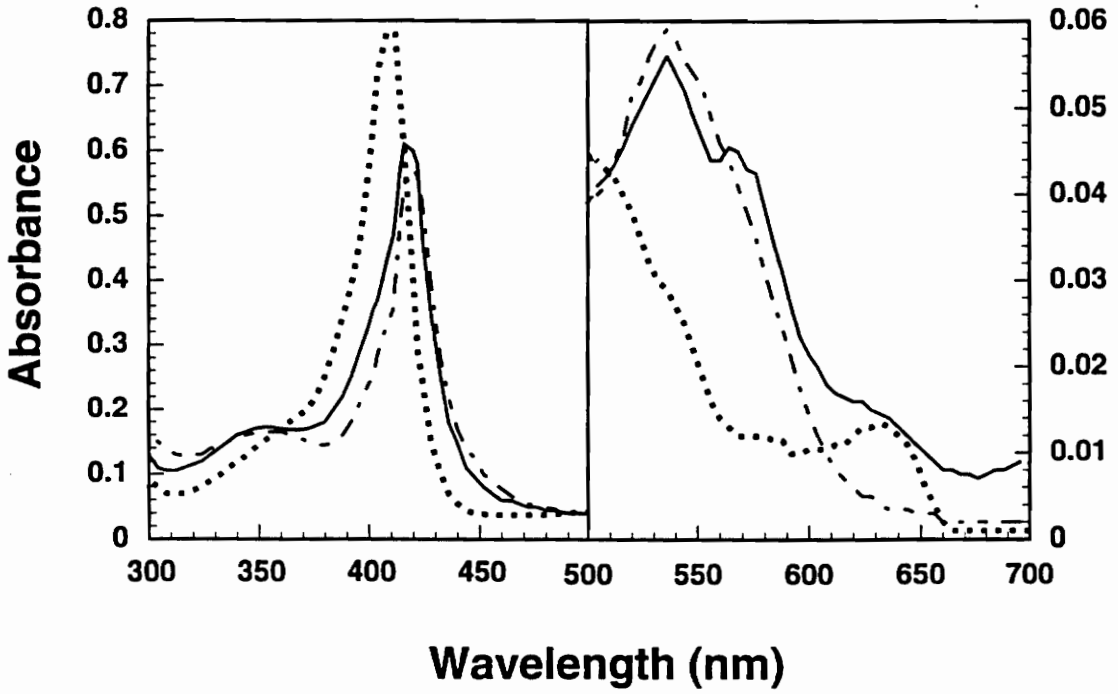
Absorbance
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.06
0.05
0.04
0.03
0.02
0.01
0
300
350
400
450
500
550
600
650
700
Wavelength (nm)

Figure 16. Soret-region CD spectra of hemoproteins in 20 mM potassium phosphate buffer pH 7. (-----) sperm whale myoglobin; (——) cyanoglobin; (--•--) leghemoglobin *a*. Molar ellipticity = degrees•cm^2• $decimol^{-1}$.

