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The Role of Linkers in the Reassembly of the 3.6 MDa Hexagonal Bilayer Hemoglobin from *Lumbricus terrestris*

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The extent and kinetics of reassembly of the four groups of linkers L1-L4 with 213 kDa subassemblies of twelve globin chains D, $(bac)_3(d)_3$, isolated from the ~3.6 MDa hexagonal bilayer (HBL) hemoglobin (Hb) of *Lumbricus terrestris*, was investigated using gel filtration. The reassembled HBL's were characterized by scanning transmission electron microscopic (STEM) mass mapping and their subunit content determined by reversed-phase chromatography. In reassembly by method (A), the linkers isolated by RP-HPLC at pH ~2.2 were added to D at neutral pH; in method (B), the linkers were renatured at neutral pH and then added to D. With method (A) the percentage of HBL reassembly varied from $\geqslant$13 % in the absence of Ca(II) to $\leqslant$75 % in 1-10 mM Ca(II). Reassembly to HBL structures whose linker contents, STEM images and masses were similar to the native Hb was observed with all the linkers ($\geqslant$75 %), with ternary and binary linker combinations (40-50 %) and with individual linkers producing yields increasing in the following order: L1 = 1-3 %, L2 $\approx$ L3 = 10-20 % and L4 = 35-55 %. The yield was two- to eightfold lower with method (B), except in the case of linkers L1-L3. Although the reassembly kinetics were always biphasic, with $t_{1/2}$ = 0.3-3.3 hours and 10-480 hours, the ratio of the amplitudes fast:slow was 1:0.6 with method (A) and 1:2.5 with method (B). These results are consistent with a scheme in which the slow HBL reassembly is dependent on a slow conversion of linker conformation at neutral pH from a reassembly incompetent to a reassembly competent conformation. Although all the linkers self-associate extensively at neutral pH, forming complexes ranging from dimers to >18-mers, the size of the complex does not affect the extent or rate of reassembly. The oxygen binding affinity of reassembled HBLs was similar to that of the native Hb, but their cooperativity was lower. A model of HBL reassembly was proposed which postulates that binding of linker dimers to two of the three T subunits of D causes conformational alterations resulting in the formation of complementary binding sites which permit lateral self-association of D subassemblies, and thus dictate the formation of a hexagonal structure due to the 3-fold symmetry of D.

Keywords: hemoglobin; *Lumbricus terrestris*; hexagonal bilayer; kinetics of reassembly; dodecamer subunits

Abbreviations used: HBL, hexagonal bilayer; Hb, hemoglobin; Chl, chlorocruorin; T, disulfide-bonded trimer subunit (chains bac); M, monomer subunit (chain d); D, dodecamer subassembly (T_3M_3=$(bac)_3(d)_3$); L, all the linker subunits (L1+L2+L3+L4) with the same stoichiometry as in native Hb; RP-HPLC, reverse-phase high pressure liquid chromatography; TFA, trifluoroacetic acid; KSiW, potassium undecatungstosilicate, $K_8[SiW_{11}O_{39}].14H_2O$; NaAsW, sodium tetraconta-tungstotetraarsenate(III), $Na_{26}[BaAs_4W_{40}O_{140}].60H_2O$; FPLC, fast protein liquid chromatography; EMG, exponentially modified gaussian; STEM, scanning transmission electron microscopy; cryoEM, cryoelectron microscopy; P_{50}, oxygen pressure at half- saturation; n_{50}, Hill constant at half-saturation; CRD, cysteine-rich domain; ESI-MS, electrospray ionization mass spectrometry.

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Introduction

Giant extracellular hexagonal bilayer (HBL) hemoglobins (Hb) occur in most terrestrial, aquatic and marine annelids and in deep sea annelids and vestimentiferans. They are ~60 S complexes of globin and non-globin, linker chains and represent a summit of complexity for oxygen-binding heme proteins (Vinogradov, 1985; Lamy *et al.*, 1996). The most extensively studied complex is the ~3.6 MDa Hb of the common North American earthworm *Lumbricus terrestris*. Based on the finding of ~200 kDa globin subassembly upon mild, partial dissociation of the Hb at neutral pH, a ''bracelet'' model of its quaternary structure was proposed to consist of 12, ~200 kDa globin subassemblies tethered to a central scaffolding of 24 to 32 kDa linker chains (Vinogradov *et al.*, 1986). The globin subassembly was determined to be a dodecamer D $(bac)_3(d)_3$, consisting of three copies each of a disulfide-bonded trimer T (bac) and a monomer M (d) (Vinogradov *et al.*, 1991). ESI-MS studies have identified four groups of linker chains, L1a and L1b (27,702 and 27,540 Da), L2 (32,104 Da), L3 (24,912 Da) and L4a, L4b and L4c (24,170, 24,102 and 24,019 Da, respectively) (Fushitani *et al.*, 1996; Martin *et al.*, 1996a). Furthermore, an overall mass of 3.56(±0.13) MDa determined by scanning transmission electron microscopy (STEM) mass mapping and sedimentation equilibrium measurements was found to be compatible with either 36 or 42 linker chains forming the central scaffolding complex (Martin *et al.*, 1996a). The essential correctness of this model was demonstrated by recent 3D reconstructions using cryoEM of *Lumbricus* Hb (Schatz *et al.*, 1995; De Haas *et al.*, 1997). Each of the 12 substructures forming the hexagonally symmetric bilayer was found to have a local 3-fold axis of symmetry, in agreement with the symmetry found for D crystals (Martin *et al.*, 1996b) and expected on the basis of its M_3T_3 structure. We report below the results of a study of the extent and kinetics of reassembly of D with linker combinations and with invidual linkers to HBL structures which appear to be similar to the native Hb structure.

Results

Linker impurities in the isolated dodecamer

The preparations of D obtained by the three methods exhibited varying amounts of linker contaminants, the order of increasing linker contamination being (ii) < (iii) < (i). Since the complex heteropolytungstate anions SiW^{8-}, SbW^{18-} and AsW^{27-} form 1:1 complexes with Mb at neutral pH with association constants in the 10^5-10^6 M^{-1} range and concomitant formation of hemichrome (Chottard *et al.*, 1992), method (iii) was used for most of the work reported here. When D exhibited measurable linker contamination (rarely exceeding ~5 %), the relative linker contents occurred in the order L2 > L1 ≈ L4, with L3 being completely absent.

HBL reassembly

Two critical conditions had to be met for the reassembly reaction to proceed without precipitation when method (A) was used: the volume of the reassembly buffer had to be ⩾12 times the volume of the acidic linker solution added, and the linker concentration had to be less than 15 mg/ml, approximately 0.5 mM. The isoelectric points of the four linker subunits occur at pH 4.7 to 5.6 (Fushitani *et al.*, 1996).

Extent of HBL reassembly from D and combinations of the linkers

Figure 1 illustrates the fast protein liquid chromotography (FPLC) elution profiles obtained with reassembly mixtures of D with a single linker, a ternary linker combination and L, the native-like linker mixture, using method (A). The extent of reassembly, i.e. the reassembled HBL peak area as percentage of total area, determined for a number of linker combinations and single linkers is provided in Table 1. Figure 2 shows HBL reassembly from D + L in the absence and presence of Ca(II). It is evident that the extent of HBL formation was critically dependent on the presence of Ca(II). When no Ca(II) was added (Figure 1(a)), the extent of reassembly was ~10-15 %; in the presence of 1 mM EDTA (Figure 1(b)) the extent of reassembly was only 5-6 %, suggesting that there was enough Ca(II) in the buffer to be bound avidly by the reassembling subunits. No effect on the extent of reassembly was observed at [Ca(II)] > 1 mM. Figure 3(a) illustrates the effect of the L:D molar ratio on the extent of reassembly of D with L and a ternary combination. In the former case, the yield increased from 58 to 71 and 95 % with increase in L:D from 2.3, to 3.3 and 4.5, respectively. For the ternary combination, the yield increased from 50 to 66 and 77 %, with increases in L:D from 3.3 to 4.5 and 5.4, respectively. Figure 4 shows some representative STEM images of unstained specimens of reassembled HBLs. The masses determined by STEM mass mapping and the total linker contents determined by RP-HPLC are collected in Table 1. The unstained STEM images and the experimental STEM masses were not affected either by the presence of Ca(II) during reassembly, the method of reassembly or the duration of reassembly. The masses and linker contents of the reassembled HBL[D + L] should be compared to the values for the native Hb, 3.56(±0.13) MDa and 27(±2.0)%, respectively (Martin *et al.*, 1996a).

