Ligand Linked Assembly of *Scapharca* Dimeric Hemoglobin*

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The assembly of *Scapharca* dimeric hemoglobin as a function of ligation has been explored by analytical gel chromatography, sedimentation equilibrium, and oxygen binding experiments to test the proposal that its cooperativity is based on quaternary enhancement. This hypothesis predicts that the liganded form would be assembled more tightly into a dimer than the unliganded form and that dissociation would lead to lower oxygen affinity. Our experiments demonstrate that although the dimeric interface is quite tight in this hemoglobin, dissociation can be clearly detected in the liganded states with monomer to dimer association constants in the range of 10^8 M^-1 for the CO-ligated state and lower association constants measured in the oxygenated state. In contrast, the deoxy dimer shows no detectable dissociation by analytical ultracentrifugation. Thus, the more highly hydrated deoxy interface of this dimer is also the more tightly assembled. Equilibrium oxygen binding experiments reveal an increase in oxygen affinity and decrease in cooperativity as the concentration is lowered (in the µM range). These experiments unambiguously refute the hypothesis of quaternary enhancement and indicate that, as in the case of human hemoglobin and other allosteric proteins, quaternary constraint underlies cooperativity in *Scapharca* dimeric hemoglobin.

To perform biological activities efficiently, protein molecules have often evolved mechanisms to couple functionally independent subunits. Such cooperative activity is known to be involved in the regulation of many protein functions including enzyme activity (1, 2), gene expression (3, 4), and oxygen transport (5, 6). Much of our understanding of this process has come from studies of mammalian hemoglobins (5, 6), for which cooperativity manifests itself as a stepwise increase in oxygen affinity (19), a clear example of quaternary enhancement.

A particularly simple system for exploring cooperative protein function is the dimeric hemoglobin found in the blood clam *Scapharca inaequivalvis*, which binds oxygen with a Hill coefficient of 1.5 and shows no change in oxygen affinity or cooperativity as pH varies from 5.5 to 9.0 (7). Although the tertiary structure of the subunits is similar to those of mammalian hemoglobins, the assembly into a cooperative complex is radically different (8). High resolution crystal structure analysis of *Scapharca* dimeric hemoglobin (HbI) 1 has shown that ligand binding is coupled with significant tertiary rearrangements but very small quaternary changes in the relative subunit dispositions (9, 10).

Intersubunit communication depends upon coupling ligand binding with interactions between subunits. As Wyman (11) recognized nearly 50 years ago, cooperative interaction energy may be present as stabilizing energy between unliganded subunits (quaternary constraint; Ref. 12) or liganded subunits (quaternary enhancement; Ref. 13). These alternate conditions can be distinguished either by measuring the strength of the subunit interface as a function of ligation or by following ligand affinity as subunit dissociation occurs. In the case of quaternary constraint (12), the deoxygenated oligomer would be more tightly assembled than the liganded form, and, since the deoxy assemblage acts to lower oxygen affinity, subunit dissociation would result in increased oxygen affinity. In the case of quaternary enhancement (13), the opposite situation exists: the liganded complex would be more tightly assembled, and lower affinity would result from dissociation into subunits.

In human hemoglobin, cooperativity results primarily from quaternary constraints in which the tight deoxy assemblage lowers the intrinsic subunit oxygen affinity, and binding of subsequent ligands leads to a stepwise reduction of these constraints (14). Analysis of equilibrium binding data has suggested that the fourth ligand is bound with higher affinity than isolated chains, an effect termed *quaternary enhancement* (13). This interpretation has, however, been questioned on the basis of kinetic experiments that appear in conflict with the equilibrium results (see Refs. 15–18). In the case of the mutant human hemoglobin, Hb Ypsilanti (δ99 Asp → Tyr), assembly of αβ dimers into noncooperative tetramers results in an 85-fold increase in oxygen affinity (19), a clear example of quaternary enhancement.

