A Cyanobacterial Hemoglobin with Unusual Ligand Binding Kinetics and Stability Properties†

Marc V. Thorsteinsson,‡ David R. Bevan, and Malcolm Potts*

Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

Yi Dou, Raymund F. Eich, Mark S. Hargrove, Quentin H. Gibson, and John S. Olson

Department of Biochemistry and Cell Biology, Rice University, Houston, Texas 77251

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ABSTRACT: The *glbN* gene of the cyanobacterium *Nostoc commune* UTEX 584 encodes a hemoprotein, named cyanoglobin, that has high oxygen affinity. The basis for the high oxygen affinity of cyanoglobin was investigated through kinetic studies that utilized stopped-flow spectrophotometry and flash photolysis. Association and dissociation rate constants were measured at 20 °C for oxygen, carbon monoxide, nitric oxide, and methyl and ethyl isocyanides. The association rate constants for the binding of these five ligands to cyanoglobin are the highest reported for any naturally occurring hemoglobin, suggesting an unhindered and apolar ligand binding pocket. Cyanoglobin also shows high rates of autoxidation and hemin loss, indicating that the prosthetic group is readily accessible to solvent. The ligand binding behavior of cyanoglobin was more similar to that of leghemoglobin *a* than to that of sperm whale myoglobin. Collectively, the data support the model of cyanoglobin function described by Hill et al. [(1996) *J. Bacteriol. 178*, 6587−6598], in which 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.

The discovery of hemoglobins (hemoproteins that reversibly bind oxygen) in bacteria renewed interest in the origins and evolution of the globin gene family (*1*−*3*). Nearly all the major divisions of Eubacteria have representatives that contain a hemoglobin-like protein. Bacterial hemoglobins include *Vitreoscilla* sp. strain C1 hemoglobin [Vhb; (*4*)]; the flavohemoglobins from *Escherichia coli* [Hmp; (*5*)], *Alcaligenes eutrophus* [FHP; (*6*)], and *Bacillus subtilis* (*7*); and FixL from *Rhizobium meliloti* (*8*).

The obligate aerobe *Vitreoscilla* sp. strain C1 is found in oxygen-poor environments in nature. Synthesis of VHb in *E. coli* rescues *cyd* (high O_2[1] affinity, *d*-type terminal oxidase) mutations under microaerobic conditions, suggesting an oxygen scavenging and storage function (*9*). The flavohemoglobins of *E. coli*, *A. eutrophus*, and *B. subtilis* are chimeric proteins that contain an oxygen binding hemoglobin domain and a flavin domain (10). There is high amino acid sequence similarity between the flavohemoglobins and Vhb, and the expression of *vhb* is also regulated by oxygen and FNR (*11*). In addition, an NADH-dependent flavin-containing enzyme copurifies with native VHb, which may suggest the heme and flavin domains evolved separately in this bacterium during evolution (*12*). The precise functions of VHb and the flavohemoglobins remain unclear.

FixL is also a chimeric hemoglobin and functions as an oxygen sensor by transmitting the oxygen signal through a kinase/phosphatase domain. Under conditions of oxygen deprivation, FixL phosphorylates a response regulator, FixJ, which activates genes required for nitrogen fixation in *Rhizobium meliloti* (*13*). Under conditions of high oxygen tension within the cell, FixL phosphatase activity predominates and desphosphorylates FixJ, which no longer activates genes required for nitrogen fixation.

Nostoc commune UTEX 584 (*Nostoc* 584) is a photoautotrophic, heterocystous cyanobacterium that is capable of aerobic nitrogen fixation. *Nostoc* 584 synthesizes a monomeric hemoglobin, named cyanoglobin (GlbN), under dual conditions of nitrogen and oxygen deprivation. Cyanoglobin has been hypothesized to be involved in some aspect of nitrogen fixation because *glbN* is juxtaposed between *nifU-nifH* in the chromosome of *Nostoc* 584 and cyanoglobin is synthesized under conditions conducive to induction of nitrogenase (*14*).

Cyanoglobin binds oxygen reversibly and with high affinity [$P_{50} \sim 0.55$ mmHg at 20 °C; (*15*)]. In vivo, cyanoglobin is specifically located at the periphery of the cell membrane of heterocysts and vegetative cells and is synthesized when the capacity to fix nitrogen is maximal (*16*). It was hypothesized that the role of cyanoglobin is to scavenge and present oxygen to, or is a part of, a terminal

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* To whom correspondence should be addressed. Telephone: 540-231-5745. Fax: 540-231-9070. Electronic mail address: geordie@vt.edu.

‡ Present address: Department of Bacteriology, University of Wisconsin−Madison, Madison, WI 53706.

[1] Abbreviations: GlbN, cyanoglobin; SWMb, sperm whale myoglobin; Lb*a*, leghemoglobin *a* (*Glycine max*); O_2, oxygen; CO, carbon monoxide; NO, nitric oxide; met, ferric (Fe^{3+}) hemoglobin.

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cytochrome oxidase complex when the capacity to fix nitrogen, and thus ATP demand, is maximal (*16*). A similar function is ascribed to the leghemoglobins, which scavenge and present oxygen to vigorously respiring rhizobial symbionts, to sustain ATP supply for nitrogen fixation (*17*). Circular dichroism studies suggested that the heme environments of cyanoglobin and soybean leghemoglobin *a* are similar (*15*).

Based on amino acid sequence similarity, cyanoglobin is more similar to the protozoan hemoglobins and the chloroplast hemoglobin of the alga *Chlamydomonas eugametos* than to sperm whale myoglobin or the leghemoglobins (*18*−*20*). Cyanoglobin was suggested to contain the three-on-three helical fold (globin fold) common to all globins *(21)*. On the basis of amino acid sequence alignments *(14, 21,* and *22)*, and NMR experiments (Yeh, Thorsteinsson, Bevan, and La Mar, unpublished data), the key first-shell residues surrounding bound ligands in cyanoglobin appear to be Ile (B10), Phe (CD1), Gln (E7), and Leu (E11).

We have examined the kinetics of ligand binding to cyanoglobin in order to understand the mechanistic origin of its high oxygen affinity. A new isolation method for cyanoglobin in the oxygenated form was developed in order to obtain adequate amounts of protein for this study. The kinetics of carbon monoxide (CO), nitric oxide (NO), and alkylisocyanide binding were also studied to probe the stereochemistry of the distal pocket. Wild-type sperm whale myoglobin (SWMb), H64Q SWMb, and H64Q/V68L SWMb were used as controls. The two altered forms of sperm whale myoglobin were constructed to have Gln and Leu residues at the E7 and E11 positions, respectively, to mimic the naturally occurring residues thought to occur in cyanoglobin based on amino acid sequence alignment (see *21* and *22*). The susceptibility of cyanoglobin to autoxidation and hemin loss also were examined. Collectively, the data allow comparison of the physiological properties of cyanoglobin, leghemoglobin *a*, and sperm whale myoglobin and provide the basis for future site-directed mutagenesis studies.

MATERIALS AND METHODS

Plasmids and Strains. The isolation of *glbN* from *Nostoc commune* UTEX 584 and the construction of the expression system in *E. coli* were described (*14*).

