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Variable Subunit Contact and Cooperativity of Hemoglobins

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Abstract. Tertiary structures of proteins are conserved better than their primary structures during evolution. Quaternary structures or subunit organizations, however, are not always conserved. A typical case is found in hemoglobin family. Although human, *Scapharca*, and *Urechis* have tetrameric hemoglobins, their subunit contacts are completely different from each other. We report here that only one or two amino acid replacements are enough to create a new contact between subunits. Such a small number of chance replacements is expected during the evolution of hemoglobins. This result explains why different modes of subunit interaction evolved in animal hemoglobins. In contrast, certain interactions between subunits are necessary for cooperative oxygen binding. Cooperative oxygen binding is observed often in dimeric and tetrameric hemoglobins. Conformational change of a subunit induced by the first oxygen binding to the heme group is transmitted through the subunit contacts and increases the affinity of the second oxygen. The tetrameric hemoglobins from humans and *Scapharca* have cooperativity in spite of their different modes of subunit contact, but the one from *Urechis* does not. The relationship between cooperativity and the mode of subunit contacts is not clear. We compared the atomic interactions at the subunit contact surface of cooperative and noncooperative tetrameric hemoglobins. We show that heme-contact modules M3–M6 play a key role in the subunit contacts responsible for cooperativity. A module was defined as a contiguous peptide segment having compact conformation and its average length is about 15 amino acid residues. We show that the cooperative hemoglobins have interactins involving at least two pairs of modules among the four heme-contact modules at subunit contact.

Key words: Cooperativity — Heme-contact module — Subunit contact — Molecular evolution — Quaternary structure — Hemoglobin — Neutral evolution — Three-dimensional structure

Introduction

Kimura (1968) and King and Jukes (1969) proposed the neutral theory of amino acid substitutions and non-Darwinian evolution, respectively. Function of a protein is tightly coupled with the three-dimensional (3D) structure of the protein, thus amino acid replacements are allowed as far as the 3D structure and function of the protein are maintained.

Hemoglobin (Hb) is now found in a wide range of organisms, not only in eukaryotes, but also in eubacteria and archaea (Hou et al. 2000). Recently a novel hemoglobin was found in human brain, which is more similar to worm hemoglobin than the human blood one (Burmester et al. 2000). The gene encoding the brain hemoglobin has three introns like the gene of plant hemoglobin, whereas two introns are observed in the gene encoding human blood hemoglobin.

Hemoglobin works in monomer, dimer, and tetramer, and in the assembly of some hundreds of subunits, depending on species (Vinogradov et al. 1993; Suzuki and Imai 1998). Human, *Scapharca inaequivalvis* (blood clam), and *Urechis caupo* (innkeeper worm) have tetrameric hemoglobins. They have, however, quite different modes of subunit contacts. Why is the quaternary

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Table 1. Subunit organization and cooperativity of hemoglobins

Species	Name of hemoglobin	Subunit organization	Cooperativity	The shortest distance between heme irons (Å)[a]	References of crystal structure
Homo sapiens	Hb	Hetero-tetramer ($\alpha_2\beta_2$)	+[b]	25.5	Shaanan 1983
Scapharca inaequivalvis	HbI	Homo-dimer	+[c]	18.4	Royer 1994
	HbII	Hetero-tetramer (A_2B_2)	+[c]	18.5	Royer et al. 1995
Caudina arenicola	HbD	Homo-dimer	+[d]	19.1	Mitchell et al. 1995
Urechis caupo	Hb	Homo-tetramer	−[e]	35.1	Kolatkar et al. 1992

[a] Crystal structures in ligand-bound states were used for distance calculation.
[b] Perutz 1970.
[c] Ikeda-Saito et al. 1983.
[d] Bonaventura et al. 1976.
[e] Garey and Riggs 1984.

Table 2. Number of hydrophilic, ambivalent, and hydrophobic residues on monomeric surface and subunit interface in hemoglobins

Species	Name of hemoglobin	Subunit organization	Subunit	Monomeric surface				Subunit interface			
				Hphlic[a]	Amb[b]	Hphbic[c]	Total	Hphlic[a]	Amb[b]	Hphbic[c]	Total
H. sapiens	Hb	hetero-tetramer	α (oxy)	36	31	7	74	10	7	5	22
			α (deoxy)	34	32	7	73	8	5	2	15
			β (oxy)	42	24	7	73	7	5	5	17
			β (deoxy)	41	23	8	72	8	5	5	18
U. caupo	Hb	homo-tetramer	—	24	29	12	65	4	1	1	6
S. inaequivalvis	Hb II	hetero-tetramer	A	39	22	10	71	8	5	3	16
			B	40	22	13	75	8	2	4	14
	Hb I	homo-dimer	—	44	21	9	74	7	2	2	11
C. arenicola	Hb D	homo-dimer	—	41	32	13	86	2	4	4	10
			Average[d]	37.8	25.9	10.2	73.9	6.5	3.6	3.2	13.3
			Percent	(51.2)	(35.0)	(13.8)	(100)	(48.9)	(26.9)	(24.2)	(100)

[a] Hphlic: hydrophilic amino acid residues (K, R, D, E, N, Q, H).
[b] Amb: ambivalent (G, A, P, S, T, C).
[c] Hphbic: hydrophobic (V, L, I, M, F, Y, W).
[d] The data of the same sequences were averaged prior to a global average among the seven different sequences of hemoglobin subunits.

structure variable during evolution? We analyzed amino acid residues involved in subunit contacts on the basis of their physico-chemical character and the 3D structures of hemoglobins.

Hydrophilic amino acid residues are major residues located on the surface of proteins. However, hydrophobic amino acid residues are also found as minor residues. Free energy gain is necessary to stabilize subunit contact thermodynamically. Such free energy is obtained by amino acid replacements during evolution. How many replacements are needed at such contact regions? Are there selective replacements in subunit contact regions? Our purpose is to solve these questions.

In many species hemoglobin shows cooperativity of oxygen binding, i.e., the first binding of the ligand makes it easier to bind a second one (Riggs 1998). Such hemoglobins have oligomeric subunit organization. However, not all the oligomeric hemoglobins have cooperativity (Table 1). It is not known why some dimeric and tetrameric hemoglobins have cooperativity but others do not. Some requirements for cooperativity might exist in subunit contact. For tetrameric hemoglobins there are two kinds of subunit contact. These are: sliding ($\alpha1\beta2$) and packing ($\alpha1\beta1$) contacts in human Hb; A1B1 and A1B2 contacts in *Scapharca* HbII; and AB-AB(E) and GH-GH(D) contacts in *Urechis* Hb (Figs. 1–3).