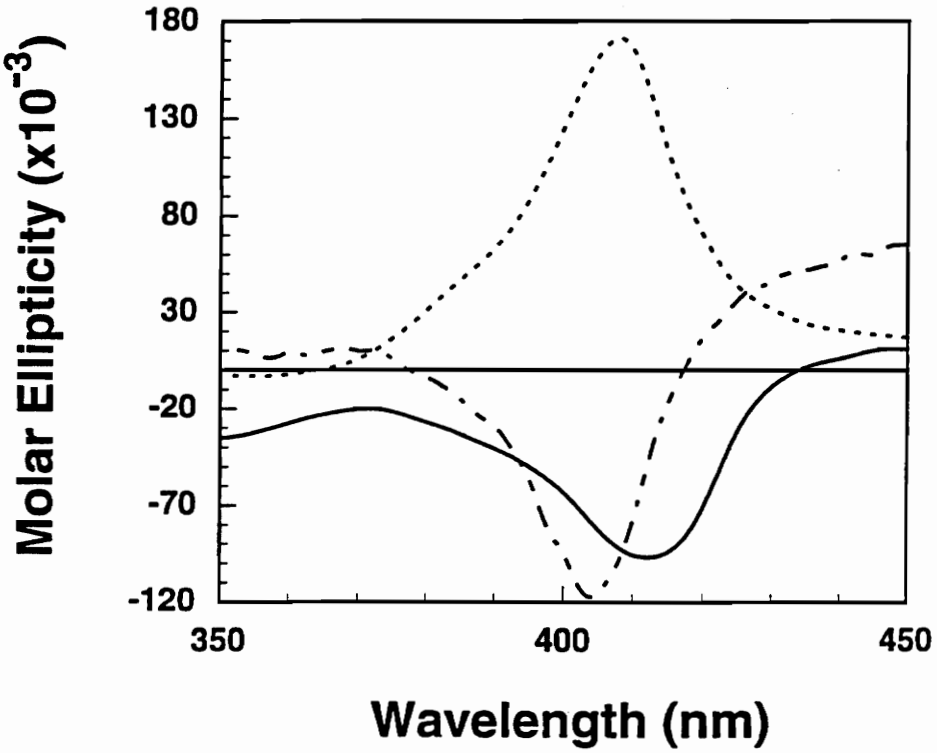
Molar Ellipticity (x10^{-3})
180
130
80
30
-20
-70
-120
350
400
450
Wavelength (nm)

Figure 17. Far-UV CD spectra of hemoproteins in 20 mM potassium phosphate buffer pH 7. (-----) cyanoglobin; (———) sperm whale myoglobin; (--•--) leghemoglobin *a*. Mean residue molar ellipticity = degrees • cm^2 • $decimol^{-1}$.

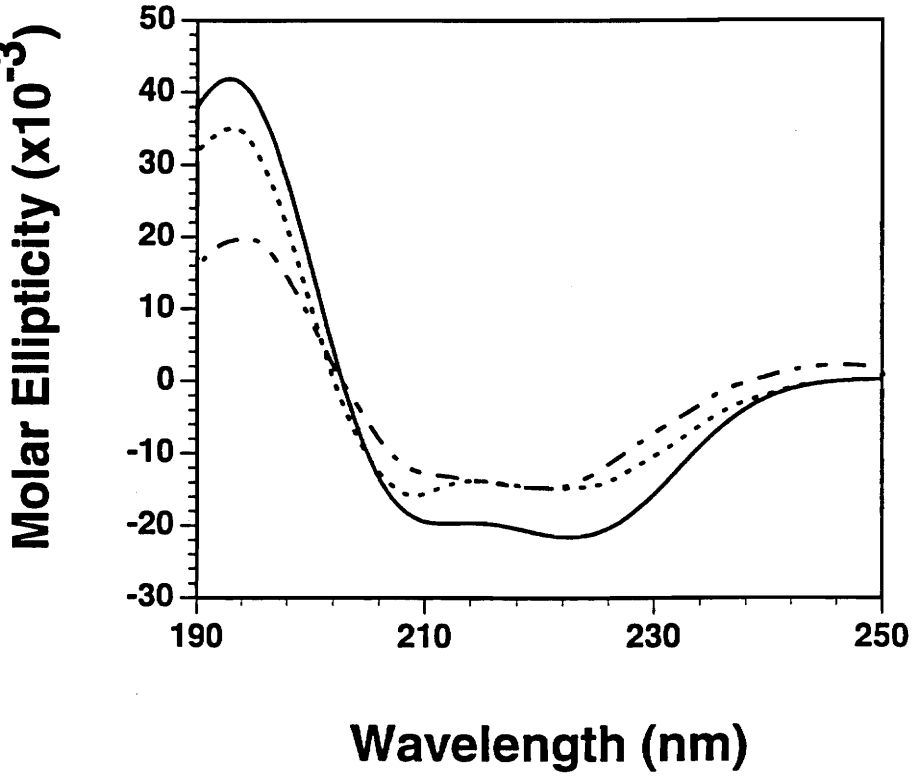
Molar Ellipticity (x10^{-3})
50
40
30
20
10
0
-10
-20
-30
190
210
230
250
Wavelength (nm)

Table 1. Analytical-scale column chromatography of GlbN.

