Identification of Residues in Human Neuroglobin Crucial for Guanine Nucleotide Dissociation Inhibitor Activity†

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ABSTRACT: Neuroglobin (Ngb) is a recently discovered vertebrate heme protein that is expressed in the brain and can reversibly bind oxygen. We previously demonstrated that ferric human Ngb binds to the α-subunits of heterotrimeric G proteins (Gα) and acts as a guanine nucleotide dissociation inhibitor (GDI) for Gα. Here we have investigated the interaction between Ngb and Gα in more detail. We report that zebrafish Ngb, which shares about 50% amino acid sequence identity with human Ngb, does not have a GDI activity for Gα. By carrying out exon swapping between zebrafish and human Ngb and site-directed mutagenesis, we have identified several residues that are crucial for the GDI activity of human Ngb.

Globins are iron porphyrin complex (heme)-containing proteins that bind reversibly to oxygen and, as such, play an important role in respiratory function. Neuroglobin (Ngb[1]) is a recently discovered globin of mammalian brain that has a high affinity for oxygen (*1*). Ngb can reversibly bind oxygen (*1*−*3*). The iron atom in the heme prosthetic group of Ngb exists in either the ferrous (Fe^{2+}) or the ferric (Fe^{3+}) redox state. In the absence of exogenous ligands, both the ferric and the ferrous forms of Ngb are hexacoordinated to endogenous distal and proximal histidine residues within the protein (*2*). Oxygen (O_2) or carbon monoxide (CO) can displace the distal histidine of ferrous deoxy-Ngb to produce ferrous oxygen-bound Ngb (ferrous-O_2 Ngb) or ferrous carbon monoxide-bound Ngb (ferrous-CO Ngb) (*2*).

Ngb is widely expressed in the cerebral cortex, hippocampus, thalamus, hypothalamus, cerebellum, and retina (*1*, *4*−*8*). Recently, it has been suggested that Ngb is involved in the neuronal response to hypoxia and ischemia (*9*, *10*). Expression of Ngb has been reported to increase in response to neuronal hypoxia in vitro and to focal cerebral ischemia in vivo (*9*, *10*). Neuronal survival following hypoxia can be reduced by inhibiting Ngb expression with an antisense oligodeoxynucleotide and can be enhanced by Ngb overexpression, supporting the notion that Ngb protects neurons from hypoxic−ischemic insults (*9*). Moreover, Ngb has been found to protect the brain from experimentally induced stroke in vivo (*10*).

The mechanism by which Ngb affords neuroprotection under conditions of oxidative stress such as ischemia and reperfusion remains unclear. Recently, we tried to clarify the neuroprotective role of Ngb under oxidative stress in vitro (*11*−*13*). We previously found that ferric human Ngb, which is generated spontaneously as a result of rapid autoxidation, binds exclusively to the GDP-bound form of the α-subunit of heterotrimeric G protein ($G\alpha_i$) and acts as a guanine nucleotide dissociation inhibitor (GDI) by inhibiting the rate of exchange of GDP for GTP on $G\alpha_i$ (*11*). The interaction of GDP-bound $G\alpha_i$ with ferric Ngb liberates $G\beta\gamma$, leading to protection against neuronal death (*14*).

Although Ngb was originally identified in mammalian species, it is also present in nonmammalian vertebrates, including the pufferfish *Tetraodon nigroviridis* and the zebrafish *Danio rerio* (*15*, *16*). Mammalian and fish Ngb proteins share about 50% amino acid sequence identity (Figure 1). Fish Ngb proteins are also hexacoordinated globins with similar oxygen-binding kinetics (*16*). The genes encoding fish Ngb reveal a mammalian-type pattern of exons and introns in the coding region (B12.2 (i.e. between codon positions 2 and 3 of the 12th amino acid of the globin helix B), E11.0, and G7.0) (Figure 1) (*15*, *16*). Exons 1, 2, 3, and 4 of Ngb correspond to compact protein structural unit "modules", termed M1, M2, M3, and M4, respectively (*1*, *16*−*19*).

The objective of this study was to investigate the interaction between Ngb and $G\alpha_i$ in more detail. To this end, we have cloned the zebrafish Ngb cDNA and have characterized properties of the zebrafish Ngb protein. We report that zebrafish Ngb does not act as a GDI activity for $G\alpha_i$. By engineering an exon-swapped chimera of zebrafish and human Ngb, as well as several site-directed human Ngb mutants, we have identified numerous amino acids in human Ngb that are crucial for its GDI activity.