Mechanism for cooperative oxygen binding was stud-

Table 3. Interactions at human oxy-Hb sliding contact

Subunit α1		Subunit β2		No. contact atoms
Module	Residue	Residue	Module	
M3	38T	97H	M6	4
	38T	99D		1
	38T	145Y	M8	1
	41T	40R	M3	4
M4	42Y	40R		3
M6	92R	36P		4
	92R	37W		6
	92R	39Q		4
	93V	37W		1
	94D	37W		7
	94D	99D	M6	1
	94D	102N		4
	95P	37W	M3	7
	96V	99D	M6	3
M8	140Y	37W	M3	10

Table 4. Interactions at human oxy-Hb packing contact

Subunit α1		Subunit β1		No. contact atoms
Module	Residue	Residue	Module	
M3	30E	124P	M7	2
	31R	122F		5
	31R	123T		2
	31R	124P		5
	31R	127Q		8
	34L	124P		3
	34L	125P		4
	34L	128A		2
	35S	127Q		3
	35S	128A		3
	35S	131Q		2
	36F	131Q		2
M6	103H	108N		11
	103H	131Q		4
M7	107V	115A		2
	107V	127Q		3
	110A	116H		2
	111A	115A		2
	111A	119G		2
	112H	120K		4
	114P	116H		8
	117F	30R	M3	5
	117F	116H	M7	3
	118T	30R	M3	2
	119P	30R		8
	119P	33V		2
	119P	55M	M4	2
	122H	30R	M3	7
	122H	34V		4
	123A	34V		2
	126D	35Y		1

Table 5. Interactions at dimeric contact of *Scapharca* HbI in CO form

Subunit 1		Subunit 2		No. contact atoms
Module	Residue	Residue	Module	
M2	30K	89D	M5	7
M4	64D	92C		2
	67R	88D		1
	67R	89D		4
	67R	92C		2
	68G	92C		3
	68G	93V		1
	71I	79N		1
	71I	83Q		1
	71I	93V		1
	72T	79N		2
	72T	93V		1
	72T	96K		1
	72T	97F	M6	1
M5	75Y	78Q	M5	1
	75Y	79N		9
	75Y	82D		5
	75Y	83Q		2
	78Q	75Y		1
	79N	71I	M4	1
	79N	72T		2
	79N	75Y	M5	9
	82D	75Y		5
	83Q	71I	M4	1
	83Q	75Y	M5	1
	88D	67R	M4	2
	89D	30K	M2	7
	89D	67R	M4	4
	92C	64D		2
	92C	67R		2
	92C	68G		3
	93V	71I		2
	95E	64D		1
	96K	72T		1
M6	97F	72T		1

ied extensively in human Hb. The interaction between C helix of subunit α1 and FG corner of subunit β2 was once proposed as the main component in cooperativity of human Hb, whereas the E and F helices are on the external surface of the tetramer (Perutz et al. 1987). However, this interaction is not observed in *Scapharca* HbI and HbII and *Caudina arenicola* (sea cucumber) HbD which show cooperative oxygen binding (Royer et al., 1995; Royer 1994; Mitchell et al. 1995). *Scapharca* HbI and *Caudina* HbD are homo-dimers having a similar subunit contact to A1B1 contact of *Scapharca* HbII (Riggs 1998). The mode of subunit contacts in *Scapharca* HbII is completely different from those of human Hb (Figs. 1, 2 and 4). The subunit contact in dimeric (HbI) and tetrameric (HbII) *Scapharca* Hbs involves the E and F helices, so that the hemes are in almost direct contact (Fig. 2). Although there does not seem to be a rule in subunit contact to determine cooperativity when human and *Scapharca* Hbs are compared, it is worthy to compare the subunit contacts of cooperative and non-cooperative tetrameric Hbs and try to find, if it exists, a common rule among the cooperative Hbs. In the non-cooperative tetrameric Hb from *Urechis*, the AB-AB(E) subunit contact involves the A, B, and E helices and the GH-GH(D) subunit contact involves the G, H, and D helices, as shown by the name of the contacts. We compared the subunit contacts in human, *Scapharca* HbII and HbI, *Caudina* HbD, and *Urechis* Hb on the basis of a structural unit, the module, which is different from the secondary structure.

Modules are defined as compact structural units of globular proteins. Soon after two introns were discovered in mouse hemoglobin genes (Tilghman et al. 1978), they were shown to be located close to the boundaries of protein structural units, later called modules (Gō 1981; Gō 1983). The module boundaries of hemoglobins were automatically identified by a computer program using the centripetal profile (Gō and Nosaka 1987; Noguti et al. 1993) which is more sensitive in detection of module boundaries than the previous distance map method (Gō 1981; Gō 1983). The hemoglobin subunit was decomposed into eight or nine modules, depending on species. Each subunit of human and *Urechis* Hbs was decomposed into eight modules, M1–M8. However, the sub-

Table 6. Interactions at A1B1 contact of *Scapharca* HbII in CO form

Subunit A1		Subunit B1		No. contact atoms
Module	Residue	Residue	Module	
M2	30E	91R	M5	2
M4	64S	94C		2
	67R	88D		1
	67R	90E		1
	67R	91R		3
	67R	94C		3
	68G	94C		2
	71I	81N		1
	71I	91R		1
	72T	81N		2
	72T	98K		2
	72T	99F	M6	2
M5	75Y	77Y	M5	1
	75Y	80Q		1
	75Y	81N		8
	75Y	84D		5
	75Y	91R		1
	79N	73I	M4	1
	79N	74T		1
	79N	77Y	M5	14
	82D	77Y		5
	86D	69R	M4	3
	88S	69R		6
	89R	69R		8
	89R	73I		3
	89R	77Y	M5	3
	92C	66S	M4	2
	92C	69R		2
	92C	70G		2
	96K	74T		1
M6	97F	74T		1
	99V	55R		1

Table 7. Interactions at A1B2 contact of *Scapharca* HbII in CO form

Subunit A1		Subunit B2		No. contact atoms
Module	Residue	Residue	Module	
M2	24V	25G	M2	1
	24V	29V		1
	27A	21R	M1	2
	27A	25G	M2	2
	28D	18D	M1	3
	28D	21R		4
M7	123K	131Y	M7	8
	124A	22M	M1	2
	124A	131Y	M7	5
	125R	22M	M1	4
	125R	26V	M2	1
	126M	26V		1
	127G	127R	M7	2
	127G	128M		2
	128N	126A		3
	128N	127R		3
	128N	129G		4
	129Y	26V	M2	3
	129Y	29V		1
	129Y	30D		1
	129Y	127R	M7	5

Table 8. Interactions at dimeric contact of *Caudina* HbD in CN form

Subunit 1		Subunit 2		No. contact atoms
Module	Residue	Residue	Module	
M4	54P	102R	M6	3
	55Q	99T	M5	3
	68R	91E		6
	68R	95E		1
	69Q	95E		13
	71H	92V		4
	72A	92V		2
	75I	83T		1
	75I	87E		1
	76R	96L		1
	76R	99T		3
	76R	100L		4
M5	80L	80L		1
	83T	75I	M4	1
	87E	75I		1
	91E	68R		6
	92V	71H		4
	92V	72A		2
	95E	68R		1
	95E	69Q		13
	96L	76R		1
	99T	55Q		3
	99T	76R		3
	100L	76R		4
M6	102R	54P		3

units of *Scapharca* and *Caudina* Hbs were decomposed into nine modules having an extra module M0 at the N-terminus (Fig. 5) (Shionyu et al., to be submitted). Module boundaries and the relationship between these modules and intron-exon genomic structures are to be published elsewhere (Shionyu et al., to be submitted). Most of the module boundaries are located on α-helices in hemoglobins. The reason we use modules instead of α-helices is that only C-terminal or N-terminal halves of α-helices are frequently involved at subunit contacts of hemoglobins. Modules M4 and M6 have distal and proximal histidines, respectively. Modules M3–M6 are characterized as heme-contact modules, since a majority of the residues in contact with heme are localized in the region consisting of modules M3–M6.