Resin	Type	Equilibration Buffer	GlbN Adsorbtion	Elution Gradient
Q-sepharose	Strong anion exchanger	50 mM Tris.HCl pH 8.0	-	1 M NaCl step
Q-sepharose	Strong anion exchanger	50 mM ethanolamine pH 11.0	+	1 M NaCl step
Mono S	Strong cation exchanger	50 mM MES pH 6.0	-	1 M NaCl step
Sulfopropyl	Strong cation exchanger	50 mM MES pH 6.0	-	1 M NaCl step
Phenyl sepharose	Hydrophobic interaction	50 mM Tris.HCl pH 8.0 w/ 2 M ammonium sulfate	+	decscending linear using buffer with no salt
G-25	Gel filtration	50 mM Tris.HCl pH 8.0 w/ 1mM DTT	-	isocratic
Superose 12	Gel filtration	50 mM Tris.HCl pH 8.0 w/ 1 mM DTT	-	isocratic

Table 2. Absorbance maxima of various hemoprotein derivatives. [1]Gb = cyanoglobin; [2]Mb = sperm whale myoglobin; [3]Lb = leghemoglobin *a*.

Derivative	Soret (γ) peak (nm)	Visible (β) peak (nm)	Visible (α) peak (nm)
metGb[1] pH 7	414	538	576
metGb pH 9	414	540	576
deoxyGb	424	554	----
oxyGb	417	540	576
carbonmonoxy Gb	419	536	564
metMb[2] pH 7	412	----	632
cyanmetMb	423	540	570
azidometMb	420	540	570
deoxyMb	434	556	----
oxyMb	418	545	580
carbonmonoxy Mb	422	540	576
metLb[3] pH 7	404	----	628
deoxy Lb	422	556	----
oxyLb	411	540	575
carbonmonoxy Lb	414	534	560

Table 3. Crystallization set-up of GlbN at pH 7.2 using 50 mM Tris.HCl. GlbN concentration = 2 mg/ml.

% D = % saturation of ammonium sulfate in drop
% R = % saturation of ammonium sulfate in reservoir
n.d. = not determined
---- = no precipitate observed
+ = very slight precipitate observed
++ = fine granular precipitate observed
+++ = coarse granular precipitate observed
++++ = clumpy precipitate observed

% D	% R				
	40	45	50	55	60
4	-----	-----	-----	-----	n.d.
6	-----	-----	-----	-----	n.d.
8	-----	-----	-----	-----	n.d.
10	-----	-----	-----	-----	n.d.
12	-----	-----	-----	-----	n.d.
14	-----	-----	-----	-----	n.d.
20	-----	-----	-----	-----	-----
25	+	+	+	+	+
30	+	+	+	+	+
35	++	++	++	++	++
40	+++	+++	+++	+++	+++
45	+++	+++	+++	+++	+++

Table 4. Crystallization set-up of GlbN at varying pH. Refer to Table 3 legend for abbreviations. GlbN concentration = 2 mg/ml.

% D/% R	pH		
	7.4	7.6	7.8
30/50	+	+	+
35/50	++	++	++
30/60	+	+	+
35/60	++	++	++

Table 5. Crystallization set-up of GlbN at varying pH. Refer to Table 3 legend for abbreviations. GlbN concentration = 3 mg/ml.

% D / % R	pH				
	7.4	7.6	7.8	8.9	9.3
25/50	n.d.	n.d.	n.d.	++	++++
30/50	n.d.	n.d.	n.d.	++	++++
35/50	n.d.	n.d.	n.d.	+++	++++
25/55	n.d.	n.d.	n.d.	++	++++
30/55	+	+	+	++	++++
35/55	++	++	++	+++	++++
30/60	++	++	++	n.d.	n.d.
35/60	++	++	++	n.d.	n.d.