Growth of Bacteria. The growth of *E. coli* strain BL21DE3 (pGlbN) and the induction of cyanoglobin synthesis were performed essentially as described (*15*) with the exception of the following: (1) δ-aminolevulinic acid was omitted, (2) 250 μM $FeCl_3$ (~50 μM Fe^{3+}; Fisher) was substituted for 12 mg/mL ferric ammonium citrate (~5 μM Fe^{3+}), and (3) the incubation temperature was lowered to 30 °C after the addition of IPTG. Cells were harvested and washed as described (*15*). The cells were then resuspended in 50 mM Tris·HCl (pH 7.5)/1 mM EDTA (4 mL/g of cells) containing protease inhibitors (*15*) and stored at −70 °C until use.

Altered Forms of Sperm Whale Myoglobin. The construction of the mutants, the expression in *E. coli*, and the isolation of sperm whale myoglobin altered proteins (H64Q and H64Q/V68L) were described by Springer, Egeberg, Sligar, and co-workers (*23* and *24*). We have adopted the helix and residue nomenclature for sperm whale myoglobin as described by Dickerson and Geis (e.g., E7 is the seventh residue along the E-helix, which is position 64 in sperm whale myoglobin and position 43 in cyanoglobin) (*25*).

Isolation and Purification of Cyanoglobin. Isolation of cyanoglobin differed from previous work (*15*) by including hydrophobic interaction chromatography and substituting S-300 gel filtration for Superose 12 gel filtration. Using the new procedure, cyanoglobin could be isolated in the oxygenated form.

The Q-Sepharose fraction (*15*) was loaded onto a phenyl Sepharose (CL-4B; Pharmacia) column (1.5 cm × 28 cm; ~50 mL), equilibrated with 2 M NaCl in 50 mM Tris·HCl, pH 7.5, at room temperature. Under these conditions, cyanoglobin eluted isocratically, with resolution of a faster-moving, red-colored fraction and a slower-moving, brown-colored fraction (data not shown). The red-colored fraction, indicating oxygenated cyanoglobin, was collected from ~100−150 mL of total effluent volume. The brown-colored fraction was collected between 200 and 250 mL effluent volume, but was not processed further. The red, fast-moving fraction (~50 mL) was then concentrated to 5 mL by ultrafiltration using nitrogen pressure and YM3 (3000 molecular weight cutoff) membranes (Diaflo).

The phenyl Sepharose fraction (5 mL) was then passed through a S-300 (Pharmacia) gel filtration resin (5 cm × 51 cm; 1000 mL) equilibrated with 0.2 M NaCl in 50 mM Tris·HCl, pH 7.5. The cyanoglobin-containing material was collected, in 10 mL fractions, from 760 to 880 mL total effluent volume. There was no discernible change in red color in the sample over the ~6 h course of the procedure. Fractions judged to contain purified cyanoglobin by Coomassie Brilliant Blue R-250 staining using 15% (w/v) SDS−PAGE slab gels (Hoefer) and a modification of the protocol of Laemmli (*26*) were pooled (~50 mL) and then concentrated to ~2 mL by ultrafiltration as described above. A UV−visible spectrum taken on purified oxycyanoglobin diluted into 0.1 M sodium phosphate, pH 7.0, buffer had absorbance ratios at 415 nm/280 nm of approximately 4, indicative of negligible apo-cyanoglobin contamination. Under these new conditions, essentially 100% of the cyanoglobin purified was in the oxygenated form (see Results and Figure 1). Metcyanoglobin was produced by adding no more than a 10% mole excess of potassium ferricyanide (Fisher) to a purified, oxygenated sample, and then removing excess ferricyanide by desalting with G-50 Sephadex gel chromatography resin (Pharmacia), equilibrated in 20 mM sodium phosphate, pH 7.0.

In this study, cyanoglobin concentrations were expressed on a heme basis. Heme content was determined using the reduced pyridine hemochromogen method (*27*). A buffered solution of cyanoglobin ($ABS_{415\ nm}$ ~ 1.0) was mixed (1:3) with 100 mM NaOH and 30% (v/v) pyridine and allowed to incubate at room temperature for 1 h. Solid sodium dithionite was added, and the spectrum of the reduced pyridine hemochromogen was collected immediately. Heme *b* (iron-protoporphyrin IX) was quantitated by measuring the absorbance at 558 nm [$\epsilon = 32\ mM^{-1}\ cm^{-1}$; (*28*)]. Values for heme quantitation for each preparation were taken as the average of three replicate determinations.

UV−Visible Absorption Spectroscopy. Optical spectra of cyanoglobin were obtained using a Hewlett-Packard photodiode array spectrophotometer using 1 cm path length quartz

cuvettes at room temperature. Absorbance readings (against a buffer blank) were taken at 2 nm intervals. The raw ASCII data were manipulated using Quattro Pro (Borland) and graphed using Sigma Plot (Jandel Scientific). The spectrum of the oxygenated form of cyanoglobin was obtained using a diluted sample (8 μM) in 0.1 M sodium phosphate, pH 7.0. This spectrum was compared to that of ferricyanide-oxidized cyanoglobin in the same buffer.

Stopped-Flow Spectrophotometry. Association rates (k') for carbon monoxide (CO) and alkylisocyanide binding to cyanoglobin were determined by rapid mixing in a Gibson-Dionex stopped-flow apparatus equipped with an On-Line-Instruments System (OLIS) Model 3820 data collection system as described by Rohlfs et al. (29). Stock solutions of hemoglobins were diluted to ~5 μM (before mixing) into anaerobic 0.1 M potassium phosphate (pH 7.0)/0.3 mM EDTA. A few crystals of sodium dithionite were then added to ensure that the hemoglobin was in the 100% reduced state. Anaerobic stock solutions of CO (1.25 mM), NO (2 mM), or the appropriate alkylisocyanide, in 0.1 M potassium phosphate (pH 7.0)/0.3 mM EDTA, were produced by either sparging the buffer with 1 atm of CO or NO for ~1 h or diluting anaerobically the neat liquid of the appropriate alkylisocyanide (*30*) into the buffer. In all the kinetic experiments, the concentration of protein was much less than that of ligand, so that the bimolecular binding processes behave as single pseudo-first-order reactions, with the observed rate constants proportional to the ligand concentration. Therefore, data were fitted with a single-exponential function according to the condition: $A_t = A_o e^{-k_{obs}t}$, where "A_o" equals the total absorbance change, "t" is the reaction time, and "k_{obs}" is the observed first-order rate constant. The traces were saved as ASCII output for later manipulation. Data were then "normalized" by setting the highest absorbance difference equal to 1. Association rate constants were calculated from the expression $k_{obs} = k'[X] + k$, where [X] is the concentration of ligand used in the stopped-flow experiment, k_{obs} is the observed rate at concentration X, k' is the association rate constant, and k is the dissociation rate constant, which is negligible at high [X].

Stopped-flow rapid mixing techniques were also used to determine rate constants for dissociation of oxygen and the alkylisocyanides from cyanoglobin using the technique of ligand replacement by CO (*31*). Carbon monoxide dissociation rates from cyanoglobin were measured using nitric oxide as the displacing ligand. Nitric oxide dissociation rates were measured by rapidly mixing a solution saturated with carbon monoxide containing excess dithionite with a solution of the NO complex of reduced cyanoglobin (*32*).

