The subunit interface of the homodimeric hemoglobin from *Scapharca inaequilvalvis*, HbI, is stabilized by a network of interactions that involve several hydrogen-bonded structural water molecules, a hydrophobic patch, and a single, symmetrical salt bridge between residues Lys-30 and Asp-89. Upon mutation of Lys-30 to Asp, the interface is destabilized markedly. Sedimentation equilibrium and velocity experiments allowed the estimate of the dimerization constants for the unliganded \(K_{1D} = 8 \times 10^4 \text{M}^{-1}\) and for the CO-bound \(K_{1L} = 1 \times 10^3 \text{M}^{-1}\) and oxygennated \(K_{1L} = 70 \text{M}^{-1}\) derivatives. For the oxygenated derivative, the destabilization of the subunit interface with respect to native HbI corresponds to about 8 kcal/mol, an unexpectedly high figure. In the K30D mutant, at variance with the native protein, oxygen affinity and cooperativity are strongly dependent on protein concentration. At low protein concentrations (e.g., 1.2 \times 10^{-6} \text{M heme}) , at which the monomeric species becomes significant also in the unliganded derivative, oxygen affinity increases and cooperativity decreases. At protein concentrations where both derivatives are dimeric (e.g., 3.3 \times 10^{-8} \text{M heme}), both cooperativity and oxygen affinity decrease. Taken together, the experimental data indicate that in the K30D mutant, the mechanism of cooperativity is drastically altered and is driven by a ligand-linked monomer-dimer equilibrium rather than being based on a direct heme-heme communication as in native HbI.

The homodimeric hemoglobin from *Scapharca inaequilvalvis* (HbI) is characterized by an unusual assembly of the globin chains that entails the juxtaposition of the heme carrying E-F helices. Dimer stability is achieved through a hydrophobic patch, a network of hydrogen bonds that is primarily mediated by structural water molecules at the subunit interface and two symmetrically related salt bridges between residues Lys-30 and Asp-89, located at the extreme edges of the interface itself. Cooperative ligand binding occurs with minor quaternary structural rearrangements and has been ascribed to the direct communication between the two heme groups that are in close contact across the subunit interface (1). The heme propionate groups provide a direct pathway for heme-heme interaction (2–4). Ligand-linked structural changes are localized to the heme proximal side in which the phenyl ring of Phe-97 is packed closely against the proximal histidine in the unliganded species and is extruded from the heme pocket to the subunit interface in the liganded derivative. The movement of Phe-97 causes relaxation of the heme-heme intersubunit contacts and the disruption of the network of ordered water molecules at the interface. In contrast, the stereochrometry of the interface salt bridge between Lys-30 and Asp-89 does not undergo significant ligand-linked changes (2).

Cooperative oxygen binding in HbI therefore is based on a completely different mechanism with respect to the vertebrate hemoglobins where the presence of two distinct quaternary structures, a low affinity (T state) and a high affinity one (R state), is a prerequisite for heme-heme cooperation. In human hemoglobin, the transition between the two states is governed by a ligand-linked structural rearrangement of the heme pocket in one subunit that is communicated to the other subunits through the so-called \(a_1b_2\) subunit interface. The quaternary rearrangements bring about the cleavage of specific intersubunit salt bridges and thereby provide a significant enthalpic contribution to the R-T conformational transition.

A third completely different mechanism is observed in hemoglobins from lower vertebrates such as Cyclostomata, in which cooperative oxygen binding is the result of ligand-linked subunit dissociation. The behavior of lamprey hemoglobins is prototypical: the deoxygenated proteins are able to form dimers (and eventually higher order polymers), whereas the ligand-bound species are monomeric. In these proteins, the subunit dissociation process that accompanies ligand binding is also linked to the release of Bohr protons. The coupling between the two processes provides the entropic driving force for the observed cooperativity (5, 6).

In the present study, the cleavage of the salt bridge Lys-30–Asp-89 at the subunit interface of *S. inaequilvalvis* Hbl is shown to bring about a marked decrease in the stability of the dimeric assemblage, which in turn results in a radical change of the mechanism of cooperativity. The direct heme-heme interaction within the homodimer, which is at the basis of cooperative oxygen binding in the native protein, is replaced in the mutant by a ligand-linked dissociation into subunits.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strain—** *Escherichia coli* strain W3110lacIq L8 was the host used for the overexpression of recombinant Hbl (7).