A key structural change upon ligand binding to *Scapharca* HbI is the extrusion of a phenylalanine (97) from the heme pocket into the subunit interface where it displaces a number of interfacial water molecules (Fig. 1). In deoxy-HbI packing of the Phe-97 side chain in the heme pocket appears to be largely responsible for the low oxygen affinity of this state, and its disposition is central to the proposed cooperative mechanism (9). The ligand-linked movement of Phe-97 and disruption of interface water molecules led to the hypothesis that cooperat-

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1 The abbreviations used are: HbI, *Scapharca* dimeric hemoglobin; HbI-CO, HbI liganded with carbon monoxide; HbI-O_2, HbI liganded with molecular oxygen; ISS, ideal single species; Mz, Z-average molecular mass.
activity in *Scapharca* HbI could result from quaternary enhancement rather than quaternary constraint. Binding of a ligand to an isolated subunit would require extrusion of Phe-97 into bulk water rather than the intersubunit interface. Since in the dimeric interface Phe-97 can interact with the methyl group of Thr-72 (from the second subunit), Royer *et al.* (8) proposed that extrusion of Phe-97 would be more favorable in the dimer than in isolated subunits. This suggests that isolated subunits would bind oxygen with lower affinity than would subunits assembled in a dimer. Then, by the linkage between ligand binding and subunit association, a liganded dimer would be more tightly associated than an unliganded dimer. This appeared reasonable given the additional water molecules in the deoxy interface whose ordering is expected to be entropically unfavorable in contrast with the liganded interface in which additional hydrophobic interactions are present involving Phe-97.

The present study was undertaken to test the hypothesis that quaternary enhancement underlies cooperativity in *Scapharca* dimeric hemoglobin. If this hypothesis were true, the liganded hemoglobin would be more tightly assembled than the unliganded hemoglobin, and oxygen affinity would decrease upon subunit dissociation. Our results demonstrate that the reverse is true: *Scapharca* dimeric hemoglobin, like human hemoglobin, is more tightly assembled in the unliganded state than in the liganded state, and subunit dissociation leads to an increase in oxygen affinity.

**EXPERIMENTAL PROCEDURES**

**Sample and Buffer Preparation**—Experiments were performed both with *Scapharca* HbI derived from the clam, kindly provided by Dr. E. Chiancone, University of Rome, and with bacterially expressed recombinant protein (20). No differences in assembly were noted for samples from these two different sources. The standard buffer consisted of 0.1 M Tris with 1 mM Na2EDTA, titrated to pH 7.2 with concentrated HCl. Titration and pH measurement were carried out at 20 °C for chromatography experiments and at room temperature (23 °C) for centrifugation and oxygen binding experiments. Tris was purchased from Sigma; EDTA was obtained from Fisher Scientific.

**Gel Chromatography**—Analytical gel chromatography experiments were carried out with CO-saturated HbI and buffers using a Sephadex G-75 column (0.9 × 40 cm) thermostatted at 20 °C. For these experiments, the CO-ligated derivative was chosen to minimize the effects of heme oxidation. The void and internal volumes of the column were determined with blue dextran and glycyl-glycine, respectively, and the quality of the column packing was determined from experiments on a variety of nonassociating proteins.

Large zone chromatography experiments (21) were carried out at a variety of HbI concentrations. For each experiment, ~25 ml of hemoglobin solution was prepared in standard buffer, filtered (Gelman 0.45 μm) and loaded onto the Sephadex G-75 column. Concentration measurements were carried out spectrophotometrically, using an extinction coefficient of 0.202 (μM heme)−1 for CO-ligated HbI (7). The flow rate of the column was controlled accurately at ~15 ml/h using a peristaltic pump. Continuous absorbance measurements in the Soret region were made using a Shimadzu UV 160U spectrophotometer and recorded on an associated analog recorder.

From experiments at each concentration, the elution volume was determined from the centroid position of the leading boundary of the column profile. The elution volume (V) was used to calculate the weight-average partition coefficient (s~w~) by the relation s~w~ = (V − V~0~)/V~i~, where V~c~ and V~i~ are the void and internal volumes of the column, respectively.