Extent of reassembly from D and individual linkers

Using method (A), the extent of reassembly from D and each of the four linkers L1-L4

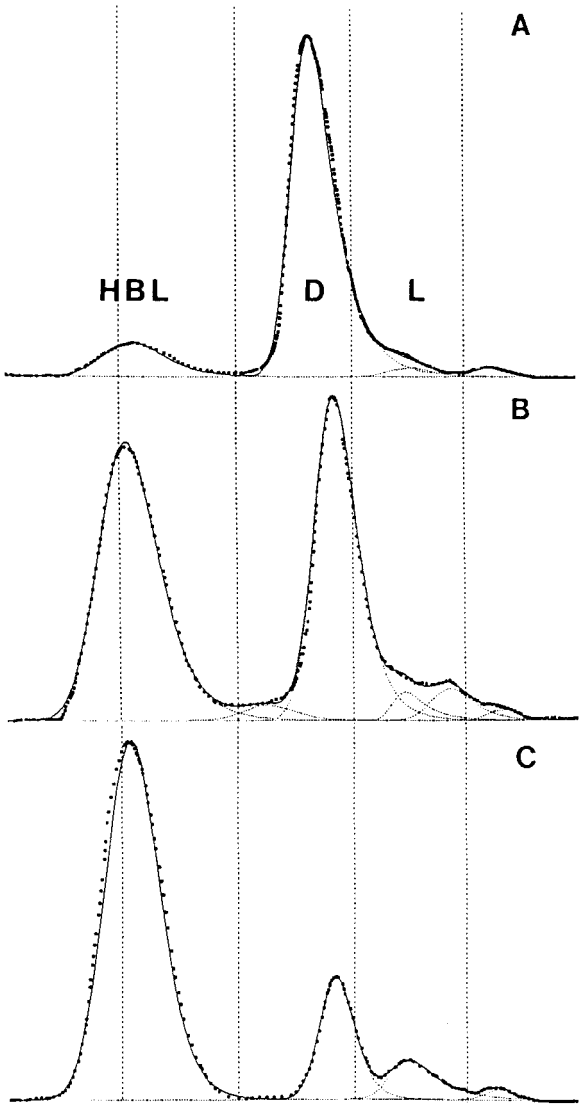

Figure 1. FPLC 280 nm elution profiles at neutral pH of the reassembling mixtures of D and linker subunit(s) using method (A). (a) D + L4 after 157 hours; (b) D + L1 + L2 + L4 after 240 hours; (c) D + all four linkers after 200 hours. HBL, D and L identify the reassembled structures, the dodecamer subassembly and the linkers L1-L4. Abcissa, elution volume. Ordinate, absorbance at 280 nm.

in 10 mM Ca(II) was found to increase in the following order: L1 = 1-3 % < L2 = 11-14 % < L3 = 13-23 % < L4 = 35-56 % (Table 1).

Preferential incorporation of linkers into reassembled HBL's

Table 2 provides some experimental data on the linker content of the reassociating solutions and of the reassembled HBLs in the case of reassembly with several binary and ternary combinations of linkers. These results illustrate the preferential incorporation of L2 and L4 over L1 and the marked tendency towards linker diversification in reassembled HBLs.

Comparison of methods (A) and (B)

Table 3 compares the extent of reassembly obtained by methods (A) and (B) using the same linker preparation and provides the total and individual linker contents of the HBLs.

Kinetics of HBL reassembly

The kinetics of reassembly from D + L, in the absence as well as in the presence of Ca(II), illustrated in Figure 1, was always found to be biphasic, requiring a sum of two exponentials for an acceptable fit, comprising a fast first-order process with $t_{1/2} \approx 0.5$ hour and a slower first-order process with $t_{1/2} \approx 21$ hours (Table 4). In addition, both processes appeared to be slowest in the absence of Ca(II) (data not shown). Representative kinetic curves for HBL reassembly from D and individual linkers obtained using method (A) are shown in Figure 5. The half-times for the two processes given in Table 4 were similar to those observed for D with different combinations of linkers, including the native L. Likewise, the ratio of the amplitudes of the fast and slow reassembly processes appear to be similar, ranging from 1:0.2 to 1:1, mean 1:0.6(±0.3) ($N = 5$). Although method (B) resulted in similar half-times for the fast and slow processes, the ratio of the amplitudes of fast:slow appeared to be substantially different, 1:2.5(±1.2) ($N = 4$). The L:D ratio exerted a strong effect on the kinetics of reassembly of D + L (Figure 3): increase in L:D from 2.3 to 3.3 and 4.5 led to decreases in $t_{1/2}$ of the fast reassembly from 0.59 to 0.47 and 0.23 hour, respectively and $t_{1/2}$ of the slow reassembly from 27 to 10 and 1.8 hours, respectively.

The self-association of linkers

The native mixture of linkers L and binary and ternary combinations were brought back to neutral pH using method (B), and subjected to FPLC: Figures 6 and 7 show the elution profiles obtained with comparable protein loads. Deconvolution of the FPLC profiles using exponentially modified Gaussian (EMG) generated overlapping peaks whose elution volumes were used to obtain estimates of the corresponding masses provided in Table 5, together with the estimated size of the multimers in parentheses. The native-like linker mixture and the ternary combinations (Figure 6(b)(d)) are much more extensively self-associated than a binary combination (Figure 6(a)) and the individual linkers (Figure 7), since the first peak eluted at the same volume as HBLs, corresponding to species >660 kDa, the exclusion limit. On the other hand, the first peak for the individual linkers eluted at volumes corresponding to 440-580 kDa (Table 5). The elution volumes of the

Table 1. Extent of reassembly, linker contents and STEM masses of reassembled HBLs

Linker combination	Extent of reassembly[a]	Linker content[b] (%)	Mass (MDa)[c]	N_j[h]
L1 + L2 + L3 + L4	6-13[d]	28, 25[d]	3.55 ± 0.24	288
			3.25 ± 0.13	137
	75 + 9	26 ± 3[f]	3.36 ± 0.15	668
	(N = 3)	(N = 9)	3.28 ± 0.13	592
	35[e]	26	3.51 ± 0.14	195
L1 + L2 + L4	50	28	3.68 ± 0.16	185
			3.42 ± 0.18	518
L1 + L3 + L4	41	25 ± 3	3.49 ± 0.17	112
		(N = 6)	3.27 ± 0.17	271
	17[e]	28	3.41 ± 0.14	
L1 + L4	39-46	22 ± 1	3.41 ± 0.16	321
		(N = 5)	3.39 ± 0.16	215
			3.41 ± 0.23	148
L2 + L3	43	25	-	-
L1	1-3	18[g]	3.51 ± 0.19	149
L2	11-14	30[g]	3.42 ± 0.18	261
L3	13-23	36 ± 7	3.19 ± 0.19	180
		(N = 3)		
L4	35-56	29[g]	3.44 ± 0.16	880

[a] Reassembly carried out using method (A), 10 mM Ca(II), except where indicated.
[b] Percentage of total area determined by HPLC.
[c] STEM mass of native *Lumbricus* Hb is 3.56(±0.13) MDa (Martin *et al.*, 1996a).
[d] In the absence of Ca(II) and 1 mM EDTA, respectively.
[e] Method (B), 10 mM Ca(II).
[f] Mean of HBLs obtained in the presence of 1 mM, 2 mM, 5 mM and 10 mM Ca(II).
[g] Mean of two determinations.
[h] Number of particles used in the STEM mass mapping.

major and minor peaks from the EMG deconvolutions were surprisingly reproducible. The extent of self-association from the elution curves appeared to decrease in the order L1 + L2 + L3 + L4 ≈ L1 + L2 + L4 > L1 + L3 + L4 ⩾ L1 + L4 > L2 ≈ L3 > L4 ⩾ L1.

