tion assays; G. Ferrari and E. Gomard for efforts to detect CTL; and J.-P. Levy for assistance. We appreciate the continuous support of J.-P. Lecocq and regret his untimely death. Supported by Pasteur Merieux Serums et Vaccins and the French National AIDS Research Agency (ANRS). M.L.G. is an American Foundation for AIDS Research–Michael Bennett Scholar.

18 February 1992; accepted 4 May 1992

Myoglobin in a Cyanobacterium

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Myoglobin was found in the nitrogen-fixing cyanobacterium *Nostoc commune*. This cyanobacterial myoglobin, referred to as cyanoglobin, was shown to be a soluble hemoprotein of 12.5 kilodaltons with an amino acid sequence that is related to that of myoglobins from two lower eukaryotes, the ciliated protozoa *Paramecium caudatum* and *Tetrahymena pyriformis*. Cyanoglobin is encoded by the *glbN* gene, which is positioned between *nifU* and *nifH*—two genes essential for nitrogen fixation—in the genome of *Nostoc*. Cyanoglobin was detected in *Nostoc* cells only when they were starved for nitrogen and incubated microaerobically.

Cyanobacteria may have figured in the evolution of Earth's oxygenic atmosphere (*1*). Many cyanobacteria have nitrogenase, an enzyme that catalyzes the reduction (fixation) of dinitrogen; as a group, these photosynthetic microorganisms are responsible for much biological dinitrogen fixation (*2*). Nitrogenase is sensitive to gaseous oxygen, and cyanobacteria use various mechanisms to achieve aerobic nitrogen fixation, including attenuation of the oxygen tension in the immediate vicinity of nitrogenase. For example, some cyanobacteria can effect either a spatial or temporal separation, or both, of their oxygen-evolving and nitrogen-fixing activities (*3, 4*), whereas others restrict their nitrogenase activity to a specialized differentiated cell, the heterocyst. During the course of its differentiation, the heterocyst is modified structurally and biochemically in order to provide a reducing environment conducive to nitrogen fixation (*5*). Marked changes in gene expression accompany heterocyst differentiation and include the induction of the *nif* operons (*6*).

Of the heterocystous cyanobacteria, species of *Nostoc* are especially prevalent in terrestial environments from the tropics to the polar regions, where they often enter into associations with a variety of higher and lower plants (*7*). One of these species, *Nostoc commune*, forms visually conspicuous colonies in the shallow natural depressions of limestone rock in karst areas. We isolated the *nifUHD* gene cluster from the strain *Nostoc commune* UTEX 584 (*8*). DNA sequence analysis revealed an open reading frame (ORF) of 118 codons, potentially encoding a 12.5-kD protein, between *nifU* and *nifH* that showed no obvious similarity in either DNA or amino acid sequence with any known *nif* genes or Nif proteins, respectively (Fig. 1A). However, marked similarity was apparent, both in size and in amino acid sequence, to the myoglobins of the ciliated protozoa *Paramecium caudatum* and *Tetrahymena pyriformis*, which contained 116 and 121 amino acids, respectively (*9, 10*). We used the polymerase chain reaction (PCR) (*11*) to amplify *glbN*, subcloned *glbN* into an expression vector (pT7-7), and overexpressed the recombinant protein in *Escherichia coli* (Fig. 1A) (*12*). A 12-kD protein was revealed by electrophoresis of whole-cell lysates from IPTG (isopropyl-β-D-thiogalactopyranoside)-induced (Fig. 1B, lanes C and D) cells carrying the cloned *Nostoc* gene (*13*); the protein was not revealed in cells uninduced by IPTG (Fig. 1B). The cell pellets from GlbN$^+$ transformants were bright red in color. Examination of induced cells with light microscopy did not reveal inclusion bodies.

The recombinant protein partitioned to the soluble fraction after disruption of GlbN-expressing *E. coli* in a French pressure cell. During the course of the purification, the color of the protein preparation changed from bright red to orange-brown, probably the result of auto-oxidation of the protein to the ferric (met)-form. This is a characteristic of myoglobins and hemoglobins isolated from other sources. The orange-brown fraction, obtained after ion-exchange chromatography, ultrafiltration, and gel-exclusion chromatography, was judged to be homogenous when examined by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining (Fig. 1B). The molecular sizes of GlbN estimated from gel filtration and SDS-PAGE were 15 kD and 12 kD, respectively. These data indicate that recombinant GlbN is a monomer.