Discussion

The presence of the *glbn* insert in *E. coli* strain BL21DE3 was apparently innocuous during both growth and induction of cyanoglobin synthesis. High expression levels (approximately 10% of the protein in the cell free lysate) of cyanoglobin were attained upon qualitative observation of the SDS-PAGE gel of the cell free lysate. Every insert is unique in its ability to be expressed with the pT7-7 system because of potential gene product toxicity, therefore comparisons to other over-expressed proteins using this and other expression systems is difficult (Studier, 1990). Indeed, when the *Vitreoscilla* hemoglobin gene is overexpressed in *E. coli* under microaerobic conditions, the *E. coli* actually grow more efficiently (higher ATP production) when compared to untransformed wild-type (Khosla and Bailey, 1989) Such detailed comparisons of growth efficiency to wild-type and potential gene product toxicity were not investigated in this study because the yield of over-expressed cyanoglobin was judged to be satisfactory.

A common feature of the pT7-7 expression system is the presence of the β-lactamase gene on the plasmid. Although this provides for a selective pressure of both plasmid replication and insert expression, caution must be exercised. Very high amounts of β-lactamase are extruded to the media during both growth and induction. Even as soon as mid-logarithmic phase growth, there is no ampicillin remaining in the growth culture medium, and thus any *E. coli* that has lost the plasmid will no longer be under selective pressure (Studier, 1990). The remaining *E. coli* that do contain the plasmid will be quickly over-grown,

leading to poor recombinant protein yields upon induction. This may explain why LB agar plates older than one week gave very low yields of recombinant cyanoglobin when colonies from these plates were used for starter cultures. In addition, cells harvested for induction in stationary growth phase also gave low yields of cyanoglobin because the population of *E. coli* BL21DE3 (pGlbN) was no longer selected. Even subculturing to fresh media with ampicillin will carry over a substantial amount of β-lactamase, causing significant hydrolysis of the ampicillin in the medium (Studier, 1990). Most of these problems, however, were circumvented by never allowing the growth culture to grow past the mid-logarithmic phase before harvesting the cells for induction of cyanoglobin synthesis.

The expression of cyanoglobin from transformed *E. coli* in this study was judged to be sufficient because of the scarlet-red appearance of the cells. Spectral analysis proved that a hemoprotein was indeed expressed by virtue of a large absorption peak in the Soret region. Furthermore, cyanoglobin was isolated, at least in part, as the oxy-form as evidenced by the characteristic visible region splitting into α and β peaks. Cyanoglobin is either expressed in *E. coli* as the oxy-form or as the deoxy-form that subsequently coordinates oxygen when the cells are disrupted and processed with oxygenated buffers. This situation is analogous to sperm whale myoglobin, which can be isolated as the oxy-form when oxygenated buffers are used (Antonini and Brunori, 1971).

The chromatographic behavior of cyanoglobin reflected its calculated isoelectric point of 9.7 (Potts, et al. 1992). At pH 8.0, there should be a net positive charge on the surface of cyanoglobin, and as expected, no adsorption to the Q-sepharose resin at this pH was

observed. Many other native *E. coli* proteins have a net negative charge at pH 8.0 and will adsorb to the resin. Thus, a sufficient enrichment of cyanoglobin was effected with this step. The step that provided the greatest enrichment of cyanoglobin was the Superose 12 gel filtration, which gave an apparently homogeneous protein preparation. During this step, the color of cyanoglobin turned from scarlet-red to brown, indicating that autoxidation had occurred. This phenomena was also observed in previous attempts at purifying cyanoglobin (Potts, et al. 1992). Sperm whale myoglobin and leghemoglobin *a* also show a propensity to autoxidize, but the rate at which this event occurs is apparently faster with cyanoglobin because within 24 hours, at room temperature, oxy-cyanoglobin was converted completely to the met-form (data not shown). Attempts to use the Superose 12 step at 4° C in the presence of DTT, a reducing agent, did not appear to slow the rate of autoxidation significantly. A relatively fast rate of autoxidation has also been observed with the cytoplasmic hemoglobin of *Paramecium caudatum*, which shares significant amino acid sequence homology near the putative heme binding site with cyanoglobin (Tsubamoto et al., 1990).