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[1] Abbreviations: Ngb, neuroglobin; ferrous-O_2 Ngb, ferrous oxygen-bound Ngb; ferrous-CO Ngb, ferrous carbon monoxide-bound Ngb; G protein, guanine nucleotide-binding protein; GDI, guanine nucleotide dissociation inhibitor; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate.

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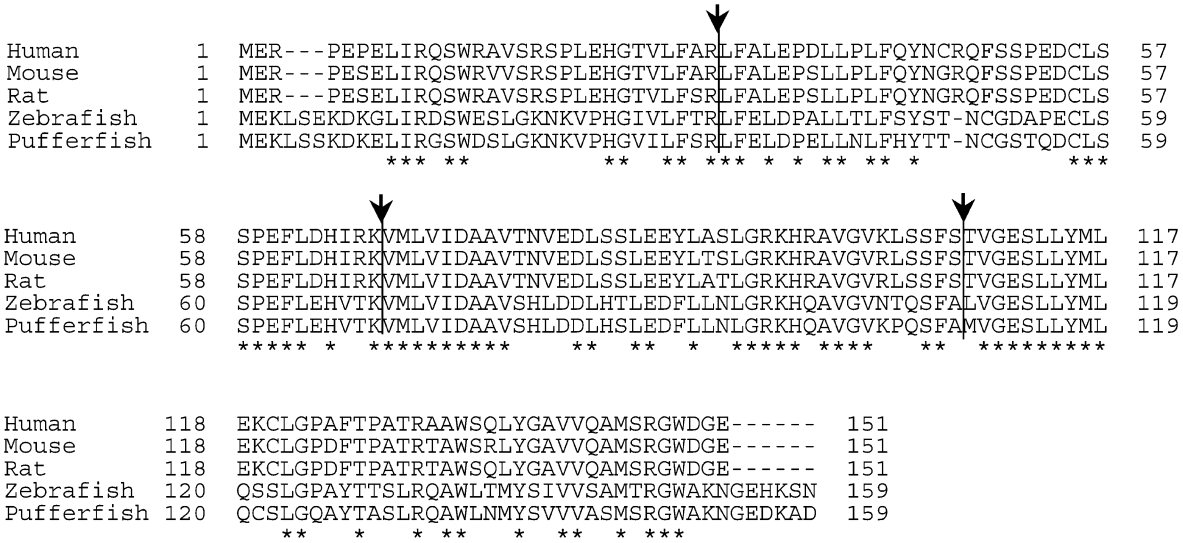

FIGURE 1: Alignment of the amino acid sequence of Ngb from various species. Multiple sequence alignment was performed by Clustal W with manual adjustments. Consensus amino acids are indicated by an asterisk. Numbers on the left and right of the sequences correspond to those at the beginning and the end of the sequences, respectively. Gaps in the sequences are indicated by dashes. Intron positions in the human, zebrafish, and pufferfish Ngb (at B12.2, E11.0, and G7.0) are indicated by arrows (*1*, *15*, *16*).

EXPERIMENTAL PROCEDURES

Samples. The rat myristoylated $G\alpha_i$-subunit ($G\alpha_{i1}$; Calbiochem, San Diego, CA) was used in GDI activity assays. [^{35}S]GTPγS (>1000 Ci/mmol) and [8-^{3}H]GDP (10−15 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Buckinghamshire, England).

Preparation of Proteins. Amplification of human Ngb cDNA was performed by PCR using human universal Quick-clone cDNA (Clontech, Palo Alto, CA) (*11*). The complete zebrafish Ngb cDNA sequence was obtained from a clone provided by Open Biosystems (Huntsville, AL) (clone ID 4200323). The human or zebrafish Ngb cDNA was cloned into plasmid PET20b (Novagen, Madison, WI) and was sequenced by an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Overexpression of Ngb was induced in *Escherichia coli* strain BL 21 (DE 3) (Novagen) by treatment with isopropyl β-D-thiogalactopyranoside. The soluble cell extract was loaded onto a DEAE Sepharose anion-exchange column equilibrated with 20 mM Tris−HCl (pH 8.0). Ngb was eluted from the column with buffer containing 75 mM NaCl and was further purified by passage through a Sephacryl S-200 HR gel filtration column. Deoxy-Ngb samples were prepared by first equilibrating Ngb with N_2 gas and then reducing the heme with sodium dithionite. Ferrous-CO Ngb samples were obtained by equilibrating Ngb with CO gas and then adding an excess of sodium dithionite.

