more than 0.014 μM AZT in sera (Personal communication with Dr S. Kimura). These doses may be used for the treatment of hemophiliacs with AIDS.

It is well known that sulfated polysaccharides act as anticoagulants as well as mitogens[6-12]. Our data suggest that these sulfated homopolysaccharides could be used in combination with AZT for the treatment of AIDS which would be advantageous because the combination significantly lowers occurrence of side effects.

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The ear-shell (*Sulculus diversicolor aquatilis*) myoglobin is composed of an unusual 39 kDA polypeptide chain

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Summary. An unusual myoglobin was isolated from the buccal mass of the ear-shell *Sulculus diversicolor aquatilis*. The myoglobin consists of a 39 kDa polypeptide chain which is about double the size of the usual myoglobin subunit, contains one heme per molecule, and has an unusual spectral property in the oxy-form. On the basis of these properties and partial amino acid sequencing, we propose that *Sulculus* myoglobin has a didomain structure, and that one of the two domains does not function as an oxygen-binding domain. So far, a myoglobin of this type has not been described in molluscs.
Key words. Myoglobin; didomain structure; *Sulculus*.

A number of molluscs have remarkable red muscles in the buccal mass and triturative stomach[1,2]. Myoglobin is abundant in such red muscle, and the subunit structure of the molluscan pigment is either a monomer or dimer consisting of a molecular weight (M_r) 15,000–18,000 polypeptide chain in all species investigated so far.

Recently, an unusual didomain structure was found in the hemoglobins of the clams *Barbatia reeveana*[3] and *Barbatia lima*[4]. Interestingly, a closely related species, *Barbatia virescens*, has no hemoglobin with such a structure[4]. Thus, molluscan globins show remarkable diversity in subunit structure and constituent chain[2], and therefore they would be an excellent source for the elucidation of the molecular evolution of globins.

We isolated myoglobin from the ear-shell *Sulculus diversicolor aquatilis* and found that the myoglobin has unique characteristics in spectral property, autoxidation and subunit structure, compared with other molluscan myoglobins.

Materials and methods

About 25 g of buccal mass of *Sulculus* was used in one preparation. All procedures were carried out at low temperature (2–4 °C) as far as possible. The water extract was fractionated with ammonium sulfate between 55 and 90% saturation at pH 7.2. The crude myoglobin fraction was dissolved in a minimum volume of 50 mM phosphate buffer (pH 7.2) and was passed through a Ultrogel AcA 44 column (3 × 110 cm) equilibrated with 50 mM phosphate buffer (pH 7.2). The myoglobin fraction was pooled and ammonium sulfate was added up to 55% saturation. This solution was then applied to a Butyl-Toyopearl 650 M column (1.6 × 13 cm) equilibrated with 50 mM phosphate buffer (pH 7.2) containing 55% ammonium sulfate, and eluted with a linear gradient of the same solution (300 ml) to 50 mM phosphate buffer (pH 7.2) (300 ml). The myoglobin thus obtained was dialyzed against 20 mM Tris-HCl buffer (pH 8), and was kept at 2 °C until used. The heme concentration of myoglobin

was determined by the pyridine-hemochromogen method using a molar extinction coefficient of 32,000 at 557 nm.

The purity of the myoglobin was checked by high-performance liquid chromatography (HPLC). After removal of heme, the apoprotein was dissolved in 6 M guanidine-HCl containing 50 mM Tris-HCl buffer (pH 8.5) and 1% 2-mercaptoethanol, incubated at 52 °C for 2 h, and then applied to a Cosmosil $5C_{18}$-300 column (4.6 × 150 mm) (Nacalai Tesque, Japan) equilibrated with 30% acetonitrile in 0.1% trifluoroacetic acid (TFA) and eluted with a linear gradient of 30–99% acetonitrile in 0.1% TFA over 60 min at a flow rate of 1 ml/min.

The molecular weight (M_r) of native myoglobin was estimated by a gel filtration column (Superose 12, 1 × 30 cm, Pharmacia) equilibrated with 50 mM phosphate buffer (pH 7.2) containing 150 mM NaCl at a flow rate of 0.5 ml/min. The calibrants used were bovine hemoglobin (65 kDa), sperm whale myoglobin (17.8 kDa) and horse cytochrome c (12.4 kDa).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 15% acrylamide gel containing 0.087% bisacrylamide, 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS. The sample was incubated in 0.75% SDS at 100 °C for 5 min in the presence of 2-mercaptoethanol, before electrophoresis.