**Mutagenesis—** The Lys-30→Asp mutant (K30D) was constructed using PCR techniques to introduce a GAT codon at the desired position in the synthetic gene for wild-type Hbl cloned into a pUHE21–2 plasmid containing the T7 promoter (7). The PCR amplification used the following primers: 5’-GAAAGTATTATCGGATCCGACAAAGATGGTAA-
C’- (Lys-30—Asp) and 5’-CAGCGGAGATCTACGGTGATATGGT
TAAC-3’. The resulting 255-bp product was digested with restriction enzymes BamHI and PstI and isolated using the QIAquick purification kit (Qiagen). This fragment was subcloned subsequently into the Hbl expression vector pUHE21–2 digested with BamHI and PstI. The changes in the DNA sequence were confirmed by dideoxy sequencing.

**Protein Expression**—Recombinant K30D was overexpressed in *E. coli* as described for the wild-type protein (7).

**Protein Purification**—After pelleting the cell debris at 15,000 × *g* for 60 min, the supernatant was dialyzed against 5 mM Tris- HCl buffer, pH 8, at 4 °C. After centrifugation (15,000 × *g*), the sample was loaded onto a DEAE-cellulose column pre-equilibrated with 5 mM Tris- HCl, pH 8. Recombinant K30D eluted at 30 mM Tris- HCl, pH 8. Thereafter the sample was dialyzed against 30 mM Tris- HCl, pH 8.5, and concentrated in an Amicon (Beverly, MA) 10-kDa molecular mass cut-off Centriprep concentrator to a final volume of 5–10 ml. The concentrated sample was loaded onto a 100-ml Toyopearl DEAE-650S (Tosohaas, Montgeryville, PA) column pre-equilibrated with 30 mM Tris- HCl, pH 8.5. The protein was purified further on a Sephadex G-50 gel-filtration column (Amersham Biosciences, Inc.) in 50 mM Tris- HCl buffer, pH 7.5. The purity of the protein was verified by SDS-polyacrylamide gel electrophoresis. UV-visible spectra were measured on a Jasco V-560 spectrophotometer. Circular dichroism spectra were measured on a Jasco J-710 spectropolarimeter. High dilution absorption and CD spectra were recorded in 10-cm optical path quartz cells (Hellma). Protein concentrations were determined using the extinction coefficient of the native protein, 14,300 M⁻¹ cm⁻¹ at 557 nm for the deoxygenated derivative (8).

Analytical Ultracentrifugation—All experiments were carried out at 10 °C and 30,000, 35,000, and 40,000 rpm (sedimentation equilibrium) or 60,000 rpm (sedimentation velocity) on a Beckman XLI analytical ultracentrifuge equipped with absorbance optics and an An60-Ti rotor. The protein concentration varied in the range 7–3300 M (heme). Oxygen equilibrium measurements were performed at 60°C at a spacing of 0.005 cm with 10 averages in a step scan mode. Equilibrium was checked by comparing scans up to 24 h. Data sets were edited with REEDIT (J. Lary, National Analytical Ultracentrifugation Center, Storrs, CT) and fitted with NONLIN (PC version provided by Emory Braswell, National Analytical Ultracentrifugation Center, Storrs, CT) according to Johnson et al. (9). Data from different concentrations and speeds were combined for global fitting. For fits to a monomer-dimer association scheme, the monomer molecular weight was fixed at the value determined from the amino acid sequence (16.5 kDa). As a control, absorbance spectra of all samples were measured in the ultracentrifuge cell before and after the run. In sedimentation velocity experiments, data were collected every 4 min at 410, 500, or 540 nm, depending on protein concentration, at a spacing of 0.005 cm with three averages in a continuous scan mode. Data were analyzed with DCDT (PC version provided by Walter Stafford, Boston Biomedical Research Institute, Boston, MA), which provides weight average sedimentation coefficients (10). The sedimentation coefficients were corrected to r₂,₀ using standard procedures. The data were fitted to a monomer-dimer equilibrium as described previously (11).