GTPγS Binding Assays. We incubated 100 nM $G\alpha_i$ for 3 min at 25 °C in buffer A (20 mM Tris−HCl, 100 mM NaCl, and 10 mM $MgSO_4$; pH 8.0) plus 10 μM GDP in the absence or presence of Ngb (5 μM). Binding assays were initiated by the addition of 50 nM [^{35}S]GTPγS (>1000 Ci/mmol). Aliquots (10 μL) were withdrawn from the binding mixtures and were passed through nitrocellulose filters (0.45 μm) (Millipore, Bedford, MA). The filters were then washed three times with 1 mL of ice-cold buffer A and were counted in a liquid scintillation counter (LSC-6100; Aloka, Tokyo, Japan). The apparent rate constant (k_{app}) values for the binding reactions were calculated by fitting the data to the following equation: GTPγS binding (%) = $100(1 - e^{-kt})$.

GDP Dissociation Assays. $G\alpha_i$ complexed with [^{3}H]GDP (0.3 μM) was prepared by incubating 0.3 μM $G\alpha_i$ with 2 μM [^{3}H]GDP in buffer A for 1.5 h at 25 °C. Excess unlabeled GTP (200 μM) was added to monitor the dissociation of [^{3}H]-GDP from $G\alpha_i$ in the absence or presence of Ngb (5 μM). Aliquots were withdrawn at the indicated times and were passed through nitrocellulose filters (0.45 μm) (Millipore). The filters were then washed three times with 1 mL of ice-cold buffer A and were counted in a liquid scintillation counter (LSC-6100; Aloka).

Module Substitution and Site-Directed Mutagenesis. We prepared human Ngb, in which the module M1 was truncated, and chimeric βHHH or ZHHH Ngb, in which the M1 module of human Ngb was replaced by that of the human hemoglobin β-subunit or zebrafish Ngb, respectively, by using PCR. The chimeric βHHH and ZHHH Ngb proteins both possessed an additional Arg (B12) to Lys mutation due to introduction of the restriction site for *Hin*dIII. A QuikChange site-directed mutagenesis system (Stratagene, La Jolla, CA) was used for site-directed mutagenesis. The sequences were confirmed by DNA sequencing using a BigDye Terminator Cycle Sequencing FS kit (Applied Biosystems) and an ABI 3100 genetic analyzer (Applied Biosystems).

^{1}H NMR Spectra. Proton NMR experiments were performed on a Bruker Avance DRX500 spectrometer at 22 °C. The PRESAT pulse sequence was used to minimize the water signal. Samples were dissolved in 50 mM sodium phosphate buffer (pH 7.4) containing 10% D_2O. Protein concentrations were 200 μM on the basis of the heme content. Proton shifts were referenced with respect to the proton resonance of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

Calculation of Relative Solvent Accessibility of Amino Acids. The water accessibility of each amino acid was computed on the basis of the crystal structures of human and mouse Ngb by using SwissPdbViewer. In brief, relative accessibility was defined as the ratio of the exposed surface area of an amino acid residue X in the actual structure to the maximum possible exposed surface area of the same