The heme content of myoglobin was estimated from heme analysis (pyridine-hemochromogen method) and amino acid analysis of the protein.

Autoxidation rate measurements were carried out in 0.1 M phosphate buffer (pH 7.2) and 0.1 M Tris-HCl buffer (pH 8.8), respectively, according to our previous method[5,6]. Myoglobin concentration was 14 μM as heme.

The methods of carboxymethylation, amino acid analysis and manual Edman degradation were the same as described previously[7,8].

Carboxymethylated apoprotein (50 nmoles) was digested with lysyl endopeptidase (Wako) in 20 mM Tris-HCl buffer (pH 8.8) at 37 °C for 6 h at an enzyme-to-substrate ratio of 1:200 (mole/mole). The peptides were purified by HPLC on an ODS-120T column (Tosoh, Japan) with a linear gradient of 0–80% acetonitrile in 0.1% TFA over 100 min at a flow rate of 1 ml/min.

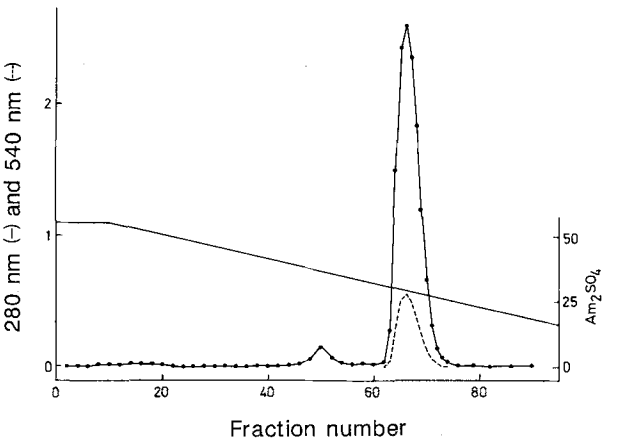

Figure 1. Hydrophobic chromatography of *Sulculus* myoglobin. The column (1.6 × 13 cm) was packed with Butyl-Toyopearl 650 M. The sample was eluted with a linear gradient of 50 mM phosphate buffer(pH 7.2) containing 55% ammonium sulfate to 50 mM phosphate buffer(pH 7.2). Fraction size, 5 ml/tube.

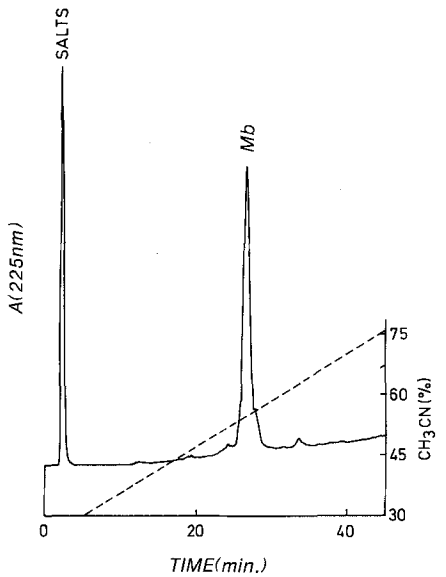

Figure 2. Reverse-phase chromatography of purified *Sulculus* myoglobin. The sample was eluted with a linear gradient of 30–99% acetonitrile in 0.1% TFA over 60 min at a flow rate of 1 ml/min.

Results and discussion

Sulculus myoglobin was purified in the oxy-form by ammonium sulfate precipitation, gel filtration and hydrophobic chromatography. We used hydrophobic chromatography (fig. 1), instead of the ion-exchange chromatography used usually, as a final purification step, because *Sulculus* myoglobin was too tightly absorbed on the ion-exchange column. The purity of the myoglobin was confirmed by reverse-phase chromatography (fig. 2). Figure 3 shows the SDS-PAGEs of marker proteins (lane A), *Sulculus* myoglobin in the presence (lane B) or absence (lane C) of a reducing agent, and carboxymethylated *Sulculus* myoglobin (lane D). The myoglobin, in all cases, migrated at almost the same position as that of RNA-polymerase alpha chain (M_r 39 kDa), and therefore the M_r of myoglobin was estimated to be 39 kDa. This value is about double the size of the usual myoglobin subunit and suggests that *Sulculus* myoglobin might have a didomain structure, like the hemoglobin of the clam *Barbatia*[3,4].

The M_r of native *Sulculus* myoglobin was estimated to be 71 kDa by a gel filtration column (fig. 4). Since the M_r of the subunit was 39 kDa, native *Sulculus* myoglobin must exist as a dimer of an unusual 39 kDa chain.

