Allosteric Effectors Influence the Tetramer Stability of Both R- and T-states of Hemoglobin A*

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The contribution of heterotropic effectors to hemoglobin allostery is still not completely understood. With the recently proposed global allostery model, this question acquires crucial significance, because it relates tertiary conformational changes to effector binding in both the R- and T-states. In this context, an important question is how far the induced conformational changes propagate from the binding site(s) of the allosteric effectors. We present a study in which we monitored the interdimeric interface when the effectors such as Cl−, 2,3-diphosphoglycerate, inositol hexaphosphate, and bezafibrate were bound. We studied oxy-Hb and a hybrid form (αFeO2)2−(βZn)2 as the T-state analogue by monitoring heme absorption and Trp intrinsic fluorescence under hydrostatic pressure. We observed a pressure-dependent change in the intrinsic fluorescence, which we attribute to a pressure-induced tetramer to dimer transition with characteristic pressures in the 70–200-MPa range. The transition is sensitive to the binding of allosteric effectors. We fitted the data with a simple model for the tetramer–dimer transition and determined the dissociation constants at atmospheric pressure. In the R-state, we observed a stabilizing effect by the allosteric effectors, although in the T-analogue a stronger destabilizing effect was seen. The order of efficiency was the same in both states, but with the opposite trend as inositol hexaphosphate > 2,3-diphosphoglycerate > Cl−. We detected intrinsic fluorescence from bound bezafibrate that introduced uncertainty in the comparison with other effectors. The results support the global allostery model by showing that conformational changes propagate from the effector binding site to the interdimeric interfaces in both quaternary states.

Hemoglobin (1) is a tetrameric protein, which plays a vital role in the transport of oxygen. It consists of two dimers of α and β subunits that reversibly bind and release oxygen (1). The description of this cooperative phenomenon has been most frequently derived from the Monod-Wyman-Changeux (MWC) two-state allosteric model (2) that attributes cooperativity to a rapid equilibrium between two conformations of distinct oxygen affinity of the whole tetramer. These distinct states are the fully unliganded T-state and the fully ligated R-state. Szabo and Karplus (3) modified the two-state model incorporating the stereochemical mechanism suggested by Perutz (4) for the T to R switch, and introduced ligation-induced tertiary changes within the T-state. In this extended model (MWC-SK), it was proposed that cooperativity still works through a ligation-induced shift in the equilibrium of states T and R, but the model attributed importance in the conformational switch to certain changes at the inter- and intrasubunit interfaces. Upon ligation in the T-state, the network of intersubunit interactions become perturbed, some (e.g. salt bridges) become broken up to release the characteristic strain of the T-state. The mechanism involves a rotation of one dimer with respect to the other, thus reaching the more relaxed R-state (5).

It has been widely reported that some molecules, referred to as heterotropic allosteric effectors, considerably lower the oxygen affinity of the T-state upon binding to HbA but not to the heme (6–9). Structural studies in the T-state showed that these allosteric effectors primarily bind to the central cavity of HbA (10–12). The modulation of the oxygen dissociation curves by allosteric effectors is addressed in the extended MWC model by the assumption that allosteric effectors bind specifically to the somewhat larger central cavity of the T-state and stabilize this conformation. This shifts the R/T equilibrium in favor of the T-state and consequently lowers the overall affinity to oxygen (8, 13). The well known Bohr effect and results reported for Cl−, also influencing the oxygen affinity of the T-state (14, 15), show that, in a broader sense, H+ and Cl− can also be considered as being members of the family of allosteric effectors.

Extended studies on the effect of allosteric effectors, however, indicated that they not only bind to the T-state but also to the R-state (16, 17). The modulation of the oxygen association constants was shown to occur at a much broader scale (65-fold change in Kf and 2000-fold change in Kd; see Ref. 18 for details)

2 The abbreviations used are: MWC, the two-state allosteric model of Monod; Wyman, and Changeux; HbA, human adult hemoglobin; oxy-Hb, native human adult hemoglobin A linked to oxygen; α-oxo-Fe2(Zn)-Hb, zinc-substituted α(FeO2)2−(βZn)n, human hemoglobin; Zn-PP-IX, zinc protoporphyrin-IX; IHP, inositol hexaphosphate; BZF, bezafibrate; DPG, 2,3-diphosphoglycerate; MPa, megapascal; GPA, gigapascal; FTIR, Fourier transform infrared spectroscopy.

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in each state than previously known. These data initiated the 
reformulation of the earlier models for the allosteric action of 
heterotropic effectors. The recently proposed “global allostery 
model” (18) supposes that the effectors can bind to both the T-
and R-states and that effectors induce direct tertiary conforma-
tional changes. These tertiary effects then lead to the detected 
changes in oxygen association (18, 19). Until now, no structural 
data have been published for the complex of R-state human HbA 
with allosteric effectors; the only available structure is that of horse 
HbA complexed to CO (20). Recently, however, a docking and 
molecular dynamics simulation study in our laboratory (21) pro-
posed models for the structure of HbA bound with the allosteric 
effectors DPG, IHP, and 2-{4-[(3,5-dichlorophenylcarbamoyl)-
{methyl}-phenoxy]-2-methylpropionoc acid. These results sup-
ported the model allowing for binding of effectors also in the 
R-state and proposed primary binding sites in the central cavity of 
the R-state tetramer.

In this paper we report the results of high pressure studies. 
We show that pressure perturbation of the structure is a valu-
able tool providing the means to measure the tetramer-dimer 
dissociation constant. This parameter reports structural 
changes at the interdimeric interface.

