High Pressure Enhances Hexacoordination in Neuroglobin and Other Globins*

Received for publication, June 8, 2005, and in revised form, July 27, 2005 Published, JBC Papers in Press, August 13, 2005, DOI 10.1074/jbc.M506253200

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The techniques of high applied pressure and flash photolysis have been combined to study ligand rebinding to neuroglobin (Ngb) and tomato Hb, globins that may display a His-Fe-His hexacoordination in the absence of external ligands. High pressure induces a moderate decrease in the His association rate and a large decrease in His dissociation rate, thus leading to an enhancement of the overall His affinity. The overall structural difference between penta- and hexacoordinated globins may be rather small and can be overcome by external modifications such as high pressure. Over the pressure range 0.1–700 MPa (7 kbar), the globins may show a loss of over a factor of 100 in the amplitude of the bimolecular rebinding phase after photodissociation. The kinetic data show that pressure induces a moderate increase of the rate for ligand binding from the correlated pair state (just after photodissociation) and a large (factor of 1000) decrease in rate for migration through the protein. The effect on the ligand migration phase was similar for both the external ligands (such as oxygen) as for the internal (histidine) ligand, suggesting the dominant role of protein fluctuations, rather than specific chemical barriers. Thus high pressure efficiently closes the protein migration channels; however, contrary to the effect of high viscosity, high pressure induces a greater decrease in rate for ligand migration toward the exterior (heme to the solvent) versus inward migration, as if the presence of the ligand itself induces an additional steric constraint.

Recent studies of new globins such as neuroglobin or cytoglobin (1–3) have revealed a second mechanism for regulating the oxygen affinity in globins. Although human Hb and Mb have an open binding site for oxygen, the heme iron in Ngb is already hexacoordinated, and oxygen binding requires the displacement of a protein histidine residue (4). Variations in the oxygen or histidine binding rates will influence the observed oxygen affinity (4–6).

The classic three-dimensional globin structure (see Fig. 1) is preserved for the various globins (7, 8), despite the fact that the sequences may vary widely, indicating an early divergence in the globin evolution (1, 2). Specific structural elements and a few highly conserved residues, rather than the similarity of the sequences, may be the critical feature for classifying these globins. Small differences in protein structure may have a large influence on the observed parameters; because small changes in structure are difficult to simulate, experimental data are still required to test the structure/function relationship. In the present case, we show that high pressure is sufficient to switch from a mainly Hb-like pentacoordinated heme to the Ngb-like hexacoordinated form.

The crystallographic studies of Ngb revealed a looser heme pocket and additional protein cavities, relative to Mb (8, 9). The cavities could reveal part of the ligand binding pathway (8–10). Alternatively, the cavities may be necessary to allow the protein to switch between conformational states. Human Ngb may form an internal disulfide bond (5, 11); the affinity for the distal histidine changes by nearly an order of magnitude between the two forms, suggesting a movement of the E-helix. Despite being monomeric, human Ngb is an allosteric protein capable of switching from a high to low oxygen affinity conformation (5).

Recent structural study of murine neuroglobin proposed that heterogeneity, observed so far in the active site (4, 12), could be explained by a sliding movement of the heme within the protein pocket (13) (see Fig. 1). The fact that Ngb switches between two forms, with and without the disulfide bond, may require a more flexible protein structure (5).

Competition with a protein residue offers a second possibility for regulation of the observed oxygen affinity, which then depends on the ratio of the intrinsic affinities of the two competing ligands (4, 5). The disulfide bond on the surface of Ngb may play such a role, because breaking this bond increases the histidine affinity (5), thereby decreasing the overall oxygen affinity. This leads to novel properties of the oxygen affinity, such as a lower temperature dependence (6), because the overall affinity will depend on the ratio of oxygen to histidine affinities. If the external (O2) and internal (His) ligands have a similar dependence on an external parameter such as temperature (T), pressure (P), or pH, the observed change in the oxygen affinity versus this parameter may be small due to a compensation effect. In this study we show that high pressure is an important parameter in controlling the degree of hexacoordination of heme proteins.