**Oxygen Equilibrium Measurements**—Oxygen equilibrium was measured in a tonometer according to the method of Rossi-Fanelli and Antonini (12). The measurements were carried out over the protein concentration range 12–3300 M (heme). Oxygen equilibrium measurements at protein concentrations higher than 500 M were carried out in a tonometer with a 0.25-mm path length cell. Full oxygen saturation was achieved under 1 atm of O₂ gas. To account for the presence of oxygen-linked monomerization equilibria, oxygen binding curves as a function of protein concentration were fitted according to Scheme 1. This scheme reduces to the following equations (Eqs. 1 and 2) in terms of a modified Adair model.

\[
Y = [K_{1} (M)(x) + K_{1,2} (M)^{2}(x)^{2}] + 2K_{2}K_{1,2} (M)^{4}(x)^{4})C;
\]

(Eq. 1)

where \( Y \) is the saturation fraction, \( (M) \) is the concentration of the deoxygenated monomer, \( (x) \) is the equilibrium concentration of the ligand, and \( C \) is the total protein concentration. The thermodynamic constants \( K_{1} \), \( K_{2} \), \( K_{1,2} \), and \( K_{1,2,3} \) are the oxygen binding constants of the unliganded dimer, the monoligated dimer, and the unliganded monomer, respectively. \( K_{1,2,1} \), and \( K_{1,2,3,1} \) are the monomer-dimer association constants of the fully oxygenated and unliganded protein, respectively.

The data sets (five records) were arranged into a data matrix, and five fitting curves were generated at each iteration. Least squares were calculated on the whole data set (global fitting procedure) by minimizing the values of four fitting parameters to Eqs. 1 and 2, namely the oxygen binding constant of the unliganded species (\( K_{1} \)), the oxygen binding constant of the monoligated species (\( K_{2} \)), and the monomer-dimer equilibrium constants of the unligated and dilitigated species (\( K_{1,2,1} \) and \( K_{1,2,3} \)). The values of \( K_{1,2,1} \) and \( K_{1,2,3} \) obtained from ultracentrifugation data were used as initial guesses.

Kinetics of Oxygen Release—The kinetics of oxygen release were measured in oxygen pulse experiments by mixing the protein solution (10–200 M) in the presence of an excess of sodium dithionite (0.02–0.1 M) with buffer solutions containing oxygen at concentrations varying from 1400 to 2 M. The experiment was carried out in 0.25 M phosphate buffer at pH 7.0 and 20 °C in an Applied Photophysics stopped flow apparatus (Leatherhead, UK). The observation wavelength was 434 or 557 nm. The measured first order rate constants were plotted as a function of oxygen saturation obtained by normalizing the observed amplitude to the measured oxy minus deoxy spectrum at 434 or 557 nm.

RESULTS

**Spectroscopic Characterization**—The visible and circular dichroism spectra of the K30D mutant measured on the oxygenated and deoxygenated derivatives are superimposable to those reported for native HbI (Fig. 1) over a wide range of protein concentration (0.1–200 μM).

**State of Association**—Sedimentation equilibrium experiments conducted on deoxygenated and carbonylated K30D HbI yielded \( K_{1,2} \) values of \((8.0 ± 0.1) × 10^{4} M^{-1}\) and \((1.2 ± 0.2) × 10^{3} M^{-1}\), respectively (Fig. 2). A direct measurement of the \( K_{1,2} \) ligand binding and association of *S. inaequivalvis* K30D Mutant
value for the oxygenated protein was not possible due to autoxidation of the heme iron over the time needed for sedimentation equilibrium measurements (>24 h). Therefore, the association-dissociation properties of the oxygenated derivative were investigated in sedimentation velocity experiments. Fitting the concentration dependence (over the range 7–140 μM) of the weight average sedimentation coefficients to a monomer-dimer model yielded a $K_{d,2}$ value of about 70 s$^{-1}$. Thus, over the protein concentration range covered in the functional studies reported below (12 μM-3.5 mM), the amount of dimers varies between 27 and 94% in deoxy K30D and between 0.1 and 15% in oxy K30D.

