Heme Proteins

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Reverse Spin-Crossover and High-Pressure Kinetics of the Heme Iron Center Relevant for the Operation of Heme Proteins under Deep-Sea Conditions**

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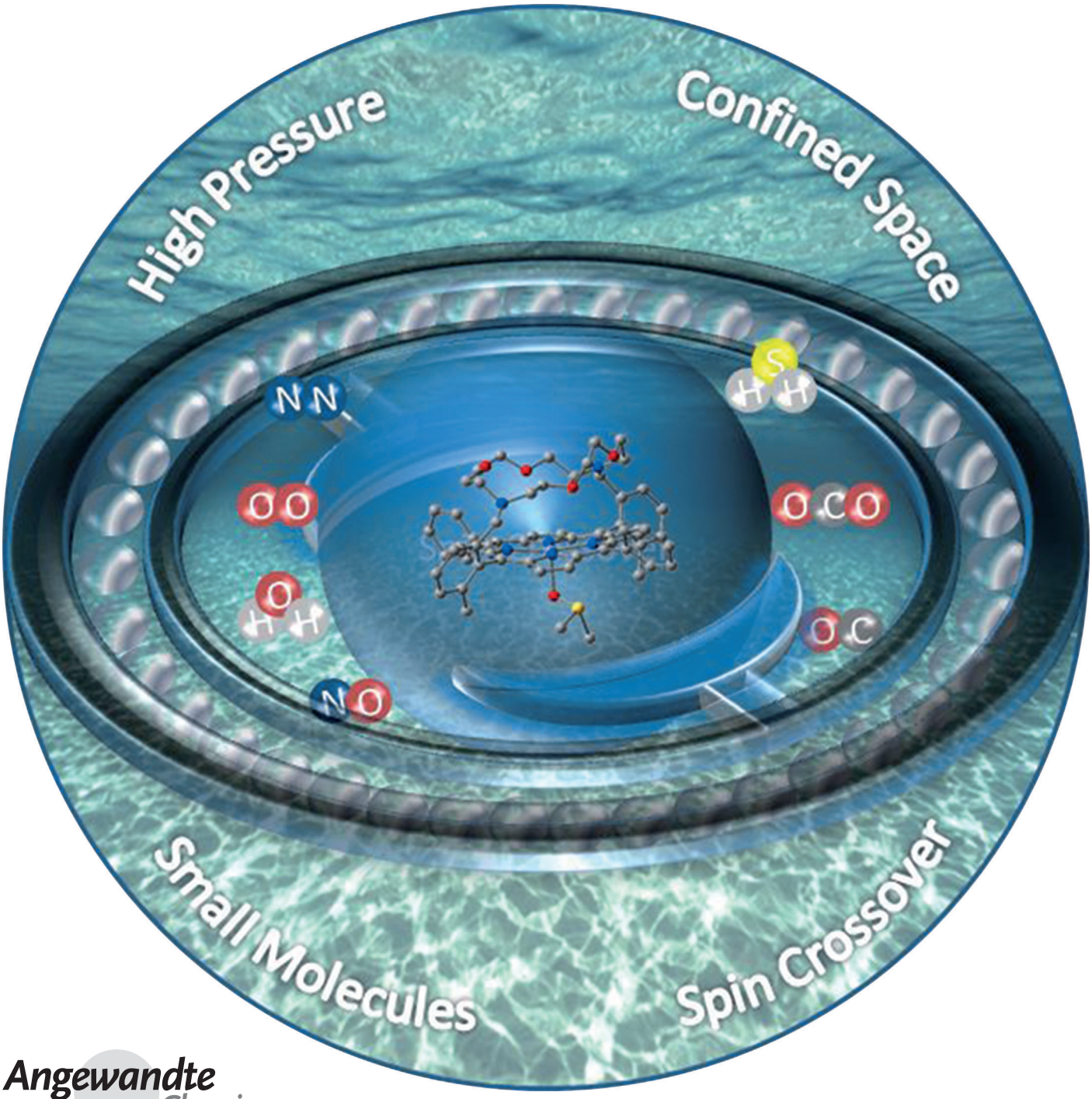