From the classic thermodynamic relation, $k = A \exp[-P(\Delta V^\theta)/RT]$, the change in the rate versus pressure allows an estimation of a difference in volume. For a rate coefficient the activation volume $\Delta V^\theta$ corresponds to the difference between the top of the barrier (transition state) to the initial state, whereas the $\Delta V$ for an equilibrium coefficient is the difference between the final and initial states. Certain overall rates or amplitudes may be a function of several microscopic rate coefficients; in this case the analogous $\Delta V$ simply reports the pressure dependence and generally will not show a simple behavior versus P.

Interpretation of these volumes is another question. In general, both positive and negative values are observed for chemical reactions, and for biomolecules the pressure dependence may involve displacement of

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*This work was supported in part by INSERM, the Délegation Générale pour l’Armement (France), the University of Paris XI, the Deutsche Forschungsgemeinschaft (Grants Ha2103/3 and Bu956/5), the University of Antwerp, and the European Commission (Contract QLG3-CT-2002-01548). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§The abbreviations used are: Hb, hemoglobin; Mb, myoglobin; Pa, pascal(s); Ngb, neuroglobin.
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water molecules or other structural rearrangements rather than purely a volume change. Note also that protein cavities may be just as expensive as extra atoms in terms of free energy. This is exemplified in the present study by the fact that ligand binding to heme proteins often shows a negative volume change (higher ligand affinity at higher pressures), despite the addition of the ligand volume.

The present study extends the pressure range by a factor of 3 for flash photolysis studies, allowing a measure of the region where ligand binding is limited by the migration through the protein. Furthermore, by use of the ligand replacement technique, we provide first results on ligand dissociation rates versus pressure. The present study offers more information on the ligand migration through the protein under conditions of high pressure which hinder such movements.

MATERIALS AND METHODS

The recombinant globins were expressed in Escherichia coli (4), and a further purification was made with a fast protein liquid chromatograph Akta Purifier (Amersham Biosciences) using a Hitrap DEAE-Sepharose Fast Flow column (5). The lyophilized myoglobin from horse heart was purchased from Sigma and purified on a G25 column.

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Because the high pressure system is all liquid, there is no change versus pressure in the total amount of protein or ligand. The samples were corrected for the solvent compressibility (15), which (for water) is initially 4% per kilobar (kbar) and a total of 15% at 6 kbar. One also needs to take into account the shift versus P of the Soret band, especially for the CO species, to determine the fraction dissociated (based on the observed signal amplitude). All samples were in 50 mM Tris-HCl buffer at pH 7, because there is a weaker pH variation versus pressure (0.02 units per kilobar) compared with 0.33 unit for potassium phosphate buffer (16).

Flash Photolysis at High Pressures—The photolysis setup consists of a 10-ns ND:YAG (neodimium-doped yttrium aluminum garnet) laser, delivering pulses of 160 mJ at 532 nm (Quantel, France) and a detection beam. Because the high pressure cell was built into the fluorometer, the light source (450-watt xenon lamp) and excitation monochromator were used for the light transmission measurement. The photolysis beam was brought to the fluorometer via an optical fiber to a window at a right angle to the transmission beam. The detection bandwidth was 2 nm for the rapid kinetics but was decreased for the slower ligand dissociation kinetics to avoid ligand dissociation with the probe beam. Light transmission, detected by a photo-multiplier, was recorded at different wavelengths for up to 10 s on a Lecroy (9400) or Waverunner LT342L oscilloscope (6, 17). For slower kinetics the light transmission was rerouted to the fluorometer detection system.

