Preparation of artificial 2-, 3-, 4- and 8-domain myoglobins and comparison of their autoxidation rates

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Abstract Although most hemoglobins and myoglobins consist of 15-kDa single-domain subunits, structurally unusual hemoglobins, such as *Artemia* 9-domain and *Barbatia* 2-domain hemoglobins, occur naturally in several invertebrates. These hemoglobins appear to be the result of gene duplication and fusion. Using cDNA coding for the open reading frame of *Aplysia kurodai* myoglobin, artificial cDNA inserts corresponding to contiguous dimer, trimer, tetramer and octamer myoglobins (2-, 3-, 4- and 8-domain myoglobins) were prepared and cloned into pMAL or pQE plasmids. These artificial myoglobins and wild-type single-domain myoglobins were successfully expressed in *Escherichia coli* in the heme-attached, oxygenated form. Myoglobin was purified partially by ammonium sulfate fractionation and gel filtration, and autoxidation rates were examined. The autoxidation rates of recombinant wild-type myoglobins with MBP or hexameric His tag were comparable to those of native myoglobin, suggesting that the recombinant proteins appear to be properly folded and that the N-terminal MBP or His tag does not have an affect on the rate. On the other hand, the rates were significantly decreased in the 2- and 3-domain myoglobins (50% and 30% of the single-domain myoglobins, respectively). The rates for 4- and 8-domain myoglobins were similar to those for 3-domain myoglobin. These results indicate that the artificial poly-domain structure of myoglobin is more stable than the usual single-domain myoglobin from the viewpoint of storage of bound dioxygen.

Keywords: Myoglobin; Poly-domain; Autoxidation; *Aplysia kurodai*

1. Introduction

Most of the myoglobins (Mbs) and hemoglobins (Hbs) consist of subunits with molecular masses of 15 kDa that contain one heme and work as a functional domain. However, several invertebrate animals abundantly express unusual poly-domain Hbs in erythrocyte or plasma: *Cardita* (blood clam), extracellular 20-domain Hb [1]; *Artemia*, extracellular 9-domain Hb [2]; *Barbatia* (blood clam), intracellular 2-domain Hb [3,4]; *Ascaris* (nematode), extracellular 2-domain Hb [5]; and *Daphnia* (water flea), extracellular 2-domain Hb [6]. These unusual Hbs, with the inter-domain amino acid identity ranging between 17% and 75%, appear to be the result of tandem gene duplication followed by fusion [3], but their functional nature is not well characterized. Among them, it is known that *Ascaris* 2-domain Hb shows an extraordinarily high oxygen affinity. However, this characteristic of *Ascaris* 2-domain Hb is maintained even when the domains are separated, showing that this unusually high oxygen affinity is not due to the two-domain formation of the Hb [7].

In addition to Hb, tandem-repeated poly-domain structure is found in several proteins and enzymes. Recently, we showed that the kinetic parameters of a 2-domain arginine kinase differ slightly, but significantly, from those of domain 2 of the 2-domain enzyme [8].

In this report, we prepared artificial, recombinant 2-, 3-, 4- and 8-domain Mbs and compared the autoxidation rates with those of wild-type single domain Mb. The autoxidation rate was slower for increased numbers of domains, showing that poly-domain Mb is more stable than the wild-type, at least from the point-of-view of storage of dioxygen.

2. Materials and methods

mRNA was prepared from the radular muscle of *A. kurodai*, a gastropodic mollusc, with a QuickPrep Micro mRNA Purification Kit (Pharmacia). The single-stranded cDNA was synthesized with a Ready-To-Go You-Prime First-Strand Beads (Pharmacia) using the oligo(dT) adaptor as a primer. The open reading frame of *Aplysia* Mb was amplified by PCR with the two primers, designed on the basis of the cDNA sequence deposited in the DDBJ database (AB003278). The products were cloned in the *Sma*I site of the plasmid pUC18 (pUC18-Mb).