Materials and Methods

Structure Data. Atomic coordinates of hemoglobins were obtained from the Brookhaven Protein Data Bank (PDB) (Bernstein et al. 1997): human Hb in the oxy- (Shaanan 1983; PDB ID: 1HHO) and deoxy-forms (Fermi et al. 1984; 2HHB), *Scapharca inaequivalvis* in both the homo-dimeric HbI (Royer 1994; 3SDH) and hetero-tetrameric HbII (Royer et al. 1995; 1SCT) of the carbon monoxy-forms, *Caudina arenicola* HbD in the cyanomet-form (Mitchell et al. 1995; 1HLM), and *Urechis caupo* Hb in the cyanomet-form (Kolatkar et al. 1992; 1ITH). In the cases of human Hb oxy-form, *Caudina* HbD, and *Urechis* Hb, their native quaternary structures were restored from their reduced structure data in PDB according to the information on symmetrically related subunits given in their PDB files and original papers.

Table 9. Interactions at AB-AB(E) contact of *Urechis* Hb in CN form

Subunit 1		Subunit 2		No. contact atoms
Module	Residue	Residue	Module	
M2	15F	66L	M4	3
	15F	67T		1
	15F	70N	M5	2
	19K	22L	M2	2
	19K	70N	M5	3
	19K	73D		1
	20G	21C	M2	2
	20G	22L		4
	20G	23Q		7
	20G	66L	M4	1
	21C	20G	M2	2
	22L	19K		3
	22L	20G		4
	23Q	20G		7
M4	63A	15F		1
	66L	15F		3
	66L	20G		1
	67T	15F		4
M5	70N	15F		1
	70N	19K		4
	74K	77D	M5	1
	77D	74K		2

Identification of Amino Acid Residues on Monomeric Surface and Subunit Interface. A residue was assigned either at the surface or interior of a subunit, then the residues on surface were further divided into two: those located on subunit interface and those remaining. This assignment was based on solvent accessibility of side-chains, defined as a fraction of solvent-accessible surface area in native conformation against the maximum value obtained in fully extended conformation (Gō and Miyazawa 1980). Solvent-accessible surface area was calculated by the method of Shrake and Rupley (1973). Residues possessing side-chain accessibility of more than 0.25 were defined as exposed. Those with accessibility of less than 0.25 were defined as buried. This threshold value led to roughly even numbers of exposed and buried residues in subunits of hemoglobins. Residues on the monomeric surface were assigned as those exposed in monomeric state of each subunit. Residues on the subunit interface were assigned as those exposed in monomeric state but buried in oligomeric state. In analyzing distribution of amino acid residues at monomeric surface and subunit interface, amino acid residues were grouped into three classes according to their chemical characters: hydrophilic (K, R, D, E, N, Q, H), hydrophobic (V, L, I, M, F, Y, W), and small ambivalent (G, A, P, S, T, C).

Identification of Residues Involved Directly in Subunit Contact. The atoms involved directly in subunit contact were identified according to the distance between atoms belonging to different subunits. A pair of atoms within 4 Å was considered to be in contact because a water molecule can not be accommodated between them. Residues that have at least one atom in contact with other subunit's atom, were assigned as residues involved in subunit contact.

Results and Discussions

Fraction of Hydrophobic Amino Acid Residues at Subunit Contact

As shown in Table 2, fractions of hydrophobic residues on the subunit interface (24.2%, on average) were found to be significantly higher than those on monomeric surface (13.8%, on average). This increase of hydrophobicity may contribute to the stabilization of subunit contact. Although few direct contacts are observed between hydrophobic residues themselves on the subunit interface, there are many van der Waals contacts of hydrophobic residues with ambivalent and hydrophilic residues. Then how many substitutions are needed to reach such an increase of hydrophobicity at a local surface area to create a subunit interface? Because the number of residues on the subunit interface of hemoglobins is small (13.3, on average for each subunit), only one or two substitutions of hydrophobic residues for small ambivalent residues are enough to raise the fraction of hydrophobic residues by about 10% (1/13.3 = 7.5% and 2/13.3 = 15.0%). Such deviation in the distribution of hydrophobic residues on the molecular surface can occur by chance because many neutral mutations can occur on the molecular surface. In fact, almost all residues on the monomeric surface of hemoglobins were substituted during animal evolution. For example, the number of substitution sites observed between human Hb α-subunit and *Scapharca* HbI is 67 out of 74 residues on the monomeric surface (90.5%) and 20 out of 22 residues on the subunit interface (90.9%). Therefore, several candidates of subunit interface can be generated by neutral mutations. This may explain variety in subunit organization of hemoglobins. Subunit arrangement may have frequently changed by a few mutations in the course of animal evolution and cooperativity was acquired in some animal lineages.

Table 10. Interactions at GH-GH(D) contact of *Urechis* Hb in CN form

Subunit 1		Subunit 3		No. contact atoms
Module	Residue	Residue	Module	
M3	31F	116Q	M7	1
	31F	117E		2
M4	53Y	120S		5
	53Y	121A		2
	53Y	122D		10
	56R	117E		3
	56R	118E		5
	56R	120S		2
M7	116Q	31F	M3	1
	117E	31F		2
	117E	56R	M4	3
	118E	56R		5
	118E	118E	M7	1
	120S	53Y	M4	5
	120S	56R		2
	121A	53Y		2
	122D	53Y		10

Atoms and Residues Involved in Subunit Contacts

The details of atomic interactions at sliding (α1β2) contact of human oxy-Hb are shown in Table 3. Similar