The elution profile of L (Figure 6(d)) was divided into three fractions A-C, corresponding approximately to the >660 kDa, the 6 to 8-mer, and the dimer complexes, respectively. Rechromatography of fraction A resulted in partial dissociation to B and C; that of fraction B showed substantial dissociation to C but no association to A, and that of fraction C showed no formation of A and B.

HBL reassembly with linker fractions A-C

Reassembly of D with each of the fractions A-C showed no differences in the extent of reassembly, the HBL linker contents or the kinetic parameters (Table 4). The L2 and L3 contents of fractions A and B were higher than fraction C, with the reverse being true of the L1 and L4 contents, in agreement with the more extensive self-association of L2 and L3 *versus* L1 and L4 (Figure 7).

Oxygen binding parameters of reassembled HBL's

Table 6 provides the oxygen affinities P_{50} and the Hill coefficients n_{50} for the native Hb, D and several reassembled HBLs. The affinity and cooperativity of D were similar to those determined by (Vinogradov *et al.* (1991). Although the affinities of the reassembled HBLs were similar to the affinity of the native Hb, the n_{50} values were substantially lower. Furthermore, the control, the reassembled HBL[D + L], did not have a cooperativity much higher than the isolated D, suggesting that the methods of reassembly require amelioration.

Discussion

Extent of HBL reassembly

Our results show the HBLs reassembled from the full complement of linkers, several ternary and binary combinations and from individual linkers, elute at the same volume as the native Hb, have STEM masses similar to each other and to the native Hb (3.56(±0.13) MDa) and have linker contents (Table 1) similar to that of native Hb, 27(±2)% (Martin *et al.*, 1996a). Furthermore, the 3D reconstruction volumes of reassembled HBLs are similar to the native Hb (J.N.L., unpublished results). The 3D reconstruction of *Lumbricus* Hb presented in the accompanying paper (Taveau *et al.*, 1999) is very similar, if not identical with the 3D reconstructions of the reassembled HBL[T + L] (De Haas *et al.*, 1997), as well as *Eudistylia* Chl (De Haas *et al.*, 1996a), the Hb from the leech *Macrobdella* (De Haas *et al.*, 1996b), the deep sea Hbs from the vestimentiferan *Riftia* (De Haas *et al.*, 1996c), and the polychaete *Alvinella* (De Haas *et al.*, 1996d).

The elution profiles of the reassembling mixtures obtained by method (A) showed little if any protein eluting between the reassembled HBL and the dodecamer subassembly D (Figure 1). Furthermore, the STEM images and masses of HBLs

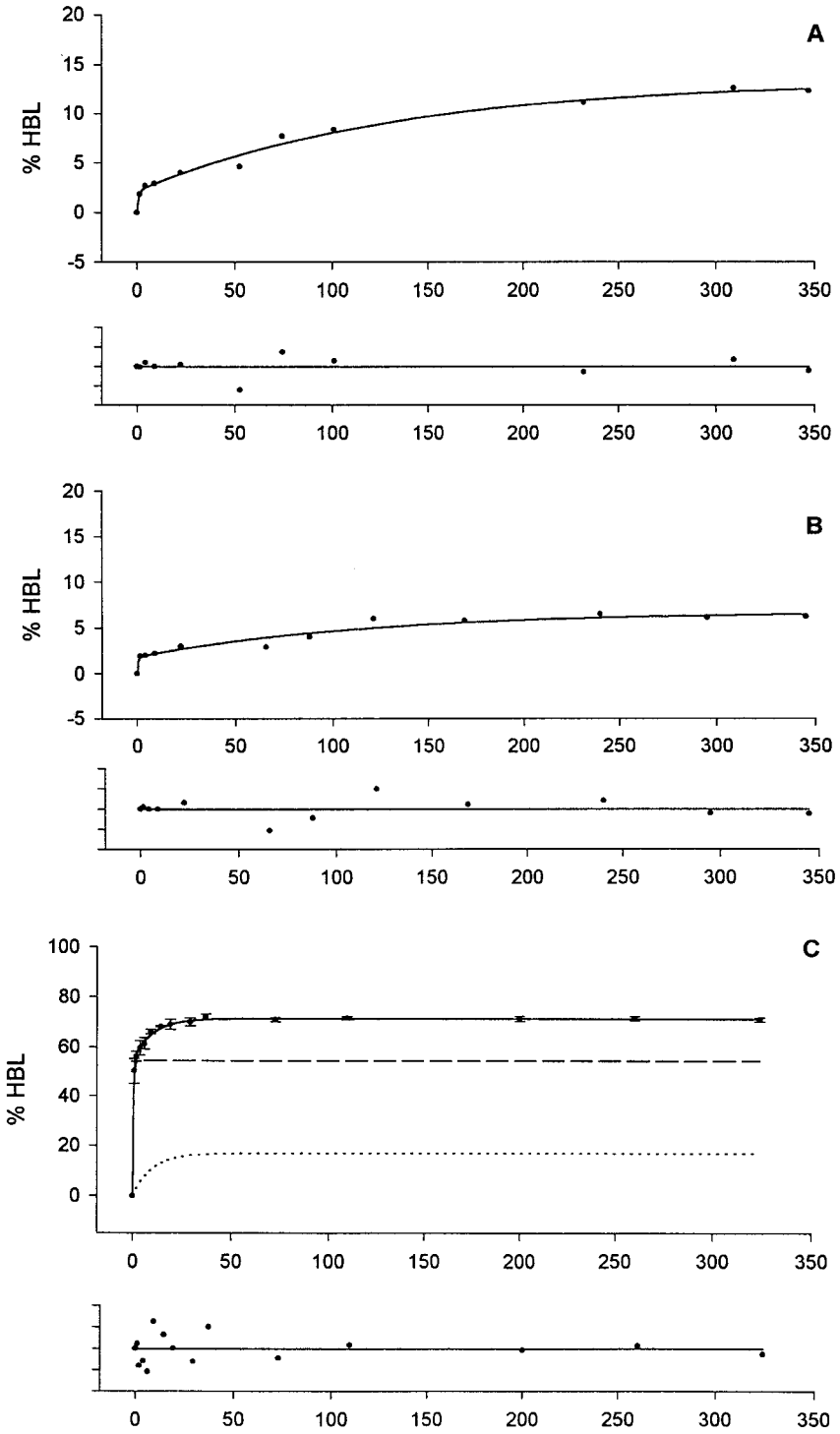

Figure 2. Kinetics of HBL reassembly from dodecamer D and all four linkers L1 + L2 + L3 + L4, obtained by method (A) at molar ratio L:D = 3.3:1; (a) in the absence of Ca(II); (b) in 1 mM EDTA; (c) in 1 mM Ca(II); Abcissa: time, hours ordinate: HBL peak area (Figure 1) as per cent of total area.

isolated early in a reassembly reaction did not differ from those isolated at a much later stage. The STEM images of reassembled HBLs (Figure 4) also showed the presence of small particles ~1 nm in diameter; these are Ds resulting from Hb dissociation during cryolyophilization of the specimens. Our results suggest that HBL reassembly is an ''all or nothing'' process.