2.1. Cloning into pMAL-c2 plasmid

Construction of wild-type single-domain Mb plasmid. The ORF (including stop codon) was amplified by PCR with the two primers, F1: 5′AGAATTC[ATG]TCTCTGTCTGCTGAAGC (*Eco*RI site added) and R1: 5′A[CTGCAG]AGCATC[TTA]TTTGCCGGCGGC (*Pst*I site added), using the pUC18-Mb as a template. Restriction sites were underlined and start or stop codon was boxed. PCR was done for 30 cycles, each cycle consisting of 0.5 min at 94 °C (denaturation), 0.5 min at 55 °C (annealing) and 0.5 min at 72 °C (primer extension). Extra *Taq* DNA polymerase (Takara, Tokyo, Japan) was used as the amplifying enzyme. The amplified products were digested with *Eco*RI and *Pst*I, separated on 1.0% agarose electrophoresis, recovered with Easy Trap Ver.2 (Takara) and cloned into the *Eco*RI/*Pst*I site of pMAL plasmid (pMAL-1DMb). The insert of the plasmid was sequenced with

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a PRISM dye terminator cycle FS ready reaction kit using a Model 373-18 DNA sequencer (Applied BioSystems).

Construction of 2-domain Mb plasmid. The cDNA for the domain 1 was amplified with the two primers, F1 (sequence shown above) and R3: 5′TTAAGCTTTTGCCGGCGGCCTTGAGGGC (stop codon removed and *Hin*dIII site added), using the pUC18-Mb as a template. The cDNA for the domain 2 was amplified with the two primers, F2: 5′TTAAGCTT ATG TCTCTGTCTGCTGCTGA (*Hin*dIII site added) and R1 (sequence shown above). PCR was done for 35 cycles, each cycle consisting of 0.25 min at 94 °C (denaturation), 0.5 min at 60 °C (annealing) and 2.0 min at 68 °C (primer extension), using KOD^+ DNA polymerase (Toyobo, Tokyo, Japan) as the amplifying enzyme. The two amplified products were digested with *Hin*dIII and ligated using DNA Ligation Kit Ver.2 (Takara). The ligated products were digested with *Eco*RI and *Pst*I, and cloned into the *Eco*RI/*Pst*I site of pMAL plasmid (pMAL-2DMb).

Construction of 3-domain Mb plasmid. The cDNA for the domain 1 was amplified with the two primers, F4: TGTCGAC ATG TCTCTGTCTGCTGAAGC (*Sal*I site added) and R5: TTGGATCCTTTGCCGGCGGCCTTGAGGGC (stop codon removed and *Bam*HI site added), using the pUC18-Mb as a template. The cDNA for the domains 2 and 3 was amplified with the two primers, F3: 5′AGGATCC ATG TCTCTGTCTGCTGAAGC (*Bam*HI site added) and R1 (sequence shown above), using the pMAL-2DMb as a template. PCR was done for 35 cycles, each cycle consisting of 0.25 min at 94 °C, 0.5 min at 60 °C and 2.0 min at 68 °C, using KOD^+ DNA polymerase as the amplifying enzyme. The two amplified products were digested with *Bam*HI and ligated. The ligated products were digested with *Sal*I and *Pst*I, and cloned into the *Sal*I/*Pst*I site of pMAL plasmid (pMAL-3DMb).

Construction of 4-domain Mb plasmid. The cDNA for the domain 1 was amplified with the two primers, F1 (sequence shown above) and R6: 5′TTGTCGACTTTGCCGGCGGCCTTGAGGGC (stop codon removed and *Sal*I site added), using the pUC18-Mb as a template. PCR was done for 35 cycles, each cycle consisting of 0.25 min at 94 °C, 0.5 min at 60 °C and 2.0 min at 68 °C, using KOD^+ DNA polymerase. The amplified products were digested with *Eco*RI and *Sal*I and ligated into pMAL-3DMb, which had been digested with *Eco*RI and *Sal*I.

Construction of 8-domain Mb plasmid. The cDNA for the domain 4 was amplified with the two primers, F2 (sequence shown above) and R7: 5′TTGAATTCTTTGCCGGCGGCCTTGAGGGC (stop codon removed and *Eco*RI site added), using the pUC18-Mb as a template. PCR was done for 35 cycles, each cycle consisting of 0.25 min at 94 °C, 0.5 min at 60 °C and 2.0 min at 68 °C, using KOD^+ DNA polymerase. The amplified products were digested with *Hin*dIII and *Eco*RI. This was ligated with the *Eco*RI/*Hin*dIII digested product of pMAL-4DMb, which contains domains 1–3 (1350 bp). The ligated products were separated on agarose electrophoresis and the 1800 bp product corresponding to 1–4 domains was recovered. Then, it was ligated with pMAL-4DMb, which had been digested with *Eco*RI (pMAL-8DMb).