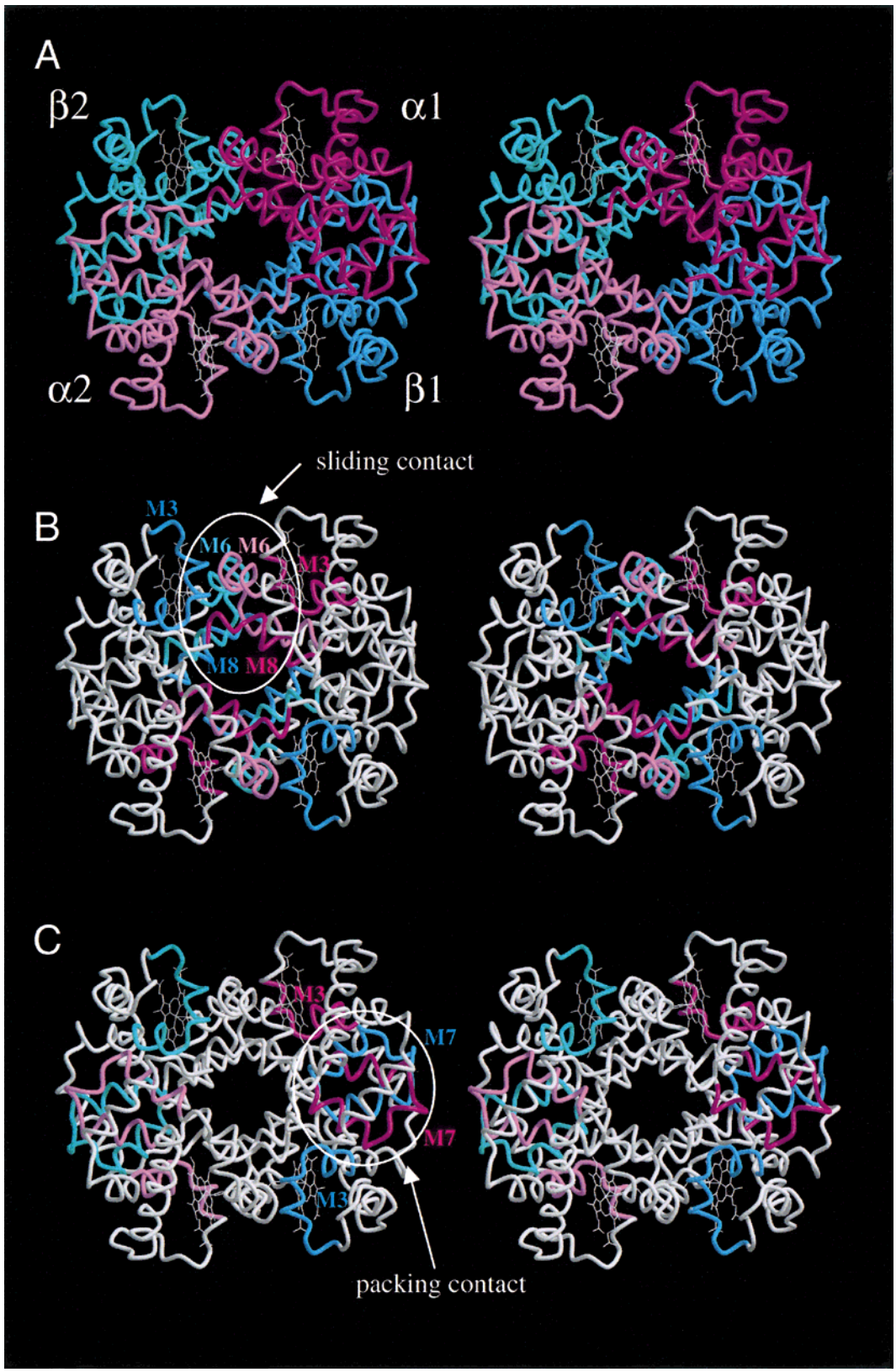

Fig. 1. Hetero-tetramer of human oxy-Hb in stereo. Hemoglobin back-bone is shown in tube and heme in white stick model. For simplicity the substance bound to heme was omitted. **A** Each subunit is drawn in different color. **B** The major modules involved in sliding contacts. **C** Those in packing contacts are shown in colors. Molscript (Kraulis 1991) and Raster3D (Merrit and Bacon 1997) were used to generate the computer graphics in this and other figures.

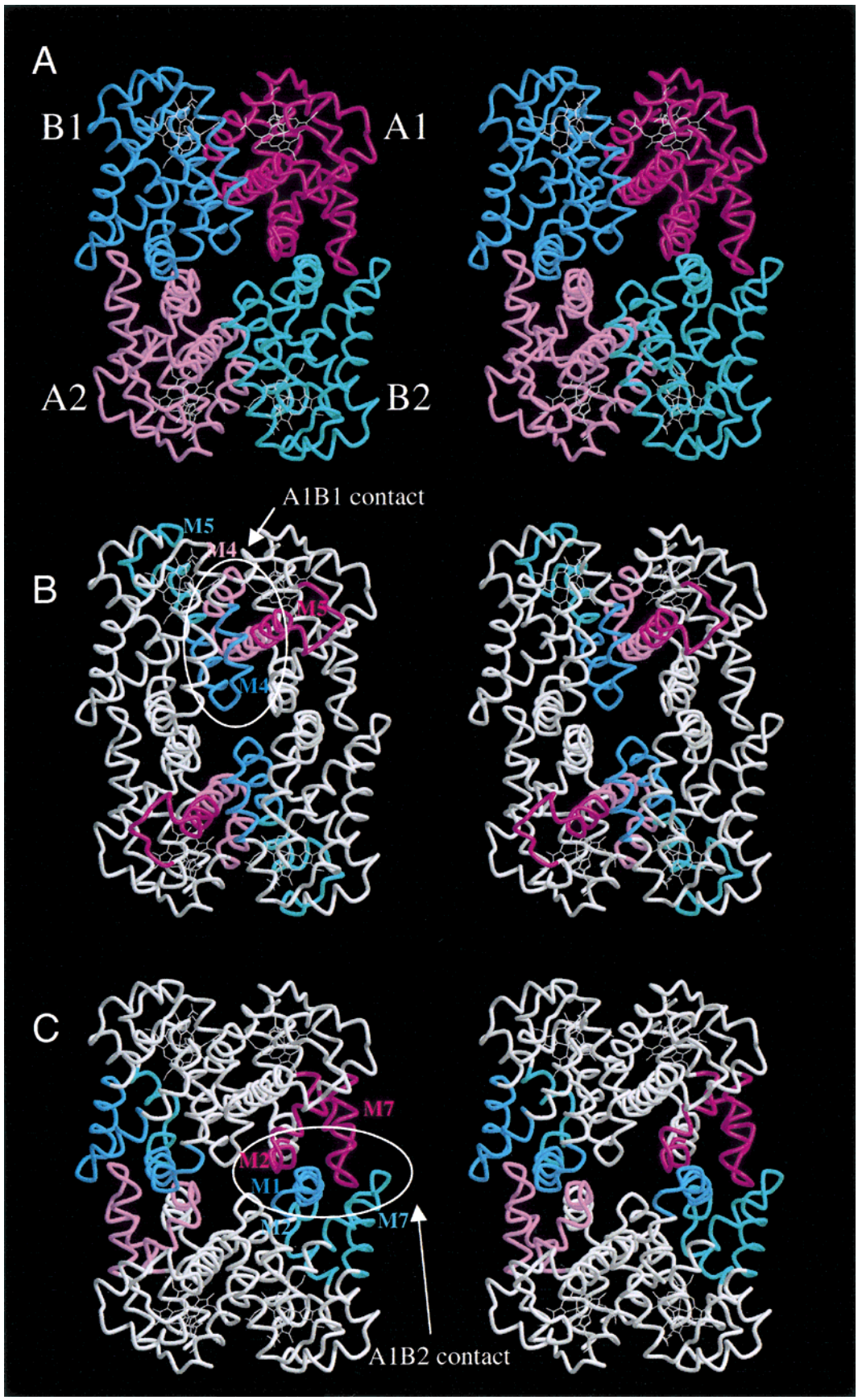

Fig. 2. Hetero-tetramer of *Scapharca* HbII in stereo. Hemoglobin is displayed as explained in Fig. 1. **A** Each of the subunits A1, A2, B1, and B2 is shown in different color. **B** The major modules involved in A1B1 contact. **C** A1B2 contact are shown in colors.