Flash Photolysis. The association rates (k') for oxygen, carbon monoxide, and nitric oxide binding to cyanoglobin were determined by flash photolysis as described by Rohlfs et al. (*29*). Concentrated stock solutions of deoxyhemoglobins were diluted anaerobically to ~100 μM into cuvettes (1 mm path length) containing a known concentration of the ligand of interest. The fully liganded samples of ferrous hemoglobins were then photodissociated by a 0.3 μs excitation pulse from a Phase-R Model 2100B dye laser using Rhodamine 575. The bimolecular rebinding time courses were collected by a digital oscilloscope and processed as described in Rohlfs et al. (*29*). Most time courses were recorded at 436 nm, near the Soret maximum for most deoxyhemoglobins examined. A minimum of five traces was collected and averaged for each experiment.

Nanosecond geminate recombination experiments were carried out as described by Carver et al. (*33*) and Quillin et al. (*34*). Photolysis of the Fe−ligand bond was initiated with a 9 ns YAG laser excitation pulse at 532 nm, and intramolecular recombination was followed by recording absorbance changes at 436 nm on 0−500 ns time scales (*33*).

Kinetics of Autoxidation. The kinetics of autoxidation of oxycyanoglobin to the metform were measured using the method described by Brantley et al. (*35*). Time courses were determined in duplicate by diluting a concentrated solution of oxycyanoglobin (0.8 mM) to 20 μM into the following, air-equilibrated (263 μM O_2), prewarmed (37 °C) buffers: 0.1 M MES, pH 5.0, 0.1 M KP_i, pH 7.0, and 0.1 M Tris·HCl, pH 9.0, containing catalase and superoxide dismutase (Sigma; 3 mmol of each/mol of cyanoglobin) to scavenge autoxidation products (O_2^- and H_2O_2). Time courses were recorded at 576 nm over 12 h at 37 °C, using a Shimadzu UV−Vis PC2100 spectrophotometer, equipped with a multicell chamber and thermostat. The data were fitted to a single-exponential decay expression to obtain the first-order rate constant, k_{ox}.

Kinetics of Hemin Loss. Hemin loss from cyanoglobin was measured using the protocol developed by Hargrove et al. (*36*). A concentrated solution (0.8 mM) of purified cyanoglobin was oxidized with a slight excess of potassium ferricyanide, which was then removed by passing material through a G-25 (Pharmacia) column equilibrated with either 0.1 M MES, pH 5.0, or 0.1 M potassium phosphate, pH 7.0. Metcyanoglobin effluents were then diluted to a final concentration of ~20 μM in a solution containing 40 μM apo-H64Y/V68F SWMb, 0.45 M sucrose, and either 0.15 M MES, pH 5.0, or 0.15 M potassium phosphate, pH 7.0. The absorbance increase at 600 nm due to formation of H64Y/V68F met-SWMb was used to follow hemin loss from cyanoglobin at 20 °C. The data were collected on the Shimadzu spectrophotometer and time courses fitted to a single-exponential expression.

RESULTS

Isolation and Purification of Cyanoglobin. The phenyl Sepharose chromatography step was essential for discriminating between oxygenated cyanoglobin and autooxidized cyanoglobin. The oxy and met forms of cyanoglobin appear to possess different surface properties under the conditions of this chromatography step. The UV−visible spectrum of the fast-moving, red effluent was characteristic of oxycyanoglobin, and that of the slow-moving, brown fraction was characteristic of metcyanoglobin (data not shown). In contrast to the previously isolated metcyanoglobin (*15*), the metcyanoglobin obtained by oxidizing a sample of oxycyanoglobin isolated by this modified procedure coordinated a variety ligands at neutral pH, including cyanide, azide, fluoride, acetate, imidazole, and nicotinate as judged by the formation of characteristic absorbance bands (data not shown).

Oxycyanoglobin and Metcyanoglobin. The spectrum of isolated oxycyanoglobin was characteristic of oxygenated hemoglobins (Figure 1), with a Soret band at 416 nm (ϵ = 131 mM^{-1} cm^{-1}) and β and α peaks at 540 nm (ϵ = 14.8 mM^{-1} cm^{-1}) and 574 nm (ϵ = 11.7 mM^{-1} cm^{-1}), respec-

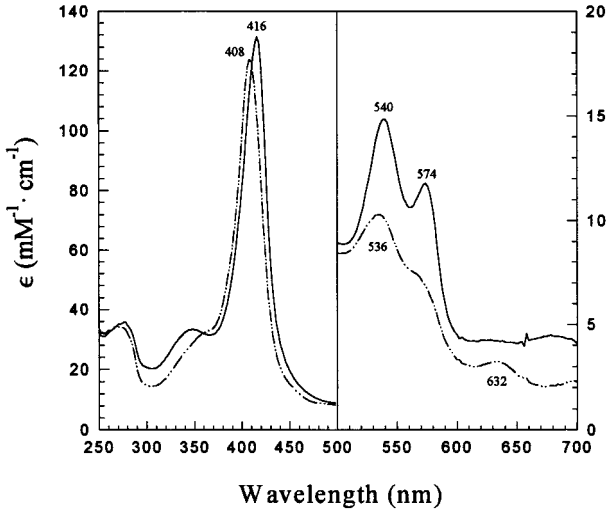

FIGURE 1: The Soret extinction coefficient of metcyanoglobin, compared to that of oxycyanoglobin, is unusual. UV−visible spectra of isolated oxycyanoglobin (—) and metcyanoglobin (– ·· –) showing the unusually low Soret extinction coefficient of the metform. Protein concentrations were 8.0 μM in 0.1 M sodium phosphate buffer, pH 7.0.

tively. The intensity of the α peak is unusually low when compared to the β peak, giving an α/β ratio of 0.8 compared to a ratio of ~1.0 for most globins. However, a lower ratio is also observed in the protozoan oxyhemoglobins, as well as the chloroplastic oxyhemoglobin of *Chlamydomonas eugametos*, all of which share high amino acid sequence similarity with cyanoglobin (*18*−*20* and *22*).

The spectrum of metcyanoglobin generated from the purified oxygenated protein (Figure 1) revealed a spectral profile not observed in past purifications (*15*). The Soret, α, and β bands are at 408 nm ($\epsilon = 123$ mM^{-1} cm^{-1}), 536 nm ($\epsilon = 10.3$ mM^{-1} cm^{-1}), and 570 nm ($\epsilon = 7.19$ mM^{-1} cm^{-1}), respectively, and there is a small high-spin peak at 632 nm ($\epsilon = 3.28$ mM^{-1} cm^{-1}). Thus, the visible spectrum of metcyanoglobin suggests a mixture of high- and low-spin ferric heme which is characteristic of water coordination. However, the Soret extinction coefficient of metcyanoglobin (123 mM^{-1} cm^{-1}) is significantly lower than that of sperm whale myoglobin [157 mM^{-1} cm^{-1}; (*28*)] and leghemoglobin *a* [151 mM^{-1} cm^{-1}; (*37*)], suggesting that the strength or extent of water coordination is less in the bacterial protein.