SDS-PAGE analysis of cyanoglobin, sperm whale myoglobin, and leghemoglobin *a* demonstrated that cyanoglobin is smaller than the other hemoproteins in molecular mass. The molecular masses derived from SDS-PAGE analysis were comparable to the literature values of all the hemoproteins studied. In addition, the molecular mass of cyanoglobin was similar to those of the protozoan cytoplasmic hemoglobins, these being 12.5 kD and 15 kD for *Paramecium* and *Tetrahymena* hemoglobins, respectively (Iwassa et al., 1989 and Iwassa et

al., 1990).

Also observed during SDS-PAGE analysis is the tendency for both cyanoglobin and sperm whale myoglobin to form dimeric aggregates. This phenomenon has been observed previously for sperm whale myoglobin, with even trimeric and tetrameric aggregates being observed (Antonini and Brunori, 1971). Precisely why this irreversible aggregation occurs is poorly understood. In sperm whale myoglobin, these multimeric forms can be separated from each other as distinct fractions that do not reaggregate, nor do they dissociate to monomeric forms, once separated. Surprisingly, all of the various multimeric forms of sperm whale myoglobin are functionally indistinguishable, sharing nearly all functional properties with the monomeric form (Antonini and Brunori, 1971). The fact that the presence of multimeric forms of sperm whale myoglobin does not affect its ligand binding characteristics was used as a basis for judging that this aggregation phenomena in cyanoglobin, which is slight in comparison to sperm whale myoglobin, should be of little concern. However, these aggregates may constitute an unacceptable amount of sample heterogeneity for the crystallization of cyanoglobin. The actual ratio of these form(s) in cyanoglobin could be assessed by the use of dynamic light scattering, that provides a measure of the hydrodynamic radius of all species present in the sample in question. The appearance of the SDS-PAGE gel developed with Coomassie R-250 shows that the amount of aggregation into dimeric forms of cyanoglobin was so low as to be undetectable.

The increased sensitivity of immunoanalysis, however, did reveal a slight aggregation of cyanoglobin into dimeric forms. Neither sperm whale myoglobin or leghemoglobin *a* cross

reacted with the rabbit anti-cyanoglobin antibodies. This indicates that the epitopes of cyanoglobin are different from those of sperm whale myoglobin and leghemoglobin *a*. Thus the hemoproteins studied are not immunologically related.

Hemoglobins were once believed to be exclusive to the eukaryotes until the discovery that the prokaryotic *Vitreoscilla* hemoprotein was a true hemoglobin (Webster, 1988). The only other prokaryotic hemoglobins characterized thus far have been from *Rhizobium meliloti* and *Nostoc commune* UTEX 584. Absorption spectroscopy analysis of cyanoglobin has revealed unique properties, most of which resemble those of the protozoan hemoglobins. Cyanoglobin was determined to bind carbon monoxide both previously and in this study (Potts, 1992). The carbonmonoxy form of cyanoglobin showed no unique differences to the carbonmonoxy derivatives of sperm whale myoglobin or leghemoglobin *a*. Since deoxycyanoglobin could bind carbon monoxide, the heme iron is penta-coordinate in the deoxy (Fe^{2+}) state and could possibly bind molecular dioxygen. This present study confirmed that cyanoglobin does indeed coordinate molecular dioxygen, but reveals spectral characteristics of the oxy-form quite unlike those of sperm whale myoglobin and leghemoglobin *a*. Specifically, α/β ratio in the visible region is atypically low when compared to sperm whale myoglobin and leghemoglobin *a*. Such low α/β ratios have been observed, however, in both the *Paramecium* and *Tetrahymena* oxyhemoglobins (Tsubamoto, 1990 and Iwaasa, 1990). This property cannot be correlated to the oxygen affinity of the hemoprotein since the P_{50} for the *Paramecium* hemoglobin is 0.6 mm Hg, a value intermediate between that of LgHb (P_{50} = 0.04 mm Hg) and SWMb (P_{50} = 8 mm Hg) (Iwassa, 1990; Appleby, 1962;

Antonini and Brunori, 1971). The differences in the oxygen affinities of these hemoproteins show no apparent correlation to the differences in their α/β ratios in the visible region of the spectrum.