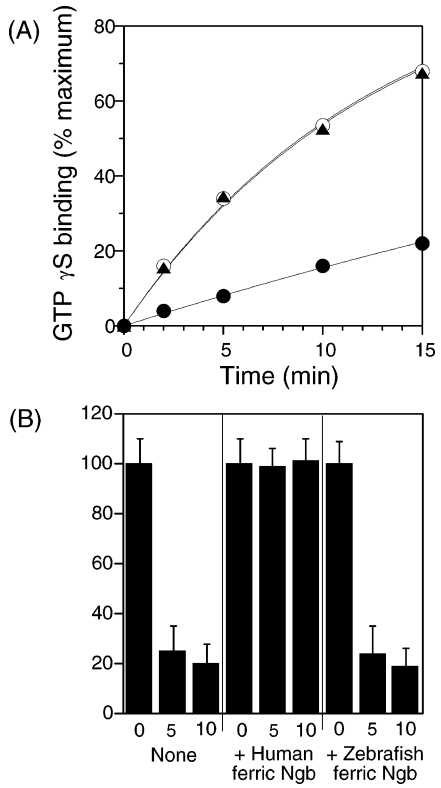

FIGURE 2: GDI activity of the human and zebrafish Ngb. (A) Effects of human and zebrafish Ngb on GTPγS binding to Gα_i. Binding of GTPγS to 100 nM Gα_i in the absence (open circle) or presence of 5 μM ferric human (closed circle) or ferric zebrafish (closed triangle) Ngb was initiated by the addition of 50 nM [^{35}S]GTPγS (>1000 Ci/mmol). Gα_i-bound GTPγS was counted by withdrawing aliquots at the indicated times and passing them through nitrocellulose filters (0.45 μm). (B) Effects of zebrafish Ngb on the dissociation of GDP from GDP-bound Gα_i. Experiments were performed in the presence of an excess amount of unlabeled GTP. Gα_i complexed with [^{3}H]GDP was obtained as described in the Experimental Procedures. An excess of unlabeled GTP (200 μM) was added to the Gα_i−[^{3}H]GDP complex in the absence or presence of 5 μM human or zebrafish Ngb. Aliquots were withdrawn at 0, 5, and 10 min and passed through nitrocellulose filters (0.45 μm). Each error bar represents the standard deviation of 3−4 independent experiments.

amino acid. This maximum surface area is the surface accessible to water of X in a pentapeptide, GGXGG, in extended conformation.

RESULTS

Functional Analyses of the Zebrafish Ngb. To determine whether ferric zebrafish Ngb functions as a GDI, we performed GTPγS (a nonhydrolyzable analogue of GTP) binding experiments. As a control, the rate of GTPγS binding to Gα_i in the presence of ferric human Ngb was reduced (Figure 2A), showing that ferric human Ngb functions as a GDI for Gα_i, as previously reported (*11*). In contrast, ferric zebrafish Ngb had no effect on the rate of GTPγS binding to Gα_i (Figure 2A). Next, to examine the effects of Ngb on the release of GDP from Gα_i, we measured the rates of GDP dissociation in the absence or presence of ferric forms of the two proteins. In the presence of an excess amount of unlabeled GTP, [^{3}H]GDP release from [^{3}H]GDP-bound Gα_i was inhibited by ferric human Ngb (Figure 2B); however, ferric zebrafish Ngb did not affect the release of [^{3}H]GDP from [^{3}H]GDP-bound Gα_i (Figure 2B).

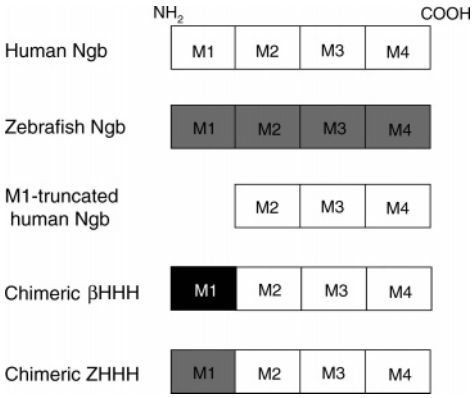

FIGURE 3: Schematic representation of the wild-type, truncated, and module-substituted Ngb proteins used in this study.

Preparation of Truncated Ngb and Module-Substituted Ngb. Gilbert and Blake previously proposed that exons encode functional and structural units and that new functional proteins evolve through the selection of various combinations of these units that are produced by unequal crossing-over on introns—a process that is termed "exon shuffling" (*20*, *21*). Furthermore, by using a diagonal plot of all of the distances between α-carbon atoms, Go demonstrated that there is a correlation between the structure of a protein and its exon pattern and found that exons correspond to compact structural unit modules (*17*).