Oxygen Binding. The oxygen association rate constant for cyanoglobin was determined to be 390 μM^{-1} s^{-1}, which is higher than the k'_{O2} for any other naturally occurring hemoglobin studied to date (Table 1). As shown in Figure 2, the reaction of oxygen with cyanoglobin is 26-fold faster than that with native sperm whale myoglobin and also much faster than the O_2 reaction with H64Q and H64Q/V68L mutants, which were designed to mimic the active site of cyanoglobin. Oxygen dissociation rate constants were determined by ligand displacement with CO. As shown in Figure 3 and summarized in Table 1, oxygen dissociation from cyanoglobin is 4-fold faster than from sperm whale myoglobin and ~14-fold faster than from leghemoglobin *a*. The sperm whale myoglobin mutants suggest that the Gln residue at position 64 is the cause of the elevated O_2 dissociation rate constant since the single H64Q shows a similar high value of k_{O2} (Table 1). In sperm whale H64Q/V68L myoglobin, the mutant leucine side chain alleviates hindrance of the bound O_2 by the γ-CH_3 moiety of the naturally occurring valine E11 residue. This change compensates partially for the weaker hydrogen bonding interaction with the Gln-E7, resulting in a k_{O2} value that is only 2-fold greater than that of wild-type sperm whale myoglobin.

Table 1: Kinetic Parameters of Gaseous Ligand Binding to Cyanoglobin and Selected Hemoglobins and Myoglobins[a]

protein	k'_{O2} (μM^{-1} s^{-1})	k_{O2} (s^{-1})	K_{O2} (μM^{-1})
SWMb wild type[b]	l5	18	0.83
SWMb H64Q[b]	24	110	0.19
SWMb H64Q/V68L[b]	33	30	1.2
SWMb (D-helix)[c]	13	31	0.42
GlbN[b]	390	79	4.8
Lb*a* (*Glycine max*)[d]	130	5.6	23
Aplysia Mb[e]	15	70	0.21
Glycera II Hb[f]	190	1800	0.11

protein	k'_{CO} (μM^{-1} s^{-1})	k_{CO} (s^{-1})	K_{CO} (μM^{-1})
SWMb wild type[g]	0.53	0.019	27
SWMb H64Q[g]	0.94	0.012	78
SWMb H64Q/V68L[g]	2.3	0.008	290
SWMb (D-helix)[c]	0.45	0.030	15
GlbN[b]	41	0.010	4100
Lb*a* (*Glycine max*)[d]	17	0.0078	2200
Aplysia Mb[e]	0.5	0.02	25
Glycera II Hb[f]	27	0.042	640

protein	k'_{NO} (μM^{-1} s^{-1})	k_{NO} (s^{-1})	K_{NO} (μM^{-1})
SWMb wild type[b]	22	0.000098	220000
SWMb H64Q[h]	43	0.00013	330000
SWMb H64Q/V68L[i]	90	0.00003	3000000
GlbN[b]	600	0.00022	2700000
Lb*a* (*Glycine max*)[d]	170	0.00002	9000000

[a] Rate constants determined in this study were in the presence of 0.1 M potassium phosphate/0.3 mM EDTA, pH 7.0, at 20 °C [b] This study. [c] Taken from Whitaker et al. (*38*). [d] Taken from Hargrove et al. (*45*). [e] Taken from Wittenburg et al. (*49*) and Wittenburg et al. (*50*). [f] Taken from Parkhurst *et al.* (*51*). [g] Dissociation rate constants taken from Rohlfs et al. (*29*). [h] Taken from Eich et al. (*52*). [i] Taken from Raymund Eich, Ph.D. Dissertation, Rice University.

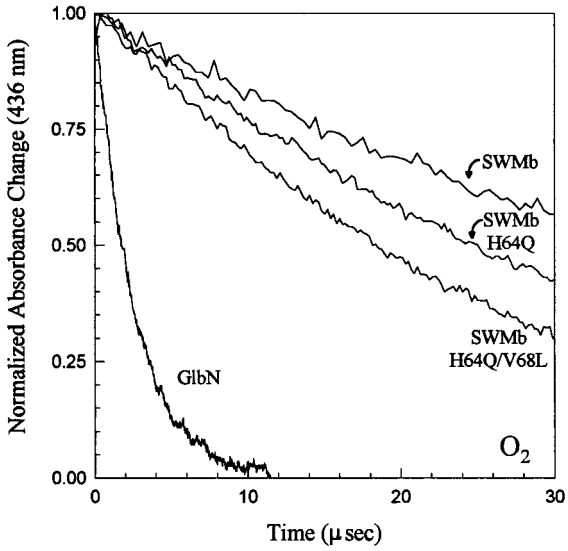

FIGURE 2: Cyanoglobin has a very rapid rate of oxygen association. Normalized absorbance traces of flash photolysis experiments of oxyhemoglobins depicting decay of deoxycyanoglobin to the oxygenated form with time after oxygen had been photolyzed with an intense laser pulse. Protein concentrations were ~10 μM, and oxygen concentration was equal to 1.25 mM (1 atm). Buffer was 0.1 M potassium phosphate/0.3 mM EDTA, pH 7.0, at 20 °C.

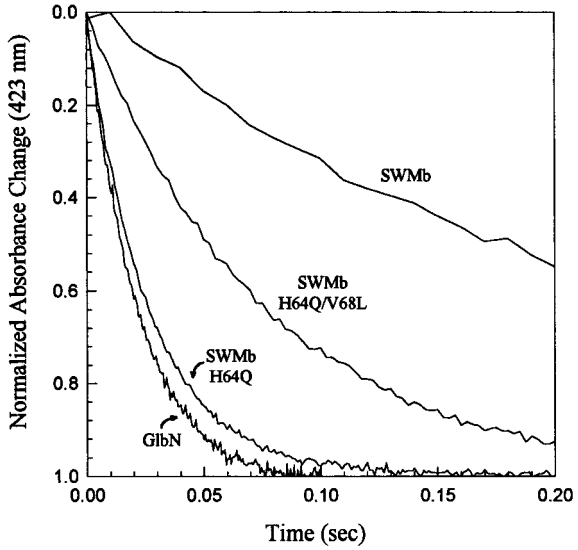

FIGURE 3: Cyanoglobin has a rapid rate of oxygen dissociation. Normalized absorbance traces of oxyhemoglobins showing oxygen displacement by CO measured by stopped-flow spectrophotometry. All traces were collected using protein concentrations of ~5 μM in the presence of 65 μM and 1 mM concentrations of oxygen and CO, respectively, before mixing. Buffer was 0.1 M potassium phosphate/0.3 mM EDTA, pH 7.0, at 20 °C.

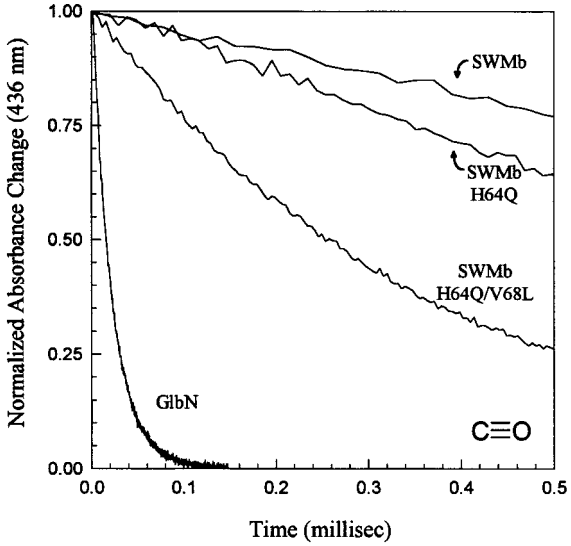

FIGURE 4: Cyanoglobin has a very rapid rate of CO association. Normalized absorbance traces of CO association to hemoglobins using flash photolysis. For all traces, the CO concentration was 1 mM (1 atm), and protein concentrations were ~10 μM. Buffer was 0.1 M potassium phosphate/0.3 mM EDTA, pH 7.0, at 20 °C.