Abstract: *By design of a heme model complex with a binding pocket of appropriate size and flexibility, and by elucidating its kinetics and thermodynamics under elevated pressures, some of the pressure effects are demonstrated relevant for operation of heme-proteins under deep-sea conditions. Opposite from classical paradigms of the spin-crossover and reaction kinetics, a pressure increase can cause deceleration of the small-molecule binding to the vacant coordination site of the heme-center in a confined space and stabilize a high-spin state of its Fe center. This reverse high-pressure behavior can be achieved only if the volume changes related to the conformational transformation of the cavity can offset the volume changes caused by the substrate binding. It is speculated that based on these criteria nature could make a selection of structures of heme pockets that assist in reducing metabolic activity and enzymatic side reactions under extreme pressure conditions.*

About 70% of the Earth's biosphere is exposed to a high-pressure environment.[1] Such conditions can extremely affect binding of small biologically relevant gaseous molecules (e.g. O_2, CO, NO, and H_2S) to a vacant coordination site of heme iron centers,[2] which is a fundamental molecular event behind versatile biological functions of heme proteins. However, we still do not know how a ligand coordination/dissociation and related spin-transition of the metal center[3] are regulated under deep-sea high-pressure (up to 110 MPa) conditions.[5] Based on classical kinetics and thermodynamics we expect that the molecule binding to a vacant coordination site within the pockets of the enzymes is accelerated at high pressures and that a low-spin state of heme proteins (usually in a form of the six-coordinate aqua species) should be favored under such conditions.[1b,6] Both effects would have lethal consequences, because they would speed-up respiration (metabolism in general) and promote undesired side-reactions.[2c] But biology shows us opposite. Namely, it is observed/proposed that under these extreme conditions some microorganisms are placed in a state of "suspended animation", that is, their enzymatic reactions are slowed-down.[1a] What is the explanation for this discrepancy between physicochemical principles and biology at a molecular level? To offer an answer to this question we need to study the solution behavior and reactivity of appropriate heme model complexes at elevated pressures because interpretation of high-pressure kinetics in the case of (heme) proteins is usually not straightforward.

The reason lies in a number of processes that accompany small-molecule binding/dissociation and result in a certain volume change, thus being pressure sensitive. The pressure effects were mostly attributed to solvation of protein surfaces and the heme pocket, changes in the conformational states of the active sites and/or the protein matrix as a whole, as well as to contributions of bond formation/cleavage and spin state changes of heme iron.[2a,e,5a] In order to distinguish between these different factors, and to concentrate more on the intrinsic mechanism of molecule binding to the iron center as the elementary reaction step, reactions of specially designed, sterically demanding porphyrin-based model complexes can be studied, where the CO ligand usually serves as an appropriate probe.[2b] Although the binding of O_2, NO, and H_2S is more physiologically relevant, it is usually accompanied by an inner-sphere electron transfer that because of the charge change and consequent solvent electrostriction causes an additional volume change along the reaction path. Thus, the application of CO as the redox innocent ligand, in the first instance, enables us to reveal a pure intrinsic contribution to the activation/reaction volume associated with the small-molecule binding independent from the contribution of solvation. Furthermore, the influence of the confined space on high-pressure behavior is expected to be crucial and can mimic the steric/conformational factors within the heme pocket at elevated pressures. Thus, it is of a particular interest to elucidate the kinetics and thermodynamics of small-molecule binding under elevated pressures in the case of model systems with a closed cavity that resembles the distal site of the heme pocket. Such investigations, however, are unknown for porphyrin model complexes (see related literature information in the Supporting Information). Therefore, in this work we have synthesized (see the Supporting Information) the Fe^{II} porphyrin complex [Fe(P1)] (Scheme 1), with the crown ether moiety as a conformational flexible cap, which generates a confined space in a very close proximity to the sixth coordination site. The solution, kinetic, and thermodynamic behavior of [Fe(P1)] at elevated pressures has been studied in dimethyl sulfoxide (DMSO) and a DMSO/CH_3CN mixture (7:3) with CO as a small-molecule probe.