2.2. Cloning into pQE30 plasmid

Construction of wild-type single-domain Mb plasmid. The ORF (including stop codon) was amplified by PCR with the following two primers, F3: 5′AGGATCC ATG TCTCTGTCTGCTGAAGC (BamHI site added) and R1 (sequence shown above), using the above pUC18-Mb as a template. The amplified products were digested with *Bam*HI and *Pst*I, and were cloned into the *Bam*HI/*Pst*I site of pQE30 plasmid (pQE-1DMb).

Construction of 2-domain Mb plasmid. The cDNA for domains 1 and 2 was amplified with the two primers, F3 (sequence shown above) and R1 (sequence shown above), using the pMAL-2DMb as a template. The amplified products were separated on 1.0% agarose electrophoresis and a 900-bp product was purified. It was then digested with *Bam*HI and *Pst*I, and cloned into the *Bam*HI/*Pst*I site of pQE plasmid (pQE-2DMb).

2.3. Expression of MBP (maltose-binding protein)/single-domain Mb in E. coli

Escherichia coli TB1 cell, transformed with pMAL-1DMb, was cultured in LB broth at 37 °C for 8 h. Then, a four-volume of fresh LB was added to the bacterial solution and cultured at 37 °C for 1 h. Protein was induced at 27 °C for 6 h by adding IPTG (final 1 mM). Then, a four-volume of fresh LB was added and cultured further at 37 °C for 18 h.

2.4. Expression of MBP/2-, 3-, 4- and 8-domain Mbs in E. coli

E. coli TB1 cell, transformed with pMAL-2DMb, pMAL-3D, pMAL-4DMb or pMAL-8DMb, was cultured in LB media at 37 °C for 8 h. Then, a four-volume of fresh LB containing 0.1 mM ALAH (aminolevulinic acid/HCl) was added and cultured at 37 °C for 1 h. Protein was induced at 27 °C for 19–27 h by adding IPTG (final 0.2 mM).

2.5. Expression of His-tag/single- and 2-domain Mbs in E. coli

E. coli TB1 cell, transformed with pQE-1DMb or pQE-2DMb, was cultured in LB at 37 °C for 8 h. Then, a four-volume of fresh LB containing 0.1 mM ALAH was added and cultured at 37 °C for 1 h. Single-domain Mb was induced at 27 °C for 19–27 h in the presence of 0.2 mM IPTG. The 2-domain Mb was expressed at 27 °C for 19–27 h in the absence of IPTG. For the latter case, induction with IPTG caused a lower yield of Mb with heme.

2.6. Purification of Mb

Mb was extracted from the cells by sonication in 50 mM Tris–HCl (pH 8.0). The extracts were fractionated with 50–75% saturated ammonium sulfate for all Mb preparations and the crude Mb solution was dialyzed with the same buffer. It was then applied to a gel filtration column of Ultrogel AcA44 (2.5×70 cm) equilibrated with the same buffer. Mb fraction was stored on ice until use. Mb concentration was determined using an extinction coefficient of 13.8 $mM^{-1}\,cm^{-1}$ at 578 nm as heme [10].

2.7. Measurements of catalase and superoxide dismutase (SOD) activities in Mb fraction

E. coli TB1 cell, transformed with pMAL-4DMb or pMAL (without insert) was cultured and the proteins were expressed as described above. The expressed proteins, one containing 4-domain Mb and the other containing no Mb, were fractionated with 50–75% saturated ammonium sulfate, and then dialyzed with 50 mM Tris–HCl (pH 8.0). Activities for catalase and SOD were measured for these two protein fractions.

Catalase activity was determined with the spectrophotometric method as described by Beers and Sizer [11]. The reaction mixture (1 ml) contained 50 mM Tris–HCl (pH 8.0), 10 mM hydrogen peroxide and the above protein fraction (the protein concentration was adjusted to that of autoxidation measurement). The reaction was followed by the decrease in absorbance at 240 nm (25 °C). Bovine liver catalase (5000–15 000 units/mg) was purchased from Wako (Osaka, Japan). In our measurement, the detection limit of catalase is 1.5–4.5 U/ml in the reaction mixture.