High pressure is known to have distinct effects on proteins 
(22) depending on the applied pressure range. Typically, 0.5–1 
GPa is needed to denature a protein, by squeezing water into 
the interior (23). Lower pressure causes elastic deformations, 
the extent of which are characteristic of the compressibility (24, 
25) of the protein. Because pressure is a thermodynamic 
parameter, pressure can shift thermodynamic equilibria of var-
ious kinds, including conformational transitions of proteins 
(26). One such equilibrium that is typically perturbed by pres-
sure is oligomer dissociation. Pressures in the range of 100–200 
MPa have been shown to be able to affect the quaternary struc-
ture of the proteins without affecting their secondary and ter-
tary structure (27). Previous pressure experiments performed 
on HbA (28–30) showed that pressure tuning affects various 
optical properties (e.g. light scattering, absorption, fluorescence 
emission, and CD spectra) of the sample. In a detailed study, Pin 
et al. (28) showed that applying pressure at pH 7 and 10^{-6}– 
10^{-4} M protein concentration will shift the tetramer-dimer 
equilibrium in favor of the dimers, and at pH 9, the observed 
dimer-monomer equilibrium will be shifted in favor of the 
monomers. Although Pin et al. (28) included IHP in their study, 
no systematic study applying pressure perturbation was con-
ducted to observe the effect of various allosteric effectors on the 
subunit interfaces in HbA.

In this study our goal was to compare the effect of allosteric 
effectors on the tetramer-dimer equilibrium in the T- and R-
states by applying high hydrostatic pressure and steady-state 
fluorescence techniques. Based on literature data (31, 32), we 
have used the intrinsic fluorescence of the tryptophan residues 
as a marker of changes at the interface region. Measurements 
under high hydrostatic pressure were, however, not feasible on 
deoxymyoglobin, because the sample holder inside the pres-
sure cell cannot be isolated in a gas-tight manner from the pres-
sure transmitter fluid (water) in the device. To overcome this 
difficulty, we used a hybrid hemoglobin, α-oxy-Fe-βZn-Hb to 
mimic the structure of the T-state. In this hybrid the hemes of 
the β subunit are replaced with Zn-protoporphyrin-IX, and the 
α subunit hemes are oxygen-ligated. Oxygen equilibrium stud-
ies and x-ray crystallography have shown that this derivative, as 
a five-coordinated metal hybrid of HbA, can be considered 
analogous to T-state HbA (33–35).

The selection of zinc substitution is supported by results that 
show a low energetic cost compared with other (for e.g. cobalt) 
metal substitutions (36). This indicates that the conformation is 
close to the native form. It can be considered questionable, 
however, for which of the T-state subpopulations the analogy 
holds. The conformation within being “T” was shown to be 
slightly tunable by environmental conditions, for example by 
gel-sol encapsulation (37, 38). This tunability was also found 
within zinc hybrids rendering the CO-ligated form the least 
tense among a variety of ligated forms and external conditions 
(39). Literature data, however, show that the zinc hybrids in 
general have lower oxygen affinity than deoxy-HbA (40). Based 
on this we considered the (αFeO_2)_{α} βZn form, which may be 
the least tense among the zinc hybrids, a sufficiently good 
model for the native T-state under physiological conditions. We 
used the oxy-form of HbA to study the R-state.

The effect of the allosteric effectors Cl\textsuperscript{–}, IHP, DPG, and BZF 
was investigated at pH 7.4 by pressure stability measurements 
based on the sensitivity of tryptophan fluorescence to structural 
changes of HbA. We observe a structural transition of the pro-
tein in the range of 100–200 MPa, and we argue for this being a 
tetramer-dimer transition. We show that the characteristic 
pressure of this transition, and thus the equilibrium constant 
(K_{eq}) of the reaction, is sensitive to the presence of allosteric 
effectors both in the R- and in the T-analogue state of HbA. 
These data characterize the dimer interface of the tetramer that 
was shown to play a decisive role in the cooperativity of oxygen 
binding. The reliability of the methodology was confirmed by 
parallel experiments with horse heart oxy-myoglobin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Myoglobin (horse heart), sodium chloride, 
HEPES, IHP, DPG, and BZF, the highest purity available, were 
purchased from Sigma.

All samples (both myoglobin and HbA) were prepared in 100 
mm HEPES, pH 7.4, with deionized water. IHP and DPG was 
used in a final concentration of 2 mm, BZF at 10 mm, and the 
sodium chloride concentration was 100 mm. The IHP, DPG, 
and BZF concentrations were selected as described previously 
(19), and these concentrations are well in excess of the 0.06 mm 
HbA concentration used in the comparative experiments thus 
ensuring the saturation of the binding sites. Myoglobin was used 
only in the oxygen-saturated form.

**Hemoglobin Preparation**—HbA was prepared from human 
blood as described previously (19) according to the method of 
Drabkin (41). HbA was promptly converted to the CO form and 
stripped of organic phosphates by the method of Berman \textit{et al.} 
(42), and further purified by ion exchange chromatography. 
The final HbA solution was eluted in 5 mm HEPES buffer, pH 
7.4. The native oxygenated HbA samples are referred to as oxy-
Hb, and these were obtained from the CO form by strong illu-
mination and a flow of pure oxygen above the sample, which 
was kept on ice. Zinc-substituted HbA was prepared from
human HbA as described previously (43). The α and β subunits were isolated from CO HbA, and the β subunit was converted to apoprotein by the acid-acetone method and then incubated with Zn-PP-IX to create β-zinc subunits. Finally, the α-Fe and β-zinc subunits were mixed and incubated in the cold for 2 days. The (αFe-CO)_2-(β-Zn)_2 form was then converted to the oxy form as described above. The (αFeO_2)_2-(βZn)_2-hemoglobin hybrid is referred to as α-oxy-Fe-βZn-Hb in this paper.

All samples were adjusted to 60 μM final heme concentration before measurement, unless stated otherwise (for concentration dependence measurements). The stripped forms (containing no organic phosphates or Cl\textsuperscript{−}) were used as reference in all measurements.

Possible denaturation or deoxygenation during experiments was controlled by recording the absorption spectrum. No significant changes were detected in the 270–700 nm wavelength range upon the addition of various effectors or upon increasing the pressure.