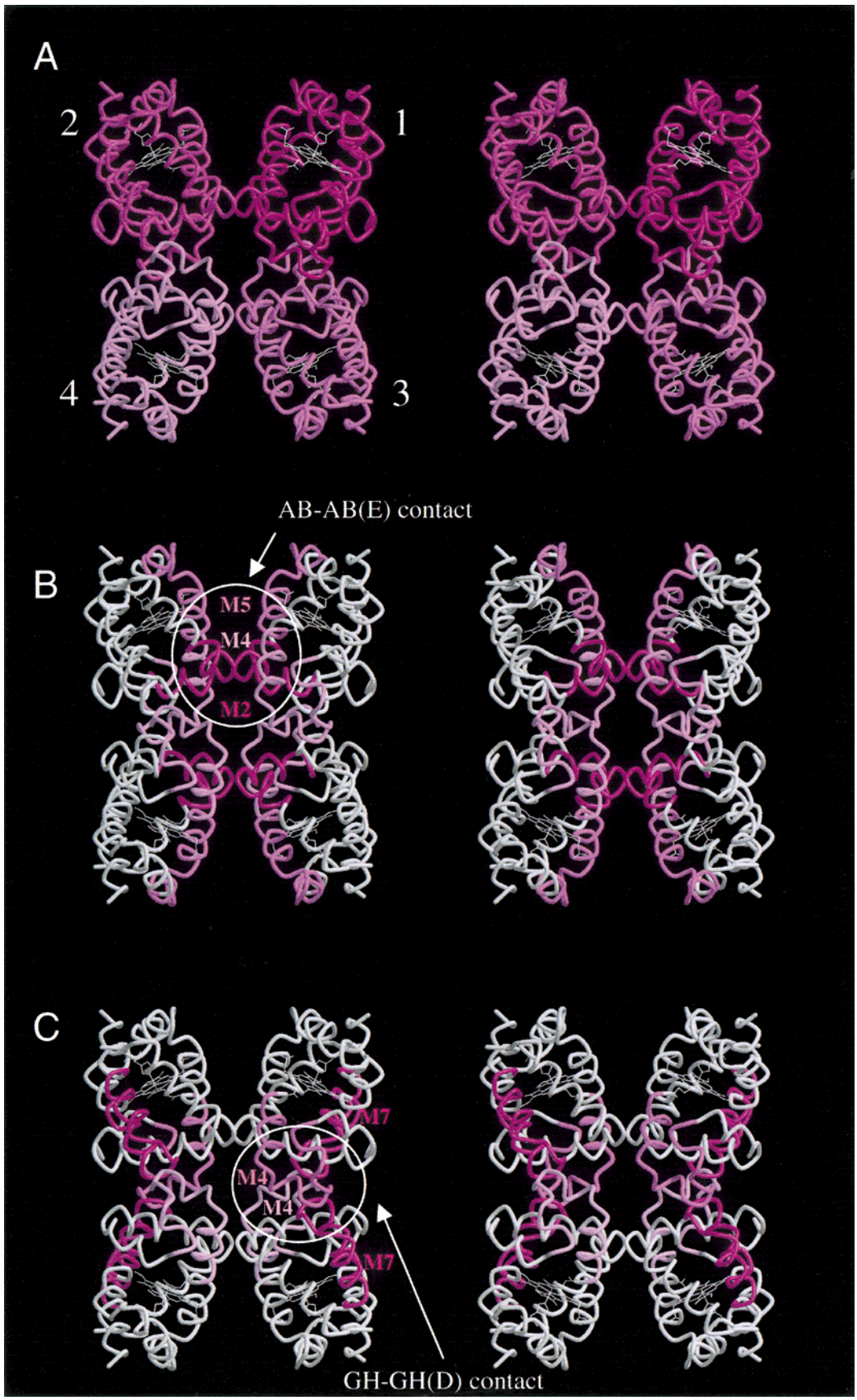

Fig. 3. Homo-tetramer of *Urechis* Hb in stereo. Hemoglobin is displayed as explained in Fig. 1. **A** Each subunit is shown in slightly different color. **B** The major modules involved in AB-AB(E) contact. **C** GH-GH(D) contact are shown in colors.

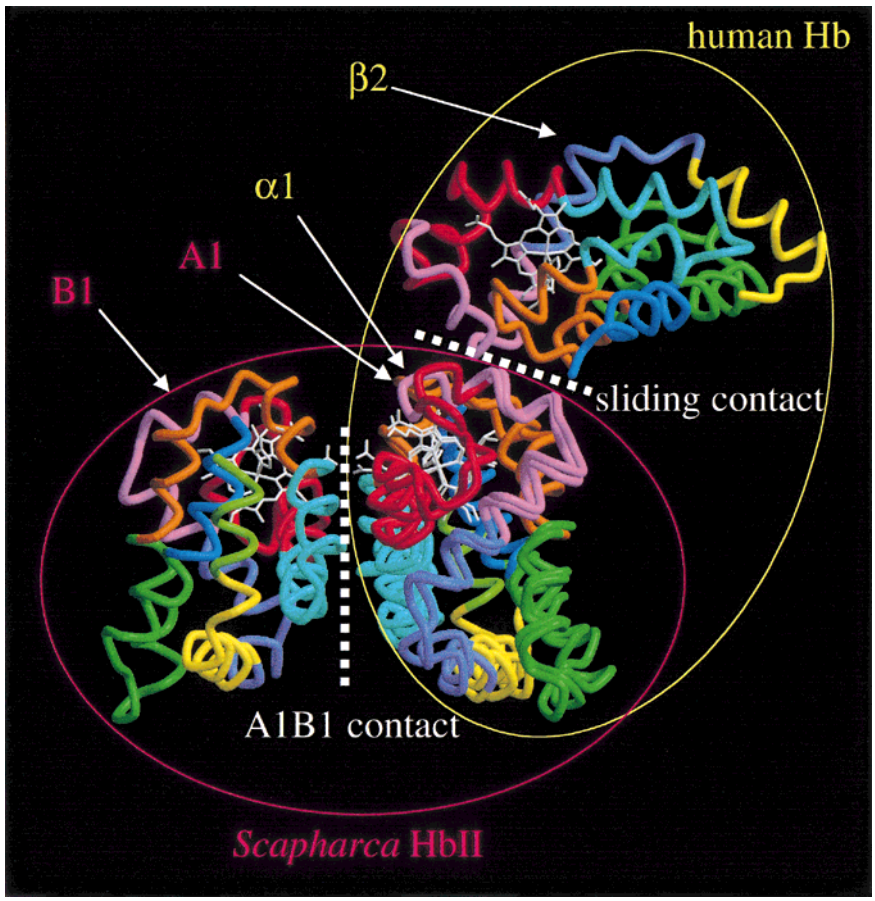

Fig. 4. Different mode of sliding (α1β2) contact of human Hb from A1B1 contact of *Scapharca* HbII. Human Hb α1-subunit and *Scapharca* HbII A1 subunit are superimposed. Each module is shown in different color. M0 in yellow green, M1 in yellow, M2 in purple, M3 in pink, M4 in red, M5 in sky, M6 in orange, M7 in green, and M8 in blue.