SOD activity was measured according to the manufacturer's protocol using SOD Assay Kit-WST (Dojindo Molecular Technologies, Tokyo, Japan). Bovine erythrocyte SOD (4712 units/mg) was purchased from MP Biomedicals (CA, USA). In our measurement, the detection limit of SOD is 0.05 U/ml in the reaction mixture.

2.8. Autoxidation rate measurement

Autoxidation rate was measured at 25 °C or 37 °C in 50 mM Tris–HCl (pH 7.9 or 7.6) with a Hitachi spectrophotometer U-2001 under air-saturated conditions [9]. The observed first-order rate constant, k_{obs}, is given by the following equation:

$$\ln[MbO_2]_t/[MbO_2]_0 = -k_{obs} \cdot t,$$

where $[MbO_2]_0$ and $[MbO_2]_t$ represent the concentration of oxyMb at time 0 and t, respectively. The concentration of oxyMb was followed by the decrease in absorbance at 578 nm.

3. Results and discussion

We succeeded in constructing cDNA inserts coding for 1-, 2-, 3-, 4- and 8-domain Mbs, cloning these into the pMAL plasmid, and expressing these proteins in *E. coli* in the soluble form with a heme. *Aplysia* Mb cDNA was used as a template to make poly-domain forms because it is well-characterized [10] and is easily expressed as a soluble protein. The 1-, 2-, 3- and 4-domain Mb cDNAs were also cloned into the pQE plasmid, but a sufficient amount of Mb was not expressed for 3- and 4-domain con-

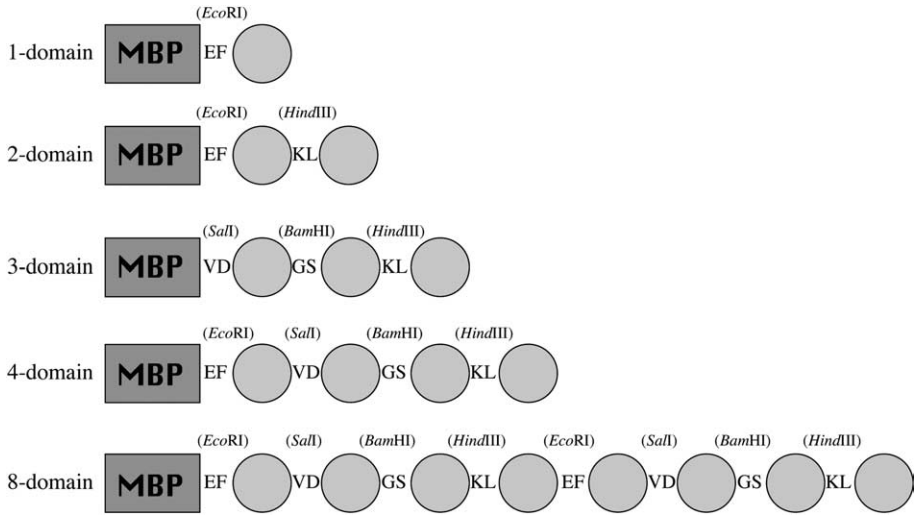

Fig. 1. Strategy for the construction of the 1-, 2-, 3-, 4- and 8-domain Mbs as a fusion protein with maltose binding protein (MBP). Each functional domain (circle) is linked by a two-amino-acid linker, EF, KL, VD or GS, derived from restriction enzyme sites, *Eco*RI, *Hin*dIII, *Sal*I or *Bam*HI, respectively.

structs. Fig. 1 illustrates the strategy for construction of the 1-, 2-, 3-, 4- and 8-domain Mbs as fusion proteins with MBP. Each domain is linked by a two-amino-acid linker, EF, KL, VD or GS, derived from the restriction enzyme sites, *Eco*RI, *Hin*dIII, *Sal*I or *Bam*HI, respectively.

Recombinant Mbs could not be purified by affinity chromatography, such as amirose-resin for MBP tag and Ni-resin for His tag; Mbs were too tightly absorbed on the column and the heme was dislodged. Consequently, we attempted to purify Mbs by ammonium sulfate fractionation and gel filtration. Further purification by DEAE ion exchange chromatography was unsuccessful as Mbs were absorbed irreversibly on the column. The Mb fraction obtained in this study was mainly isolated in the oxygenated form, a physiological active form.