**Analysis of Gel Chromatography Experiments**—For HbI, which undergoes monomer-dimer assembly

\[ s_w = s_1 + f_d (s_1 - s_2) \]  
(Eq. 1)

where s~w~ is the weight-average partition coefficient; s~1~ and s~2~ are the partition coefficients for monomer and dimer, respectively; and f~d~ is the weight fraction of monomer in solution. The weight fraction monomer (f~d~) is given by

**FIG. 1. Scapharca HbI interface water molecules.** These diagrams depict the structures of (panel a) deoxy-HbI at 1.6 Å resolution (PDB entry code 3SDH) (9) and (panel b) HbI-CO at 1.4 Å resolution (PDB code 4SDH) (9). Included is a ribbon diagram showing the tertiary structure of each subunit, bond representations for the heme group, and Phe-97 side chain and spheres representing the approximate van der Waals radii of oxygen atoms for core interface water molecules. Note the cluster of 17 ordered water molecules in the interface of deoxy-HbI for which Phe-97 is packed in the heme pocket. Upon ligation, by either CO or O_2, Phe-97 is extruded into the interface and disrupts this water cluster, expelling six water molecules from the interface. This plot was produced with the program MOLSCRIPT (31).
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Physiological studies of shellfish hemoglobins, particularly Scapharca dimeric hemoglobin (HbI), have been of interest in the context of understanding the evolution of hemoglobin structures. HbI is a dimeric hemoglobin composed of two monomer subunits that can exist in a monomeric or dimeric state, with the dimeric state being more prevalent at lower concentrations.

**Chemical and Physical Properties**

The equilibrium association constant for monomer-dimer assembly, $K_a$, is given by:

$$K_a = \frac{[\text{dimer}]}{[\text{monomer}]^2}$$

where $C_i$ is given in units of molar heme (monomer), and $K_a$ is the equilibrium association constant for monomer-dimer assembly.

**Data Analysis**

Data analysis was carried out using a modified least squares method. The analysis program uses a modified Gauss-Newton algorithm to obtain parameter values that correspond to a minimum in variance. For experiments on HbI, an independent estimate of the monomer partition coefficient was obtained from experiments using myoglobin (horse heart). This value was fixed to the experimental value of 0.4204 in all data analysis. An estimate of $\sigma_2$ (dimer partition coefficient) of 0.2828 ± 0.0047 was obtained from the data analysis.

**Results of the Sedimentation Equilibrium Experiments**

Analysis of Centrifugation Data—The concentration distribution at equilibrium was analyzed using a nonlinear least squares program to test various models. A model assuming an ideal single species (ISS) was used to fit subsets of data from different sample loading concentrations and speeds of rotation. This provides the value of the molecular mass if an ideal species is present and $M_z$ if the material is nonideal or a mixture of species. Many other models were then examined: most notable for this experiment is the monomer-dimer model, which yields the molecular mass ($M_z$) of the associating monomer and its association constant. Criteria for goodness of fit is that the root mean square error be small (usually less than 0.01 AU, or about 1 µg/ml) and that it be random (small systematic error in the residuals).

Calculation of molecular mass and equilibrium constant from the pattern of concentration with radius in the cell requires the knowledge of the partial specific volume of the protein ($\upsilon$) in its solvent and the density of the solvent ($\rho$). Based on the amino acid sequence, the partial specific volume was calculated as 0.745 cm$^3$/g. The density of the solvent does not include the heme group, which would be expected to increase its value. Values of $\rho$ for human oxy- and deoxyhemoglobin have been reported to be 0.746 and 0.749, respectively, at 20°C and 0.90 m NaCl (28). For the results reported here, a value of 0.75 was used. The density of the solvent was measured to be 1.004 g/cm$^3$ with a Paar DMA 602 density meter at 20°C. (This was corrected to 1.006 and 1.003 g/cm$^3$ at 1 and 25°C, respectively.)