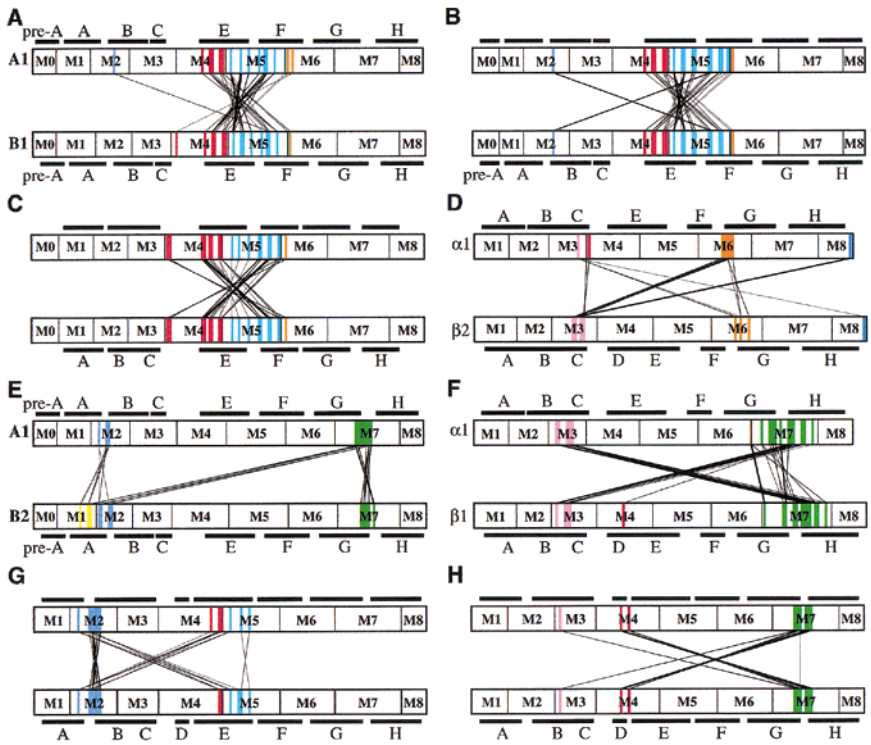

Fig. 5. Module organization of hemoglobin subunits and inter-residue interactions at subunit contacts. **A** A1B1 contact of *Scapharca* HbII. **B** Dimer contact of *Scapharca* HbI. **C** Dimer contact of *Caudina* HbD. **D** Sliding contact of human Hb. **E** A1B2 contact of *Scapharca* HbII. **F** Packing contact of human Hb. **G** Ab-AB(E) contact of *Urechis* Hb. **H** GH-GH(D) contact of *Urechis* Hb. Modules of each subunit are shown with the letters M0–M8 in the rectangles showing the peptide sequence schematically. Amino acid residues involved in the subunit contact are shown as the bars in the color assigned to each module in Fig. 4. The interacting residue pairs are shown in black lines across the interacting subunits. Horizontal black bars above and below the rectangles are the α-helices.

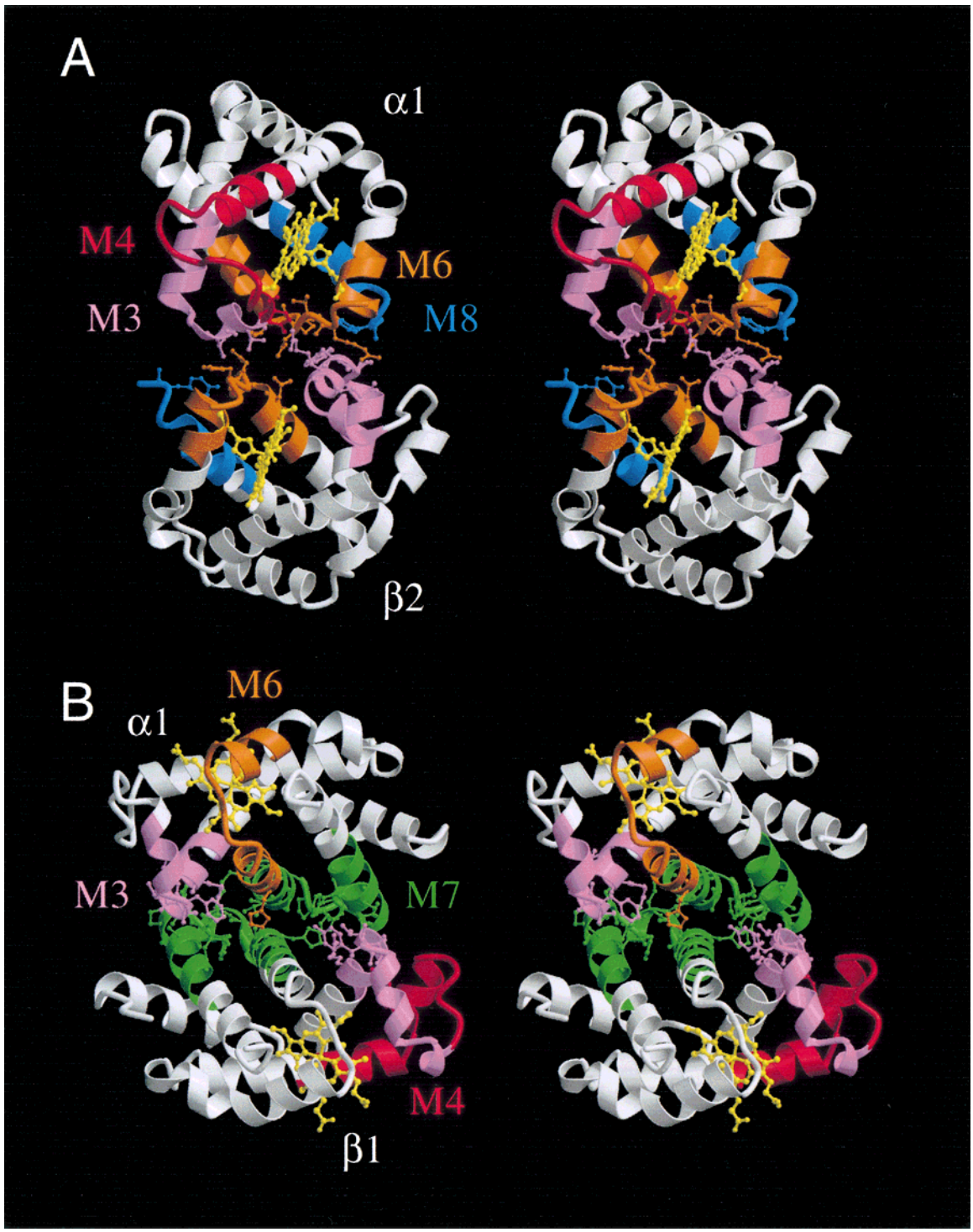

Fig. 6. Interactions at subunit contacts of human oxy-Hb in stereo. **A,** sliding contact and **B,** packing contact are shown in ribbon model from a different angle than Fig. 1. The modules involved in subunit contact are shown in colors the same as those in Fig. 4. Heme and proximal histidine are shown as a yellow ball-and-stick model and the interacting residues at the subunit contact are shown in colors that coordinate to the modules to which they belong.