Geminate versus Bimolecular Kinetics—After photodissociation of the iron-ligand complex, there are globally two kinetic phases. A certain fraction of the dissociated ligands rebind to the same iron atom without leaving the protein; this phase, which occurs on the nanosecond timescale under normal conditions (room temperature and low viscosity), is the geminate recombination resulting from the correlated pair produced by photolysis. The fraction of ligands that escape for the protein will compete with other solvent ligands for binding sites forming the bimolecular phase, which is generally on the microsecond to second timescale. For measurements of the amplitude versus pressure, it is important to avoid an excessive intensity of the photolysis beam, which may photolyze certain proteins more than once.

To describe overall kinetics (18), we consider a simplified A ⇔ B ⇔ S reaction scheme, where state B is the correlated (geminate) pair formed by the photodissociation. Because photodissociation places the ligand in state B, the bimolecular yield will depend on the relative rates for rebinding (B to A) or escape to the solvent (B to S). The inner barrier (BA) corresponds to the “chemical” binding step to the iron atom, and the rate constant $k_{BA}$ generally increases with $T$, but is nearly independent of solvent viscosity (19).

$N$ Barriers—The internal migration from B to the solvent S may involve several barriers (18). We first considered a three-state (A-B-S) versus four state (A-B-C-S) model, as well as $N$ identical barriers. In the case of large $N$ (10) and a fast rate for jumping these barriers relative to the step B to A, the geminate kinetics takes on the same form as the solution of the diffusion equation (slope of one-half on a log-log plot); the advantage of the discrete barriers is that one can also consider the case of a very rapid rate $k_{BA}$ ($\gg k_{BS}$) such as NO rebinding, where the geminate phase may then take on a more exponential shape. For the present study, we simply note that extraction of the overall features of the $P$ dependence in not critical on the details of this model.

Observed Rates versus Microscopic Parameters—The overall rate is usually denoted as $\lambda$ rather than $k$ to indicate that it involves several steps or barriers. Thus $\lambda_{on}$ refers to the overall (S to A) bimolecular on-rate of ligand binding, and $\lambda_{off}$ the ligand dissociation (A to S) or off-rate. The fraction that escapes the bimolecular yield $f_{bimol}$, also called $N$-out (18, 19), depends on the same rates, reflecting the fact that the photodissociated ligand, or one arriving from the solvent, is confronted with the same choice of binding or escaping from state B.

The change in overall rate versus pressure $P$ will depend on the “region”; that is, whether the overall rate is limited by the internal “chemical” barrier ($k_{BA} \ll k_{BS}$) or by the migration through the protein matrix ($k_{BA} \gg k_{BS}$). For the observable rates (of reactions going to completion) and amplitude, the dependence on the rate coefficients is given in Equations 1–4.

$$f_{bimol} = k_{BS}/(k_{BA} + k_{BS})$$

$\rightarrow 1$ (barrier limited) (Eq. 1)

$$\lambda_{on} = k_{SB}^*k_{BA}/(k_{BS} + k_{BS}) = k_{SB}^*(1 - f_{bimol})$$

$\rightarrow k_{BA}/k_{BS}^*$ (barrier limited) (Eq. 2)

$$\lambda_{off} = k_{AB}/(k_{BA} + k_{BS}) = k_{AB}^*f_{bimol}$$

$\rightarrow k_{AB}$ (barrier limited) (Eq. 3)

$$\lambda_{gem} = k_{BA} + k_{BS}$$

(Eq. 4)
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There is some compensation between the fitting parameters. A measurement of the rate of geminate recombination $A_{gem}$ versus $P$ provides a more direct measure of $k_{BA}$ and a unique set of activation volumes. When the reaction is barrier-limited, the amplitude of the geminate phase approaches zero. In general the geminate phase is not a simple exponential kinetics.