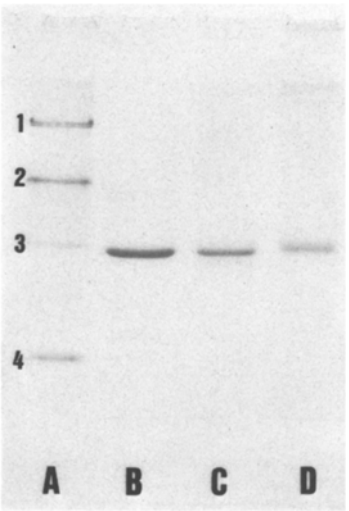

Figure 3. SDS-PAGE of *Sulculcus* myoglobin. Lane A, Marker proteins (RNA-polymerase beta(160 kDa, 1) and alpha(39 kDa, 3) chains, bovine serum albumin (68 kDa, 2) and trypsin inhibitor (21.5 kDa, 4)). Lane B, reduced *Sulculus* myoglobin. Lane C, unreduced *Sulculus* myoglobin. Lane D, carboxymethylated *Sulculus* myoglobin.

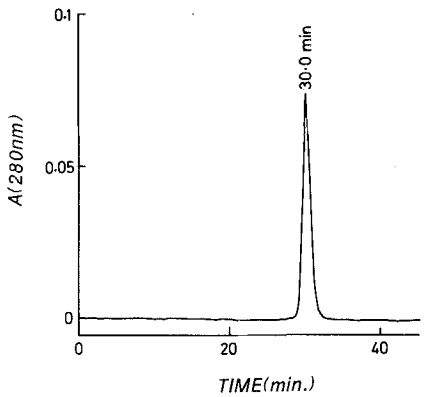

Figure 4. Gel filtration of native *Sulculus* myoglobin. The column (Superose 12) was equilibrated and eluted with 50 mM phosphate buffer (pH 7.2) containing 150 mM NaCl at a flow rate of 0.5 ml/min. The retention time of *Sulculus* myoglobin was 30.0 min (average of three runs). The calibrants used are bovine hemoglobin (30.2 min), sperm whale myoglobin (32.5 min) and horse cytochrome c (32.9 min).

Table 1. Amino acid composition of *Sulculus* myoglobin

Amino acid	Moles per 39,000 g protein
Asp	42.4
Thr	17.1
Ser	19.8
Glu	32.6
Pro	18.5
Gly	33.1
Ala	40.5
Cys*	2.6
Val	26.5
Met	10.9
Ile	11.9
Leu	52.3
Tyr	12.2
Phe	8.3
Lys	22.6
His	5.6
Arg	9.1
Trp	ND
Total	366

*Determined as carboxymethylcysteine. ND, not determined.

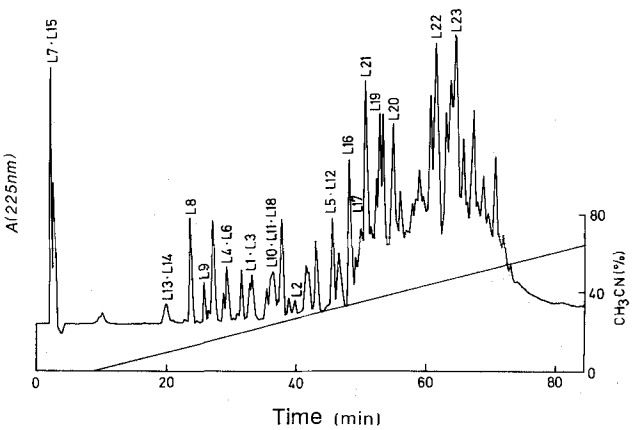

Figure 5. Separation of lysyl endopeptidase peptides of *Sulculus* myoglobin on a reverse-phase column. The peptides were eluted with a linear gradient of 0–80% acetonitrile in 0.1% TFA over 100 min at a flow rate of 1 ml/min.

Amino acid composition of *Sulculus* myoglobin is shown in table 1. The composition did not show any strong similarity with those of other molluscan globins. Calculating from the M_r of the subunit, *Sulculus* myoglobin is composed of about 365 amino acids.

The heme and amino acid analyses showed that *Sulculus* myoglobin contains one gram-equivalent of heme per 40,000 g protein, and therefore it was concluded that there is one heme per molecule. The heme content corresponds to just one-half of the value expected from a didomain structure that can bind two heme groups. Such a low heme content has only been found in the 37 kDa myoglobin from the nematode *Ascaris*[9], besides *Sulculus* myoglobin.