data for other subunit contacts in human oxy-Hb, *Scapharca* HbI and HbII, *Caudina* HbD, and *Urechis* Hb are shown in Tables 4–10. The atoms at the sliding contact of human oxy-Hb are localized on modules M3, M4, M6, and M8 (Table 3, Figs. 5 and 6), and those at the packing ($\alpha1\beta1$) contact are on M3, M4, M6, and M7 (Table 4, Figs. 5 and 6). This is also observed in human deoxy-Hb (data not shown). The atoms at subunit contacts of *Scapharca* HbI are localized on modules M2, M4, M5, and M6 (Table 5, Fig. 5). These modules are observed at A1B1 contact in *Scapharca* HbII (Table 6, Figs. 5 and 7). The atoms of subunit A1

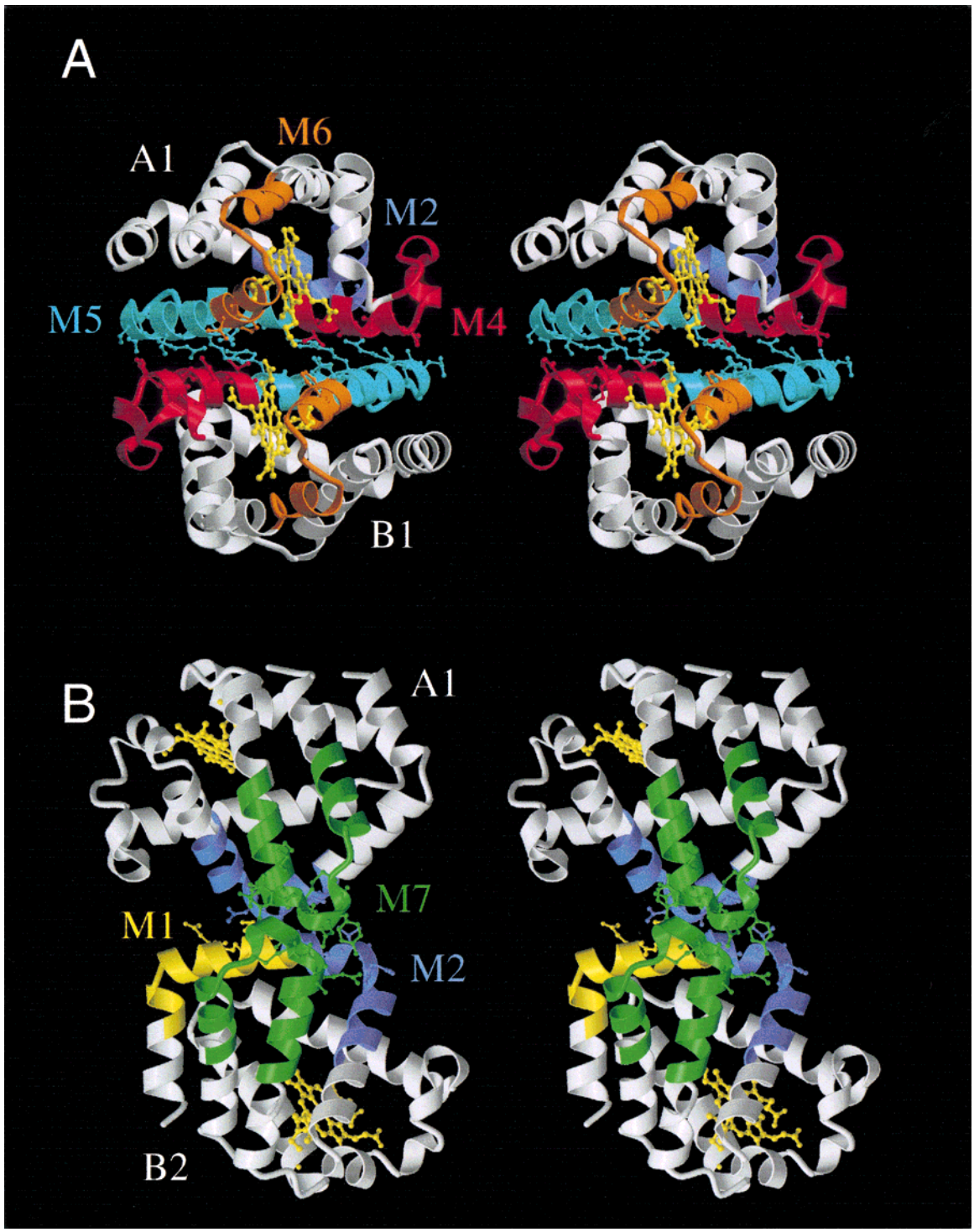

Fig. 7. Interactions at subunit contacts of *Scapharca* HbII in stereo. **A,** A1B1 contact and **B,** A1B2 contact are shown in ribbon model from a different angle than Fig. 2. The coloring is the same as that of Fig. 6.

at A1B2 contact are localized on M2 and M7, and those of subunit B2 at A1B2 contact are located on M1, M2, and M7 (Table 7, Figs. 5 and 7). In *Caudina* HbD, the atoms at the dimeric contact are localized on modules M4–M6 (Table 8, Fig. 5). In *Urechis* Hb, the atoms at AB-AB(E) contact are localized on M2, M4, and M5 (Table 9, Figs. 5 and 8), and those at GH-GH(D) contact are on M3, M4, and M7 (Table 10, Figs. 5 and 8). The atomic interactions at subunit contacts are presented as residue interactions in Fig. 5. Figure 5A–D shows the subunit interactions at the A1B1 contact of *Scapharca* HbII, the dimeric contacts of *Scapharca* HbI and *Caudina* HbD, and the sliding contact of human Hb, respectively. These contacts are thought to