The ultraviolet (UV)-visible spectrum of the recombinant GlbN was consistent with that of a high-spin ferric hemoprotein, with a maximum absorbance in the Soret region at 412 nm (Fig. 2). Reduction with sodium dithionite shifted the Soret peak to 422 nm, whereas addition of carbon monoxide to the reduced protein caused a shift in the Soret peak to 419 nm. These data demonstrate that the reduced protein has an open ligand position that can bind carbon monoxide and probably molecular oxygen. As no cysteine residues are present in

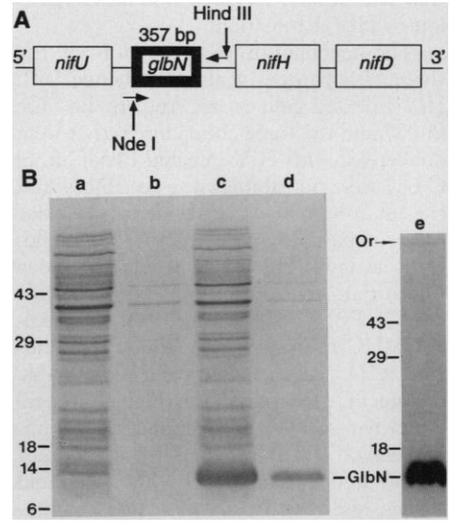

Fig. 1. Chromosomal location of *glbN* and purification of recombinant GlbN protein. (**A**) The position of *glbN* in the *nifUHD* cluster of *Nostoc commune* (*8*) and the region amplified by PCR are represented schematically. Note that the *nifU-nifH* intergenic region of the related cyanobacterium *Anabaena sp.* strain PCC7120 is devoid of any ORF (*22*). The two oligonucleotides used were 5′-CTGGCATATGAGCACATTGTACG-3′ and 5′-GCGAAGCTTCGATAGGACTTGAT-3′. These are complementary to the 5′ end of *glbN* and to a region 50 bp downstream of its 3′ terminus, respectively. Recognition sites for Nde I and Hind III were introduced into the oligonucleotides to permit subsequent subcloning of the PCR product in the desired orientation in pT7-7. The sequence of *glbN* is 357 bp; the engineered PCR product was 422 bp. The first five amino acid residues of GlbN—represented by bases 8 to 22 in the first oligonucleotide—are Met, Ser, Thr, Leu, and Tyr. The sequence of *glbN* has been submitted to GenBank (accession number M92437). (**B**) Lysates from uninduced (lanes a and b) and induced cells (lanes c and d); purified GlbN prepared by liquid chromatography and examined by SDS-PAGE (lane e). Lanes b and d were loaded with one-tenth the amount of total protein present in lanes a and c. Molecular sizes in kilodaltons are shown at the left. Proteins were detected with Coomassie blue stain (lanes a to d) or silver stain (lane e). GlbN, recombinant cyanoglobin; Or, origin.

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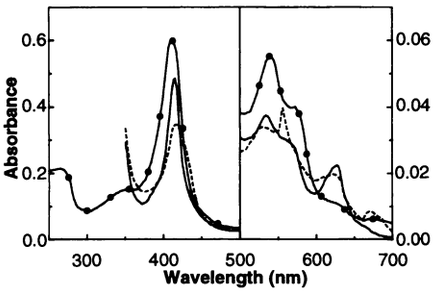

Fig. 2. Soret and visible spectra of recombinant GlbN. Spectra of purified native recombinant GlbN were obtained from a solution in 50 mM Hepes (pH 7.5) (solid curve with symbols); from the identical solution after the addition of crystalline sodium dithionite (dashed curve); and after saturation of the solution with carbon monoxide (solid line only). Spectra were obtained with a Cary 219 spectrophotometer (Varian, Palo Alto, California) and 1-cm pathlength quartz cuvettes.