As shown in Figure 1, the genes of human and zebrafish Ngb are made up of four exons interrupted by three introns, and exons 1, 2, 3, and 4 correspond to modules M1, M2, M3, and M4, respectively (*1*, *16*−*19*). On the basis of the results of homology searches modules M2−M4, but not module M1, of human Ngb share 25−35% amino acid sequence homology with RGS (*11*). To narrow down the regions that are important in the human-specific contacts between Ngb and Gα, we generated expression constructs encoding a human Ngb variant in which module M1 was truncated, termed M1-truncated Ngb, as well as two human Ngb variants in which module M1 was substituted, termed chimeric βHHH and ZHHH (Figure 3). The chimeric βHHH and ZHHH proteins consist of the N-terminal M1 module of the human hemoglobin β-subunit or the zebrafish Ngb jointed to the C-terminal M2−M4 modules of human Ngb, respectively.

M1-truncated Ngb and the chimeric βHHH and ZHHH Ngb proteins were expressed in *E. coli* and were subjected to purification by the same protocols used for wild-type Ngb. We succeeded in purifying chimeric ZHHH Ngb; however, M1-truncated Ngb and chimeric βHHH were not purified because of structural instability.

Structural Analyses of M1-Substituted Ngb. Visible absorption spectra of the ferric, ferrous deoxy, and ferrous-CO forms of the chimeric ZHHH Ngb were almost identical to those of human and zebrafish wild-type Ngb (data not shown), suggesting that the substitution of module M1 does not perturb the electronic state of the heme. To examine the effects of module M1 substitution on the globin structure, we measured the far-UV circular dichroism (CD) spectra of the ferric forms of the Ngb proteins. The α-helical content of the chimeric ZHHH Ngb was estimated as 68%, which is the same as that of human Ngb (68%) (data not shown).

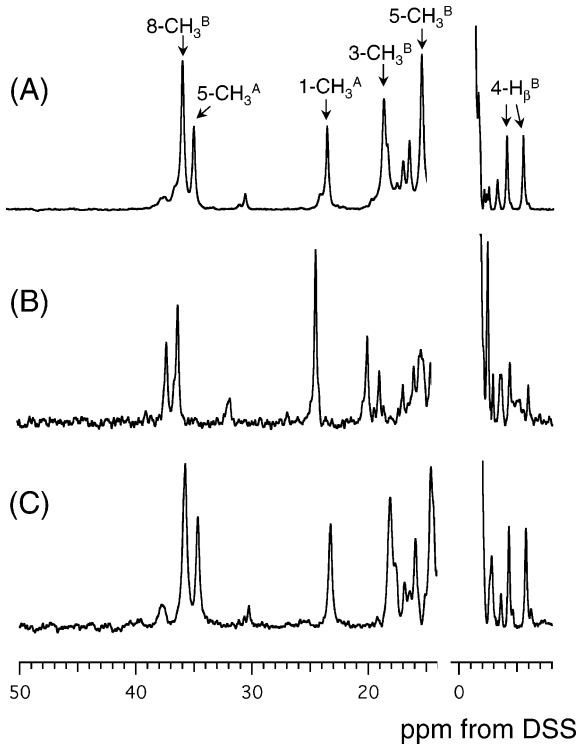

FIGURE 4: ^{1}H NMR spectra (500 MHz) of ferric Ngb: (A) human Ngb, (B) zebrafish Ngb, (C) chimeric ZHHH Ngb. Spectra were recorded in 50 mM sodium phosphate buffer (pH 7.4) containing 10% D_2O at 22 °C. The concentration of each Ngb was 200 μM on the basis of the heme content. Superscript A or B denotes the heme orientation isomer A or B, respectively.