Fluorescence Emission Spectra under High Pressure—A high pressure cell with sapphire windows (Unipress, Warsaw, Poland) was mounted in a Fluorolog-3 (Yobin Ivon Inc. Longjumeau, France) spectrofluorometer. High pressure was created by a manually driven pump (Nova Swiss, Effretikon, Switzerland), and pressure was monitored with a pressure transducer (DMS-580.4018 Nova Swiss, Effretikon, Switzerland) equipped with a digital meter, with an accuracy of 10 bar. The sample was allowed to equilibrate at each pressure for 3 min. Spectra were acquired by integrating for 1 s at each wavelength, with slits adjusted at 5 nm excitation and 2 nm emission bandpass. The spectrofluorometer was equipped with a 450-watt xenon lamp (Osram, München, Germany) as light source. The temperature was maintained by a thermostat at 20 °C and was directly controlled in the sample.

The HbA sample was injected into a quartz tube (UV-fused silica) with a 2.4-mm inner diameter with 1.3-mm wall thickness and 19-mm height. The sample volume was 110 μL. This tube was covered with a rubber stopper and placed into the pressure cell. The pressure transmitter fluid was water.

Spectral shift under pressurization was determined based on either the maximum position of the emission band determined by a Savitzky-Golay algorithm or the center of gravity of the band in the 300–400-nm range. Both kinds of evaluation lead to the same information. We report data based on the maximum positions.

Absorption Spectra at High Pressure—The high pressure cell was mounted in a Cary 4E UV-visible spectrophotometer (Varian Inc, Palo Alto, CA) and kept at 20 °C. Spectra were acquired at a scan rate of 1 nm/s, using the full height slit mode, allowing for 1 nm bandpass. Control spectra before pressurization were acquired in the quartz tube, covered with a rubber stopper, and placed centered in the light beam.

FTIR Spectroscopy Under Pressure—The infrared spectra were obtained with a Bruker IFS66 FTIR spectrometer equipped with a broad band MCT solid-state detector cooled with liquid nitrogen. During data acquisition, 256 interferograms were co-added at a resolution of 2 cm\textsuperscript{−1}. High pressure was generated in a diamond anvil cell (Diacell Products, Leices-

RESULTS

Oxy-Hb and α-oxy-Fe-βZn-Hb samples corresponding to the R- and T-analogue state HbA, respectively, were studied in stripped condition as reference and then bound to various allosteric effectors. The effect of allosteric effectors was monitored by registering the conformational perturbation effect of high pressure using fluorescence and absorption spectroscopy.

Effects Shown by Tryptophan Fluorescence—In Fig. 1, the solid lines show the fluorescence emission spectra of stripped
oxy-Hb (a) and α-oxy-Fe-βZn-Hb (b) at ambient pressure; the dashed lines are spectra at a pressure of 350 MPa. The excitation wavelength was 290 nm for both samples to directly excite the Trp residues. It is seen that the intrinsic Trp fluorescence is easily measured under the experimental conditions, and a pressure effect can be observed. In the case of oxy-Hb, the emission peak characteristic for the Trp fluorescence can be observed around 330–340 nm, as seen in Fig. 1a. For α-oxy-Fe-βZn-Hb (Fig. 1b), characteristic Zn-PP-IX emission peaks also appear at 594 and 645 nm, indicative of energy transfer from Trp to the Zn-PP-IX. This is also seen in Fig. 1b, inset, where it is directly shown that Zn-PP-IX fluorescence can be excited by Trp absorption. Energy transfer from Trps to the hemes can also be considered in the case of oxy-Hb. A proof for this is a slight dip in the emission spectrum (Fig. 1a) around the wavelength of the Soret maximum (~420 nm).

It can be seen that increased pressure induces a red shift (cf. Fig. 1a, inset), broadening of the Trp fluorescence emission band, and a significant increase of emission intensity.

The red shift of the Trp emission versus pressure has a sigmoidal character in all kinds of Hb samples. Fig. 2 shows the maximum positions of the emission peaks versus pressure in both quaternary states in the presence of 100 mM chloride. The fluorescence intensity showed the same feature when plotted as a function of increasing pressure (Fig. 3). Both kinds of evaluations lead to a feature that is indicative of a pressure-induced conformational transition. The pressure at 50% transition of a specific sample is very similar when determined from the maximum position or from the increase of quantum yield of Trp emission. In the comparative analysis of the effect of allosteric effectors, we used the data obtained from the red shift of the maximum position. Table 1 shows that the characteristic pressures were sensitive to the presence of bound allosteric effectors in both quaternary states. For comparison we used the data obtained at 60 μM concentration.

Pressure-induced Changes in Tryptophan Fluorescence of Myoglobin—To assess pressure-induced changes at the monomeric level, we report fluorescence data for myoglobin, which has a structure similar to a Hb monomer. The results are shown in the insets of the figures reporting data for HbA. Fig. 2a and Fig. 3a, insets, show the maximum position and the intensity of the Trp emission maximum of myoglobin in the function of pressure, respectively. In contrast to HbA, myoglobin shows a different behavior. The shift of the maximum position is relatively small (few nm/100 MPa) and is strictly linear. The intensity linearly decreases with increasing pressure, in contrast to the sigmoidal intensity increase observed in HbA (Fig. 2a).
Pressure Effect on the Porphyrin Spectra

The electronic transition energy of the porphyrin, monitored either by fluorescence of α-oxy-Fe-βZn-Hb or by the absorption spectra of oxy-Hb, did not show a structural transition upon applying high pressure, unlike that of the Trps. A linear dependence of the maximum position could be measured in the function of increasing pressure up to 350 MPa as shown in Fig. 4. Both the Soret band position in the absorption spectrum of oxy-Hb seen in Fig. 4a and the Q-band position in the fluorescence spectrum of α-oxy-Fe-βZn-Hb seen in Fig. 4b show a linear dependence. Similarly, linear shifting effects of pressure were observed in all samples. This observation corresponds to the finding of others in the case of cytochromes (47), where a linear shift was also observed in a similar pressure range. It was concluded that in this pressure range the heme pocket of hemoproteins is affected only by elastic deformations resulting in a linear (red) shift of the electronic transition energy. In our experiments the peak positions and the linear shifting effect were not significantly influenced by binding of allosteric effectors.