SDS–PAGE showed that Mb fractions contained a considerable amount of protein contamination, especially in the 3- and 4-domain fractions (Fig. 2). In the 8-domain fraction, Mb was estimated to be roughly less than 5% of the total protein eluted (data not shown).

It is well established that catalase and SOD affect on the autoxidation rate of oxyMb [12,13]: addition of catalase (400 U/ml) decreased the rate by 40% and addition of SOD (400 U/ml) decreased by 10% [13]. Since our Mb fractions have a considerable amount of protein contamination, we measured the activities for catalase and SOD to the expressed protein fraction containing MBP/4-domain Mb and to that containing no Mb (reference to the former). No catalase activity was found for both the fractions and the SOD activity was detected only at a level of 0.5–1.0 U/ml. Thus, we conclude that our Mb fractions contain no significant activities for catalase and SOD, which have an affect on the autoxidation rate of oxyMb.

Measurement of autoxidation rate of Mb is a good index to evaluate the stability of the oxygenated state; lower rates indicate that the oxyform is more stable and favorable as an oxygen storage protein. Fig. 3 shows the spectral changes of 1-, 2-, 3- and 4-domain oxyMbs during autoxidation at intervals of 2.5 h (25 °C and pH 7.9). Two isosbestic points are clearly observed in the four autoxidation reactions, indicating that the

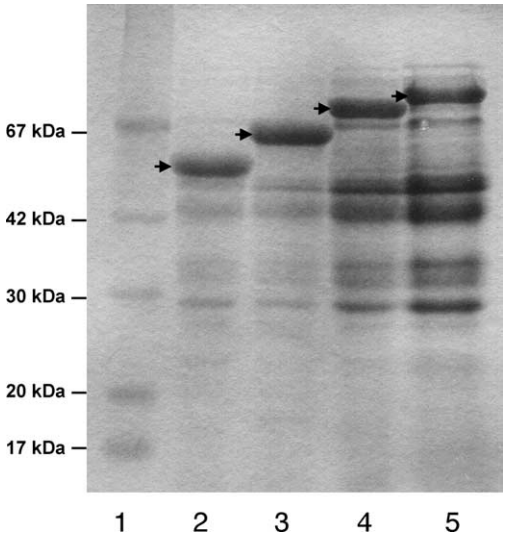

Fig. 2. SDS–PAGE of 1-, 2-, 3- and 4-domain Mbs, passed through a gel filtration column. Mbs were expressed as a fusion protein with MBP (40 kDa): lane 1, molecular weight marker proteins; lane 2, 1-domain Mb (55 kDa, indicated by arrow); lane 3, 2-domain Mb (70 kDa); lane 4, 3-domain Mb (85 kDa) and lane 5, 4-domain Mb (100 kDa).

reaction from the oxyform ($Mb[Fe^{2+}O_2]$) to the metform ($Mb[Fe^{3+}]$) proceeds simply without any detectable intermediate.

The observed first-order rate constant, k_{obs}, of autoxidation reaction was obtained for 1-, 2-, 3-, 4- and 8-domain Mbs from the slope of each straight line of the plot for $\ln[MbO_2]_t/[MbO_2]_0$ vs time (Fig. 4). The plot for 8-domain Mb is not shown, but it is similar to that of 3- and 4-domain Mbs.

The autoxidation rates of 1-, 2-, 3-, 4- and 8-domain Mbs with MBP tags, 1- and 2-domain Mbs with His tags, and native (1-domain) Mb are compared in Table 1. The autoxidation rate (10.0) for native Mb at 25 °C is comparable to those for