To enable adequate comparisons, the same studies were performed for the analogue complexes [Fe(P2)] (with a semi-confined space) and [Fe(P3)] (without a sterically defined binding cavity; Scheme 1), previously synthesized and studied by us regarding reactivity towards superoxide.[4,7,9]

In the DMSO solutions (pure or DMSO/CH_3CN mixtures) of the studied Fe^{II} complexes an equilibrium between the high-spin (HS, $S=2$) mono- and the low-spin (LS, $S=0$) bis-DMSO complexes, $[Fe(P)(DMSO)_n]$ ($n=1$, 2), exists (Scheme 2).[4,7–9] Using pressure- (in the range 2–150 MPa)

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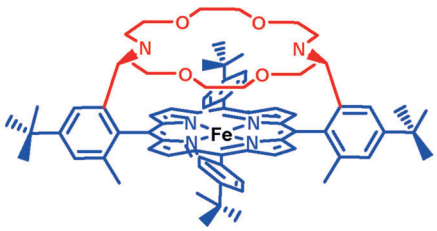
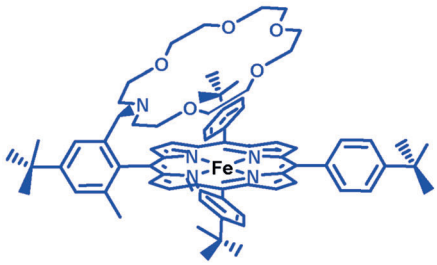
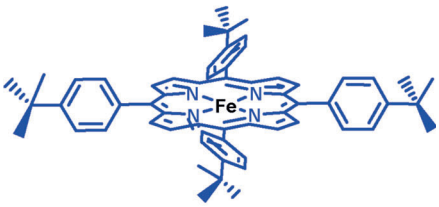


[Fe^{II}(P1)]



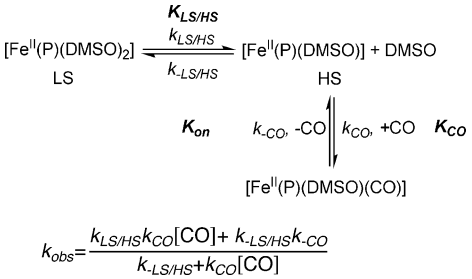
[Fe^{II}(P2)]



[Fe^{II}(P3)]

Scheme 1. Structural formula of the studied complexes.

and temperature- (in the range 298–333 K) dependent ^{1}H NMR spectrometry[7] (see Figure S6–S10 and Tables S1 and S2 in the Supporting Information) we have, for the first time, quantified the effect of a confined binding site on the thermodynamics of such type of coordination-induced spin-transition equilibria.[3a] For comparison, the corresponding equilibrium constants $K_{LS/HS}$ and the reaction volumes $\Delta V^0_{LS/HS}$ are tabulated in Table 1 (for the reaction enthalpies $\Delta H^0_{LS/HS}$ and enthropies $\Delta S^0_{LS/HS}$ see Table S2). In contrast to [Fe(P2)] and [Fe(P3)],[4,7] the ^{1}H NMR signals for the paramagnetic pyrrole protons of [Fe(P1)] are profoundly shifted, for about 40 ppm, towards lower field (Figure S10), leading to a calculated high-spin ratio of 80.3 % at 298.2 K. This value is markedly higher than those obtained for [Fe(P2)] (3.0 % of HS) and [Fe(P3)] (7.6 % of HS) resulting in the LS-to-HS transition equilibrium constant that is two orders of magnitude higher for [Fe(P1)] (Table 1). The stabilization of the high-spin state of [Fe(P1)] can be ascribed to the small confined space over the porphyrin plain, hampering the accessibility of the sixth coordination site for DMSO molecules. The response of the spin equilibrium (Scheme 2) in the case of [Fe(P1)] on temperature and pressure changes was quite surprising. For the first time we could observe the equilibrium shift towards low-spin state by a temperature increase (Figures S6 and S8 and Table S1), which is contrary to what is generally expected for spin-crossover processes and for a normally dissociative character of the underlying LS/HS transition.[6] Pressure-dependent ^{1}H NMR measurements support this unique behavior of [Fe(P1)] and instead of resulting in a big positive reaction volume, with values normally ranging between +10 and +30 cm^3 mol^{-1},[7] they lead to a small negative $\Delta V^0_{LS/HS}$ (Table 1). Again, opposite to what was observed for [Fe(P2)] and [Fe(P3)], where elevated pressure favors DMSO coordination and the low-spin state, a small but visible downfield shift of the paramagnetic pyrrole protons of [Fe(P1)] upon a pressure increase (Figures S7 and S9) revealed a shift of the equilibrium rather towards the high-spin state (Table S1).



$$k_{obs}=\frac{k_{LS/HS}k_{CO}[CO]+k_{-LS/HS}k_{-CO}}{k_{-LS/HS}+k_{CO}[CO]}$$

Scheme 2. Simplified scheme of the investigated processes[8] and corresponding rate law.