**Oxygen Equilibria**—The oxygen equilibrium measurements reported in the Hill plots of Fig. 3 indicate that oxygen affinity and cooperativity depend on protein concentration over the range $1.2 \times 10^{-5}$–$3.3 \times 10^{-3}$ μM heme. In contrast, the oxygen binding properties of the native protein are independent of protein concentration over the same range ($\log P_{50} = 0.88$ and $n_{50} = 1.45$) (Fig. 3, dashed curve). In the mutant, at $8 \times 10^{-4}$ μM heme, oxygen affinity is similar, and cooperativity is slightly decreased ($\log P_{50} = 0.84$ and $n_{50} = 1.35$) with respect to native HbI. At 3.3 mM heme, the change in oxygen binding behavior is more evident because both a decrease in oxygen affinity and a further decrease in cooperativity are observed. At the lowest protein concentration studied (12 μM), oxygen binding is non-cooperative ($n_{50} = 0.94$), whereas oxygen affinity is 4–5-fold higher than in the native protein ($\log P_{50} = 0.18$).

The curves of Fig. 3a were calculated according to Eqs. 1 and 2 (see “Experimental Procedures”). The fitting procedure used yields both the oxygen binding constants ($K_1$ and $K_2$) and the dimerization constants ($K_{1,2L}$ and $K_{1,2D}$). The experimental values obtained from ultracentrifugation experiments of $K_{1,2L}$ and $K_{1,2D}$ and the values of the $K_1$ and $K_2$ of the native protein (0.044 and 0.39 torr$^{-1}$, respectively) were used as initial guesses. Convergence of the fitting parameters is obtained after a few iterations and is accompanied by a 10-fold decrease of the $K_2$ value, which therefore reaches essentially the same value as $K_1$. Fig. 3e shows that the dependence of $\log P_{50}$ and $n_{50}$ on protein concentration calculated using the fitted values of the thermodynamic parameters is in good agreement with the experimental value of each single binding isotherm of Fig. 3a.

**Kinetics of Oxygen Release**—The kinetics of oxygen release were measured in parallel on the native protein and on the K30D mutant in oxygen pulse experiments (Fig. 4). In this experiment, the observed rate for oxygen release is measured as a function of oxygen concentration. In native HbI, the ob-
Ligand Binding and Association of *S. inaequivalvis* K30D Mutant

![Diagram](http://www.jbc.org/)

**Fig. 4.** Kinetics of oxygen release as a function of oxygen saturation in native HbI and in the K30D mutant. Data were obtained in 0.25 M phosphate buffer at pH 7.0 by mixing the oxygenated proteins at a concentration of 10 μM (□, ○) and 200 μM (■) heme in the presence of 1–100 mM sodium dithionite with oxygen-containing buffer at 20 °C. The time courses, observed by following the absorbance decrease at 434 (□, ○) or 572 (■) nm, were fitted to monoexponential curves.

The present study provides a unique example of a mutation that introduces a dramatic change in the mechanism of cooperative oxygen binding in a hemoglobin. The mutation Lys-30→Asp abolishes the only salt bridge (Lys-30→Asp-89) present at the two extremes of the subunit interface in native HbI (1, 2). The charge repulsion introduced by the mutation destabilizes the interface, but the change is more significant than expected. Native HbI in the ferrous state is a highly stable dimer characterized by a dimerization free energy of −10.5 kcal/mol (K_{dimer} = 6 × 10^7 M^{-1}) for the oxygenated derivative (14). The stability of the deoxygenated derivative is even higher, such that a direct measurement of the equilibrium constant is not feasible by analytical ultracentrifugation methods. The K30D mutation leads to a 10^6-fold decrease in the value of K_{dimer} in the oxygenated derivative, i.e. to an 8–9 kcal/mol increase in the free energy of dimerization with respect to the native protein (14). In the deoxygenated derivative, the decrease of the dimerization constant is such that it can be measured accurately by means of sedimentation equilibrium experiments (K_{dimer} = 8 × 10^8 M^{-1}).