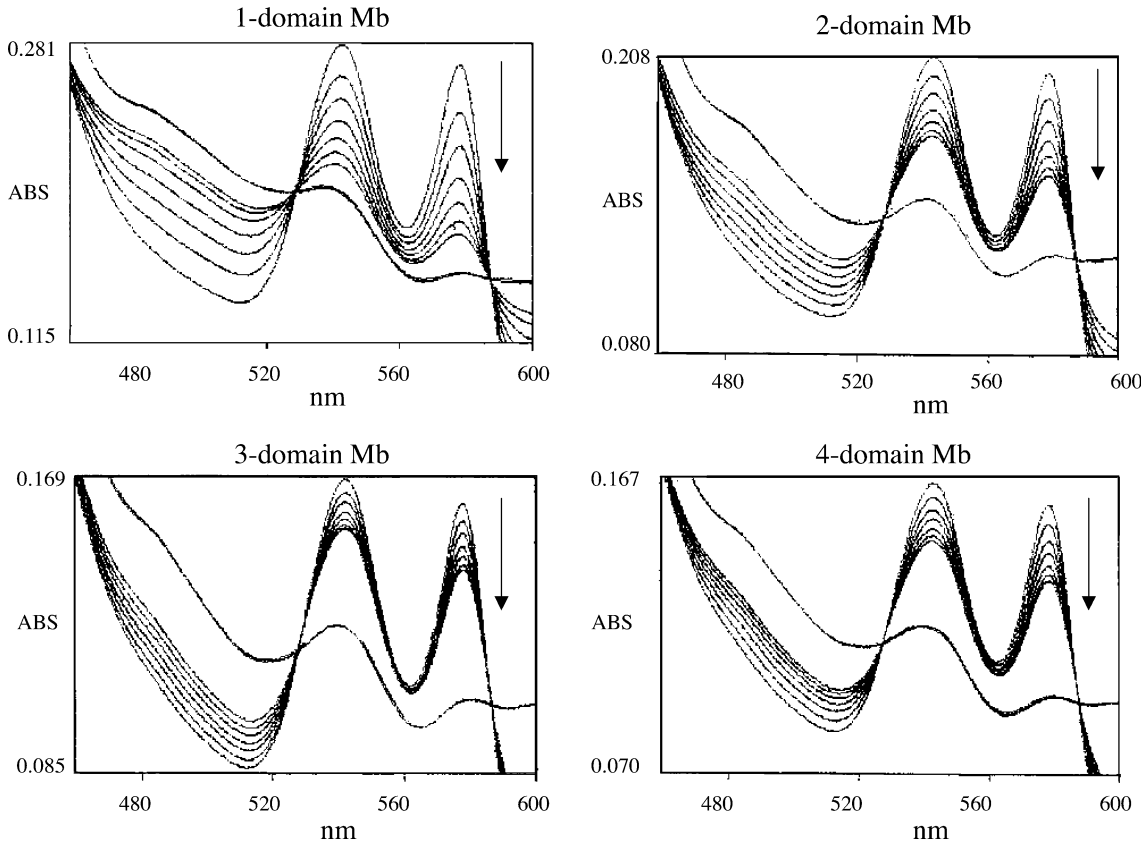

Fig. 3. Spectral changes of 1-, 2-, 3- and 4-domain oxyMbs during autoxidation. Reactions were carried out at 25 °C and pH 7.9 under air saturated conditions. Spectra were taken at intervals of 2.5 h. The final metform was obtained by the addition of ferricyanide. Mb concentration: 20, 15, 12 and 12 μM as heme for 1-, 2-, 3- and 4-domain Mbs, respectively.

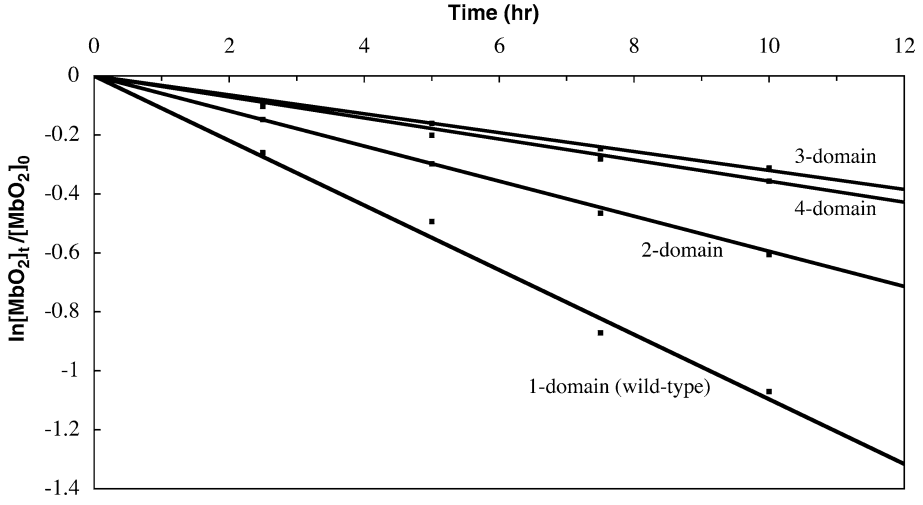

Fig. 4. First-order plots ($\ln[MbO_2]_t/[MbO_2]_0$ vs time) of autoxidation of 1-, 2-, 3- and 4-domain Mbs. The concentration of MbO_2 was followed by the decrease in absorbance at 578 nm. The rate constant, k_{obs}, was obtained from the slope of each straight line.