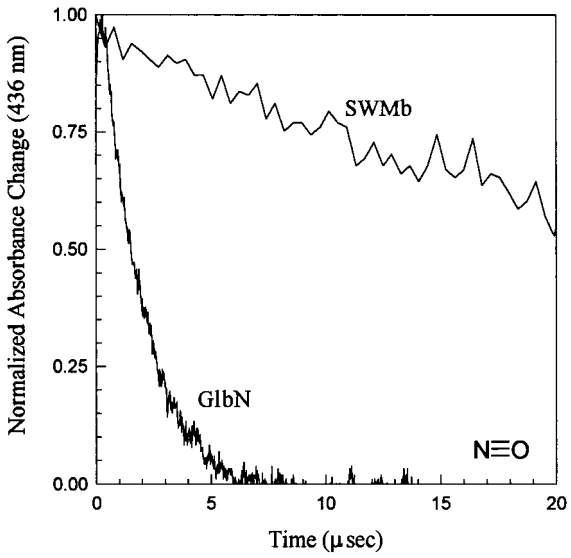

FIGURE 5: Cyanoglobin has a very rapid rate of NO association. Normalized absorbance traces of NO association to cyanoglobin and sperm whale myoglobin using flash photolysis. For all traces, the NO concentration was 1 mM (0.5 atm), and protein concentrations were ~10 μM. Buffer was 0.1 M potassium phosphate/0.3 mM EDTA, pH 7.0, at 20 °C.

The oxygen affinity calculated from the ratio of k'_{O2}/k_{O2} for cyanoglobin is 4.8 μM^{-1}, which is intermediate between that of sperm whale myoglobin (0.83 μM^{-1}) and leghemoglobin *a* (23 μM^{-1}). Although the rate of oxygen dissociation in cyanoglobin is moderately large, the very high rate of oxygen association gives cyanoglobin a moderately high overall oxygen affinity. According to amino acid sequence alignments, cyanoglobin does not possess a D-helix (*21*). As shown by Whitaker et al. (38), removal of the D-helix in sperm whale myoglobin has only a small effect on O_2 binding. The results for sperm whale myoglobin show that the O_2 binding properties of cyanoglobin cannot be mimicked either by E7 and E11 substitutions or by removal of the D-helix (Table 1).

Carbon Monoxide (CO) and Nitric Oxide (NO) Binding. The kinetics of association of carbon monoxide (and alkylisocyanides; see below) to cyanoglobin were investigated by two different methods: stopped-flow spectrophotometry and flash photolysis. The rate constants obtained by the two methods were comparable and are reported as the average of both experiments. As in the case of O_2 binding, the CO association rate constant for cyanoglobin is markedly higher than that for the native and altered forms of sperm whale myoglobin used in this study (Figure 4 and Table 1). In contrast, cyanoglobin has a CO dissociation rate that is comparable to nearly all hemoglobins and myoglobins. The net result is that cyanoglobin has a remarkably high overall affinity for CO (Table 1). Compared to the reference myoglobins and hemoglobins, cyanoglobin also shows a very rapid rate of NO association (Figure 5 and Table 1). The rate of NO dissociation is very slow and typical of most native myoglobins and hemoglobins.

Geminate Recombination of O_2. As is true for both leghemoglobin and sperm whale myoglobin, the extent of CO geminate recombination to cyanoglobin is too small (≤5%) at room temperature to allow deconvolution of internal bond formation and ligand escape rate constants. In the case of NO, the rate of geminate recombination is too fast to measure on nanosecond time scales (*39*). The fraction of NO molecules that escape from cyanoglobin, although very small, is roughly 2 times greater than that observed for wild-type sperm whale myoglobin (traces not shown). Only in the case of the O_2 complexes are both the rate and fraction of geminate recombination readily measured and analyzed on nanosecond time scales.

Normalized time courses for intramolecular O_2 rebinding to deoxycyanoglobin and wild-type sperm whale deoxymyoglobin are shown in Figure 6. Geminate recombination of O_2 is roughly 5 times faster in the bacterial protein with $k_{geminate} = 60\ \mu s^{-1}$. The fitted fraction of rebinding is ~0.30 for cyanoglobin and 0.45 for sperm whale myoglobin. Note that the secondary phase of cyanoglobin rebinding is the

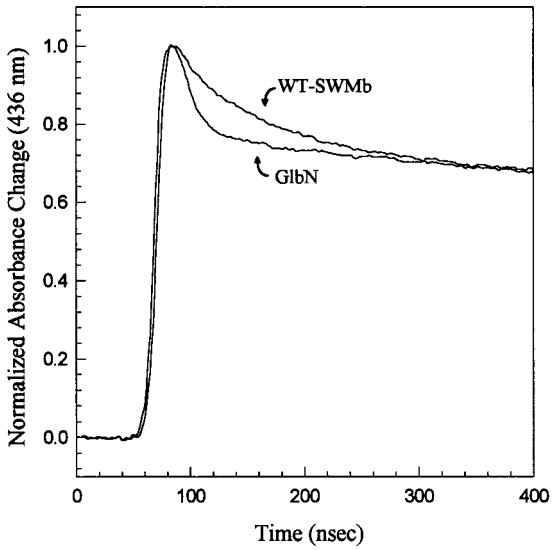

FIGURE 6: Oxygen has a very rapid rate of entry into, and escape from, the distal pocket of cyanoglobin. Oxygen recombination traces to cyanoglobin following a very short (9 ps) laser pulse as described under Materials and Methods. Oxygen concentration was 1.25 mM (1 atm), and protein concentrations were ~100 μM. Buffer was 0.1 M potassium phosphate/0.3 mM EDTA, pH 7.0, at 20 °C.

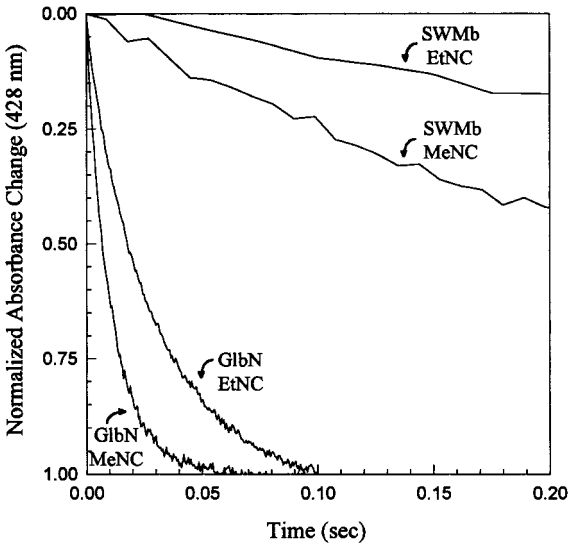

FIGURE 7: Cyanoglobin has very rapid rates of alkylisocyanide association. Normalized absorbance traces of methyl isocyanide (MeNC) and ethyl isocyanide (EtNC) association to cyanoglobin (left curves) and to sperm whale myoglobin (right curves) determined by stopped-flow spectrophotometry. Protein concentrations were ~5 μM and R-NC concentrations were 20 μM, before mixing. Buffer was 0.1 M potassium phosphate/0.3 mM EDTA, pH 7.0, at 20 °C.