Such unique spectral profiles in the visible region also cannot be correlated to the propensity to autoxidize to the met-form. The oxyhemoglobin of *Aplysia* sp. has a nearly identical α/β peak ratio in the visible region when compared to sperm whale myoglobin, yet the rate of autoxidation to the met-form is approximately 100-times faster (Shikama and Matsuoka, 1986). Autoxidation of the oxy to the met-form is believed to occur because of a lack of stabilization of the bound dioxygen on the distal side of the heme iron. Sperm whale myoglobin and leghemoglobin *a* have distal histidines that are believed to stabilize the iron-dioxygen complex. The protozoan hemoglobins and cyanoglobin may have glutamine residues at the distal side of the putative site of the heme iron, which might be a factor in the stability of the bound dioxygen complex. Further investigation into the kinetics of oxygen binding and the kinetics of autoxidation of cyanoglobin could reveal if the putative distal glutamine imparts unique characteristics to the equilibrium.

The met-forms of sperm whale myoglobin and leghemoglobin *a* have different spectral properties from cyanoglobin. The visible spectrum of sperm whale myoglobin and leghemoglobin *a* (pH 7.0) are consistent with a high-spin heme (Fe^{3+}) iron. The presence of water, a weak-field ligand, as the sixth coordinating ligand to the Fe^{3+} is consistent with the interpretation. Met-cyanoglobin at pH 7 is clearly different, exhibiting a spectral profile of a low-spin heme complex. Thus the presence of water at the sixth-coordination position is

unlikely. Met-cyanoglobin showed no pH dependence of spectral properties when examined at pH 7 and at pH 9.0. This suggests that a hydroxide ion cannot occupy the sixth-coordination position in met-cyanoglobin, and reinforces the idea that water probably cannot occupy the sixth position either. The low-spin met spectra of cyanoglobin and *Paramecium* hemoglobin, specifically a peak at 540 nm and a shoulder at 570 nm, suggests the presence of a hemichrome in the met-form. A hemichrome is defined as a hemoprotein that possesses an internal protein ligand in the sixth-coordination position as well as the fifth position in the met-form (Rachmilewitz, 1971). Kinetic studies of autoxidation events in *Paramecium* hemoglobin have suggested the presence of hemichrome as the final product, with no hydroxymet intermediate detectable (Tsubamoto, 1990). In addition, the most likely candidate for the sixth ligand in autoxidized *Paramecium* methemoglobin is the imidazole ring of a histidine residue located close to the heme binding site. Corroborating this fact is the formation of an identical spectral profile of a hemichrome when imadazole is added to the acidic-met form of *Paramecium* hemoglobin (Tsubamoto, 1990).

As stated earlier, shifts in spectral character are not observed in met-cyanoglobin at pH 7 and pH 9. A possible interpretation is the formation of a hemichrome under both neutral and alkaline conditions in the met-form, which is unique compared to all hemoglobins studied to date. The fact that at both pH 7 and at pH 9 cyanoglobin fails to bind the high affinity, strong-field ligands, cyanide and azide, reinforces the idea that an internal residue is occupying the sixth-coordination position. Interestingly, both *Paramecium* hemoglobin and cyanoglobin have histidine residues (excluding the proximal histidine) at the same position,

when aligned, close to the putative site of the heme iron (Potts, 1992).

The key structural differences between cyanoglobin, sperm whale myoglobin, and leghemoglobin *a* are in the heme binding site. Little difference was observed in the alpha helical contents by CD analysis. Large differences, however, were seen in the CD analysis in the Soret region. Both leghemoglobin *a* and cyanoglobin have strong negative ellipticities, suggestive of a similar pattern of protein side chain contacts, in contrast to the strongly positive ellipticity of sperm whale myoglobin (Nicola, 1975). X-ray analysis of leghemoglobin *a* revealed that the heme binding pocket is more open than that of sperm whale myoglobin (Appleby, 1984). *Paramecium* hemoglobin has also been proposed to have a more open heme binding pocket by kinetic analysis of autoxidation events. Analysis of the oxygen binding affinities of cyanoglobin and the kinetics of autoxidation events could provide information about the openness of the cyanoglobin heme binding site.