RESULTS AND DISCUSSION

Hexacoordination versus Pressure—It is known that, under certain conditions, especially near denaturing points, Hb and Mb may also take on the hexacoordinated form (20), previously denoted as hemochrome (or hemichromes for the ferric species). NMR or Raman studies (21, 22) have described the formation of hemichromes of Mb or Hb resulting from a reorganization of the heme pocket involving a collapse or degradation of the protein. As can be seen in Fig. 1, a small change in structure may be sufficient for such a change in the iron coordination. We therefore applied pressure to various heme proteins to test for evidence of hexacoordination. Both the $\alpha$ and $\beta$ chains of human Hb display a transition within 400 MPa, whereas Mb showed the start of such a transition at 600 MPa (data not shown).

Tomato Hb is a good example for the visualization of the pressure effect on the coordination state at the distal position, because this globin is only partially hexacoordinated under normal conditions, as reported for rice Hb (23). As seen in Fig. 2, pressure increases the fraction of hexacoordination, as evidenced by the enhanced alpha absorption band. There is not an observable spectral difference of this band for Ngb, because the protein is already $>99\%$ hexacoordinated at 1 atm.

Kineti cs—We have investigated the ligand binding kinetics of CO, O$_2$, and the (E7) histidine for the hexacoordinated globins such as Ngb, Mb, and Tomato Hb under high pressure using flash photolysis. The application of high pressure to study the ligand binding kinetics of heme proteins has contributed to a better understanding of the protein dynamics for pentacoordinated globins such as Mb (24–28), the isolated chains of human Hb, and cytochrome P450 (29, 30).

When the pressure is increased, there is a decrease in the amplitude of the bimolecular phase, indicating a larger fraction of the rapid geminate rebounding (Fig. 3). This is in agreement with previous studies reporting a lower bimolecular yield and a higher rate of geminate binding (29).

Mb is known to have a high bimolecular yield after photolysis (barrier-limited), and there is thus little observed change $f_{bimol}$ of pressure in the low pressure region; at sufficiently high pressures, the rate for protein migration becomes the limiting step, and one observes a dramatic decrease in the fraction bimolecular (Fig. 3). Ngb shows about 50% bimolecular phase at 1 atm ($k_{BA} = k_{BA}$) and the large change in $k_{BA}$ influences the fraction bimolecular over the entire pressure range. Note that the simulations (solid lines in Fig. 3) employ a single $\Delta V^s$ for each rate coefficient over the entire pressure range (TABLE ONE, and see Fig. 7).

Recombination kinetics after photodissociation of CO are shown for tomato Hb in Fig. 4. At low pressures, the kinetic curve is nearly monophasic as for pentacoordinated Mb. At higher pressures the His dissociation rate greatly decreases and the kinetics take on the biphasic shape characteristic of hexacoordinated Ngb.

Unlike the “pentacoordinated” Mb, which displays a single exponential bimolecular phase, the ligand competition for hexacoordinated globins such as Ngb leads to a biphasic shape (4). After photodissociation, the bimolecular kinetics of Ngb occurs as two distinct phases, differing by about a factor of 1000. The rapid bimolecular phase involves competitive binding of the external ligand (CO) and the internal protein ligand (the distal histidine). For the fraction that binds His, the replacement reaction to return to the initial CO form requires about 1 s; note that one should use a low repetition rate to measure the kinetic curves to allow for full sample recovery.

One obtains information about the association rate for both ligands, and the dissociation rate for the histidine. A full separation of these three parameters is best done by considering a series of kinetic curves at different CO concentrations (4–6).