The Fluorescence of BZF—We observed that BZF became fluorescent upon binding to HbA. Fig. 5 shows the excitation spectrum and the emission spectrum of BZF excited at 290 nm when bound to oxy-Hb. As seen from the comparison of the excitation and emission spectra, excitation of the Trps may lead to energy transfer to BZF. In Fig. 5a, a broad emission band around 450 nm represents the contribution of protein-bound BZF to the fluorescence emission. A decrease in the fluorescence emission signal at the Soret band (~420 nm) because of the absorption of the porphyrins can also be seen in the figure. The intensity of the BZF peak around 450 nm varied with pressure and

### Table 1

| HbA type          | Concentration | Effector  | $p_{1/2}$ |
|-------------------|---------------|-----------|-----------|
| α-Oxy-Fe-βZn-Hb   | 0.68          | None (stripped) | 200 |
| α-Oxy-Fe-βZn-Hb   | 0.23          | None (stripped) | 160 |
| α-Oxy-Fe-βZn-Hb   | 0.02          | None (stripped) | 135 |
| α-Oxy-Fe-βZn-Hb   | 0.06          | 100 mM Cl | 113 |
| α-Oxy-Fe-βZn-Hb   | 0.06          | 2 mM DPG | 95 |
| α-Oxy-Fe-βZn-Hb   | 0.06          | 2 mM IHP | 72 |
| α-Oxy-Fe-βZn-Hb   | 0.06          | 10 mM BZF | 165 |
| Oxy-Hb            | 7.7           | None (stripped) | 225 |
| Oxy-Hb            | 0.77          | None (stripped) | 157 |
| Oxy-Hb            | 0.06          | None (stripped) | 100 |
| Oxy-Hb            | 0.06          | 100 mM Cl | 137 |
| Oxy-Hb            | 0.06          | 2 mM IHP | 144 |
| Oxy-Hb            | 0.06          | 10 mM BZF | 170 |

**FIGURE 4.** a, Soret absorption peak maximum position versus pressure of stripped oxy-Hb. Solid line is a linear fit to the data, with a slope of 4.7 nm/GPa. b, zinc-PPIX fluorescence emission peak maximum position versus pressure (excited at 290 nm) of α-oxy-Fe-βZn-Hb with chloride. Solid line is a linear fit to the data; the slope is 6.1 nm/GPa.

**FIGURE 5.** a, fluorescence emission (excited at 290 nm) and excitation (registered at 460 nm) spectra of oxy-Hb with BZF bound. Solid line, emission spectrum at ambient pressure; dashed line, emission spectrum at 125 MPa. Arrow shows the emission of bound BZF. Dotted line, excitation spectrum registered at 460 nm. b, BZF emission intensity excited at 290 nm and registered at 450 nm versus pressure. The solid line, sigmoid fit to the data, $p_{1/2}$ is 177 MPa. a.u., arbitrary units.

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Pressure Effect on the Porphyrin Spectra—The electronic transition energy of the porphyrin, monitored either by fluorescence of α-oxy-Fe-βZn-Hb or by the absorption spectra of oxy-Hb, did not show a structural transition upon applying high pressure, unlike that of the Trps. A linear dependence of the maximum position could be measured in the function of increasing pressure up to 350 MPa as shown in Fig. 4. Both the Soret band position in the absorption spectrum of oxy-Hb seen in Fig. 4a and the Q-band position in the fluorescence spectrum of α-oxy-Fe-βZn-Hb seen in Fig. 4b show a linear dependence. Similarly, linear shifting effects of pressure were observed in all samples. This observation corresponds to the finding of others in the case of cytochromes (47), where a linear shift was also observed in a similar pressure range. It was concluded that in this pressure range the heme pocket of hemoproteins is affected only by elastic deformations resulting in a linear (red) shift of the electronic transition energy. In our experiments the peak positions and the linear shifting effect were not significantly influenced by binding of allosteric effectors.
showed a sigmoidal character, as shown in Fig. 5b. Characteristic pressures obtained by fitting a sigmoid curve agreed well with the data from the emission maximum of the Trp signal. A linear increase superimposed on the sigmoid change can be observed before and after the transition. These were corrected in the fitting.

Optical Absorption and FTIR Spectroscopy to Assess Possible Unfolding Effects of High Pressure—As a control of sample quality, optical absorption spectra were recorded at ambient pressure and, in some cases, also at 250–350 MPa hydrostatic pressure. In the case of the stripped reference, samples of several absorption spectra were recorded from ambient pressure up to 350 MPa. No signs of denaturation, met-Hb formation, or deoxygenation were observed in these spectra. This was the case for both oxy-Hb and α-oxo-Fe-βZn-Hb stripped or with effectors. As an example, the absorption spectra of stripped oxy-Hb at ambient and at 350 MPa pressures are shown in Fig. 6a.

To further ensure the absence of any unfolding in this pressure range, we recorded FTIR spectra of oxy-Hb and α-oxo-Fe-βZn-Hb up to 902 MPa. Fig. 6b shows the FTIR spectra of oxy-Hb at ambient, 325 MPa, and 902 MPa pressures. In Fig. 6b, an arrow indicates the band characteristic for α-helical structure, best seen after deconvolution. The change of the position of this band is shown in Fig. 6b, inset. It is seen that the change is negligible in the 0.1–250-MPa pressure range used in our studies.

Effect of Protein Concentration—The pressure stability measurements were performed at various protein concentrations in the stripped case. Table 1 summarizes the corresponding transition pressures, and in Fig. 7 we show the data for stripped oxy-Hb and α-oxo-Fe-βZn-Hb. The transition pressure strongly depends on the concentration of the protein in both quaternary states, with the concentration dependence being larger for oxy-Hb. The transition pressure versus the total protein concentration in a semi-logarithmic plot is linear as expected from the influence of concentration on a tetramer-dimer equilibrium.