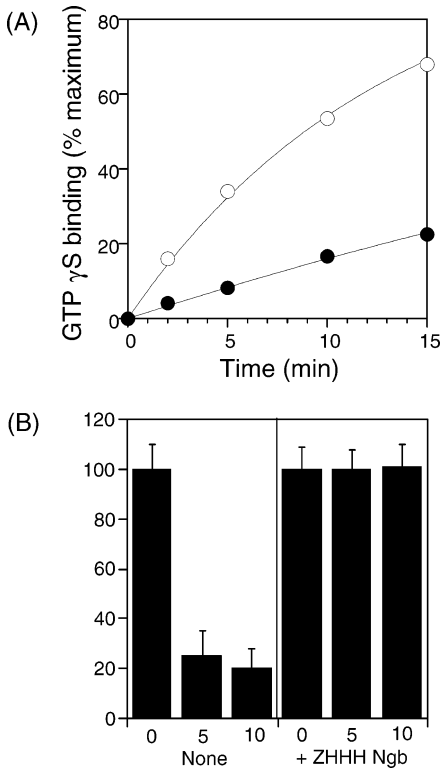

FIGURE 5: GDI activity of chimeric ZHHH Ngb. (A) Effects of M1-substituted Ngb on GTPγS binding to Gα_i. Binding of GTPγS to Gα_i in the absence (open circle) or presence (closed circle) of ferric chimeric ZHHH Ngb was initiated by the addition of [^{35}S]-GTPγS. Gα_i-bound GTPγS was counted by withdrawing aliquots at the indicated times. Experimental conditions were the same as in Figure 2A. (B) Effects of chimeric ZHHH Ngb on dissociation of GDP from GDP-bound Gα_i. Experimental conditions were the same as in Figure 2B.

These results show that the secondary structure of Ngb is insensitive to substitution of the M1 module.

To gain further insight into the environment surrounding the heme in chimeric ZHHH Ngb, we examined the NMR spectra of the ferric forms of the Ngb proteins. As depicted in Figure 4, several prominent hyperfine-shifted signals from the heme peripheral groups were observed for the ferric Ngb. We assigned the hyperfine-shifted signals of human Ngb on the basis of spectra recorded for mouse Ngb (*22*), as labeled in Figure 4. Hyperfine-shifted resonances from the heme methyl groups in the chimeric ZHHH Ngb were observed at almost the same position as those in human Ngb, suggesting that the chimeric ZHHH Ngb forms almost the same structure around the heme environment as the human Ngb.

M1-Substituted Ngb Acts as a GDI. To determine whether ferric chimeric ZHHH Ngb functions as a GDI, we performed GTPγS binding experiments. As shown in Figure 5A, Gα_i bound GTPγS, as assessed by the spontaneous exchange of guanine nucleotide ($k_{app} = 0.075$ min^{-1}). In the presence of ferric chimeric ZHHH Ngb, the rate of GTPγS binding to Gα_i was reduced 5-fold ($k_{app} = 0.016$ min^{-1}) (Figure 5A), implying that ferric chimeric ZHHH Ngb functions as a GDI for Gα_i as does human Ngb.

We considered that ferric chimeric ZHHH Ngb might inhibit GTPγS binding to Gα_i via a mechanism involving a reduction in the rate of nucleotide exchange. Therefore, to examine the effects of ferric chimeric ZHHH Ngb on the release of GDP from Gα_i, we measured the rates of GDP dissociation in the absence or presence of ferric Ngb. In the presence of an excess amount of unlabeled GTP, [^{3}H]GDP release from [^{3}H]GDP-bound Gα_i was inhibited by chimeric ZHHH Ngb (Figure 5B). The inhibition of GDP dissociation by ferric Ngb suggests that ferric Ngb diminishes the rate of spontaneous GTPγS binding to Gα_i by blocking the release of GDP.

Functional Analyses of Site-Directed Ngb Mutants. To delineate the region in modules M2−M4 of the human Ngb that is responsible for GDI activity, we pinpointed key differences between the human and zebrafish Ngb sequences, with a particular focus on exposed residues with positive or negative charges. We identified Arg47, Glu53, Arg97, Lys102, Glu118, Lys119, Asp149, and Glu151 (amino acid numbering is based on that of human Ngb) as potential candidate residues, and prepared the site-directed Ngb mutants R47A, E53Q, R97Q, K102N, E118Q, K119N, D149A, and E151N. The electronic absorption spectra and CD spectra of these mutants verified that the mutations did not induce significant structural changes (data not shown).

To determine whether the mutant Ngb proteins retained GDI activity, we measured GTPγS binding to Gα_i and GDP dissociation from Gα_i in the absence or presence of the ferric form of each mutant. As shown in Figure 6, like human wild-type Ngb, R47A, K102N, K119N, and D149A Ngb inhibited GTPγS binding to Gα_i and inhibited [^{3}H]GDP release from [^{3}H]GDP-bound Gα_i in the presence of an excess amount of unlabeled GTP, implying that ferric forms of these mutants retained GDI activity toward Gα_i. In contrast, E53Q, R97Q, E118Q, and E151N Ngb did not function as a GDI (Figure