It is noteworthy that the fine structure of the heme pocket appears to be unaffected by the mutation. In fact, the CD and absorption spectra of both the deoxy and oxygenated derivatives are superimposable to those of the native protein (Fig. 1) and do not change even at high dilution (0.1 μM), at which the mutated protein is essentially monomeric (>95%) even in the deoxygenated state. In particular, the unusually intense band at 590 nm (Qe band) in the visible spectrum of the deoxygenated derivative is identical in K30D HbI and in the native protein. This feature, which is paralleled by an unusually high ellipticity of the Qo band also in the CD spectrum, has been ascribed to the lowering of the C_{4v} heme symmetry; it reflects the strain induced by the Phe-97 residue on the iron proximal histidine linkage (13). The persistence of the Qo band in the high dilution spectrum of deoxygenated K30D HbI indicates that monomerization, though accompanied by exposure of the heme edge to the solvent, does not alter the stereochemistry of the heme pocket. The same reasoning applies to the spectra of the liganded derivatives.

The identity of the CD and absorption spectra therefore demonstrates the very high similarity in the tertiary structure of native HbI and of the K30D mutant in both the ligated and the unligated derivatives. In contrast, the dependence of oxygen affinity and cooperativity on protein concentration, which reflects the linkage between oxygen binding and the association-dissociation equilibria, differs in the two proteins. In native HbI, oxygen affinity decreases upon an increase in protein concentration up to about 2–3 μM, and cooperativity increases in a similar fashion; a further increase in concentration has no effect on oxygen affinity and cooperativity (14). In K30D HbI, oxygen affinity steadily decreases upon increase in protein concentration (Fig. 3A), whereas cooperativity passes through a maximum around 4–8 × 10^{-4} M heme (Fig. 3B).

The linkage scheme proposed by Royer et al. (14) for native HbI (see “Experimental Procedures”), when applied to the K30D mutant, brings out that the different behavior of the two proteins at high concentrations is accounted for by intriguing differences in the values of the thermodynamic parameters. The oxygen binding properties of native HbI at very high dilution and of the K30D mutant below 12 μM concentrations are indicative of the presence of a ligand-linked monomer-dimer equilibrium. In native HbI, monomer formation occurs at submicromolar concentrations and is readily identified by an increase in oxygen affinity accompanied by a decrease in cooperativity (14). Nevertheless, at concentrations higher than 2 μM, at which both the liganded and unliganded species are fully dimeric, the native protein displays high cooperativity with n_{50} = 1.5. Cooperative ligand binding at equilibrium is fully explained in terms of a simple two-site Adair scheme with apparent binding constants of 0.044 (K_{1}) and 0.39 (K_{2}) torr^{-1}, respectively. In the K30D mutant, the fitting of the oxygen equilibrium data to the linkage scheme reveals unexpected consequences on the fitted values of the Adair constants for the first (K_{1}) and second (K_{2}) oxygenation step. In fact, K_{1} and K_{2} converge to about the same value of about 0.038 torr^{-1}, corresponding to the low affinity asymptote of the Hill plot. The 10-fold increase in the overall oxygen affinity at low protein concentrations can be accounted for entirely by the dissociation of the low affinity dimers into high affinity monomers. This is in contrast with native HbI, where the K_{1}/K_{2} ratio of about 10 is indicative of a higher oxygen affinity of the monoligated dimer with respect to the unligated species (13, 15). In the K30D mutant, therefore, cooperativity is no longer based on intradimer heme-heme communication but appears to be driven by the ligand-linked monomer dimer equilibrium. In turn, the latter mechanism of cooperativity predicts that, at protein concentrations where the liganded form contains a significant proportion of dimer, cooperativity and oxygen affinity should start to drop. The experimental data meet such a prediction (Fig. 3). A further outcome of the present thermodynamic analysis is that the monomeric species is endowed with high oxygen affinity, as proposed by Royer et al. (14), for the native protein. It follows that not only the structural but also the functional properties of the native and mutated K30D monomer are identical.

The different origin of cooperativity in native HbI and in the K30D mutant is apparent also in the oxygen release experiments (Fig. 4). In native HbI, the increase of the apparent first
order rate constant upon decrease in oxygen saturation represents the kinetic manifestation of cooperativity, as is seen in vertebrate hemoglobins (13). In K30D HbI, this effect is absent. The value of the rate constant of oxygen release is centered around 100 s\(^{-1}\) and is independent of oxygen saturation and protein concentration. In the interpretation of this result, one must take into account the possible contribution of several factors. A major factor is that the oxygen binding or release kinetics in self-associating systems are necessarily affected by the contribution of the monomer-dimer equilibration rates. In this connection, it is worth recalling that in native ferric HbI, the rate of dimer dissociation has been estimated to be about 50–60 s\(^{-1}\), a value in the same range as the observed rates of oxygen release (16). Thus, it is premature to incorporate the oxygen release data of native and K30D HbI in the framework of a quantitative model that takes into account the whole set of kinetic pathways underlying the oxygen release process. Qualitatively, the lack of kinetic cooperativity can be explained if one considers that the oxygenated protein is monomeric and associates to form dimers only after oxygen dissociation. As a whole, the body of thermodynamic and kinetic data demonstrates that cooperativity in K30D HbI is driven by the dissociation of the oxygenated protein into monomers and that the heme-heme communication pathway characteristic of the native protein is no longer operative.