The major conclusion from our study is that every one of the four linker subunits can replace another; based on the extent of HBL reassembly, L1 is the least efficient in forming HBLs and L4 is the most efficient one, with L3 and L2 being intermediate. Although the binary and ternary combinations did not appear to provide higher yields than L4 alone, the native-like combination of all

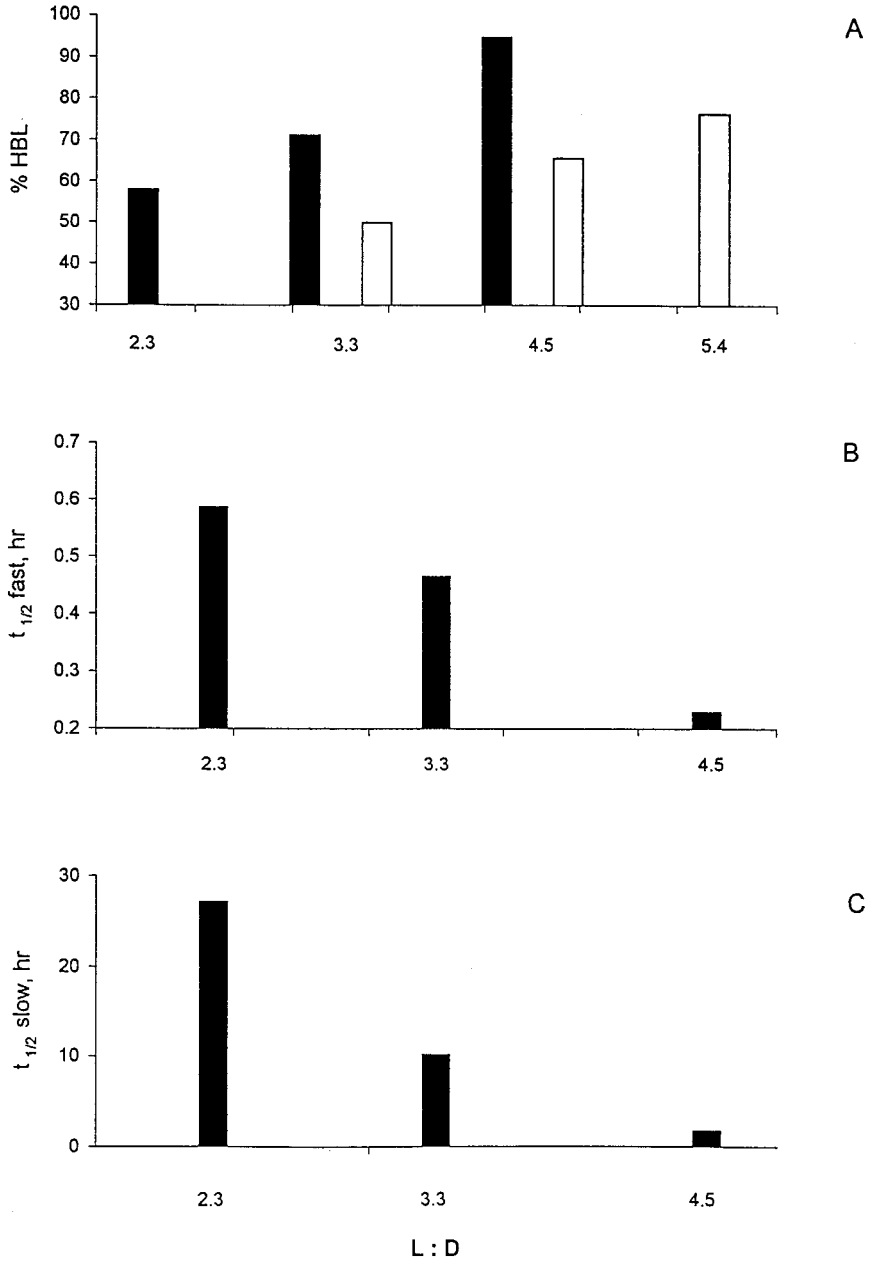

Figure 3. Effect of the molar ratio L:D on the extent of HBL reassembly using all four linkers L1 + L2 + L3 + L4 (solid black bars) and the ternary combination L1 + L2 + L4 (a) and on the kinetic parameters of reassembly, the $t_{1/2}$ values of the (b) fast and (c) slow processes.

four linkers was the most efficient (Table 1). A necessary consequence of the structural interchangeability of the linker subunits is that they all must have globin-binding and linker-binding domains. The results shown in Table 2 illustrate the synergistic effects of linker incorporation into the reassembled HBLs; these results can be summarized as follows. (i) L1 was generally underrepresented and L2 was always incorporated preferentially in the reassembled HBL's; there was no clear trend in L3 or L4 incorporation. (ii) Although reassembly by method (A) resulted in preferential incorporation of L4, usually at the expense of L1, the opposite occurred when method (B) was used, in agreement with the results of reassembly with individual linkers by the two methods: L4 had the highest affinity for HBL formation using method (A) but not method (B) (Table 3). (iii) The extent of reassembly always increased with an increase in linker diversification, the highest yield occurring when the linker composition approximated that of the native Hb: in these cases, no significant differences were observed in the linker contents.

Oxygen binding of reassembled HBLs

One of our aims was to determine whether the linker subunits played a role in determining the