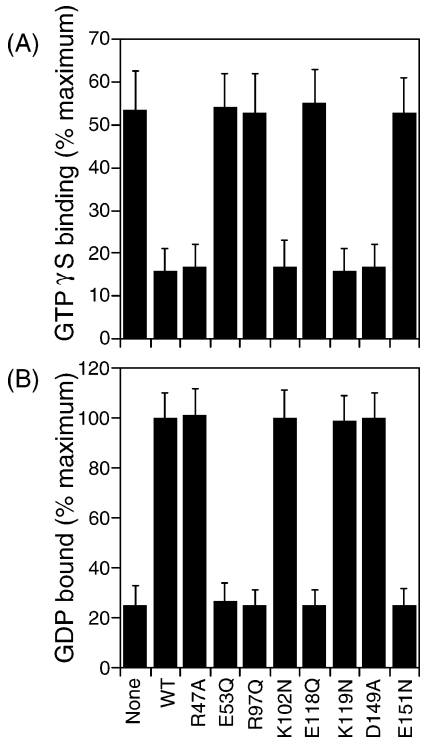

FIGURE 6: GDI activity of site-directed human Ngb mutants. (A) Effects of site-directed Ngb mutants on GTPγS binding to Gα_i. The binding of GTPγS to Gα_i in the absence or presence of the ferric forms of each mutant Ngb was initiated by the addition of [^{35}S]GTPγS. Gα_i-bound GTPγS was counted by withdrawing aliquots at 10 min. Experimental conditions were the same as in Figure 2A. (B) Effects of site-directed Ngb mutants on dissociation of GDP from GDP-bound Gα_i. Percentages of [^{3}H]GDP-bound Gα_i at 5 min are shown. Experimental conditions were the same as in Figure 2B.

6), suggesting that residues Glu53, Arg97, Glu118, and Glu151 are crucial for the GDI activity of human Ngb.

DISCUSSION

Binding Sites of Human Ngb with Gα_i. In the present study, we have shown that zebrafish Ngb, which shares about 50% amino acid sequence identity with human Ngb, does not have a GDI activity for Gα_i. Furthermore, by module substitution between zebrafish and human Ngb and site-directed mutagenesis, we have identified several residues that are essential for GDI activity in modules M2−M4 of the human Ngb (Figure 7).

Previously, we found that a human Ngb double mutant (C55S, C120S) homodimer that is linked by an intermolecular disulfide bond at Cys^{46}−Cys^{46} cannot function as a GDI, whereas its DTT-reduced monomeric form has GDI activity (*11*), suggesting that the CD-D region in which Cys^{46} exists is important for protein−protein interaction between ferric human Ngb and Gα_i and may be involved in the GDI activity for Gα_i.

X-ray structural analyses of the ferric forms of human and mouse Ngb have clarified that the CD-D region of mammalian Ngb is very flexible (*18*, *19*). In addition, the CD-D region connecting helices C and E of *Vitreoscilla* Hb, which is synthesized under hypoxic conditions, is also disordered in the crystal structure (*23*). Mutagenesis experiments on this protein have shown that a negatively charged Glu residue in the CD-D region is involved in *Vitreoscilla* Hb binding

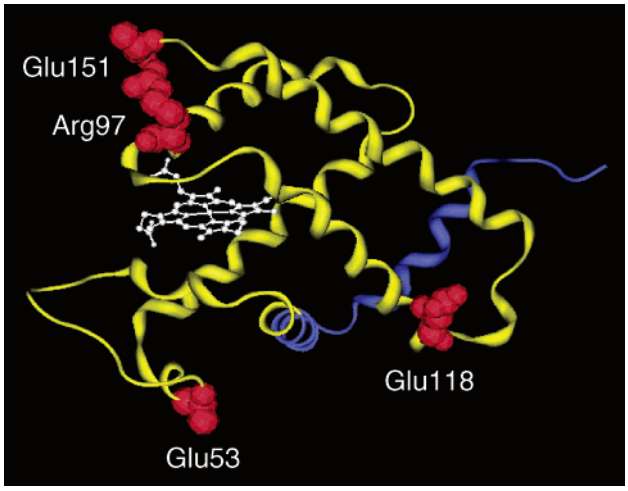

FIGURE 7: Surface residues of human Ngb that are required for its GDI activity. The essential residues (Glu53, Arg97, Glu118, and Glu151) of human Ngb are indicated as red space-filled balls. Module M1 and modules M2−M4 are highlighted in blue and yellow, respectively.

to a flavin domain of 2,4-dinitrotoluene (DNT) dioxygenase (*24*).