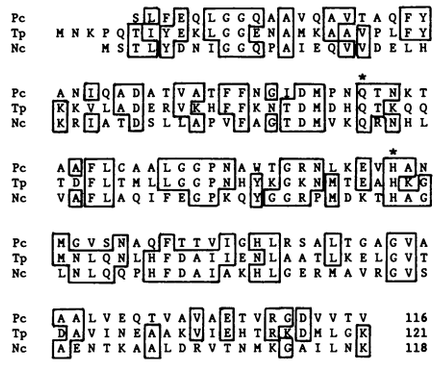

Fig. 4. Deduced amino acid sequence (*23*) of cyanoglobin and comparison with other globins. Alignment of GlbN with the sequences of the monomeric hemoglobins of *Paramecium* (Pc) and *Tetrahymena* (Tp). Nc, *Nostoc* cyanoglobin. For presentation of the comparison of Nc, Pc, and Tp, only exact correspondences are boxed. Numbers = the total residues in each protein. Residues in Pc and Tp thought to function in heme-binding (*9, 10*) are indicated with an asterisk.

the amino acid sequence of GlbN, the heme group is probably noncovalently attached to the monomeric apoprotein—a characteristic of myoglobins and hemoglobins (*14*). The UV spectrum of the ferric form of the protein and the spectra in the Soret region of the ferrous and carbon monoxy forms of the protein are typical of myoglobins and hemoglobins isolated from other sources, including those from the two protozoans. However, the spectral characteristics of the ferrous and carbon monoxy forms in the visible region are atypical. To reflect the globin-like properties of GlbN, we refer to this cyanobacterial hemoprotein as cyanoglobin.

Cyanoglobin, with a molecular size identical to that of the recombinant protein (12 kD), was detected in cells of *Nostoc commune* UTEX 584 only when they were grown in the absence of combined nitrogen (when they had differentiated heterocysts) and then only after a 24- to 56-hour period of incubation under microaerobic conditions (Fig. 3) (*15*). Under these conditions, induction of cyanoglobin synthesis was demonstrated with cultures in the early and late logarithmic phase of growth (Fig. 3).

Nostoc cyanoglobin is smaller than other known myoglobins but appears to be related to the monomeric protozoan globins for which, at present, no functions have been assigned. The calculated isoelectric points for the *Nostoc* and *Tetrahymena* proteins are virtually identical (9.7 and 9.5, respectively), and all three proteins show very similar hydropathic profiles. These three monomeric globins constitute a distinct homogenous group when their sequences are aligned (*16*) and compared with the mammalian globins as well as with the dimeric hemoglobin reported from a species of the bacterium *Vitreoscilla* (*17*) (Fig. 4). Cyanoglobin shares only limited sequence similarity with the mammalian and *Vitreoscilla* proteins and no obvious similarity with other hemoproteins including the leghemoglobins and cytochromes.

The marked conservation between cyanoglobin and related protozoan myoglobins, in contrast to the limited similarity between cyanoglobin and the eubacterial *Vitreoscilla* hemoglobin, raises questions of whether these different monomeric hemoproteins have a common origin and whether they can provide clues to early globin evolution. The observation that the synthesis of cyanoglobin is induced by nitrogen starvation, and the positioning of *glbN* between *nifU* and *nifH*, may suggest that GlbN is involved in some aspect of nitrogen fixation. That aspect may involve a scavenging of oxygen because the induction of cyanoglobin synthesis required incubation of *Nostoc* cells under low oxygen tension.

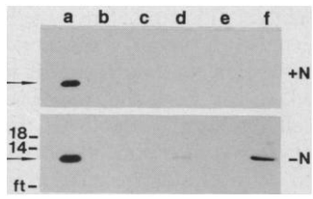

Fig. 3. Cyanoglobin in *Nostoc*. Immunoblotting was used to detect cyanoglobin in cells at different developmental stages grown in the presence (+N) or absence (−N) of combined nitrogen. Lane A, 20 ng of recombinant cyanoglobin; lane B, hormogonia (24 hours of aerobic growth); lane C, 48 hours of aerobic growth (heterocysts differentiated in −N culture); lane D, 48 hours of aerobic growth and 24 hours of anaerobic induction; lane E, 9 days of aerobic growth; and lane F, 9 days of aerobic growth and 56 hours of anaerobic induction. Equivalent amounts of *Nostoc* proteins (approximately 5 μg) were loaded in each lane of two gels, which were processed under identical conditions. Molecular size standards in kilodaltons are at the left; ft, unresolved protein front. Arrows, 12-kD cyanoglobin.