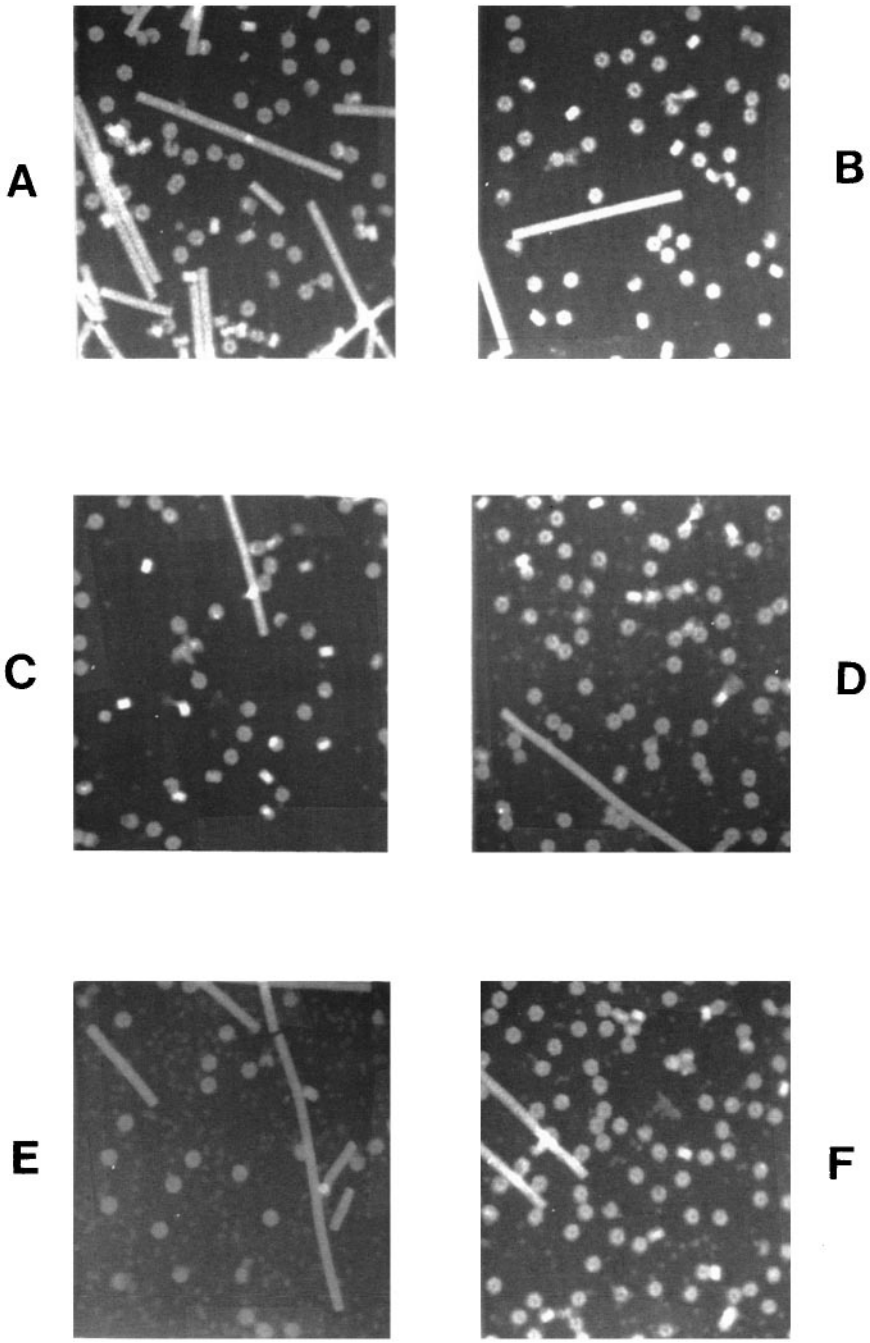

Figure 4. STEM scans (0.512 μ) of unstained specimens of the HBLs isolated by FPLC of reassembling mixtures of dodecamer D with the native-like mixture of (a) all four linkers, (b) L1 + L2 + L4, (c) L1 + L3 + L4, (d) L1 + L4, (e) L1 and (f) L4. The small ~1 nm diameter particles are dodecamers resulting from the dissociation of HBL's during cryolyophilzation of the specimens.

higher cooperativity of oxygen binding of the native Hb relative to the isolated D subassembly (Vinogradov *et al.*, 1991). The results given in Table 6 show that all the reassembled HBLs, including the control HBL[D + L], bound oxygen cooperatively but with an n_{50} value not much greater than that of the isolated D. These disappointing results suggest that our methods of reassembly require amelioration, since it is likely that the full cooperativity of the reassembled HBLs is critically dependent on the proper packing of the linker chains in the central linker complex, and that the latter was impaired by the isolation of the linker chains under the harsh conditions of RP-HPLC.

Kinetics of HBL reassembly

The kinetic curves shown in Figures 2 and 5 and the results provided in Table 4, demonstrate the occurrence of a fast initial phase of HBL reassembly with $t_{1/2}$ ranging from 0.3 to 3.3 hours, and a subsequent slow phase with $t_{1/2}$ ranging from 7.3

Table 2. Preferential incorporation of linkers into reassembled HBLs

Linker combination	Percentage reassembly	Total linker content of HBL[a]	Individual linker percentage[b]	
			Reassoc.	Mixture → HBL
L1 + L4	46[c]	22	L1	51 → 38
			L4	46 → 56
			L2	3 → 7
L1 + L4	39	24	L1	48 → 40
			L4	48 → 55
			L2	4 → 5
L1 + L4	15	22	L1	74 → 31
			L4	26 → 69
L1 + L3 + L4	40	22	L1	31 → 23
			L3	39 → 39
			L4	29 → 32
			L2	1.5 → 5
L1 + L3 + L4	66[c]	25	L1	29 → 18
			L3	36 → 32
			L4	26 → 30
			L2	9 → 21
L1 + L2 + L4	77[d]	28	L1	34 → 18
			L2	38 → 42
			L4	29 → 41
L1 + L2 + L4	66[e]	28	L1	23 → 13
			L2	51 → 49
			L4	27 → 39

Reassembly carried out using method (A).
[a] Sum of all the linker peak areas as percent of total RP-HPLC elution profile.
[b] As a percentage of total linker peak area in the RP-HPLC elution profile.
[c] Dodecamer obtained by dissociation of the Hb in the presence of SiW.
[d] Carried out at L:D = 5.4:1.

to 480 hours, independent of the reassembly method used. In contrast, the ratio of the amplitudes fast:slow varied with the method of reassembly: 1:0.6(±0.3) for method (A) and 1:2.4(±1.2) for method (B). The significantly greater proportion of the slow phase relative to the initial fast phase when D was reassembled with L brought to neutral pH, is in accordance with the notion that the linker conformations in the reassembled, and by implication in the native HBL, are different from their conformations in solution in the absence of subassembly D.

Table 3. Comparison of HBL reassembly obtained by methods (A) and (B)

Linker combination	Extent of reassembly[a]		Total linker content of reassembled HBLs[a]		Individual linker content of reassembled HBLs[b]	
	Method (A)	Method (B)	Method (A)	Method (B)	Method (A) (%)	Method (B) (%)
L1	3	11	19	21	-	-
L2	12	10	21	22	-	-
L3	16	11	29	21	-	-
L4	40	5	24	15	-	-
L1 + L4	39	8	23	23	31 L1	62 L1
					69 L4	38 L4
L1 + L2 + L4	50	27	23	24	38 L1	38 L4
					43 L2	35 L2
					19 L4	20 L4
L1 + L3 + L4	41	17	22	28	26 L1	18 L1
					40 L3	52 L3
					27 L4	23 L4
					7 L2	7 L2
L1 + L2 + L3 + L4	76	35	26	25	20 L1	28 L1
					40 L2	38 L2
					24 L3	22 L3
					16 L4	12 L4

Using the same preparation of linkers.
[a] Sum of all the linker peak areas as percent of total HPLC elution profile.
[b] As a percentage of total linker peak area in HPLC elution profile. The results should be compared with the linker composition of native *Lumbricus* Hb: 21% L1, 37% L2, 22% L3 and 19% L4.

Table 4. Kinetics of HBL reassembly from dodecamer and linker combinations

Linker	Ratio of amplitudes fast:slow	Fast $t_{1/2}$, h^{-1}	Slow $t_{1/2}$, h^{-1}
L1 + L2 + L3 + L4[a] ($N = 5$)	1:0.6 ± 0.4	0.5 ± 0.3	21 ± 18
L1 + L2 + L3 + L4[b]	1:2.9[c]	0.3	16
L1 + L2 + L4[a]	1:0.3	0.4	480
L1 + L2 + L4[b]	1:0.6	0.9	10
L1 + L3 + L4[a]	1:0.6[c]	1.5	52
L1 + L3 + L4[b]	1:3.2	1.9	81
L1 + L4[a]	1:1′	3.3	51
L1 + L4[b]	1:3.1	0.6	340
L2 + L3[a]	1:0.5	0.25	15
L1[a]	1:0.6	0.3	23
L2[a]	1:0.6	0.3	9.9
L3[a]	1:0.75	0.4	7.3
L4[a]	:0.2[c]	1.1	38
Peak A[b]	1:1.6	0.96	69
Peak B[b]	1:1.2	0.93	35
Peak C[b]	1:0.7	1.7	55

[a] Using method (A).
[b] Using method (B).
[c] Average of two values.

The observed biphasic kinetics is in contrast to the single phase, with $t_{1/2} \approx 1.5$-3 hours found in our earlier study of HBL reassembly from Hb completely dissociated at neutral pH to M, T and L in the presence of urea (Sharma *et al.*, 1996). Since the latter $t_{1/2}$ is similar to the $t_{1/2}$ for the initial phase, the additional, slow reassembly could have reflected control by a slow alteration in linker conformation from a reassembly incompetent to a reassembly competent, native HBL-like conformation. This interpretation is consistent with the increased amplitude of the slow process when method (B) was used, since the conformation of the linker chain(s) renatured to neutral pH does not correspond to their conformation in HBLs, whereas when the Hb was dissociated by urea at neutral pH, the dissociated linker(s) retained the conformation they had in the Hb. Our kinetic observations can be explained by the following simple scheme:

(1) Fast formation of HBL using method (A): $D + L^+ \rightarrow HBL + L^*$.
(2) Slow interconversion at neutral pH: $L^* \leftrightarrow L$.
(3) Formation of additional HBL: $L + D \rightarrow HBL$.

where L^+ is the linker conformation at acid pH, probably at least partially denatured, L* is the linker conformation at neutral pH that is reassembly incompetent, and L is the reassembly competent, native HBL-like conformation. Reassembly using method (A) results in a rapid and considerable formation of HBL, suggesting that the D subassemblies exert a ''chaperonin-like'' action on L^+, promoting the formation of the reassembly-competent conformation L. The substantial increases in