beginning of the bimolecular O_2 rebinding phase which has an observed half-time of ~2 μs in 1 atm of oxygen. Assuming a simple two-step rebinding scheme, the overall association and dissociation rate constants for O_2 rebinding are given by

$$k'_{\text{overall}} = \frac{k'_{\text{entry}} k'_{\text{bond}}}{k_{\text{bond}} + k_{\text{escape}}} \qquad k_{\text{overall}} = \frac{k_{\text{escape}} k_{-\text{bond}}}{k_{\text{bond}} + k_{\text{escape}}} \quad (1)$$

where k'_{entry} and k_{escape} represent the rate constants for rapid oxygen entry into and escape from the distal pocket and k'_{bond} and $k_{-\text{bond}}$ represent the rate constants for internal bond formation and dissociation (*34* and *39*). The geminate recombination parameters are given by

$$F_{\text{gem}} = \frac{k_{\text{bond}}}{k_{\text{bond}} + k_{\text{escape}}} \qquad k_{\text{gem}} = k_{\text{escape}} + k_{\text{bond}} \quad (2)$$

Thus, measurements of F_{gem}, k_{gem}, and k'_{O2} and k_{O2} allow computation of the individual parameters for a single two-step ligand binding scheme.

The values of k'_{entry}, k_{escape} and K_{entry} ($k'_{\text{entry}}/k_{\text{escape}}$) for cyanoglobin were estimated to be 1300 μM^{-1} s^{-1}, 40 μs^{-1}, and 33 M^{-1}, respectively. All three of these parameters are much greater than the corresponding values for wild-type sperm whale myoglobin which are 34 μM^{-1} s^{-1}, 6.3 μs^{-1}, and 5 M^{-1}, respectively (*34*, *40*, and E. E. Scott, 1998, Ph.D. dissertation, Rice University). The larger rate and equilibrium constants for ligand entry into the distal pocket of cyanoglobin are similar to those observed for apolar distal histidine mutants of sperm whale myoglobin ($k'_{\text{entry}} = 100-300$ μM^{-1} s^{-1} and $K_{\text{entry}} = 10-40$ M^{-1}; *33*) and for leghemoglobin *a* ($k'_{\text{entry}} \sim 200$ μM^{-1} s^{-1} and $K_{\text{entry}} = 10$ M^{-1}; *41*). Thus, the geminate recombination data for cyanoglobin are consistent with an unhindered apolar distal pocket.

The rate constant for iron−O_2 bond formation, k'_{bond}, in cyanoglobin is estimated to be ~20 μs^{-1}, which is 4-fold greater than that observed for wild-type sperm whale myoglobin, indicating a more accessible or reactive iron atom. The thermal bond disruption rate constant, $k_{-\text{bond}}$, is ~110 s^{-1}, which is also ~4-fold greater than that for native sperm whale myoglobin. As a result, the equilibrium constant for bond formation within the distal pocket of cyanoglobin is roughly equal to that of sperm whale myoglobin. The higher affinity of cyanoglobin for ligands is almost completely a result of the larger equilibrium constant for noncovalent ligand binding in the distal pocket of the bacterial protein. Leghemoglobin also shows large values of k'_{entry}, K_{entry}, and k_{bond}, which are all very similar to those observed for cyanoglobin. However, the plant protein also has a low rate of thermal bond disruption ($k_{-\text{bond}} = 6$ s^{-1}), accounting for its very high overall affinity for oxygen.

Alkylisocyanide Binding. The rates of methyl and ethyl isocyanide association with cyanoglobin are also very large when compared to those for sperm whale myoglobin and other hemoglobins (Figure 7 and Table 2). The ethyl and methyl isocyanide dissociation rate constants for cyanoglobin are more similar to those of leghemoglobin *a* than to those of sperm whale myoglobin, also suggesting a very open and unhindered distal pocket as is observed for leghemoglobin *a* (*42*−*45*).

Kinetics of Autoxidation. Upon standing at room temperature, oxygenated hemoglobins and myoglobins autoxidize spontaneously to the metform. Previous observations during the isolation of cyanoglobin revealed that cyanoglobin autooxidizes rapidly [$t_{1/2} < 12$ h; (*15*)]. This problem accounted for our previous difficulties obtaining a single population of purified protein. The new phenyl Sepharose chromatography step allows rapid separation of oxygenated cyanoglobin from its oxidized forms.

Autoxidation of oxyhemoglobins is known to be influenced by pH. At pH 7, the rate of autoxidation of cyanoglobin is 4-fold higher than that for sperm whale myoglobin and is similar to that of leghemoglobin *a* and the Gln(E7) mutants

Table 2: Kinetic Parameters of Alkylisocyanide Ligand Binding to Cyanoglobin and Selected Hemoglobins and Myoglobins[a]

protein	k'_{MeNC} (μM^{-1} s^{-1})	k_{MeNC} (s^{-1})	K_{MeNC} (μM^{-1})
SWMb wild type[b]	0.12	4.3	0.027
SWMb H64Q[c]	0.20	5.6	0.036
GlbN[d]	8.4	0.31	27
Glycera I Hb[e]	0.45	0.65	0.69
Aplysia Mb[f]	0.60	72	0.0083
Lb*a* (*Glycine max)*[g]	2.3	0.095	24
protein	k'_{EtNC} (μM^{-1} s^{-1})	k_{EtNC} (s^{-1})	K_{EtNC} (μM^{-1})
SWMb wild type[b]	0.073	0.3	0.24
SWMb H64Q[c]	0.071	0.15	0.47
GlbN[d]	6.9	0.064	110
Glycera I Hb[e]	0.16	0.035	4.5
Aplysia Mb[f]	0.54	15	0.036
Lb*a* (*Glycine max)*[g]	2.0	0.026	77

[a] Rate constants determined in this study were in the presence of 0.1 M potassium phosphate/0.3 mM EDTA pH 7.0, at 20 °C. [b] Dissociation rate constants taken from Rohlfs et al. (*29*). [c] Taken from Rohlfs et al. (*29*). [d] This study. [e] Taken from Mims et al. (*43*). [f] Taken from Di Iorio et al. (*53*). [g] Taken from Stetzkowsky et al. (*42*).