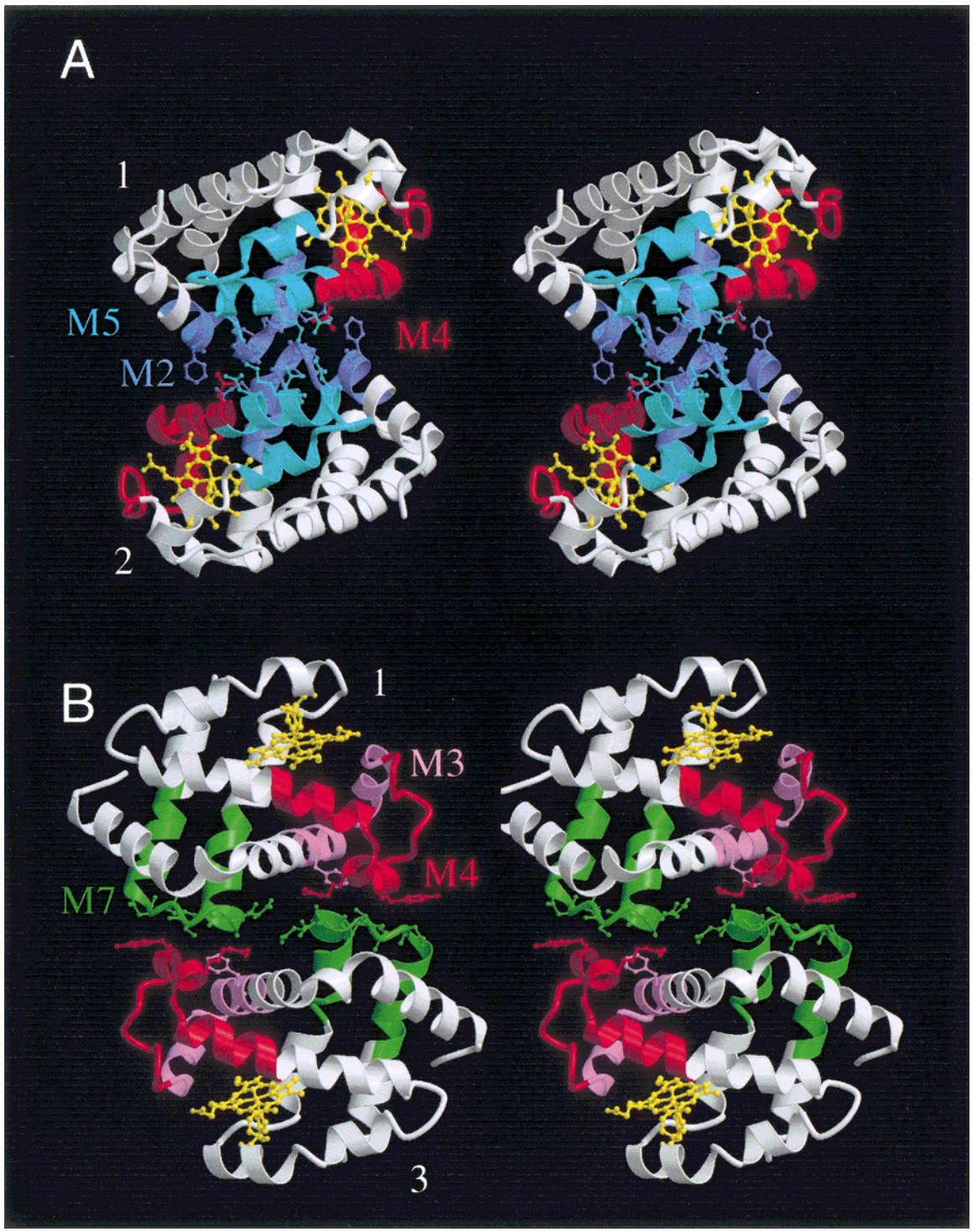

Fig. 8. Modules at subunit contacts of *Urechis* Hb in stereo. **A,** AB-AB(E) contact, and **B,** GH-GH(D) contact are shown in ribbon model from a different angle from Fig. 3. The coloring is the same as that of Fig. 6.

determine the cooperative oxygen binding of these hemoglobins (Royer et al. 1995; Royer 1994; Mitchell et al. 1995; Perutz et al. 1987). Figure 5E–H shows the A1B1 contact of *Scapharca* HbII, the packing contact of human Hb, and the AB-AB(E) and GH-GH(D) contacts of *Urechis* Hb, respectively. These contacts are thought to contribute little to cooperativity. Modules which have the residues involved in subunit contacts are shown in different colors (Figs. 1–3). Amino acid residues involved in subunit contacts are shown together with the modules to which the residues belong (Figs. 6–8).

Relationship Between Cooperativity and Subunit Contact Mode

In *Scapharca* and *Caudina* Hbs the main modules involved in subunit contacts responsible for cooperativity are M4 and M5, and the minor ones are M2, M3, and M6. In human Hb, M3 and M6 are the major modules involved in sliding contact (Fig. 5). At least two modules out of the four heme-contact modules, M3–M6, are involved in the subunit contact responsible for cooperativity in *Scapharca* HbII and HbI, *Caudina* HbD, and the sliding contact of human Hb. Furthermore, at least two pairs of modules interacting with each other among these four heme-contact modules are observed at the subunit contacts responsible for cooperativity, but not in those without cooperativity. We hypothesize that the interactions involving more than two modules among the four heme-contact ones from each subunit are responsible for cooperative oxygen binding. The heme-contact modules M3, M4, M5, and M6 make a contiguous polypeptide segment and surround the heme group. When any two out of the four modules from each subunit are involved at subunit contact, two hemes are positioned in a short distance as seen in *Scapharca* HbII and HbI, *Caudina* HbD, and human Hb. The shortest distances between iron atoms of hemes in various hemoglobins are shown in Table 1. It is clear that the distances between iron atoms of hemes are smaller in the subunit contacts responsible for cooperativity than in those not responsible, suggesting that the inter-iron distance is a factor which determines the cooperative oxygen binding.

Correlation of the intron positions of genes with module boundaries of the proteins encoded by the genes was observed not only in hemoglobins but also in other proteins (Gō 1985; Gō and Noguti 1995; Sato et al. 1999). The enzymatic function of a single module and the role of modular structure in protein folding were also studied by experimental and computational methods (Yanagawa et al. 1993; Ikura et al. 1993; Takahashi et al. 1997). We designed a mini-protein by deleting one module at the middle region of barnase and showed that the designed mini-barnase took a similar form to that of the native barnase in water (Takahashi et al. 1999, 2001). The 3D structures of the hemoglobin subunit and the α- and β-subunits of phycobiliprotein are similar to each other. It is notable that the subunits of phycobiliprotein have extra X-Y helices at the N-terminus of the globin fold (Schirmer et al. 1986). We reported that the X-Y helices are composed of one or two modules, depending on the subunit, and these modules are involved in the intersubunit interactions leading to the creation of a supramolecular assembly for the light-capturing function of photosynthesis in cyanobacteria and red algae. Furthermore, the X-Y helices correspond to a module in pyruvate phosphate dikinase (PPDK), in which the module is involved in the interactions of two domains (Kikuchi et al. 2000). This type of module functions as an adaptor to make subunit contact in phycobiliprotein as well as to make domain contact in PPDK. In the present study we suggest for the first time that inter-module interactions have an important role in the cooperativity of hemoglobin.

In conclusion, evolution of new subunit organization in hemoglobin could occur by chance. However, in order for the evolution of cooperativity to occur, we suggest that the characteristic interactions involving two pairs of modules among the heme-contact modules M3–M6 at subunit contacts are necessary to position hemes within a short distance, making communication possible between the heme and subunit interface.

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