Association Rates—As indicated in Reaction 2, the ligand association rate depends on several microscopic parameters. Thus the variation with $T$ or $P$ may not show a simple dependence. The CO on-rate for Mb versus pressure is an example (Fig. 5). In the low pressure region, the on-rate increases, because the overall on-rate depends mainly on the barrier B to A; however, at sufficiently high pressure, the outer barrier becomes the limiting factor, and this step has an opposite $P$ dependence. As for the fraction bimolecular, the simulations (solid lines in Fig. 5) employ a single $\Delta V^s$ for each rate coefficient. The change in slope of the observed parameter (Fig. 5) does not indicate a change in the protein, but is a consequence of the switch from barrier to migration limited reaction; the transition in slope occurs when the fraction bimolecular (Fig. 3) is about 0.5. To better extract the $\Delta V^s$ for each rate coefficient, constrained simulations were made on the three observables: $f_{bimol}$ as well as the overall ligand on and off rates. The geminate rate for Mb was independently used to better determine the values for $k_{BA}$.
Histidine Dissociation—Dissociation rates versus pressure are difficult to measure, because mixing reactants under pressure (to start a replacement or scavenger reaction) is not practical. However, the replacement reaction is a natural consequence of the competitive binding in the case of Ngb. Thus Ngb offers a unique case to study ligand dissociation versus pressure, because of the competition between the external ligand and the internal protein ligand (the distal histidine). After photodissociation of CO, a certain fraction of the hemes bind the protein distal histidine, and the slower replacement reaction (see Fig. 4) provides information on the histidine dissociation rate. Note that Ngb has two major conformations: when the disulfide bond is broken, the His off-rate is about 9 times slower, which influences both the His affinity and the observed affinity for the external ligands. The values reported here refer to this case, because the mutant without cysteines was studied.

We measured the kinetics versus $P$ (at several CO concentrations) to obtain the histidine rate parameters; however, at high $P$, the bimolecular yield was greatly decreased (Fig. 3), and the amplitude of the reaction in question approached zero. To increase the signal for the replacement reaction, we employed a method similar to photo bleaching: because the histidine dissociation is slow (1 s at 1 bar and decreases with higher $P$) relative to the maximum laser repetition rate (10 Hz), one can use a multiple flash method to populate the meta-stable His-bound form. We employed the series of pulses to obtain a high population of the Fe–His state; the laser was then stopped and the transition from the Fe–His to Fe–CO form could be followed.

Because the His dissociation rate is greatly decreased at high pressure, it became impractical to use the usual transient recorder on the slow timescale. We therefore re-routed the transmission signal to the fluorometer detector, where the high pressure cell was installed. The kinetics displayed a time coefficient of 15 min at 500 MPa. For such low rates, the kinetics may be long relative to the actinic rate of CO photodissociation. The detection beam intensity was progressively decreased to avoid perturbing the kinetics by a light-induced CO dissociation reaction. We preferred to accept a higher noise level rather than a perturbed (or time-dependent) baseline. Even under these conditions it was not practical to measure the kinetics over the full pressure range, because the reaction at our limit of 700 MPa would require hours.

The oxygen dissociation rate can be obtained by a similar method. In this case a mixed atmosphere of CO and O$_2$ was used. Because CO binds more tightly, but oxygen binds more rapidly, photodissociation of the CO leads to a transient Fe–O$_2$ population. As for the histidine, the on and off rates of the transient ligand can be extracted.

The variation of the ligand binding rates versus pressure is shown in Fig. 6. The dissociation rates display a much larger change than for ligand association. Based on an equilibrium study of CO binding, we expected the CO dissociation rate to behave in a similar fashion. Surprisingly, the dependence of the dissociation rate on pressure was nearly independent of the ligand. This would indicate a more mechanical process of ligand migration dependent on protein fluctuations, as opposed to ligand specific chemical interactions.

Ligand Migration—The study of ligand binding kinetics has provided much information on the structure/function relationship of heme proteins. The initial measurements versus temperature revealed multiple reaction steps for the ligand migration between the solvent and the iron atom; the reaction was previously modeled as a series of internal barriers requiring some protein movement to allow the ligand to pass (18, 19). The rates for these transitions are dependent on the solvent viscosity, indicating a strong coupling of the internal fluctuations with the external environment. All the reactions were slower at high viscosity, except the final chemical-binding reaction ($B\rightarrow A$ in the original model).