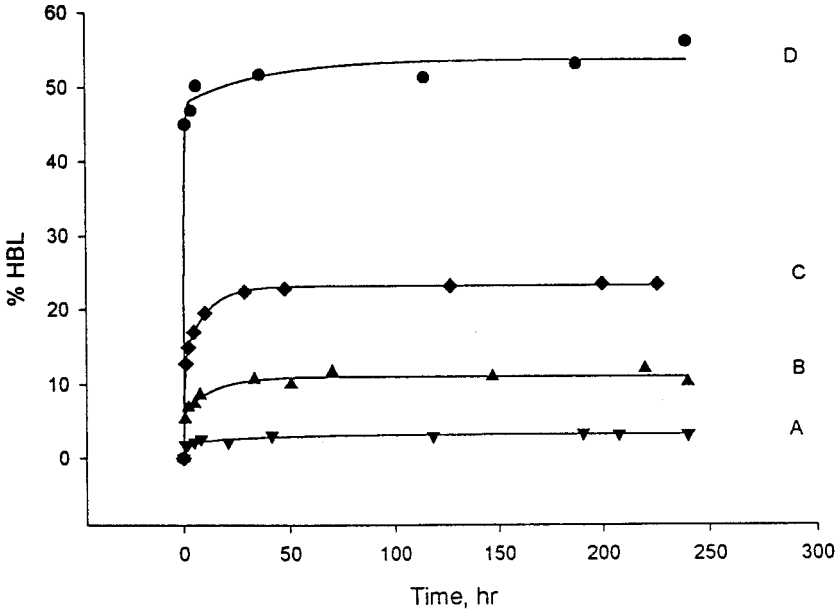

Figure 5. Kinetics of HBL reassembly from dodecamer D and individual linkers obtained by method (A), in 10 mM Ca(II), molar ratio L:D = 2.7-3.3:1; (a) D + L1; (b) D + L2; (c) D + L3; (d) D + L4.

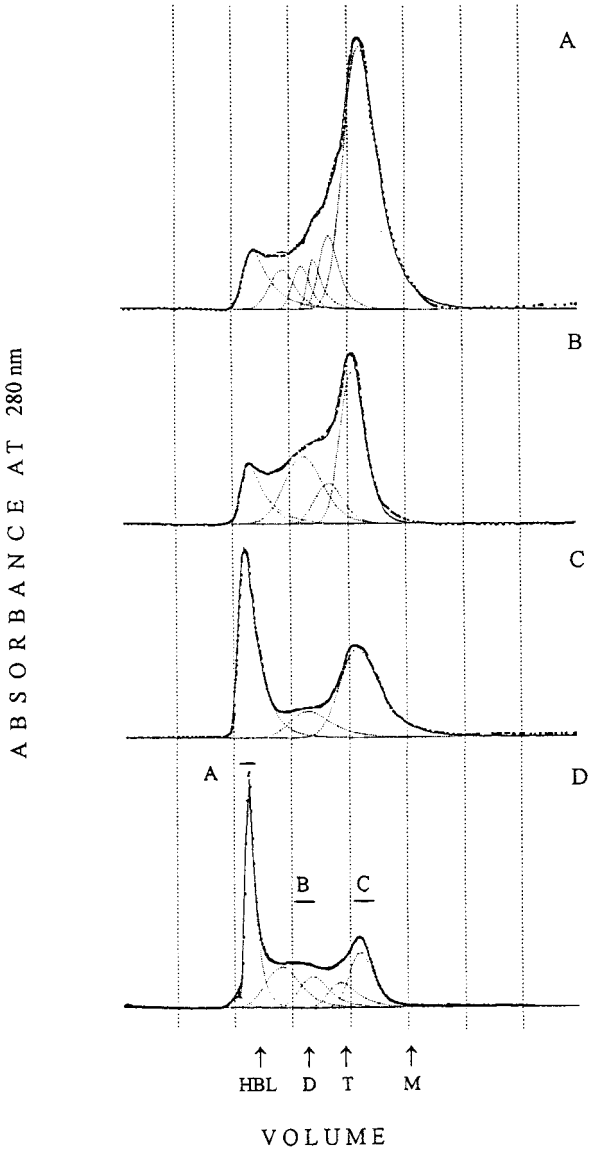

Figure 6. FPLC elution profiles at 280 nm and neutral pH of linker combinations (a) L1 + L4, (b) L1 + L3 + L4, (c) L1 + L2 + L4 and (d) of the native mixture of linkers L1 + L2 + L3 + L4. The elution profiles were deconvoluted as sums of EMG functions. The approximate molecular masses corresponding to the elution volumes of the peaks are provided in Table 5.

the half-times for both processes with modest increase in L:D (Figure 3) supports this idea. Reassembly using method (B), results in a much smaller initial formation of HBL which is dependent on the small amount of the reassembly competent form L in equilibrium with L* in the linker(s) brought back to neutral pH. The two- to eightfold better yield of HBL obtained using method (A) relative to method (B) is in accordance with this interpretation. Recent results on the association-induced folding of Staphylococcal nuclease support the concept of a chaperonin-like action of D *via* association with linkers in the L^+ conformation (Uversky *et al.*, 1998).

Self-association of linkers

The FPLC elution profiles of the four linker subunits show extensive self-association at neutral pH (Figure 7), suggesting the presence of dimers and higher multimers (Table 5). The binary and ternary combinations and the native-like mixture exhibited much more extensive aggregation (Figure 6): large complexes with estimated masses >660 kDa in addition to 6-mers and dimers (Table 5). STEM images of stained and unstained specimens of the higher aggregates did not reveal any discrete, interpretable structures. Reassembly using each of the three fractions A-C of L (Figure 6(d))

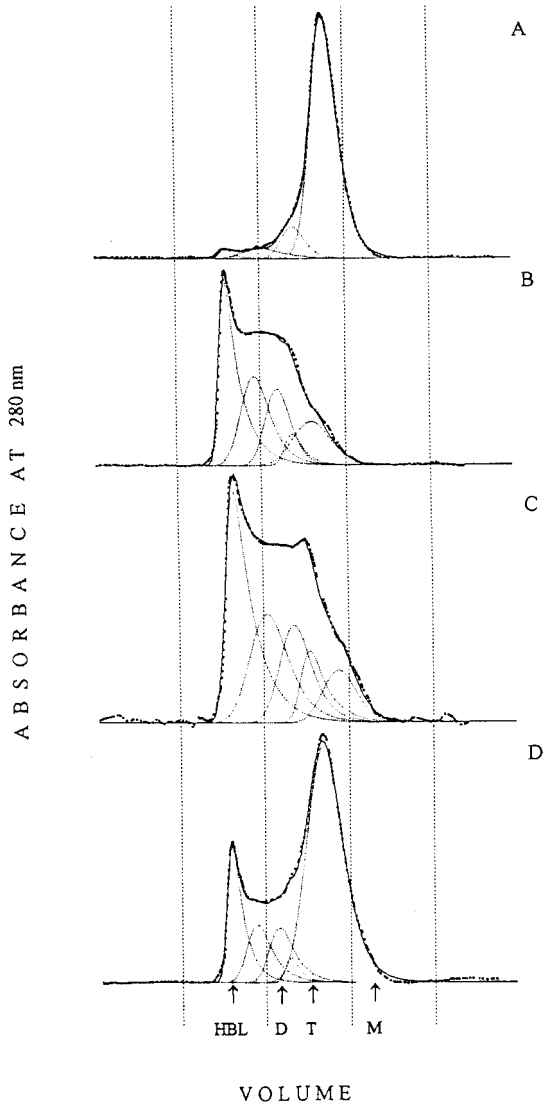

Figure 7. FPLC elution profiles at 280 nm and neutral pH, of the individual linkers: (a) L1, (b) L2, (c) L3 and (d) L4. The elution profiles were deconvoluted as sums of EMG functions. The approximate molecular masses corresponding to the elution volumes of the peaks are provided in Table 5.