The obtained results suggest that the elevated pressure leads to compression, whereas an increase in temperature stabilizes a more open conformation of the crown ether cap. Therefore, the position of the spin equilibrium for [Fe(P1)] depends on two antithetical effects, with the temperature and pressure dependent size and conformational changes of the cavity and variable accessibility of the pocked for DMSO, evidently dominating over the expected behavior for dissociation of the sixth ligand accompanied by spin transition.

The obtained reaction volume, $\Delta V^0_{LS/HS}$, of almost zero (Table 1) demonstrates that with the appropriate size of the confined binding site that is conformational flexible, that is, strongly compressible, it is possible to almost completely offset the effect of high-pressure and preserve a position of the spin equilibrium of iron heme. In other words, by preserving the high-spin state, an appropriate confined space also protects the heme-pocket from increased contact with the solvent molecules (i.e. water), which chemical potential (i.e. availability) increases under elevated pressures

Table 1: Relevant thermodynamic and kinetic parameters.

	$K_{LS/HS}^{313}$	$\Delta V^0_{LS/HS}$ [cm^3 mol^{-1}]	K_{CO}^{293}[d] [M^{-1}] × 10^6	k_{CO}[e] [M^{-1} s^{-1}]	k_{-CO} [s^{-1}]	$\Delta V^{\neq}_{CO}$ [cm^3 mol^{-1}][f]	$\Delta V^{\neq}_{-CO}$ [cm^3 mol^{-1}][f]
[Fe(P1)]	4.20 ± 0.03[a]	−0.3 ± 0.1[a]	0.025 ± 0.001	(2.38 ± 0.08) 10^3	0.09 ± 0.04	+23 ± 2	−17 ± 2
[Fe(P2)]	0.030 ± 0.001[b]	+26 ± 2[b]	5.6 ± 0.1	(2.63 ± 0.03) 10^6	2.49 ± 0.03	−21 ± 2	+14.4 ± 0.4
[Fe(P3)]	0.082 ± 0.002[c]	+16 ± 2[c]	2.6 ± 0.1	(1.24 ± 0.03) 10^6	2.82 ± 0.03	−13 ± 2	+11.4 ± 0.2

[a] This work. [b] Ref. [4]. [c] Ref. [7]. [d] $K_{CO} = K_{on}/K_{LS/HS}$. [e] $k_{CO} = k_{on}/K_{LS/HS}$. [f] At 294 K.

because of the thermodynamic rules.[10] This has a biological relevance because a controlled accessibility of the heme active sites in cytochrome P450s for water molecules is crucial in order to prevent undesired side processes which can generate cytotoxic reactive oxygen species.[2c]

Having clarified the underlying spin pre-equilibrium ($K_{LS/HS}$, Table 1) for [Fe(P1)], we have performed thermodynamic and kinetic studies on the CO binding to all three Fe^{II} complexes. A characteristic hypsochromic shift of the Soret bands in the UV/Vis spectra observed upon CO coordination (Figure S12–S14) was used for the spectrophotometrical titrations and determination of the overall equilibrium constant $K_{on} = K_{LS/HS} K_{CO}$ (Scheme 2 and Table S2). The analyses of the obtained spectroscopic data (Figure S15–S17; for the experimental procedure, data treatment, and characterization of the obtained carbonyl complexes see the Supporting Information) resulted in the CO binding constants K_{CO} given in Table 1.

The lowest K_{CO} value was found for [Fe(P1)] and is in agreement with the increased steric hindrance that originates from the tightly bound crown ether in the vicinity of the sixth coordination site. The pseudo first order kinetics of the reactions with variable CO concentrations (a high excess of CO over the complex concentrations) was monitored by time resolved UV/Vis stopped-flow measurements as a function of temperature (272.2–300.2 K) and pressure (0.5–140 MPa) (for the experimental procedure see the Supporting Information).