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12. The PCR reactions were performed in a Coy Tempcycler model 50. Before its expression in *E. coli*, the nucleotide sequence of the PCR product was determined and found to be identical with the genomic sequence of *glbN*. Ligation of the Nde I–Hind III–digested PCR product to Nde I–Hind III–digested pT7-7 created the recombinant plasmid pGlbN (2895 bp), in which the translational initiation codon of GlbN was positioned 7 bp downstream from the ribosome-binding site, an orientation that favored efficient translation. The pGlbN was used to transform *E. coli* BL21DE3 (F^-, *ompT*), a strain that contains a chromosomal copy of the gene for T7 DNA–dependent RNA polymerase, with expression of that gene under the control of the *lac* repressor. IPTG was used to induce transcription from the T7 promoter upstream of pGlbN, with concomitant overproduction of the GlbN protein.
13. The *glbN* transformants were grown in Luria-Bertani liquid medium in the presence of ampicillin (100 μg/ml) at 37°C to the logarithmic phase of growth monitored at an absorbance of 550 nm. When the absorbance at 550 nm reached 0.4, IPTG was added to a final concentration of 0.4 mM, and cells were collected after 2 hours at 37°C. For analytical purposes, cells from induced, and uninduced cultures were lysed in Laemmli buffer (*18*) and boiled for 5 min, and the clarified protein extracts were examined directly by SDS-PAGE (12% w/v polyacrylamide). Protein concentrations were determined with a protein determination kit (Pierce) with bovine serum albumin as the standard. For preparative purposes, the colored cell pellet harvested from induced cultures was washed in 50 mM Hepes (pH 7.5), resuspended in the same buffer (containing 1 mM phenylmethylsulfonyl fluoride), and then passed through a chilled French pressure cell at 100 MPa. The suspension was clarified by centrifugation at 30,000*g* (SS-34 rotor; Sorvall, Wilmington, DE) for 30 min. The cleared lysate was applied directly to a Mono S HR 5/5 FPLC (fast protein liquid chromatography) column (Pharmacia LKB) equilibrated with 50 mM Hepes (pH 7.5) with a flow rate of 1 ml/min. Absorbance was monitored simultaneously at 280 and 418 nm. Under these conditions, GlbN appeared in the flow-through fraction. This orange-colored fraction was concentrated with Centriprep 10 cartridges (Amicon, Danvers, MA)

and applied to a Superose 12 HR10/30 column equilibrated with the same buffer. The fractions that showed maximum absorbance at 418 nm were collected and concentrated again by ultrafiltration. Aliquots were denatured in Laemmli buffer (*18*) and applied to 12% w/v polyacrylamide mini-gels (Hoefer) to assess their purity. The fractions were judged to be >95% homogenous when examined by SDS-PAGE and silver staining. To determine the native molecular size of the recombinant protein, we equilibrated the Superose 12 column with 50 mM tris-HCl (pH 8.0) and 100 mM NaCl and calibrated it after individual resolution of the following standards: carbonic anhydrase (29 kD), equine-heart myoglobin (18.8 kD), cytochrome c (12.4 kD), and aprotinin (6.5 kD) (Sigma). Purified recombinant GlbN (Fig. 1B) was used to generate polyclonal antibodies in rabbit.

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15. Cells of *Nostoc commune* UTEX 584 in stationary phase were subcultured and grown in the presence or absence of combined nitrogen, in BG-11 or BG-11_0 liquid media, respectively (*19*). Incubation was at 32°C, with a photon flux density at the surface of the culture vessels of 60 μmol photons m^{-2} s^{-1} and with continuous and vigorous sparging with sterile air. Under these conditions, induction of hormogonia (motile filaments that lack heterocysts) occurred within 22 hours. After 48 hours, those hormogonia that were induced in BG-11_0 cultures had completed heterocyst differentiation. Aliquots of the cultures were harvested, resuspended in fresh BG-11 or BG-11_0, and transferred to Erlenmeyer flasks provided with gas-tight Suba seals (Fisher, Pittsburgh, PA). To achieve microaerobic conditions, we flushed the gas phase once with argon (100 v/v) and continued incubation for 24 hours under the same conditions of light and temperature. Cultures that had grown to a higher cell density (over 9 days) were also subjected to microaerobic conditions. In this case, the gas phase was flushed intermittently (for 5 min at approximately 12-hour intervals) during a 56-hour period of incubation. The developmental growth stage of cultures was monitored by light microscopy, and proteins were extracted when necessary by grinding of cells in liquid nitrogen and then in Laemmli buffer (*18*). Protein extracts were processed for SDS-PAGE and immunoanalysis as described (*20*). To reduce nonspecific cross-reactions in immunoblotting analysis, we diluted the antiserum 1:10 in tris-buffered saline buffer and incubated it overnight with nitrocellulose filters that had been saturated with protein extracts from *E. coli* BL21DE3 (pT7-7).
16. Using a published alignment of sperm whale myoglobin (SMb), the alpha (Ha) and beta (Hb) polypeptides of human hemoglobin, and *Vitreoscilla* dimeric hemoglobin (Vb) (*17*), we compared protein sequences with the alignment of the monomeric hemoglobins of *Paramecium* (Pc) (*9*) and *Tetrahymena* (Tp) (*10*). The derived sequence of the *Nostoc* cyanoglobin (Nc) was added to the alignment after sequence comparison of Nc with Pc and Tp with the use of the FASTA program (*21*), available through GenBank.
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23. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
24. Supported by the National Science Foundation (grants DCB 8803068 and DCB 9103232 to M.P.). We thank T. Larson for the synthesis of oligonucleotides and for critically reading the manuscript, D. Dean for pT7-7, and D. Hill for photography.