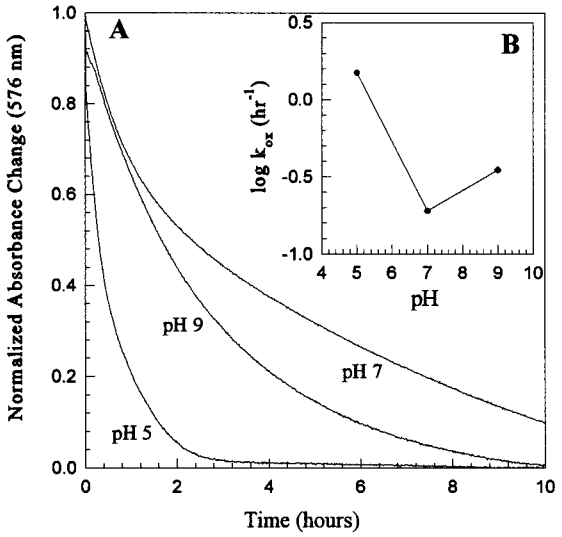

FIGURE 8: Rapid rate of oxycyanoglobin autoxidation is pH-dependent. Panel A: Normalized absorbance traces for the decay of oxycyanoglobin to the metform as a function of time at pH 5 (0.1 M MES), at pH 7 (0.1 M potassium phosphate), and at pH 9 (0.1 M Tris·HCl). All experiments were performed in air-equilibrated buffers, at 37 °C, in the presence of catalase and superoxide dismutase. (Inset; Panel B) pH stability profile of cyanoglobin showing log of the observed rate constant of autoxidation (k_{ox}) vs pH. Protein concentrations were ~20 μM.

of sperm whale myoglobin (Figure 8 and Table 3). Decreasing the pH to 5 increased the rate of autoxidation to ~1.5 h^{-1} in all of the native proteins. Cyanoglobin also exhibited a higher rate of autoxidation at pH 9 than at pH 7 (Figure 8). Although analyzed as a first-order process, the absorbance traces for cyanoglobin autoxidation are complex due to the high rate of hemin loss (data not shown) which results in irreversible precipitation of both the prosthetic group and the apoprotein [p*I* of cyanoglobin ~9.5; (*14*)]. Thus, caution should be taken when interpreting the autoxidation rate constants, particularly at basic pH. From these data, we conclude that cyanoglobin does not have an unusually high rate of autoxidation when compared to sperm whale myoglobin and its mutants and to leghemoglobin *a*.

Table 3: Kinetic Parameters of Autoxidation and Hemin Loss of Cyanoglobin and Selected Hemoglobins and Myoglobins

protein	k_{ox} (h^{-1})	k_{loss} (h^{-1})
SWMb wild-type, pH 5.0	1.2[a]	1.0[b]
SWMb wild-type, pH 7.0	0.05[c]	0.01[b]
SWMb (D-helix), pH 5.0	6.0[a]	46[a]
SWMb H64Q, pH 7.0	0.22[c]	0.12[b]
SWMb H64Q/V68L, pH 7.0	0.21[d]	0.4[e]
GlbN, pH 5.0[f]	1.5	62
GlbN, pH 7.0[f]	0.2	28
GlbN, pH 9.0[f]	0.4	nd[h]
Lb*a*, pH 5.0[g]	1.6	1.3
Lb*a*, pH 7.0[g]	0.2	0.7

[a] Taken from Whitaker et al. (*38*). [b] Taken from Hargrove et al. (*47*). [c] Taken from Brantley et al. (*35*). [d] Taken from Raymund Eich, Ph.D. Dissertation, Rice University. [e] Hargrove, unpublished data. [f] This study. [g] Taken from Hargrove et al. (*45*). [h] nd = not determined.

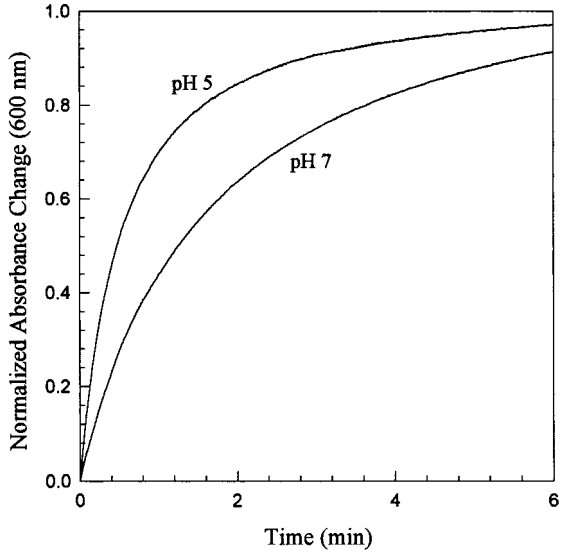

FIGURE 9: Cyanoglobin has a very rapid rate of hemin loss. Normalized absorbance traces of hemin loss from cyanoglobin to apo-H64Y/V68F SWMb, at 20 °C. Protein concentrations were ~20 μM in the presence of 0.15 M MES, pH 5, or 0.15 M potassium phosphate, pH 7, supplemented with 0.45 M sucrose and 40 μM apo-H64Y/V68F SWMb.

Kinetics of Hemin Loss. Retention of heme is influenced by the number and types of contacts that the protein makes with the porphyrin ring and the iron atom. The rate of hemin loss from cyanoglobin is very large when compared to other hemoglobins (Figure 9 and Table 3). Similar high rates of hemin loss are observed for a sperm whale myoglobin mutant that is missing the D-helix (*38*).

DISCUSSION

Isolation and Purification of Cyanoglobin. Recombinant cyanoglobin was expressed in *E. coli* (pGlbN) and isolated in the oxygenated form using a new method. The charge and surface properties of the oxy and the met forms of cyanoglobin are sufficiently different to allow complete resolution during the isolation procedure. This difference may be the result of exposure of more hydrophobic patches of amino acids in autoxidized cyanoglobin.

At pH 7, the optical spectrum of metcyanoglobin had an appearance that suggests a mixture of high- and low-spin ferric heme, with low-spin bands at 536 and 570 nm and a high-spin band at 632 nm. Unlike sperm whale myoglobin

and its altered form, H64Q/V68L, which exhibit pK_a values of ~9 for deprotonation of coordinated water, no significant changes in the visible spectrum for metcyanoglobin were observed from pH 5 to 9 (data not shown). At pH 7.0 under cryogenic temperature, the metcyanoglobin has an electron paramagnetic resonance spectrum indicative of high-spin ferric heme (data not shown), suggesting the existence of a spin equilibrium, with the high-spin state dominating at neutral pH and room temperature.

Ligand-Binding Pocket of Cyanoglobin. The UV−visible spectrum of aquometcyanoglobin is unusual in that its Soret extinction coefficient, at pH 7, is low compared to that for sperm whale metmyoglobin and aquometleghemoglobin. Moreover, the extinction coefficient for oxycyanoglobin is slightly higher than that calculated for the metform. This result is not observed in sperm whale myoglobin or leghemoglobin *a*, which exhibit much higher Soret extinction coefficients for the met form when compared to the oxygenated form. According to the criteria of Shikama and Matsouka (*46*), the spectral characteristics of metcyanoglobin, when compared to those of oxycyanoglobin, are indicative of hemoglobins that possess a non-histidyl amino acid residue at the E7 position. Sequence alignment (*14*, *21*, and *22*) suggests that Gln is at this position in cyanoglobin, which is consistent with this spectral observation.

Kinetics of Ligand Binding to Cyanoglobin. Current models of ligand binding to sperm whale myoglobin indicate that the distal histidine (His E7) residue discriminates in favor of O_2 against CO binding by first requiring displacement of distal pocket water and then selectively forming a hydrogen bond with bound O_2, but not bound CO. Mutations of the E7 residue that decrease distal pocket polarity in sperm whale myoglobin cause a 5−10-fold increase in the O_2 association rate constant and a 100−1000-fold increase in the O_2 dissociation rate constant. The latter effect is due to lack of stabilization of the O_2−Fe complex. The distal valine (Val E11) in sperm whale myoglobin contributes to the size and hydrophobicity of the distal pocket. Alterations of Val to larger amino acid residues [e.g., Leu (E11) and Phe (E11)] in sperm whale myoglobin can cause a decrease in the O_2 dissociation rate constant by preventing ligand movement away from the heme iron (*34*).