Besides the fact that the CO coordination to [Fe(P1)] is also kinetically less favorable (three orders of magnitude lower k_{CO}) than the coordination to [Fe(P2)] and [Fe(P3)], its k_{CO} (Table 1) is one of the smallest rate constants obtained for the CO binding to any heme model system.[2d,11] Only for the 3,5-pyridine-5,5-hemecyclophane Fe^{II} complex comparably slow CO binding ($k_{CO} = 6 \times 10^2\ M^{-1} s^{-1}$) was reported.[12] This is because that complex also possesses a binding cavity of a similar small size as [Fe(P1)][12] and ligand approach experiences significant steric hindrance in both cases. Most importantly, the obtained data demonstrate an absolutely unique kinetic response of [Fe(P1)] on high-pressures, where the coordination of CO to its five-coordinate form (an associative process) is strongly decelerated (reflected in a large positive activation volume, $\Delta V^{\neq}_{CO}$) and the CO dissociation is accelerated (reflected in large negative $\Delta V^{\neq}_{-CO}$) by pressure increase (Figure S42, discussions in the Supporting Information). This behavior is in line with also unique pressure effect on spin pre-equilibrium described above, opposing well-established axioms in the kinetics and physical chemistry of spin-crossover. Normally, an associative reaction between uncharged reactants is always accelerated and consequently its back dissociation reaction is slowed down by pressure.[2i] This is exactly what we observed in the case of [Fe(P2)] and [Fe(P3)] (Figure S43 and S44, see the Discussions section in Supporting Information) and is reflected in negative activation volumes for CO binding, $\Delta V^{\neq}_{CO}$, and positive activation volumes for CO release, $\Delta V^{\neq}_{-CO}$ (Table 1). The nature of the transition state can be visualized in the corresponding volume profiles that illustrate the volume changes along the overall reaction paths for all three complexes (Figure 1 and Figure S45). The $\Delta V^{\neq}_{CO}$ values

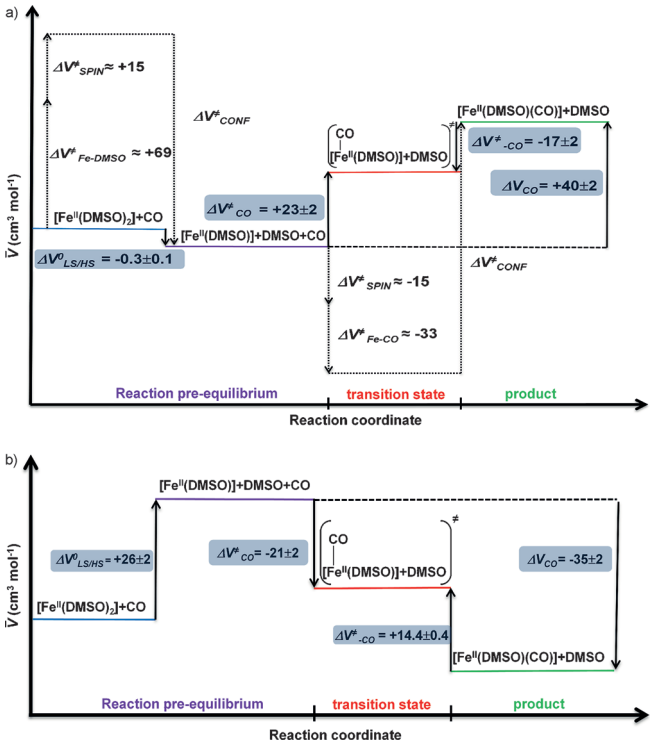


Figure 1. Volume profile analyses. Volume profiles obtained for the overall processes, that is, the spin-change pre-equilibrium and subsequent CO binding to five-coordinate a) [Fe(P1)] and b) [Fe(P2)]. (Experimentally obtained values are highlighted in gray.)

for [Fe(P2)] and [Fe(P3)] (Table 1) suggest that on the way to the transition state a partial Fe-CO bond formation takes place causing a volume collapse. A somewhat more negative $\Delta V^{\neq}_{CO}$ for [Fe(P2)] suggests that a bigger portion of the partial molar volume of CO "disappears" from bulk solution within the semi-confined space of [Fe(P2)] in the transition state. After the transition state was approached (Figure 1b and Figure S45), the volume continues to decrease because of the completion of the Fe-CO bond formation, which is accompanied by high-spin to low-spin change and related solvent reorganization. Consequently, going from the product to the transition state there is a significant volume increase (positive $\Delta V^{\neq}_{-CO}$). By way of comparison, the partial molar volume of CO is about 33 $cm^3 mol^{-1}$,[13] and the low-spin-to-high-spin transition of the iron center is usually coupled to a volume change of about 15 $cm^3 mol^{-1}$.[14] The overall reaction volume for the formation of carbonyl complexes, starting from the five-coordinate species, is smaller (about $-35\ cm^3 mol^{-1}$ and $-25\ cm^3 mol^{-1}$ for [Fe(P2)] and [Fe(P3)], respectively) than the sum of these two effects (Figure 1b and Figure S45). This is due to the positive volume contribution caused by desolvatation, which is more prominent for "unprotected" porphyrins,[11a] such as [Fe(P3)]. The volume profile for the reaction of [Fe(P1)] (Figure 1a) is contrary to that obtained for [Fe(P2)], [Fe(P3)] and a non-heme cyclidene complex[13] that is the only complex with the confined pocket for which pressure-dependent binding of CO was reported (see related literature information in the Supporting Information). The striking feature of the overall addition