So far, a number of molluscan myoglobins have been isolated and all the subunit structures were found to be either a monomer or dimer consisting of 15–17 kDa polypeptide chain[1,2]. However, in this study, we found that *Sulculus* myoglobin consists of an unusual 39 kDa chain. We also confirmed that the closely related ear-shell *Nordotis madaka* contains the same type of myoglobin as *Sulculus*. To elucidate the evolutionary origin of this unusual chain, we determined the partial amino acid sequence of *Sulculus* myoglobin. No N-terminal amino acid of *Sulculus* myoglobin was detected by the Edman method, suggesting that the N-terminus is blocked, as in most molluscan globins. Therefore we digested the myoglobin with lysyl endopeptidase (fig 5), and isolated 23 peptides (about 305 residues) which represented about 83% of the whole protein. The peptides were purified further by rechromatography. Furthermore, we sequenced about 220 residues of the isolated peptides.

Table 2. Amino acid sequences of lysyl endopeptidase peptides of *Sulculus* myoglobin

Peptide	Total residue	Yield (%)	Sequence determined
L1	7	74	Blocked-A-D-I-Q-L-S-K.
L2	8	36	Q-L-L-A-D-L-T-K.
L3	9	46	V-P-Q-I-V-A-E-A-K.
L4	7	12	Y-I-V-T-A-S-K.
L5	11	14	V-C-G-X-V-
L6	8	26	S-L-A-Y-G-D-T-K.
L7	8	42	E-V-R-D-D-T-Q-K.
L8	5	42	Y-H-V-S-K.
L9	7	16	L-A-G-Y-R-Q-K.
L10	9	38	E-M-P-L-L-D-S-S-K.
L11	10	30	S-L-P-D-L-V-A-S-H-K.
L12	10	12	P-L-W-N-V-
L13	4	16	M-P-R-K.
L14	6	20	D-A-N-L-S-K.
L15	2	12	A-K.
L16	16	40	Q-A-F-L-D-E-I-S-N-Y-M-I-P-A-H-K.
L17	15	22	S-D-L-I-P-F-L-X-E-V-R-D-D-
L18	13	32	G-A-Q-S-V-Q-N-V-L-D-G-A-K.
L19	19	12	P-L-G-N-V-X-N-D-G-
L20	25	58	M-T-G-G-L-T-E-L-T-G-T-I-A-N-M-Q-A-A-
L21	19	22	G-G-D-I-E-V-M-Y-N-
L22	27	54	D-I-G-F-L-L-E-P-L-Q-D-V-L-P-D-Y-F-A-P-G-N-
L23	61	40	M-N-D-N-L-T-P-D-H-F-Y-N-V-L-E-P-F-L-G-G-F-G-G-P-A-X-P-I-X-G-X-L-I-Y-

X, unidentified residue.

Table 3. Absorption maxima, absorption coefficients and characteristic absorbance ratios of oxymyoglobins. The extinction coefficients are based on heme analysis by the pyridine hemochromogen method using a millimolar extinction coefficient at 557 nm of 32.0.

Source	Reference	Absorption maximum (nm) (absorption coefficient(mM^{-1} cm^{-1}))			alpha/beta
		alpha	beta	gamma	
Sulculus	This work	579 (15.3)	544 (13.4)	414 (117)	1.14
Aplysia	16	578 (13.8)	543 (13.4)	418 (120)	1.03
Dolabella	12	578 (13.4)	543 (13.1)	418 (117)	1.02
Shark	21	579 (15.7)	543 (14.8)	418 (127)	1.06
Sperm whale	18	581 (15.4)	543 (14.3)	418 (129)	1.08

The sequencing results are shown in table 2. The peptide L1 appeared to be located at the N-terminus of the intact protein, since the N-terminus was blocked. This peptide was sequenced, after digestion with acylamino-acid releasing enzyme to remove the N-terminal blocked residue. The peptides L2, L3 and L4 showed 40–60% homology with the sequences corresponding to the positions 9–12, 57–65 and 119–125, respectively, of the dimeric hemoglobin from the blood clam *Anadara*[10], rather than with the sequences in the same positions of the molluscan myoglobins sequenced so far[11–14]. In two peptides, L22 and L23, more than 20 residues were sequenced, but no strong homology was found with other molluscan globins. Our partial sequence study suggested that *Sulculus* globin is slightly, but significantly, related to molluscan globins. In addition, we could not find any obvious homology between the amino acid sequences of the peptides. This implies that *Sulculus* myoglobin is rather different from the usual didomain hemoglobin, for example, *Barbatia* hemoglobin[4,15], because the latter has 70–80% homology in the sequences between the two domains.