DISCUSSION

Sensitivity of Trp Fluorescence to Pressure-induced Structural Changes at the Dimer–Dimer Interface—The HbA tetramer has 6 Trp residues, one in each α and two in each β subunits. It was...
realized some time ago that Trp fluorescence could be used to monitor conformational changes, i.e. T to R transition, and this sensitivity of the signal was assigned mostly to Trp-β37, which are located at the αβ2 and αβ3β1 subunit interfaces (31, 32). In resonance Raman spectra obtained by UV excitation, the contribution assigned to these Trp residues can be separately analyzed and used to monitor local structural changes in the interface region (see for example Refs. 37 and 38). In a fluorescence study, the sensitivity of the signal to structural changes relies significantly on the conditions for quenching by Förster-type energy transfer to the hemes. The Trps in Hb are within short distances from one or more hemes of the structure; thus the distance requirements of energy transfer are fulfilled. Table 2 lists distance data for all Trp to the hemes in the R- and T-state average structures after 2 ns molecular dynamics simulations.

### Table 2

| Distance data of Trps to the hemes in the R- and T-state average structures after 2 ns molecular dynamics simulations |
|---|
| Heme | Trp | HbR stripped | HbR + DPG | HbT stripped | HbT + DPG |
|---|---|---|---|---|---|
| α1 | 16.68 | 16.40 | 16.57 | 19.17 |
| α2 | 16.68 | 16.40 | 16.57 | 19.17 |
| α3 | 16.68 | 16.40 | 16.57 | 19.17 |
| β1 | 16.68 | 16.40 | 16.57 | 19.17 |
| β2 | 16.68 | 16.40 | 16.57 | 19.17 |
| β3 | 16.68 | 16.40 | 16.57 | 19.17 |
| β4 | 16.68 | 16.40 | 16.57 | 19.17 |
| β5 | 16.68 | 16.40 | 16.57 | 19.17 |
| β6 | 16.68 | 16.40 | 16.57 | 19.17 |

The Changes in Trp Fluorescence in the Studied Pressure Range Are Not Because of Local Conformational Changes, Unfolding, or Denaturation—Before reaching conclusions from our results concerning the tetramer-dimer equilibrium of R- and T-state HbA associated with allosteric effectors, we have to consider the possibility of other mechanisms having an effect on the Trp emission. Unfolding or denaturation under pressure could also cause changes in the Trp emission. We argue to rule out these possibilities, as follows:

a) The 100–200-MPa pressure range is very low compared with the typical pressures needed for protein unfolding. We have shown previously that myoglobin is pressure-stable up to 650 MPa (22, 49, 50). Our results presented in this paper also show no signs of denaturation in the pressure range up to 250 MPa, as can be seen in the insets of Fig. 2, Fig. 3, and Fig. 6b. Recently (51), a denaturation pressure of 400 MPa was reported for the isolated subunits of HbA; 100–200 MPa used in this study is significantly lower, and thus it is not reasonable to expect denaturation at this low level of pressure.

b) A second argument comes from the absorption spectrum at 350 MPa (see Fig. 6), where the Trp emission is already red-shifted with respect to the initial emission spectrum. No significant changes, except a slight red shift, can be seen in the Q-band and in the Soret region. Denaturation of the protein would be clearly seen in the absorption spectrum of the porphyrin, and absorption spectra recorded after releasing the pressure also show no signs of sample denaturation. Also, as observed in the Q-band region, no change in the spin state or in the coordination state of the heme occurs in the pressure range used. Adlen et al. (30) reported resonance Raman and optical absorption data in the function of pressure that they interpreted as resulting from changes in the spin state of the heme in oxy-Hb at 150 MPa and from further coordination changes at 350 MPa. Hamdane et al. (51) found that high pressure up to 700 MPa enhanced hexacoordination in globins. We observed neither of these effects in our samples within the studied pressure range. These reported effects could arise from different sample preparation methods, different pH values, or from different buffers used, which can all change the behavior of the sample under pressure.

The spectra shown in Fig. 6 differ in a small red shift of the Soret band around 420 nm. This effect is the same as shown in Fig. 4 and can be explained by the change of the electronic transition energy due to applying high pressure that decreases the distance between the chromophore and the interacting units (atomic groups). This change of the electronic transition energy is supposed to be proportional to the solvent shift at atmospheric pressure. If the interaction of the chromophore with the atomic groups of the protein matrix is dominated by one kind of interaction with a potential energy of 1/Rn distance dependence, one observes a linear shift of the transition energy as a function of pressure (52–54). We observed a linear red shift in both cases that has also been reported for other heme proteins (47, 49). The linear shift observed in the studied pressure range shows that
the structure of the heme pocket in general was not affected, and we only see an overall elastic effect.

c) It is well known that pressures of this range typically affect intermolecular interactions and cause the dissociation of oligomers (55). An earlier HbA fluorescence study (28) found reduced molecular volumes calculated from the fluorescence lifetime and anisotropy of a covalently attached fluorescent label 5-dimethylaminonaphthalene-1-sulfonyl under pressures up to 250 MPa. The authors interpreted these changes in terms of dissociation and not denaturation.

d) Besides conformational changes induced by pressure, we have to consider the possibility of deoxygenation because of pressure. This, however, would lead to almost a magnitude stronger shift of the Soret band (an almost 20 nm red shift of the Soret band would occur upon deoxygenation) than the shift observed. Thus, we can rule out pressure-induced deoxygenation in this pressure range.

e) FTIR spectroscopy does not show significant changes in the amide-I band position (characteristic for \( \alpha \)-helical structure) up to 350 MPa, as can be seen in Fig. 6b. Because Hb is a predominantly \( \alpha \)-helical structure, this rules out unfolding of any helices in this pressure range. To demonstrate pressure-induced unfolding, we present the FTIR spectrum acquired at 902 MPa, which clearly shows an unfolded state. This spectrum is, however, very different from the ones corresponding to the pressure range in our studies.