reaction is its positive reaction volume ($\Delta V^{\neq}_{CO}=\Delta V^{\neq}_{CO}-\Delta V^{\neq}_{-CO}=40\ cm^3\,mol^{-1}$ Figure 1a). This is because the overall volume change ($\Delta V^{\neq}_{CO}$) is composed not only of bond formation ($\Delta V^{\neq}_{Fe\text{-}CO}$) and HS-to-LS transition ($\Delta V^{\neq}_{SPIN}$) that have negative contributions, but also of a positive contribution related to the conformational change ($\Delta V^{\neq}_{CONF}$; Figure 1a), that is, expansion/opening of the crown cavity, which is inevitably coupled to the rate-limiting CO entering into the pocket. This conformational reorganization (opening) of the crown pocket that controls the binding of the sixth ligand, was visualized by DFT calculations for the five-coordinate, as well as six-coordinate CO- and DMSO-bound forms (Figure 2).

In general, CO binding to heme models and proteins was characterized by negative activation volume, because of the either solely existence of the negative $\Delta V^{\neq}_{Fe\text{-}CO}$ and $\Delta V^{\neq}_{SPIN}$ factors or its domination in the rate determining event.[2d,13]

The only exception, besides [Fe(P1)], is known for cytochrome P450cam in the presence of specific substrates, where positive $\Delta V^{\neq}_{CO}$ values are attributed to a very compressible, that is, conformationally flexible active site.[2c] Thus, the flexibility and size of the confined space, that define the heme active site, control the actual pressure effect on small molecule binding, which we have here directly demonstrated and quantified on the molecular level for the first time. A significant contribution from desolvation processes is not expected for [Fe(P1)], because it has previously been established that the binding site of protected porphyrins, in principle, does not face significant solvation effects.[11a] Expecting that the CO binding contributes with about $-33\ cm^3\,mol^{-1}$ (i.e. $\Delta V^{\neq}_{Fe\text{-}CO}\approx\bar{V}_{CO}$, because of the complete disappearance of CO within the confined space of [Fe(P1)]) and the spin-change with $\Delta V^{\neq}_{SPIN}\approx-15\ cm^3\,mol^{-1}$, it follows that the conformational contribution largely outweighs these two effects with the $\Delta V^{\neq}_{CONF}=\Delta V^{\neq}_{CO}-(\Delta V^{\neq}_{Fe\text{-}CO}+\Delta V^{\neq}_{SPIN})$ value of ca. $88\pm4\ cm^3\,mol^{-1}$. This value is also in agreement with the neutralization of the high-pressure effect on the low-spin/high-spin pre-equilibrium ($K_{LS/HS}$ Scheme 2 and $\Delta V^{0}_{LS/HS}$ in Table 1) involving the similar conformational change. Starting from five coordinate mono-DMSO species [Fe(P1)-(DMSO)], the entering of DMSO into the binding pocked leading to its complete "disappearance" ($\Delta V^{\neq}_{Fe\text{-}DMSO}\approx\bar{V}_{DMSO}\approx-69\ cm^3\,mol^{-1}$)[15] and again HS to LS transition $\Delta V^{\neq}_{SPIN}\approx-15\ cm^3\,mol^{-1}$ should result in an overall volume decrease of about $-84\ cm^3\,mol^{-1}$. However, as we have demonstrated above, this equilibrium almost does not result in the volume change ($\Delta V^{0}_{LS/HS}=-0.3\ cm^3\,mol^{-1}$, Table 1) suggesting that these $-84\ cm^3\,mol^{-1}$ are completely compensated by the positive conformational contribution, above predicted to be $\Delta V^{\neq}_{CONF}\approx88\pm4\ cm^3\,mol^{-1}$. Consequently, the volume difference that we have observed between the starting ([Fe(P1)$(DMSO)_2$] + CO) and product ([Fe(P1)-(DMSO)(CO)] + DMSO) mixtures of $+40\pm4\ cm^3\,mol^{-1}$ corresponds to the difference between the partial molar volumes of DMSO and CO ($+36\ cm^3\,mol^{-1}$).