Table 5. Molecular masses and multimes observed in FPLC profiles of linker subunits

Linker subunits	Calculated mass (kDa) (multiple of subunit mass)[a]								
L1 + L2 + L3 + L4	>660		330 (12)	180 (6.4)			90 (3.2)	50 (1.8)	
L1		500 (18)		200 (7.2)			81 (2.9)	38 (1.4)	
L2		470 (15)		230 (7.2)		120 (3.7)	87 (2.7)	54 (1.7)	
L3		440 (18)		190 (7.6)		93 (3.7)		58 (2.3)	28 (1.1)
L4		470 (20)	250 (10)		130 (5.4)	87 (3.6)		44 (1.8)	
L1 + L4		580 (22)	290 (11)	190 (7.2)	140 (5.3)	100 (3.8)		50 (1.9)	
L1 + L2 + L4	>660			180 (6.3)				54 (1.9)	
L1 + L3 + L4	>660			190′ (7.4)		110 (4.3)		62 (2.4)	
Peak A	>660			220 (7.9)		110 (3.9)		50 (1.8)	
Peak B				220 (7.9)		120 (4.3)		62 (2.2)	
Peak C								71 (2.5)	31 (1.1)
Average multimer		18	11	6-8	~5	3.9	2.9	2.0	

Corresponding to the elution volumes of peaks identified by deconvolution of FPLC profiles as sums of EMG functions.

[a] Calculated from the linear regression Log Mass = $4.17 - 1.34(V_e/V_o)$. In parentheses is the mass divided by the weighted mean mass, 27.9 kDa for L1 + L2 + L3 + L4, 26.4 kDa for L1 + L4, 28.7 kDa for L1 + L2 + L4, 25.8 kDa for L1 + L3 + L4 and 27.9 kDa for peaks A, B and C, based on 27.6 kDa for L1, 32.1 kDa for L2, 24.9 kDa for L3 and 24.1 kDa for L4.

corresponding approximately to the three complexes above, produced similar yields of HBLs and similar kinetic results that were experimentally indistinguishable from the other results (Table 4). Hence, the presence of complexes larger than dimers appears to have no effect on HBL reassembly.

Possible globin-binding domain in linkers

Although only the amino acid sequence only of L1 is available (Suzuki & Riggs, 1993), the cDNA sequences of the other three linkers have been determined (A. Riggs, personal communication). The linker chains from *Lumbricus* and other HBL Hbs share a single 39 residue cysteine-rich domain (CRD) in the N-terminal moiety, C-X_6-C-X_6-C-X_6-C-D-G-X_2-D-C-X_4-D-E-X_4-C, with overlapping disulfide bond connectivities similar to the CRDs found in members of the scavenger receptor cysteine-rich superfamily, including the LDL receptor protein family and other diverse proteins such as complement factors, the proteoglycan perlecan and renal glycoprotein GP3. The structure of the ligand-binding CRD of the seven found in LDL receptor protein has been determined (Blacklow & Kim, 1996; Fass *et al.*, 1997): it requires Ca(II) for folding and has a high Ca(II) affinity, ~70 nM. The latter property is likely shared by the *Lumbricus* linkers thus explaining the requirement for Ca(II) in HBL reassembly. Furthermore, it appears likely that the CRD of the linkers represents the domain that binds the T subunits in the D subassembly.

Table 6. Oxygen binding parameters of *Lumbricus* Hb, dodecamer and reassembled HBLs

Protein	P_{50}, torr	n_{50}
Native HBL Hb	11.8 ± 1.3	3.3 ± 0.3
Dodecamer	14.6	2.1
	12.1 ± 0.2[a]	2.1 ± 0.2[a]
HBL[D + L1 + L2 + L3 + L4]	13.0	2.6
	13.7[b]	1.6[b]
HBL[D + L1 + L2 + L4]	15.5	1.8
HBL[D + L1 + L3 + L4]	12.2	1.4
HBL[D + L2 + L3]	14.2	2.5
HBL[D + L1 + L4]	12.7	1.7

Reassembly of all the HBLs was carried out by method (A) in 10 mM Ca(II), except where noted.

[a] Dodecamer obtained by dissociation of the Hb in 4 M urea at neutral pH 7.

[b] HBL reassembled by method (B) in 10 mM Ca(II).

A model of linker-mediated HBL assembly

Our findings that linkers are structurally interchangeable coupled with the structural similarity of reassembled HBLs to the native Hb (J.N.L. *et al.*, unpublished results) imply that each linker has separate globin and linker-binding domains. Since the T but not M globin subunits can form reassembled HBL structures (Kapp *et al.*, 1987; De Haas *et al.*, 1997), the linkers must bind to each of the three T subunits in D. Given that 3D reconstructions suggest a common quaternary structure for all the HBL complexes examined, we propose a general model of HBL assembly, shown schematically in Figure 8. The first stage comprises the binding of one L and an L dimer to D to give a subassembly DL_2L (top) or the binding of three L dimers to two Ds to provide the subassembly DL_2L-LL_2D (bottom), or both. The key assumption is that the binding of a linker dimer (but not monomer) to two of the three T subunits in D causes conformational alterations (steps (2) and (3)) leading to the formation of complementary binding sites permitting lateral association of D. It is well known that homo- as well as heterodimerization of protein ligands bound to a target macromolecule can lead to conformational alterations in the latter. Given the 3-fold symmetry of D, lateral self-association of DL_2L (step (4)) and of DL_2 L-LL_2 D (step (5)) must result in the formation of a hexagonal structure, an HBL in the latter case and a hexagonal subassembly $(DL_2L)_6$ in the former case, which could then dimerize to an HBL (step (6)). Our early bracelet model of the HBL structure, with twelve Ds tethered to a linker complex (Vinogradov *et al.*, 1986) provided no rational explanation for its

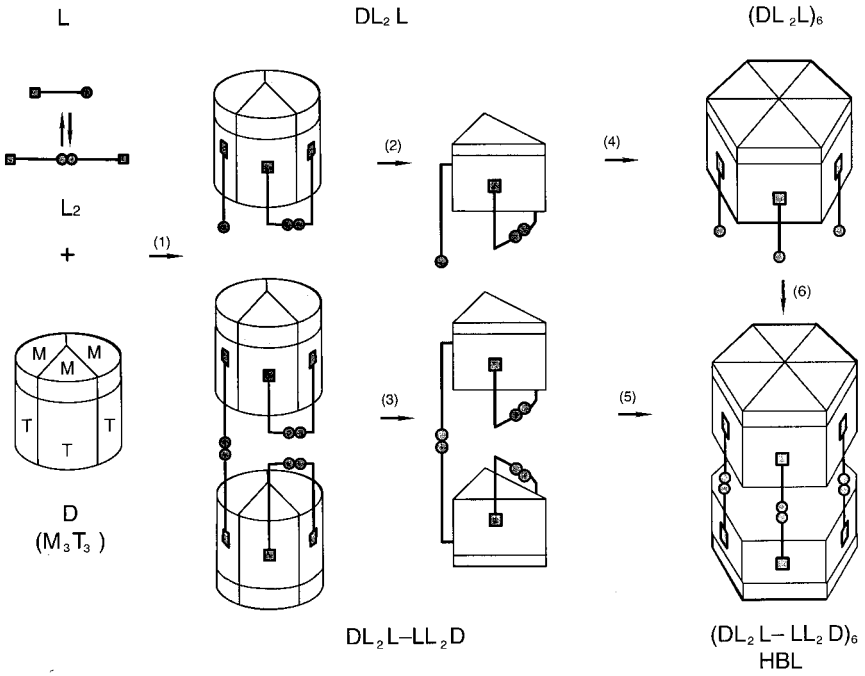

Figure 8. Schematic model of HBL assembly from D subassemblies and L subunits. Each D consists of three trimer (T) subunits with three monomer (M) subunits on top, consistent with previous findings (De Haas *et al*., 1997). Each L is represented as having a globin-binding domain (square) and a linker-binding domain (circle). The initial stage is the binding of an L monomer and an L dimer to D to form a subassembly DL_2L (top) or the binding of three L dimers to two Ds to form a subassembly DL_2L-LL_2D (bottom), or both. We propose that the binding of an L dimer to D leads to conformational alteration(s) (indicated by change in shape from cylindrical to a triangular pyramid in steps (2) and (3)) resulting in the formation of complementary binding sites, permitting lateral self-association of subassemblies DL_2L and DL_2L-LL_2D (steps (4) and (5)). The 3-fold symmetry of D dictates that lateral self-association of subassemblies DL_2L and DL_2L-LL_2D must result in hexagonal structures: an HBL in the latter case and in the former case, a single layer hexamer $[DL_2L]_6$ which can then form an HBL (step (6)). Note that this model does not predict any specific structure for the central linker complex, whose formation must accompany the formation of the native HBL structure.