High pressure also greatly slows the migration steps; however, unlike viscosity, it had an asymmetric effect (see Fig. 7). Viscosity had an equal effect for migration crossing the outer barriers from either direction, whereas pressure had a much greater effect for ligand migration toward the solvent. In addition the geminate kinetics for Mb was more exponential compared to the high viscosity samples using cosolvents. As might be expected, pressure closed the migration channels, but the presence of the ligand seemed to add an additional constraint. Based on typical solvent compressibilities (15% decrease in the volume of water at 600 MPa, and ~8% for glycerol), high pressure would further close the pathway by only a few percent. Because the ligand is larger than the channel size, or equivalently, the protein must fluctuate to allow the ligand passage (19, 31), the ligand itself induced an additional steric factor. This physical description was previously described (see Footnote 4 of Ref. 19), comparing the entry of the ligand in the protein to a “cow in the tent.”

The values of the pressure dependence of the ligand binding parameters are summarized in TABLE ONE. Both static and kinetic methods were combined to determine the various rates and their pressure dependence. For example, the equilibrium coefficient $K$ for His binding to Mb and tomato Hb could be determined from static absorption spectra versus $P$; however, Ngb is already saturated with His at 1 atm, so $K$ was determined from the flash photolysis rate coefficients. Note that the His ligand was not photodissociable in Ngb, so competition after dissociation of CO was employed to determine the His rate coefficients.

In addition, both pressure ranges (of high and low bimolecular yield) were employed to better extract the microscopic rate coefficients. This was possible for Mb, but was less clear for Ngb. The values for $k_{BA}$ and $k_{AB}$ for Ngb were thus not yet well determined; however, the changes in the observable on and off rates, as well as the amplitudes, were similar for the two proteins.

CONCLUSIONS

Stability—The pressure effects on Mb and Ngb were fully reversible to 700 MPa. Tomato Hb showed a shift to another state at 400 MPa but was still reversible. Other proteins such as the Hb chains, or cytochrome P450, did not show reversible changes versus pressure.

Hexacoordination—Pressure induced hexacoordination, because the His off-rate decreased more than the His on-rate. The observed pressure effect depended on whether reaction was barrier- or diffusion-limited, and a change in slope may be observed when the protein...
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Pressure dependence of the ligand binding parameters for horse heart Mb and human Ngb

Experimental conditions were 50 mM Tris-HCl, pH 7, at 25 °C for the flash photolysis and equilibrium techniques. The volumes are for the high pressure region where the fraction bimolecular is low. For Mb, a second set of parameters could be determined when the fraction-bimolecular is high, which allows a better determination of the volumes for the microscopic rate coefficients: for the model A-B-S, the activation volumes were 11, 12, 16, and 18 cm³/mol for the steps AB, BA, BS, and SB, respectively (see Fig. 7). The volumes were calculated as: \( \Delta V = -RT \ln \lambda / \lambda P \) for any parameter \( \lambda \) (for example, the overall on or off rates \( \lambda \), or the fraction-bimolecular) versus pressure. A slope of a factor 2 per kbar (100 MPa) would yield a volume of 17 cm³/mol; note that a volume of 20 cm³/mol = 30 Å³/molecule is equivalent to the volume of a water molecule. The intrinsic ligand affinity of the pentacoordinated form is \( K_{\text{His}} \), which is dimensionless. For the hexacoordinated forms, the expression of the observed affinity for the external ligand is \( K_{\text{obs}} = K / (1 + K_{\text{His}}) \). The observed volume will then depend on whether the presence of the competing ligand: \( \Delta V_{\text{obs}} = \Delta V - (K_{\text{His}}/1 + K_{\text{His}}) \Delta V_{\text{His}} \).