4 February 1992; accepted 7 May 1992

The Motor Cortex and the Coding of Force

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The relation of cellular activity in the motor cortex to the direction of two-dimensional isometric force was investigated under dynamic conditions in monkeys. A task was designed so that three force variables were dissociated: the force exerted by the subject, the net force, and the change in force. Recordings of neuronal activity in the motor cortex revealed that the activity of single cells was directionally tuned and that this tuning was invariant across different directions of a bias force. Cell activity was not related to the direction of force exerted by the subject, which changed drastically as the bias force changed. In contrast, the direction of net force, the direction of force change, and the visually instructed direction all remained quite invariant and congruent and could be the directional variables, alone or in combination, to which cell activity might relate.

One problem in motor physiology concerns the relation between cell activity in the motor cortex and the force exerted by a subject. This problem has been studied extensively under static conditions—that is, when a constant isometric force is exerted. In this case, the rate of motor cortical cell discharge varies with the magnitude (*1–3*) and direction (*4*) of the force exerted. In contrast, the relation of motor cortical cell activity to force under dynamic conditions—that is, when the force changes—has not been studied adequately; for example, such studies have been restricted to one dimension (*1, 2, 5, 6*) or have been complicated by concomitant movement (*7*). In general, cell activity relates to the change in force (*2, 5*), although in several studies that involved movement, forces were not measured (*4, 8*).

We use the term "static force" (*9*) to refer to postural control and "dynamic force" to refer to changing force patterns. The usual experimental situation is a combination of a changing force in the presence of a constant bias force (for example, gravity). In this case, the desired outcome depends not only on the force exerted by the subject but also on the force bias: the crucial variable is the net force acting on the object, which is the vector sum of the force exerted by the subject and the force bias. We assume that the force exerted by the subject consists of a dynamic and a static component. Therefore

$$\text{Net force} = \text{subject force} + \text{force bias} \quad (1)$$

$$= \text{dynamic force} + \text{static force} + \text{force bias} \quad (2)$$

We assume that static force compensates for and is therefore equal and opposite to force bias, so that net force = dynamic force; we use these terms interchangeably. Finally, we define the change in force as the difference between successive force vectors at times t and $t + 1$:

$$\text{Force change} = \text{net force}\ (t + 1) - \text{net force}\ (t) \quad (3)$$

or, given Eq. 1,

$$\text{Force change} = \text{subject force}\ (t + 1) - \text{subject force}\ (t) \quad (4)$$

Therefore, the change in force is the same for both the net force and the force exerted by the subject. These forces change in time when a net force pulse is produced in a specified direction and in the presence of a constant force bias (Fig. 1). The various forces are dissociated, especially dynamic force and the force exerted by the subject; the time course of the change in force is similar to that of dynamic force. We used these dissociations to examine the relation of motor cortical activity to these different forces under isometric conditions and to determine which one is specified by the motor cortex.

For this purpose, we trained a monkey to grasp an isometric handle (*10*) with its hand pronated and to exert force pulses so that the net force was in eight visually specified directions. These directions were indicated by a target on a display placed 45 cm in front of the animal, and a force feedback cursor displayed the net force on the handle. A steady deflection of the force feedback cursor was used to produce a constant bias force. In the task, the visual target first appeared in the center of the

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