Oxygen Binding Measurements—Oxygen binding to HbI at low concentrations (0.2–8 µM) was followed by tonometric measurements in the Soret region. Samples were deoxygenated at 4°C by flushing with nitrogen and were then equilibrated at 23°C. Air was introduced with the use of a 2.5-ml gas-tight Hamilton syringe through a rubber septum and allowed to equilibrate at 25°C for 10 min. Absorption readings were recorded at 416, 422, 424, and 434 nm. Significant loss of material was observed with samples at low concentration apparently due to adsorption to the glass tonometer. This problem was eliminated by coating the glass with bovine serum albumin (3 mg/ml) for 30 min prior to oxygen binding measurements. To minimize heme oxidation, the tonometer was also coated for 10 min with a solution containing an enzymatic reduction system (27). Following rinsing, residual remaining components were sufficient to eliminate problems with oxidation. This method was used to minimize the strong absorption in the Soret region by catalase, which would obscure the hemoglobin contribution at very low concentrations.

**RESULTS**

Large zone gel chromatography experiments were performed on HbI-CO with solutions ranging in concentration from approximately 5 to 0.05 µM heme. Although the dimeric assemblage is quite stable, a clear increase in elution volume results as concentration is lowered, indicating detectable dissociation in this concentration range. Fig. 2 shows the weight average partition coefficients plotted as a function of HbI concentration along with a theoretical curve based on a best fit of the equilibrium constant and end points. This fit yielded a monomer-dimer association constant ($K_a$) of $7.6 \times 10^4$ M$^{-1}$ (Table I).

Sedimentation equilibrium experiments were performed in several runs of liganded (O$_2$ or CO) HbI and two runs with unliganded HbI. For each run samples were loaded at three different protein concentrations and centrifuged at two different speeds, providing a total of six data sets/run. The centrifugal depletion effect at the meniscus and concentrating effect at the bottom of the cell result in a range of concentrations studied from lower than 0.06 µM heme to as high as 10 µM heme.

Results of the sedimentation equilibrium experiments are presented in Table I. Values are included for the $M_z$ based on the ISS model and for association constants based on a monomer-dimer equilibrium. Experiments were performed meas-
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uring absorbance at two wavelengths to observe any wavelength dependence of molecular mass which could indicate the presence of oxidized hemoglobin (28). The values at the two wavelengths were found to be within the confidence interval of the measurements, therefore averages of the values at both wavelengths are reported in Table I. For liganded species, fits obtained with the monomer-dimer model were significantly better than the fits obtained with the ISS model and far better than any of the other models considered. Although this can be seen from the lower degree of systematic error shown in Fig. 3, a more demanding measure of the superiority of the monomer-dimer model fit to the data is that the ratio of the variance of the two models favored the monomer-dimer fit over the ISS model to greater than a 99.7% confidence limit. In contrast to the liganded species, ultracentrifugation of the deoxygenated samples showed no measurable dissociation and indicated Mz values close to the molecular mass (33.1 kDa) of the dimer. In one experiment (labeled reoxy) following a run of deoxy sample, the cell was opened and allowed to equilibrate with oxygen, then resealed and centrifuged again. This experiment is in good agreement with the other oxyhemoglobin runs (Table I).

As is clear from Table I, there is good agreement for the monomer-dimer association constant obtained by gel chromatography and sedimentation equilibrium for HbI-CO. However, lowerassociation constants are observed for the oxygenated samples compared with the HbI-CO. This may reflect a real difference between HbI-CO and HbI-O₂, despite their very similar structures (10), or it could result from a slight oxidation of the heme iron which was not detected. (Oxidation of HbI results in a hemichrome species that shows extensive dissociation, with an association constant of about 3 × 10⁸ M⁻¹ at pH 7.0 (29).)