In general terms, it is surprising that the abolition of a single, though symmetrically related, salt bridge at the dimer interface results in such a large increase in the dimerization free energy. The observed free energy difference for dimer association between the native and the mutated protein corresponds to about 8 kcal/mol (for the liganded derivative), a value that cannot be assigned simply to an electrostatic charge separation within an aqueous solvent. We attribute the large free energy difference to the disruption of the ordered network of water molecules at the interface, and more importantly, to the extensive hydration in correspondence of the juxtaposed carboxyl residues (Asp-30 and Asp-89) that face each other across the dimer interface.

REFERENCES
1. Royer, W. E., Jr., Hendrickson, W. A., and Chiancone, E. (1990) Science 249, 518–521
2. Royer, W. E., Jr. (1994) J. Mol. Biol. 235, 657–681
3. Rousseau, D. L., Song, S., Friedman, J. M., Boffi, A., and Chiancone, E. (1993) J. Biol. Chem. 268, 5719–5723
4. Chiancone, E., Elber, R., Royer, W. E., Jr., Regan, R., and Gibson, Q. H. (1993) J. Biol. Chem. 268, 5711–5718
5. Andersen, M. E., and Gibson, Q. H. (1971) J. Biol. Chem. 246, 4790–4799
6. Qu, Y., Maillet, D. H., Knapp, J., Olson, J. S., and Riggs, A. F. (2000) J. Biol. Chem. 275, 13517–13528
7. Summerford, C. M., Pardanani, A., Betts, A. H., Poteete, A. R., Colotti, G., and Royer, W. E., Jr. (1995) Protein Eng. 8, 593–599
8. Chiancone, E., Vecchini, P., Verzili, D., Ascoli, F., and Antonini, E. (1981) J. Mol. Biol. 152, 577–592
9. Johnson, M., Correia, J. J., Yphantis, D. A., and Halvorson, H. (1981) Biophys. J. 36, 575–588
10. Stafford, W. F. (1992) Anal. Biochem. 203, 295–301
11. Giangiacomo, L., D’Avino, R., Di Prisco, G., and Chiancone, E. (2001) Biochemistry 40, 3062–3068
12. Rossi-Fanelli, A., and Antonini, E. (1958) Arch. Biochem. Biophys. 77, 478–492
13. Antonini, E., Ascoli, F., Brunori, M., Chiancone, E., Verzili, D., Morris, R. J., and Gibson, Q. H. (1984) J. Biol. Chem. 259, 6730–6738
14. Royer, W. E., Jr., Fox, R. A., Smith, F. R., Zhu, D., and Braswell, E. (1997) J. Biol. Chem. 272, 5689–5694
15. Ikeda-Saito, M., Yonetani, T., Chiancone, E., Ascoli, F., Verzili, D., and Antonini, E. (1988) J. Mol. Biol. 170, 1009–1018
16. Spagnuolo, C., De Martino, F., Boffi, A., Rousseau, D. L., and Chiancone, E. (1994) J. Biol. Chem. 269, 20441–20445
The Mutation K30D Disrupts the Only Salt Bridge at the Subunit Interface of the Homodimeric Hemoglobin from Scapharca inaequivalvis and Changes the Mechanism of Cooperativity

Pierpaolo Ceci, Laura Giangiacomo, Alberto Boffi and Emilia Chiancone

J. Biol. Chem. 2002, 277:6929-6933.
doi: 10.1074/jbc.M107422200 originally published online December 12, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107422200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 16 references, 8 of which can be accessed free at http://www.jbc.org/content/277/9/6929.full.html#ref-list-1