Cyanoglobin binds molecular oxygen faster than all other naturally occurring hemoglobins but has only a moderately large oxygen dissociation rate constant, accounting for its high oxygen affinity. The H64Q and H64Q/V68L altered forms of sperm whale myoglobin have Gln and Leu residues at the analogous E7 and E11 positions in cyanoglobin based on amino acid sequence alignment. However, the properties of these altered forms of sperm whale myoglobin cannot be correlated easily with the ligand binding behavior of cyanoglobin. There is a modest increase in the association rate constant going from H64Q to H64Q/V68L sperm whale myoglobin, which is expected due to decreases in both distal pocket polarity and steric restriction (*29* and *34*). Combined with previous observations, the lack of correspondence with altered forms of sperm whale myoglobin suggests that cyanoglobin has a much more open distal pocket compared to that of sperm whale myoglobin.

Leghemoglobin *a* and *Glycera* Hb II also have very high oxygen association rate constants which are comparable to that of cyanoglobin. *Glycera* hemoglobin possesses an E7 leucine, which decreases the polarity of the distal pocket and facilitates entry of oxygen into the heme pocket. However, the lack of a Gln or His at the E7 position prevents stabilization of bound oxygen in *Glycera* hemoglobin, causing it and the corresponding H64L altered form of sperm whale myoglobin to have very large oxygen dissociation rate constants, ~2000 s^{-1} (*29*).

Table 4: Ability of Cyanoglobin To Discriminate against Carbon Monoxide Binding Is Relatively Low

protein	K_{CO} (μM^{-1})	K_{O2} (μM^{-1})	K_{CO}/K_{O2}
chelated protoheme			
in benzene[a]	330	0.015	22000
in 2% soap micelles[a]	300	0.55	550
SWMb wild type[b]	27	0.83	33
SWMb H64Q[b]	78	0.19	410
SWMb H64Q/V68L[b]	290	1.2	240
GlbN[b]	4100	4.8	850
Lb*a* (*Glycine max*)[c]	2200	23	96
Aplysia Mb[d]	25	0.21	120
Glycera Hb[e]	640	0.11	5800
SWMb (D-helix)[f]	15	0.42	36

[a] Taken from Traylor et al. (*54*−*56*). [b] This study. [c] Taken from Hargrove et al. (*45*). [d] Taken from Wittenburg et al.(*49*) and Wittenburg et al. (*50*). [e] Taken from Parkhurst et al. (*51*). [f] Taken from Whitaker et al. (*38*).

The large association rate constant of cyanoglobin suggests a relatively apolar and unhindered active site. The absence of internal water molecules in the distal pocket of deoxycyanoglobin remains speculative. However, the large rate and equilibrium constants for ligand entry in cyanoglobin strongly suggest that the distal pocket is unhindered and that water displacement is not required for ligand entry. This conclusion is supported by the remarkably high affinities of cyanoglobin for ethyl and methyl isocyanides. The oxygen dissociation and autoxidation rate constants are only 2- and 5-fold, respectively, greater than those of sperm whale myoglobin, implying significant stabilization of the oxygen−heme iron bond by hydrogen bonding to the E7 Gln residue.

The oxygen affinity of cyanoglobin (~5 μM^{-1}) is intermediate between that of sperm whale myoglobin (0.83 μM^{-1}) and that of leghemoglobin *a* (23 μM^{-1}). Leghemoglobins are known to sequester oxygen and then provide it to a terminal cytochrome oxidase (*17*). The oxygen affinity of cyanoglobin is still sufficiently high to allow cyanoglobin to participate in a similar mechanism of oxygen sequestration and presentation to a terminal cytochrome oxidase in *Nostoc* 584. Such a role would be consistent with the positioning of cyanoglobin in vivo at the periphery of heterocysts and vegetative cells in *Nostoc* 584 (*16*).

Cyanoglobin also possesses very large association rate and equilibrium constants for CO binding. Like oxygen, k'_{CO} for cyanoglobin is higher than that of any other naturally occurring hemoglobin studied to date. The ratio of CO to O_2 affinities is ~25 in sperm whale myoglobin, whereas K_{CO}/K_{O2} for cyanoglobin is ~1000, a value similar to those of model heme compounds and sperm whale myoglobin mutants with apolar E7 residues (K_{CO}/K_{O2} = 2000−20 000; Table 4). The K_{CO}/K_{O2} for leghemoglobin *a* is significantly lower, ~100, probably reflecting a stronger hydrogen bond to the bound oxygen by the His (E7) residue in the plant protein.

Cyanoglobin Stability. Rates of autoxidation of oxycyanoglobin at pH 5 and 7 are moderately faster (3−4-fold)

than those of native sperm whale myoglobin and are similar to those of leghemoglobin *a* (Table 3). Notably, the rate of autoxidation of cyanoglobin at pH 7 is similar to that of the H64Q and the H64Q/V68L altered sperm whale myoglobins, which is probably due to weaker hydrogen bonding between a Gln64 residue and bound oxygen. Brantley et al. (*35*) showed that hydrogen bonding between E7 His64 and bound oxygen in sperm whale myoglobin is crucial in inhibiting protonation of the $Fe^{2+}-O_2$ complex and subsequent release of the hydroperoxy radical and Fe^{3+} heme iron. In support of this hypothesis, cyanoglobin exhibited a marked increase in the autoxidation rate constant with decreasing pH, presumably due to protonation of the bound O_2.

The instability of cyanoglobin is due to a very large rate of hemin loss, which is ~100-fold faster than that of sperm whale myoglobin. Hargrove, Olson, and co-workers showed that the two most important factors dictating hemin loss rates are accessibility of the distal pocket to water and the strength of the heme−iron protein bond (*47*). Increases in the polarity and size of the heme pocket enhance solvation and result in significant increases in the rates of hemin dissociation (*47*). Sequence alignments (*21*) suggest that cyanoglobin does not possess a D-helix, which is required for heme retention in sperm whale myoglobin (*38*). In addition, cyanoglobin apparently contains a Gln residue at position 76 (FG5) whereas sperm whale myoglobin contains an Ile residue in the analogous position. Alteration of Ile99 (FG5) to more polar residues in sperm whale myoglobin results in increases in hemin loss (~10-fold) as a result of higher solvation of the proximal heme pocket (*47*). Thus, the high rate of hemin loss from cyanoglobin may be the result of the size and solvation of both the proximal and distal portions of the heme pocket.

CONCLUSIONS

Cyanoglobin reacts more rapidly with all ferrous ligands than any other naturally occurring hemoglobin studied to date. Thus, cyanoglobin appears to have a highly accessible but relatively apolar distal pocket. The unique ligand binding behavior of cyanoglobin may be reflective of the unique environments in which cyanobacteria are found. As a result of its high O_2 affinity, and high rate of O_2 release, cyanoglobin may function to sequester and then present O_2 to a terminal oxidase complex, when the capacity to fix nitrogen, and thus the demand for ATP, is maximal. This idea is supported by the similarities of the ligand binding properties of cyanoglobin and leghemoglobin *a (48)*. Crystallization of cyanoglobin is currently underway to determine if there are any structural similarities with leghemoglobin *a*. In addition, site-directed mutagenesis strategies are being developed to further understand the unique ligand binding properties of cyanoglobin.

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