Thermodynamic coupling between ligation and subunit dissociation (14) predicts that given a tighter assemblage in the deoxy state compared with the oxy state, oxygen affinity of HbI should increase upon subunit dissociation. To test this prediction, we carried out oxygen binding experiments on solutions with hemoglobin concentrations between 0.2 and 8 μM heme. In this concentration range, significant dissociation of liganded species is expected, based on the chromatography and sedimentation results discussed above. As concentration is lowered, a clear increase in oxygen affinity is observed as indicated by the decrease in p₅₀ (Fig. 4). The observed increase in affinity is coupled with a decrease in cooperativity as evidenced by the Hill coefficient declining from 1.45 at 8 μM heme to 1.25 at 0.2 μM heme, indicative of partial dimer dissociation.

**DISCUSSION**

Our experiments demonstrate that Scapharca HbI is more tightly assembled into a dimer in the absence of a heme ligand than it is once oxygen or carbon monoxide is bound. Consequently, the energetic coupling of subunit assembly and ligand binding, depicted in Fig. 5, requires that monomers will bind oxygen with higher affinity than dimers. This has been verified by oxygen binding measurements at low hemoglobin concentration which show increased oxygen affinity as a result of partial subunit dissociation. Thus, as in the case of human hemoglobin and other allosteric proteins, quaternary constraint underlies the cooperative mechanism.

An important ramification of these experiments is that the more hydrated interface of deoxy-HbI is also the form with the greater subunit interface stability. It is relevant to note that no large quaternary structural changes occur upon ligand binding to HbI. Thus, differences in the stability of the dimer can be interpreted in light of the rather localized ligand-linked structural changes at the subunit interface. As shown in Fig. 1, the high resolution crystal structures reveal the presence of 17 well ordered water molecules arranged in a cluster in the core of the deoxy interface compared with 11 water molecules in HbI-CO or HbI-O₂. This crystallographic observation has recently been extended by measurements of oxygen binding as a function of osmotic pressure which indicate that oxygen binding is coupled with the release of approximately six water molecules/heme globin dimer (30). Significantly, the water molecules in the deoxy interface are more well ordered and provide more favorable hydrogen bonding than those in the liganded interfaces (9, 10). Our present results suggest that these water molecules could contribute to interface stability, despite the entropic cost of their ordering. Removal of just two hydrogen bonds from the

![Image](https://example.com/image.png)

FIG. 2. Concentration dependence of elution of HbI-CO by gel chromatography. Weight-averaged partition coefficients (σ_w) are plotted as a function of HbI concentration (μM heme). The curve represents the best fit equilibrium constant and end points.

**Table I**

| Method                  | Ligand | Temperature | Mz  | ²Kₐ [95% confidence limits] |
|-------------------------|--------|-------------|-----|-----------------------------|
| Chromatography          | CO     | 20          | 32.7 ± 1.3 | 7.6 × 10⁶ [4.1–12.1 × 10⁶] |
| Ultracentrifugation (XLA)| CO     | 20          | 32.0 ± 1.5 | 2.2 × 10⁸ [0.4–13.3 × 10⁸] |
| Ultracentrifugation (XLA)| O₂     | 1           | 28.7 ± 1.0 | 1.1 × 10⁹ [0.3–4.4 × 10⁹] |
| Ultracentrifugation (E) | O₂     | 1           | 29.1 ± 0.8 | 3.3 × 10⁸ [2.2–4.9 × 10⁸] |
| Ultracentrifugation (E) | O₂     | 25          | 25.8 ± 0.6 | 4.9 × 10⁸ [1.6–14.8 × 10⁸] |
| Ultracentrifugation (XLA)| Deoxy  | 1           | 34.5 ± 0.6 | 1.5 × 10⁹ [1.2–1.8 × 10⁹] |
| Ultracentrifugation (XLA)| Deoxy  | 1           | 34.5 ± 0.5 | No dissociation |
| Ultracentrifugation (XLA)| Reoxy  | 1           | 30.3 ± 1.0 | 5.5 × 10⁸ [2.5–12.2 × 10⁸] |

* The reported sedimentation values were converged upon using a fixed value of 33 kDa for the molecular mass of the dimer.
protein to the deoxy water cluster by mutation of Thr-72 to Val results in the loss of two water molecules and a 40-fold increase in oxygen affinity (30). This suggests a direct link between the integrity of the interface water cluster and maintenance of the low affinity deoxy conformation, further supporting a contribution by these water molecules to interface stability.