| Parameter | \( \Delta V \) Value at 0.1 MPa (1 atm) |
|-----------|----------------------------------------|
|           | Mb          | Ngb         | Mb            | Ngb          |
| On-rate (\( \lambda_{\text{on}} \)) |                 |             |               |               |
| CO        | 16          | 6           | 0.65          | 50           |
| \( O_2 \) | 14          | 12          | 15            | 170          |
| His-E7    | 8.5         |             |               | 2000         |
| Off-rate (\( \lambda_{\text{off}} \)) |                 | (s⁻¹)       | (s⁻¹)         |               |
| CO        | 38          | 41          | 0.02          | 0.004        |
| \( O_2 \) | 30          | 44          | 10            | 0.8          |
| His-E7    | 43          |             | 0.8           |               |
| Equilibrium |             | (\( \mu \text{M}^{-1} \)) | (\( \mu \text{M}^{-1} \)) |               |
| CO        | -22         | -35         | 32.5          | 12,500       |
| \( O_2 \) | -16         | -32         | 1.5           | 212          |
| His-E7    | -35         | -34.5       | 2.5           | 2,500        |
| CO, \( \Delta V_{\text{obs}} \) | -22         | -0.5        | 32.5          | 5            |
| \( O_2 \), \( \Delta V_{\text{obs}} \) | -16         | 2.5         | 1.5           | 0.08         |
| Fraction-bimolecular |            |             | \(~1\)       | 0.6          |

FIGURE 4. Ligand rebinding kinetics after photodissociation of CO from tomato Hb.

Higher pressure favors a higher affinity for the internal histidine residue, leading to a biphasic shape of the ligand recombination, where the slower phase corresponds to the fraction that binds histidine.

switches between these regions (as evidenced by the CO on rate for Mb). Note that the condition for the transition from barrier- to migration-limited kinetics is the same as for transition from a high to low bimolecular yield. For proteins such as Hb or Ngb, which already showed a lower bimolecular yield at 1 atm, the barrier did not limit the reaction.

For these reasons the observed on-rate (S to A) may show opposite trends for different proteins. Mb showed an initial increase in on-rate versus \( P \), whereas Ngb showed a decrease. As the \( P \) was further increased, the rate for CO binding to Mb reversed direction. This was not due to a change in thermodynamic parameter (\( \Delta V \)), but simply the transition from the barrier- to diffusion-limited kinetics. The decrease of a factor of 100 in bimolecular yield (Fig. 3), and the reversal of the on-rate (Fig. 5) can be simulated with a single set of parameters (TABLE ONE and Fig. 7).

Ligand Migration—High pressure lowered the rates for ligand migration through the protein, as for high viscosity samples. At very high pressure, this was the dominant effect and the ligand escape probability became very small (Fig. 3).

Asymmetry—For the systems studied, there was larger pressure effect in the outward steps (BS versus SB), unlike viscosity, which produced a symmetric change, leaving the ligand affinity unchanged. Note that even at 1 atm there were no open ligand channels; the protein must move to allow the ligand to cross the protein matrix (18, 19, 31).

For Mb, the initial increase reflects the acceleration of the geminate rate (B to A step) in the region where the kinetics are barrier-limited; at higher pressures, the migration through the protein matrix becomes rate-limiting, and this process is greatly slowed by high pressure. Solid lines are simulations using a single \( \Delta V \) value for each rate coefficient in the multiple barrier (A-B-S) model.
the $P$ effect of the ratio of the rates will therefore disappear, leaving only the effect on the BS migration, which depends on the protein fluctuations. However, simulations could also be made with a larger $\Delta V^d$ for $k_{AB}$. In any case, as for temperature and pH, the overall oxygen affinity of neuroglobin will be little changed by high pressure ($\Delta V_{obs}$ values in TABLE ONE).

The dominant effect of pressure is the enhanced hexacoordination and a decrease of protein fluctuations necessary for ligand migration. A main difference with the viscosity effect is the asymmetry of the reaction. Viscosity tends to slow all the outer barriers in a similar fashion, leaving the ligand affinity little changed. Pressure slows these barriers as well; however, the rate to leave the heme pocket (step B to S) decreased by over a factor of 1000, as if the presence of the ligand further stressed the protein and decreased the migration channel.

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