f) Myoglobin is a monomeric heme protein, and its tertiary structure closely resembles the structure of a HbA subunit. To model the effect of high pressure on a single HbA monomer, we acquired Trp emission spectra of myoglobin in the same pressure range as used in the HbA studies. This shows striking differences as follows: both the shift of the maximum position and the intensity change are strictly linear, as shown in Fig. 3a and Fig. 2a, respectively. It has been reported in the literature how pressure itself influences the fluorescence spectrum of Trp in Tris buffer, in the presence of alcohol or glycerol, and when it was incorporated in a small peptide (56). In all cases the authors observed a linear, continuous red shift induced by pressure. This effect, attributed to the increase of water density and dielectric constant under pressure, would cause a shift of 1–2 nm in the pressure range studied. The shift of the maximum position in our measurements is in this range, although somewhat larger, which can be attributed to the presence of the protein matrix. It is interesting to note that this red shift is comparable with the shift of the Soret peak and of the fluorescence maximum position of the Zn-porphyrin emission (cf. Fig. 4) of Hb, showing the presence of the same elastic compression in Hb. None of the transition-like features of Hb appear in the fluorescence of myoglobin. It should also be considered that an elastic compression, which shortens the heme-Trp distance, would itself decrease the intensity of the Trp fluorescence because of an increase in energy transfer. This latter effect is expected to occur also in Hb but is overwhelmed by another effect, which we attribute to pressure-induced dissociation of the tetramer.

The Pressure Effect on Trp Fluorescence Emission Shows Pressure-induced Dimerization—The emission spectrum of the Trps undergoes clear changes when the HbA samples are subjected to high pressure (Figs. 1 and 2). Because we ruled out pressure-induced unfolding, we present our argumentation for dimerization as follows.

a) A red shift of the Trp emission is usually assigned to a local environment of increasing polarity, as in the case of exposure to water (57, 58); however, changes in the electric field and in the dipole moments around the Trp residue can also result in the shift of the Trp emission spectrum (57). Because pressure-induced unfolding is very unlikely under our experimental conditions, this rules out significant electric field or dipole moment changes in Trp surroundings due to effects other than exposure to water. In the structure of HbA, two of the Trps, the Trp–β37, are located at the inter-subunit contact surface. These residues are somewhat buried in the tetramer but become solvent-exposed in the dimer form. Thus a tetramer-dimer transition and a consequent water exposure could explain the observed red shift.

b) Besides the red shift, the intensity of the fluorescence emission is increasing during the pressurization, in full agreement with the mentioned red shift of the emission maximum (Figs. 2 and 3). We attribute the increase of the Trp fluorescence intensity to a change in the efficiency of the energy transfer because of the tetramer dissociation. This effect would mostly influence Trps–β37, for these Trps the nearest extra subunit heme is the adjacent \( \alpha \)-heme of the other dimer (i.e. \( \alpha_2 \) heme for Trp–β37 and \( \alpha_1 \) heme for Trp–β37). Published energy transfer rates (59) indicate that these Trps are the only ones having energy transfer rates to a heme in the adjacent dimer comparable with the heme in their own monomer (cf. Table 5 in Ref. 59). The known strong dependence of energy transfer efficiency on the donor-acceptor separation explains the significant quantum yield effect.

c) This argument is further supported by literature data, available for Trp fluorescence lifetimes of HbA (60). The ratio of a long component of the Trp lifetimes was shown to increase with pressure and was attributed to pressure-induced changes in the quaternary structure. In the case of the multimeric Hb of Lumbricus terrestris, a similar change in Trp fluorescence was also observed (61).

d) The concentration dependence of the transition pressure (Fig. 7) clearly supports the tetramer-dimer transition model. Dissociation reactions are known to depend strongly on the concentration of the initial molecule, whereas this dependence would not be expected for unfolding or denaturation reactions. Also, if the observed effect would be due to a possible shift in the ratio of possible different subpopulations in the T-state (see for e.g. Refs. 37–39), a concentration dependence would not be expected.

e) High pressure dissociates protein oligomers by squeezing water molecules into the voids. This model was experimentally verified by H/D exchange experiments (62). Theoretically, this is also expected because ordered water molecules have a smaller volume than those in the bulk solution, and thus based on the LeChatelier-Braun principle, the disintegration is favorable under increasing pressure. High pressure-induced dimerization and also monomerization of HbA have been demonstrated previously based on fluorescence labeling and anisotropy measurements (28). In our opinion, however, these data are not fully comparable with our results, because differences in the experimental condi-
Table 3

Dissociation constants and volume changes of Hb with various allosteric effectors

| Type           | Effector | $K_d$ (10^{-5} M) | $\Delta V$ |
|----------------|----------|-------------------|------------|
| Oxy-Hb         | None (stripped) | 1.41              | -99.5      |
| Oxy-Hb         | 100 mM Cl | 1.22              | -83.2      |
| Oxy-Hb         | 2 mM IHP  | 0.47              | -97.7      |
| $\alpha$-Oxy-Fe-$\beta$-Zn-Hb | None (stripped) | 0.44              | -105.3     |
| $\alpha$-Oxy-Fe-$\beta$-Zn-Hb | 100 mM Cl  | 0.78              | -106.6     |
| $\alpha$-Oxy-Fe-$\beta$-Zn-Hb | 2 mM DPG   | 2.06              | -103.6     |
| $\alpha$-Oxy-Fe-$\beta$-Zn-Hb | 2 mM IHP   | 12.90             | -83.4      |

Allosteric Effectors Influence Hemoglobin Tetramer Stability

Type is the quaternary state, oxyHb or $\alpha$-oxyFe-$\beta$-Zn-Hb, stripped to without allosteric effectors. Protein concentration was 60 $\mu$M in all cases. $K_d$ and $\Delta V$ values were calculated by fitting the model described in the text to the emission maximum position data. Uncertainty of the fitted values is typically 5%.