In summary, for the first time we offer an explanation at the molecular level for some basic physicochemical principles of life under high-pressures by demonstrating two unique phenomena (not observed until now) that 1) binding of a small molecule to the vacant coordination site of a heme model complex can be strongly decelerated by pressure and 2) that high pressure can shift the spin-state equilibrium towards high-spin and not just towards low-spin, both opposing well-established axioms in kinetics and chemistry/physics of spin crossover. These phenomena are caused by our design of a heme-binding cavity that forms a confined space around the sixth coordination site of heme iron, with exactly appropriate size and conformational flexibility that can offset the high-pressure effects. Furthermore, a deceleration of the small-molecule (substrate) binding at elevated pressures depends on the ratio between the substrate volume and the volume change originating from conformational reorganization of the cavity. The smaller the substrate and the bigger the conformational changes, the more prominent pressure deceleration of the molecule binding will be. This principle, we demonstrated here, can be used both by chemists and by nature to tune the

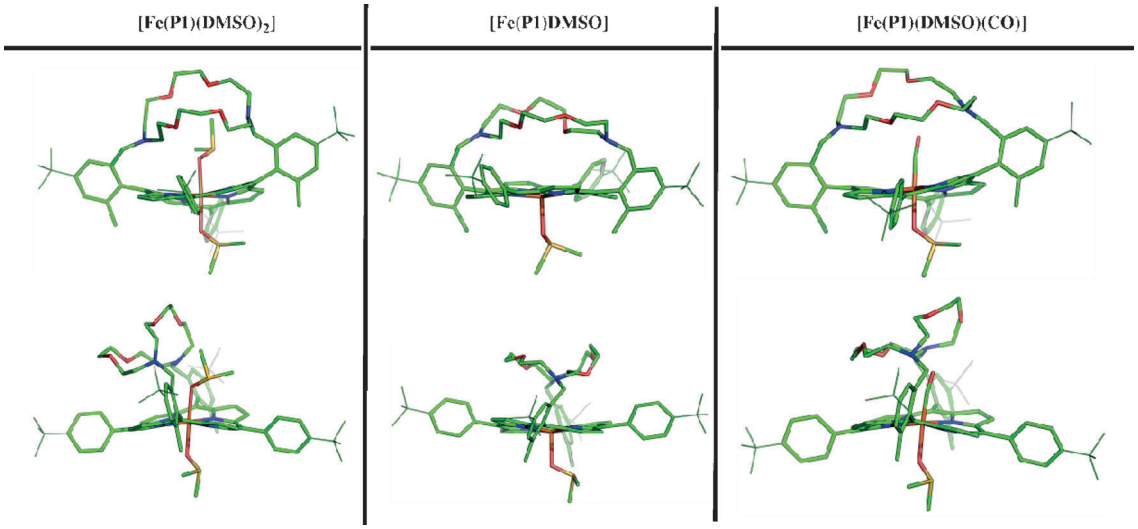


Figure 2. Breathing of the heme binding pocket. Two different views on the calculated structures of LS [Fe(P1)$(DMSO)_2$], HS [Fe(P1)(DMSO)], and LS [Fe(P1)(DMSO)(CO)] (hydrogen atoms are omitted for clarity).

flexibility of the distal pocket around metal centers that depending on the volume of the particular substrate will be sufficient to neutralize or reverse the pressure effects on thermodynamic/kinetic behavior of the metal complexes in general and heme centers in particular. It could even provide new ideas for how we can use this confined-space phenomenon to engineer spin(magnetic) switches that will operate in reverse mode. The herewith quantified molecular phenomena caused by the confined space, can assist in adaptation of microorganisms to high-pressure conditions by 1) preventing increased access of water molecules to the heme pocket and related undesired processes as well as 2) by slowing-down certain enzymatic reactions thus, placing these organisms in a state of "suspended animation"[1a] until the pressure is normalized.

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[8] Small molecules can bind to either side of the porphyrin. However, since the pressure effect on the binding to the non-capped side of P1 and P2 is expected to be the same, in the overall experimentally observed effect, which is a sum of contributions of all possible processes, only a portion related to the binding to the caped side is responsible for a (huge) difference in the high-pressure behavior between [Fe(P1)] and [Fe(P2)].

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