The lack of data concerning the oxygen binding characteristics of cyanoglobin precludes a detailed analysis of a functional role for cyanoglobin function *in vivo*. The fact that cyanoglobin is not synthesized constitutively under nitrogen-limiting conditions in *Nostoc commune* casts doubt on cyanoglobin functioning solely as an oxygen scavenger to protect the nitrogenase complex. In addition, the small amount of cyanoglobin that is produced *in vivo* when induced (M. Potts, personal communication) is in contrast to leghemoglobin *a*, which is produced in large amounts in legumous root nodules. However, the expression of low intracellular amounts of cyanoglobin is analogous to the expression of hemoglobins in actinorhizal roots (Tjempkema and Asa, 1987). A possible role that must be investigated

further is the function of cyanoglobin as an effector of other promoter(s) that exist in *Nostoc commune*. Such an "oxygen-sensing" role has been proposed for the Fix L hemoprotein of *Rhizobium meliloti* and the hemoglobin of *Frankia* sp. (Gilles-Gonzales, 1991; Silvester and Harris, 1990). Along these lines, cyanoglobin may be modulated *in vivo* in response to microaerobic growth conditions, possibly effecting the expression of other gene(s). Whether or not any of the *nif* genes of *Nostoc* are effected by cyanoglobin expression is currently unknown. Further investigations, primarily deletion mutagenesis of the cyanoglobin gene in intact *Nostoc* cells and the resultant physiological responses, could reveal clues to cyanoglobin function. To date, cyanoglobin is the only cyanobacterial hemoglobin, and the third prokaryotic hemoglobin (besides *Vitreoscilla* and *Rhizobium meliloti*) to be characterized. Until these hemoglobins were discovered, it was generally accepted that the globin gene originated from eukaryotic genetic lines. New interest in globin gene evolution has been sparked by the discovery of globins from the lower eukaryotic organisms, yeast, and the protozoans. Phylogenetic analysis of the heme-containing domain of the *Saccharomyces* hemoprotein show *Vitreoscilla* hemoglobin as the putative ancestral globin gene (Zhu and Riggs, 1992). Although high amino acid sequence homology exists between *Vitreoscilla* hemoglobin and leghemoglobin *a*, the latter was found to retain a third central intron missing from animal hemoglobins, which refutes the theory of an ancestral globin gene transfer event through symbiosis (Appleby, 1984). The protozoan hemoglobins are apparently unique, occupying a branch divergent from the yeast hemoglobin. Where does cyanoglobin fit into this scheme? Recently, percent difference analysis (cladogram) of aligned globin sequences

has established cyanoglobin and the protozoan hemoglobins as a unique assemblage of hemoproteins that, as a group, are very much different from the other prokaryotic hemoglobins (Angeloni and Potts, in press). The high amino acid sequence homology of cyanoglobin to that of the protozoan hemoglobins and the fact that cyanoglobin is of prokaryotic origin warrants phylogenetic analysis to explore the ongoing enigma of prokaryotic and eukaryotic globin gene divergence.

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Vita

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Professional Societies

Gamma Sigma Delta Agricultural Honor Society (Virginia Tech Chapter)
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The Protein Society (Student Member)
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Publications

Oxenrider, K.A., Rasche, M.E., Thorsteinsson, M.V., and Kennelly, P.J. (1993) Inhibition of an archeal protein phosphatase activity by okadaic acid, microcystin-LR, or calyculin. *FEBS Letters* 331:291-295.

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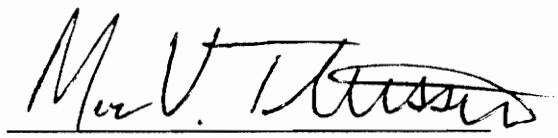

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