assembly. The model proposed above answers the basic question ''why an HBL structure?'' and explains several observations made from recent 3D reconstructions. First, it explains the twelve lateral connections c1 and c2 as consequences of lateral association of Ds. Second, it explains the six interlayer connections c4 as corresponding to the linker interlayer dimers. Third, it provides a plausible explanation for the unique lack of rotation of the two layers in the *Alvinella* 3D reconstruction (De Haas *et al*., 1997) as being probably due to a shorter c4 connection, i.e. a shorter inter-layer linker dimer. Furthermore, ESI-MS of *Arenicola* Hb has shown it to have the same globin subunits as *Lumbricus* Hb and two linker chains existing only as disulfide-bonded homodimers (Zal *et al*., 1997), thus preserving the linker dimerization necessary for HBL formation.

The foregoing model makes no predictions concerning the central linker complex. The accompanying article (Taveau *et al*., 1999) presents convincing evidence that there are 42 linkers instead of 36 in the native *Lumbricus* Hb, with the six additional linkers being part of the central complex. Work is proceeding on the incorporation of the lateral association model proposed above within the general pathways of assembly proposed by Taveau *et al*. (1999).

Conclusion

The results obtained in this study have demonstrated that: (i) Ca(II) is a requirement for HBL reassembly: (ii) the four linker subunits of *Lumbricus* Hb are structurally interchangeable *vis á vis* reassembly to an HBL structure similar to that of the native Hb; (iii) the extent of reassembly with individual linkers increased in the order $L1 \ll L2 \approx L3 < L4$; (iii) reassembly was favored when more than one linker was available and was most extensive in the presence of all four linker subunits, (iv) HBL reassembly had a half-time of approximately 0.5 hour; and (v), the extent and kinetics of reassembly are not dependent on the state of aggregation of the linkers.

Materials and Methods

Materials

L. terrestris Hb was prepared as described (Vinogradov & Sharma, 1994) in 0.1 M Tris-Cl buffer (pH 7.0), 1 mM EDTA. The dodecamer subassembly

D was isolated from partially dissociated Hb obtained by exposure to 4 M urea (Sigma) or 10-30 mM SiW or AsW, and the passage through a 2 cm × 30 cm column of a mixed-bed ion-exchange resin Amberlite MB-1 (Sigma), followed by FPLC. The Hb and D concentrations were determined from the absorbance of the cyanmet forms at 540 nm, using the extinction coefficients 0.442 and 0.656 ml mg^{-1} cm^{-1}, respectively (Vinogradov & Sharma, 1994; Vinogradov *et al.*, 1977). The linker fractions were isolated by RP-HPLC of the Hb, subjected to water pump evaporation to remove the acetonitrile and concentrated by ultrafiltration in a 200 ml model 8200 stirred concentrator (Amicon, Inc.) equipped with a PM10 membrane. The resulting stock solution (~15 mg/ml) was either used directly for reassembly or brought to neutral pH by dialysis first against 0.1 M Tris-Cl buffer (pH 8.7) (dilution factor ~500) and then against 0.1 M Tris-Cl buffer (pH 7.0). The linker concentrations were estimated from a calibration of RP-HPLC with known amounts of Hb, assuming a molar ratio D:L = 12:36.

Reversed-phase chromatography

RP-HPLC was performed using a Synchropak C_{18} RP-P column, 4.6 mm × 260 mm (Micra Scientific Inc.), an L205 binary pump (Perkin Elmer) with a Hitachi 250 dual wavelength detector, employing linear gradients of water/acetonitrile in 0.1 % trifluoroacetic acid, with flow rates of 2 ml/minute. The absorbance of the eluate was monitored at 220 nm.

Analytical gel filtration and fitting of elution profiles

Low pressure, isocratic gel filtration was carried out at room temperature (20(±2)°C) employing an FPLC system (Pharmacia) and 1 cm × 30 cm columns of Superose S12 or S6 (Pharmacia). Flow rate was 0.5 ml/minute and the eluate was monitored at 280 nm. A constant amount of protein in a constant sample volume, ~800 ug/200 ml, was loaded each time. The elution curves were acquired using the Easyest System 8 (Keithley Instruments, Inc.) and an IBM PC. The digitized data was fitted to a sum of several EMG functions employing least squares minimization (Peak Fit version 2.0, Jandel Scientific; Sharma *et al.*, 1996). The column was calibrated with a set of standard proteins using the relationship $\log(\text{molecular mass}) = a - b(V_e/V_o)$, where V_e is the elution volume and V_o is the elution volume of Blue Dextran.

Reassembly of HBL structures from dodecamers and linker subunits

Reassembly was carried out using two methods. In method (A), 250-500 ml of the linker stock solution (ca15 mg/ml) pH ~2.2 was added to 3-6 ml of D (3-5 mg/ml) in 0.5 M Tris-Cl (pH 7), containing 0-10 mM Ca(II), in a 10 ml model 8400 concentrator (Amicon, Inc.) equipped with a PM10 membrane. The volume of the reassociation mixture was reduced to ~0.5 ml until the total protein concentration reached 30-40 mg/ml and the solution kept at 4 °C. In method (B), linkers in 0.1 M Tris-Cl buffer (pH 7), were added to the solution of D in the same buffer containing 0-10 mM Ca(II) at a total protein concentrations of 30-40 mg/ml. Aliquots of protein taken at time intervals from ten minutes to 300 hours were subjected to FPLC to follow the course of the reassembly. The molar ratio of L:D in the reassociating mixtures was kept at ~3.3:1.

Kinetics of HBL reassembly

The area of the HBL peak expressed as percentage of total area was plotted *versus* time and fitted to sums of two exponentials:

$$f(t) = a_1\{1 - \exp[-k_1 t]\} + a_2\{1 - \exp[-k_2 t]\}$$

employing Sigma Plot 3.0 software (Jandel Scientific) and the Marquardt-Levenburg method. The fit was judged acceptable only in the absence of systematic trends in the plot of residuals with time.

STEM imaging and mass measurement of unstained protein

The mass measurements were performed at the STEM facility at Brookhaven National Laboratory (Wall & Hainfeld, 1986). Preparation of the unstained specimens with TMV fibers as internal mass standards was carried out as described by Kapp *et al.* (1990). The STEM was operated at 40 kev, a dose level <10 e/0.1 nm^2 and a resolution of ca 0.2 nm. Two computer programs were used for analysis of STEM data, an interactive program and an automatic program as described (Martin *et al.*, 1996a).

Oxygen binding measurements

Oxygen binding equilibria were measured at 25 °C in 50 mM Tris-Cl buffer, using a modified gas diffusion chamber (Weber, 1992). The data were analyzed in terms of the two-state MWC equation as described (Weber *et al.*, 1995). The P_{50} and n_{50} were interpolated from linear plots of $\log[S/(1-S)]$ *versus* $\log P_{O_2}$ for S (fractional saturation) between 0.3 and 0.7.

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