1-domain/MBP (11.24) and 1-domain/His tag (10.68) Mbs, indicating that the recombinant proteins are properly folded and that the N-terminal tag (MBP or His) does not affect the autoxidation rate. Consistent with this finding, the rates for 2-domain/MBP (6.04) and 2-domain/His (5.70) are also comparable.

The comparison of autoxidation rates at 25 °C (pH 7.9) for 1- (11.24), 2- (6.04) and 3-domain/MBP (3.30) Mbs is of special interest. The rate apparently decreases with increasing number of domains; the rate for 1-domain Mbs is 1.9 and 3.4 times larger than those for 2- and 3-domain Mbs, respectively. This trend is also confirmed at 35 °C (pH 7.6) as the rate for 1-

Table 1
Comparison of autoxidation rates for 1-, 2-, 3-, 4-, and 8-domain myoglobins

Temperature and pH	Autoxidation rate (k_{obs}, h^{-1}) × 100						
	Native		Recombinant				
	1-domain	(Fusion state)	1-domain	2-domain	3-domain	4-domain	8-domain
25 °C (pH 7.9)	10.0[a]						
		MBP tag	11.24 ± 1.40	6.04 ± 0.70	3.30 ± 0.43	3.34 ± 0.41	3.72 ± 0.02
		His tag	10.68 ± 0.35	5.70 ± 0.49			
35 °C (pH 7.6)		MBP tag	40.62 ± 2.76	31.49 ± 2.88	25.34 ± 1.06	ND	ND

Autoxidation reaction was measured at least three times under the same conditions.
[a] These data were read from the pH profile of autoxidation of *Aplysia kurodai* myoglobin [10].

domain Mbs is 1.3 and 1.6 times larger than those for 2- and 3-domain Mbs, respectively. But the differences in autoxidation rate are not so marked when compared with those at 25 °C (pH 7.9). We cannot explain this reason exactly.

These results indicate that addition of domains decreases the autoxidation rate; that is, additional domains increase the stability of the oxygenated form. Since the N-terminal tag does not affect the autoxidation reaction as shown above, Mb domains with an additional domain at its C-terminus, such as domain 1 of 2-domain Mb and domains 1 and 2 of 3-domain Mb (see Fig. 1), may reduce its autoxidation rate. While the precise mechanism is not known, it is likely that structural flexibility of the functional domain is suppressed in an unusual, contiguous poly-domain structure.

The autoxidation rates of 3-, 4- and 8-domain Mbs are slightly increased (3.3–3.7) at 25 °C, but are essentially comparable (Table 1). This indicates that the autoxidation rate at 25 °C is minimized to about 1/3 that of the wild-type Mb in 3-, 4- and 8-domain Mbs.

Even in naturally occurring Hb, it appears that the autoxidation rate of poly-domain Hb is lower than that of single-domain Hb. The blood clam *Barbatia lima* contains three types of Hbs in its erythrocytes, a 30-kDa dimer consisting of two δ chains, a 60-kDa tetramer consisting of α and β chains, and a 400-kDa polymeric Hb consisting of unusual 2-domain and δ chains [4]. The 2-domain chain appears to have evolved by gene duplication and fusion from the δ chaingene, and these two have an amino acid identity of 71–74%. We examined the autoxidation rates for the Hbs and found that the δ-chain dimer is autoxidized 5-fold more easily than the polymeric Hb containing 2-domain and δ chains. Of course, these data cannot be linked directly to the data for artificial poly-domain Mbs (Table 1) because the aggregation states differ greatly in the two *Barbatia* Hbs. However, these data are not inconsistent with the idea that polydomain formation both reduces the autoxidation rate and stabilizes the storage of bound dioxygen. In the future, we are planning to measure the oxygen equilibrium curves for the artificial polydomain Mbs to know whether these Mbs can bind oxygen just like native, single-domain Mb.

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