Judging from the M_r(39 kDa), the characteristics of *Sulculus* myoglobin may result from gene duplication. A low heme content (one-half of the expected value) and a low homology between the domains suggest that one of the two domains of *Sulculus* myoglobin lost the ability to bind heme at one time, and has evolved with higher amino acid substitution, resulting from the absence of functional limitation.

Another interesting feature of *Sulculus* myoglobin is its spectral properties in the oxy-form. Table 3 shows a comparison of the spectroscopic properties of the isolated *Sulculus* myoglobin with those of myoglobins from other sources. So far, all the oxymyoglobins isolated in highly purified state, including molluscan myoglobins such as those of *Aplysia*[16] and *Dolabella*[12], have the common characteristic that the absorbance ratio of alpha- to beta-peak (alpha/beta ratio) ranges from 1.02–1.08. In this respect, it is noteworthy that the ratio of *Sulculus* oxymyoglobin shows the much higher value of 1.14, although the extinction maxima of alpha- and beta-peaks were the same as those of other myoglobins. Since it was suggested that the alpha/beta ratio reflects the electronic

Table 4. Comparison of autoxidation rates (k_{obs} in h^{-1}) of molluscan and vertebrate myoglobins, at 25 °C in 0.1 M buffer

Sources	Reference	k_{obs}(pH 7.2)	k_{obs}(pH 8.8)	k_{obs}(pH 8.8)/k_{obs}(pH 7.2)
Sulculus	This work	0.030	0.59	19.7
Aplysia	16	0.158	0.100	0.63
Dolabella	12	0.044	0.048	1.09
Cerithidea	14	0.022	0.020	0.91
Sperm whale	18	0.010	0.0012	0.12

structure of the bound oxygen [17], it seemed that it would be of great interest to examine the stability of *Sulculus* myoglobin showing an unusual alpha/beta ratio.

Table 4 shows the first-order rate constant (k_{obs}) for the autoxidation of *Sulculus* oxymyoglobin at two different pHs, together with those of *Aplysia* [16], *Dolabella* [12], *Cerithidea* [14] and sperm whale [18] myoglobins. The autoxidation rate of *Sulculus* myoglobin at pH 7.2 was comparable to those of *Dolabella* (monomeric myoglobin) and *Cerithidea* (dimeric myoglobin), but at pH 8.8, where autoxidation is minimized in most of oxymyoglobins, the rate for *Sulculus* myoglobin was extremely accelerated. This can be clearly seen from the ratio of k_{obs}(pH 8.8)/k_{obs}(pH 7.2). As shown in table 4, the ratio for *Sulculus* myoglobin shows a higher value of 19.7, compared with the ratios (0.12–1.09) for the other myoglobins. Since autoxidation is understood to take place through a nucleophilic displacement mechanism by nucleophiles such as H_2O or OH^- [19, 20], this indicates that the heme pocket of *Sulculus* myoglobin might offer extremely easy access to the OH^- ion, resulting in very rapid autoxidation. These characteristics of autoxidation and also the spectral properties could be caused by structural distortion inherent in the unusual structure of *Sulculus* myoglobin.

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The growth of human fibroblasts and A431 epidermoid carcinoma cells on gamma-irradiated human amnion collagen substrata

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Summary. Human fibroblasts and A431 human epidermoid carcinoma cells were cultured on gamma-irradiated human amnion collagen as well as on plastic dishes and non-irradiated collagen coated dishes. The morphology, attachment, growth and short-term cytotoxicity of these culture conditions have been determined. Both irradiated and non-irradiated amnion collagen enhanced the attachment and proliferation of fibroblasts as compared to the plastic dishes. No differences in these properties were observed for A431 cells cultured on irradiated collagen when compared with culture on non-irradiated collagen substrates. Cytotoxicity assays showed that irradiated and non-irradiated collagens were not cytotoxic for either fibroblasts or A431 cells. The results demonstrated that amnion collagen irradiated at doses of 0.25–2.0 Mrads is optimal for cell growth.
Key words. Gamma irradiated collagen; injectable collagen; collagen substrata; gamma irradiation; amnion collagen; tissue culture.