We suppose that both the wavelength change of the Trp fluorescence maximum and that of the quantum yield of fluorescence are proportional to $\alpha$. By choosing the maximum position of the Trp spectra, it can then be expressed as shown in Equation 5,

$$\lambda_{max} = \lambda_0 + \Delta \lambda \cdot \alpha$$

where $\lambda_0$ is the initial Trp emission maximum position corresponding to atmospheric pressure, and $\Delta \lambda$ is the shift in the maximum position corresponding to total dimerization. Substituting Equation 4 into Equation 5 yields Equation 6,

$$\lambda = \lambda_0 + \Delta \lambda \left[ \frac{K_d \cdot e^{-\frac{p \Delta V}{RT}} + 16 C_0 K_d \cdot e^{-\frac{p \Delta V}{RT}} - K_d \cdot e^{-\frac{p \Delta V}{RT}}}{8 C_0} \right]$$

which can be directly fitted to the experimental data points and yield $K_d$ and $\Delta V$ parameters.

The $p_{\lambda_0}$ (where $p_{\lambda_0}$ indicates characteristic transition pressure at 50% transformation) values shown in Table 1 can also be expressed from the model, because they correspond to $\alpha = \frac{1}{2}$. In this way we render typical pressure values to the studied cases (where $C_0$ is constant), when their $K_d$ values are identical. The difference in $p_{\lambda_0}$ means distinct $K_d$ parameters. Thus the data in Table 1 and Table 3 are characteristic for the same phenomenon (through Equation 3). The fitting procedure to yield $p_{\lambda_0}$ from the data points, however, is less sensitive to the experimental error than the fitting of Equation 6 to obtain $K_d$ and $\Delta V$. In the case of the complexes with BZF where the uncertainty of the data points was higher, we characterized the effect only by the $p_{\lambda_0}$ values.

The data in Tables 1 and 3 show that the quaternary state (R or T) and the binding of allosteric effectors influence the tetramer-dimer dissociation constant in HbA. The effect of allosteric effectors is distinct in the R-state and in the T-state analogue.

Verifying the Validity of the Tetramer-Dimer Dissociation Model—A control of the model is to measure the concentration dependence of the phenomenon. Solving Equation 4 for $\alpha$ and substituting $\alpha = \frac{1}{2}$ yields a logarithmic function of $C_0$ as shown in Equation 7.

$$p_{\lambda_0} = \frac{RT}{\Delta V} \log \left( \frac{K_d}{2 C_0} \right)$$

To validate our model, we conducted a concentration dependence study for both the R- and the T-analogue states in the stripped condition. We have observed a logarithmic dependence of $p_{\lambda_0}$ on $C_0$ in both cases, as shown in Fig. 7, that supports our interpretation of pressure-induced dimerization. We note that the transition pressure changes only by...
Allosteric Effectors Influence Hemoglobin Tetramer Stability

A few tens of MPa in our experiments, remaining in the range of 100 to 160 MPa, which is characteristic for subunit dissociation.

The tetramer-dimer transition interpretation is further supported by the fact that besides the Trp fluorescence signal, the BZF fluorescence intensity also showed a sigmoid change in accordance with the Trp signal. It was shown (12, 21) that BZF and similar derivatives bind primarily to the central cavity in HbA. This region is expected to be affected in a structural transition like dimerization. The increased quantum yield in this transition may originate from a structural change during dimerization, which makes new hydrophobic areas accessible to the already bound BZF molecules or for those in the solvent.

The Effect of Allosteric Effectors—A HbA solution is always a dynamic mixture of tetramers and dimers; however, the dimer concentration is usually very low. The dimer-tetramer equilibrium was extensively studied along with the kinetics of the dissociation (63–68). Under our conditions the dimer ratio is estimated to be less than 1% in the R-state and less than 0.1% in the T-state. Physiological conditions (high Hb concentration and close to neutral pH) shift the equilibrium toward the tetrameric form. Table 1 shows the characteristic $p_{1/2}$ values observed for the stripped samples and for the samples with CI$^-$, IHP, and BZF, both in oxy-Hb and $\alpha$-oxy-Fe$\beta$Zn-Hb samples. We also studied DPG binding to $\alpha$-oxy-Fe$\beta$Zn-Hb. These data show a clear change in the $p_{1/2}$ values upon the binding of allosteric effectors and, consequently, also in the $K_{d0}$ and $\Delta V$ values as listed in Table 3.

Considering the data for the R-state, the determined dissociation constants ($K_{d0}$) agree well with previous reports on R-state HbA (65), where tetramer to dimer dissociation constants were measured in the case of stripped human oxy-HbA and also in the case of the IHP-complexed HbA. A dissociation constant of 1 $\mu$M was found for the stripped case, which decreased to 0.18 $\mu$M when complexed to IHP. These results are in excellent correlation with our results shown in Table 3. In a study of the Bohr effect (65), the authors found a value of 0.5–7.9 $\mu$M for human oxy-HbA depending on the pH and protein concentration, which is again in good agreement with our results.

Compared with the R-state, in the case of the T-analogue we found an overall opposite effect: the allosteric effectors decreased the transition pressures as shown in Table 1. This trend points to an increase in the dissociation constant, as shown in Table 3. For deoxyhemoglobin, $K_{d0}$ values 4–6 orders of magnitude lower than values for the R-state were reported in the literature (36, 65, 69). We also observed the same trend but the effect was smaller. This we attribute to the fact that we studied a T-state analogue, which, by having a more tense conformation (40), may be less sensitive to the binding of the effectors. This finding supports our choice of a semi-hybrid structure as a T-analogue and not a tetra-zinc-substituted Hb, which might be so tense that no effect could have been observed. To validate our $K_{d0}$ values, it is useful to consider the $\Delta G$ value for the same two systems (oxy- and $\alpha$(FeO)$_2\beta$(Zn)$_2$-Hb) found in Huang et al. (36). The ratio of the two tetramer to dimer dissociation constants derived from this value ($\Delta G = 0.2$ kcal/mol) is $\sim 0.7$, which is very close to our experimental result. This good agreement we consider as an internal control of our method.