Our experiments also reveal that isolated subunits of HbI bind oxygen with higher affinity than those in the dimeric assemblage. The high resolution crystal structures reveal a number of interactions between deoxy subunits which could stabilize their low affinity conformations. A key determinant of oxygen affinity appears to be the conformation of Phe-97 whose side chain packs tightly in the heme pocket in the deoxy state but is extruded into the subunit interface upon ligation (9). When packed in the heme pocket, Phe-97 is thought to reduce oxygen affinity by restricting movement of the iron into the heme plane and by lengthening a hydrogen bond involving the proximal histidine (9). Coupled with the deoxy disposition of Phe-97 is a sharp bend of the F helix and a displacement of the heme group toward the subunit interface. The dimeric assemblage of deoxy-HbI appears to contribute to the stability of these conformations by providing hydrogen bond partners for main chain atoms of the bent F helix (from water molecules) and the heme propionate groups (from Lys-96, Asn-100, and water molecules). Thus, the interactions present in the subunit interface appear important for the stability of the low affinity conformation, which is consistent with our present results demonstrating increased oxygen affinity as partial subunit dissociation occurs.

The assembly of subunits of Scapharca HbI into a highly stable dimer makes complete elucidation of the thermodynamic linkage between assembly and ligand binding difficult. The results presented here provide estimates for the dissociation constants of liganded forms, but the lack of any observable

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**FIG. 3. Residuals of the fit for sedimentation of HbI-CO.** This plot shows the residuals of the fit of nine sets of sedimentation equilibrium data for HbI-CO at 30 and 40 krpm at 1 °C. Panel a, ISS model; panel b, monomer-dimer model. Note the better fit of the data with the monomer-dimer model, which is most evident at the low concentrations found at lower radii (R) where greater proportions of monomer will be present.

**FIG. 4. Concentration dependence of oxygen affinity for HbI.** The p50 values from equilibrium oxygen binding measurements are plotted as a function of concentration (µM heme). The decrease in p50 is coupled with a decrease in cooperativity as evidenced by the Hill coefficient, which is estimated at 1.45 at 8.3 µM heme and 1.25 at 0.19 µM heme. In this range significant dissociation of the liganded species would be predicted from the sedimentation and chromatography experiments. For instance, using the association constant of 7.6 × 10^7 M^-1 (obtained for HbI-CO by gel chromatography) 17% monomer would be expected at a concentration of 0.19 µM, and 3% monomer would be predicted for 8.3 µM heme.

**FIG. 5. Linkage scheme for oxygenation and assembly of HbI.** The thermodynamic linkage between oxygen binding and assembly is shown schematically along with approximate values for the free energy for certain parameters. The free energy for binding the first and second oxygen molecules to the HbI dimer is calculated from the oxygen binding data at 20 °C of Ikeda-Saito et al. (32). The estimate for assembly of the liganded dimer is taken from the dimer association constant obtained for HbI-CO at 20 °C, although the free energy for assembly of HbI-O_2 may be higher. Assembly of the deoxy dimer is significantly more favorable than in the liganded form, thus the free energy must be significantly less than -10.5 kcal/mol. Likewise, binding of oxygen to a dissociated monomer is more favorable than to subunits assembled in the dimer.
dissociation of the deoxygenated form precludes an estimate of its stability. As well, the strength of the dimeric interface prevents an accurate estimate of the oxygen affinity of dissociated monomers. To address these issues, we intend to use site-directed mutagenesis of HbI (20) to probe the assembly reaction.

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