In the present study, we have demonstrated that a negatively charged residue Glu53 in the CD-D region of human Ngb is involved in the interaction with Gα_i (Figure 7). Moreover, site-directed mutagenesis experiments clarified that some Ngb residues (Arg97, Glu118, and Glu151) in modules M3 and M4 are also involved in the regulation of Gα_i by Ngb (Figure 7). Further studies by using zebrafish Gα will be necessary to elucidate the molecular evolution of Ngb as a GDI.

Module M1 Substitution of Ngb. Our study has shown that a chimeric ZHHH Ngb variant, in which module M1 of human Ngb is replaced by that of zebrafish Ngb, forms almost the same structure as human Ngb and acts as a GDI for Gα_i as does human Ngb. By contrast, M1-truncated human Ngb and the chimeric βHHH were very unstable.

Previous studies of myoglobin (Mb) by using NMR spectroscopy, mutagenesis, and computer graphics suggested that packing around the A (in module M1), G, and H helices (in module M4) plays an important role in the protein folding and stability, because the folding and packing of the A, G, and H helices are considered to be the first step in the dominant folding pathway of apomyoglobin (*25*−*28*).

Our previous results of module substitution among the hemoglobin (Hb) α- and β-subunits and Mb also demonstrated that the interactions among the modules are crucial for folding and stability of the proteins (*29*−*31*). For example, it was previously conjectured that the substitution of module M1 of Mb for that of the Hb α- or β-subunit to form an Mb$\alpha\alpha\alpha$ or Mb$\beta\beta\beta$ chimera, respectively, would have no influence on the association properties of the proteins because the amino acid residues that are crucial for subunit contacts between the α- and β-subunits are not located in M1 (*32*); however, the chimeric Mb$\alpha\alpha\alpha$ and Mb$\beta\beta\beta$ variants were subsequently found to have a low α-helical content and to show altered association characteristics as compared to the wild-type globins (*31*). These results suggest that intermodular packing between module M1 and other modules (M2, M3, and/or M4) is essential for folding and stability in globins.

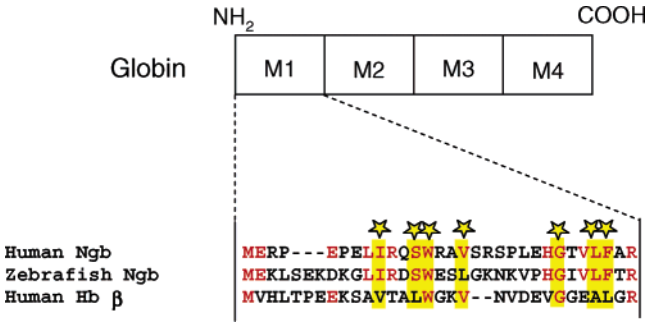

FIGURE 8: Correlation between the evolutionarily conserved residues and residues with a low solvent accessibility in module M1. Consensus amino acids between human and zebrafish Ngb and between human Ngb and the hemoglobin β-subunit are indicated in red. Residues with a relative water accessibility of less than 5% in human Ngb are highlighted in yellow.

As shown in Figure 8, all except one of the amino acid residues that are buried by intermodular packing in module M1 of human Ngb (i.e., Ile9, Ser12, Trp13, Gly24, Leu27, and Phe28) are evolutionarily conserved residues between human and zebrafish Ngb. The only exception, Val16 of human Ngb, is a hydrophobic amino acid residue and thus is similar to the corresponding Leu residue of zebrafish Ngb (Figure 8). In contrast, some residues that are buried in M1 of human Ngb, in particular Ser12 and Leu27, differ from the corresponding residues in the Hb β-subunit (Figure 8). The instability of M1-truncated Ngb and chimeric βHHH Ngb suggests that the folding of these variants is perturbed by the loss of some intermodular interactions that are central to protein packing. Our present findings on chimeric ZHHH Ngb allow us to conclude that module substitutions will be useful for designing and producing novel functional proteins if we can reproduce the stable intermodular packing in the module-substituted proteins.

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