In the T-state analogue we could compare the efficiency of allosteric effectors of different chemical structures. DPG is structurally related to IHP; however, IHP is known to be a stronger effector in oxygen binding experiments (19). Our data show that DPG has the same kind of effect (increase of the dissociation constant) on the $\alpha$-oxy-Fe$\beta$Zn-Hb than IHP but with less efficiency. This shows that difference in effector potency is manifested in the strength of interactions at the dimer interface. In a UV resonance Raman study where the Trp-$\beta$37–Asp-94 H-bond strength could be monitored, no effect was observed when IHP was added to $\alpha$(FeCO)$_2\beta$(Zn)$_2$-Hb in a proportion similar to our experiments (39). The resonance Raman study, however, monitors a local effect involving the Trps-$\beta$37, whereas in our study we report overall changes at the whole interdimeric interface. Thus the two findings cannot be directly connected. It would have been interesting to compare the effect of the phosphates with that of an aromatic molecule such as BZF. The intense fluorescence of this effector in the bound state, however, did not allow us to derive reliable dissociation constants for this complex. It can be seen in Fig. 5 that the fluorescence of BZF interferes with the Trp emission, making the evaluation of Trp emission maximum difficult, especially at high hydrostatic pressures, where the BZF fluorescence outweighs the Trp emission signal. We can, however, conclude that changes in the BZF emission also indicate significant structural changes at the dimer interface in HbA. The effect of BZF seems to be different from that of DPG and IHP (see Table 1). In both R- and T-states BZF has a stabilizing effect on the interdimeric interface, in a way that the stability will be the same in the two states in the presence of BZF. This effect can be related to the results reported for an effector of similar structure, 2-[4-[[3,5-dichlorophenyl]amino]-carbonylamino]phenoxyl-2-methylpropanoic acid, to cause the decrease of CO affinity when added together with IHP to $\alpha$(FeCO)$_2\beta$(Zn)$_2$-Hb (39). The authors of this work attributed this affinity change to a conformational effect of the aromatic effector resulting in a more tense T conformation.

Changes in dissociation constants in the T-state analogue show that allosteric effectors induce conformational changes that propagate to the interface also in this form. It is interesting that changes caused by IHP or BZF binding were not observed in the heme pocket by resonance Raman spectroscopy in the fully deoxy-state of HbA (70). It can be that propagation of conformational changes from the interface to the heme pocket is inhibited in the entirely unligated state but not in partially or fully ligated states. The communication pathway from the interdimeric interface to the heme pockets is a topic of further detailed studies.

Until now we discussed the observed changes in the dissociation constants. It is also interesting to estimate how significant the direct effect of the change in $K_{d0}$ may be on the oxygen association. This we can do for the R-state based on characteristic half-oxygen saturation ($P_{50}$) values from the literature (see for example Atha and Riggs (65), $P_{50} = 0.4$ mm Hg for the dimer form and 1.78 mm Hg for the tetramer). As an approximation,
we assume that the proportion of the dimers is low enough (less than 1%) such that the measured \( P_{50} \) values can be considered as a linear combination of the tetramer and dimer \( P_{50} \) values. From \( K_{d0} \) we know the proportion of the dimers, and so we can estimate the change in \( P_{50} \) caused only by a change in the tetramer-dimer equilibrium. Under our experimental conditions (i.e. 60 \( \mu \text{M} \) total HbA concentration) and for the IHP binding to oxy-Hb as an example, we obtain a 10% increase in \( P_{50} \) as an estimate. In the literature, the reported changes in \( P_{50} \) are more than a magnitude higher (18) as an effect of binding IHP, i.e. changes in the dissociation constants cannot account for the total effect of allosteric effectors on oxygen binding. However, there are cases, for instance that of the mutant Hb Howick, where dimerization plays a central role in the control of Hb function (71). In such a case, the changes in \( K_{d0} \) may have more significance. In the interpretation of the Bohr effect, the role of dimerization was also emphasized (65). We note, however, that in the T-state we can totally neglect the direct effect of \( K_{d0} \) on the \( P_{50} \) values, because \( K_{d0} \) is by orders of magnitude lower than in the R-state.

An overview of the effects of allosteric effectors on the tetramer-dimer equilibrium is presented in Scheme 1. Double-ended arrows in Scheme 1 represent the equilibrium, and dashed arrows show the influence of allosteric effectors. The changes in these equilibria are a direct consequence of tertiary structural changes propagating from the effector binding sites to the dimer interfaces in both quaternary states.

The volume changes during the dimerization process are also listed in Table 3. These changes arise from the following two factors: 1) from the change in the molecular volume of the protein, and 2) from the change in the volume of the water shell around the protein. In the oxy-Hb samples, volume changes do not correlate with the changes in dissociation constants and remain essentially the same in all cases. In the \( \alpha \)-oxy-Fe–\( \beta \)-Zn-Hb samples, a 20% variation in the volume changes can be observed for the strong effector IHP. Further analysis of this effect may enable us to distinguish between the two factors contributing to this effect.

Conclusions—1) The good agreement with available data obtained by other techniques show that the dissociation constants for the tetramer to dimer transition of Hb determined by the pressure perturbation method yield reliable data. We note that among the possible techniques (e.g. size exclusion chromatography, light scattering, sedimentation velocity techniques, and CO binding experiments), the high pressure approach is outstanding in its simplicity.

2) The decrease in the dissociation constants upon binding of allosteric effectors in the R-state in itself leads to lower oxygen affinity. This direct effect, however, represents only a minor contribution to the reported change in oxygen affinity.

3) Allosteric effectors induce conformational changes in both the oxy-Hb and the \( \alpha \)-oxy-Fe–\( \beta \)-Zn-Hb samples corresponding to the R- and T-states of HbA, respectively. These changes affect the structure of the interdimeric interface, resulting in changes of the dissociation constants. This finding supports the global allostery model by showing that conformational changes of the tertiary structure propagate from the effector binding site(s) to the interdimeric interfaces.

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