Pressure Induced Structural Fluctuations in Hemoglobin, Studied by EPR-Spectroscopy

J. Paul, E. v. Goldammer

Institut für Biophysik, Universität Witten/Herdecke, Stockumer Straße 10, D-5810 Witten, Bundesrepublik Deutschland

and

H. R. Wenzel

Fakultät für Chemie, Universität Bielefeld

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A quartz based cavity for pressure dependent EPR measurements on liquid samples allowing pressures up to 0.6 GPa was constructed. First investigations with this setup were done on spin labeled horse hemoglobin derivatives both in ferric and ferrous state of oxidation. The second derivative EPR spectra show changes of the label's mobility, which are not correlated with spin state changes of the Fe-porphyrin complex, but which point out structural fluctuations inside the globin protein matrices.

Introduction

High pressure EPR studies on proteins in aqueous solutions are strongly hampered by the high dielectric constant of water, that causes a dissipation of microwave energy in the cavity, which will decrease considerably its Q-factor. An X-band vessel for pressure and temperature dependent EPR investigations on liquid systems has been developed with a Q of 885 for the water filled cavity.

The attainable pressures and temperatures lie within the regions of 0.1 MPa up to 0.6 GPa and of 270 K up to 340 K.

The first applications of the method were done on aqueous solutions of various spin labeled hemoglobin derivatives. The reason why to use hemoglobin is twofold:
First, the relationship between structure and function is fairly well understood. The second reason lies in the existence of several pressure dependent investigations on this system, done with optical and NMR methods, which can be taken for comparison. Therefore hemoglobin was taken as reference system in order to test the method of pressure dependent EPR.

Pressure induced structural changes on hemoproteins have already been reported in the literature. These studies basically deal with changes of the optical spectra caused by chemical exchange of axially coordinated ligands at the Fe-porphyrin ring system, which happens to occur parallel to the proposed pressure induced structural changes within the protein matrix. No such changes in the optical spectra could be observed for corresponding hemoprotein derivatives with ligands such as CN^- or CO fixed at the Fe-porphyrin, which are known to form stable complexes in those systems. This, however, does not necessarily exclude the possibility of pressure induced structural changes in these systems. It was the aim of the present study to see, whether pressure induced structural changes can be observed in systems where optical changes are not detectable for reasons as discussed above.

Instrumentation

One of the main technical problems of pressure dependent EPR is the combination of four energy currents, namely the mechanical energy (pressure), the microwave energy, the magnetic field modulation energy, and the energy from the static magnetic field itself.

Due to the homogenity of the static magnetic field an NMR magnet system was used, which allows wider magnet gaps with less lowering of field homo-

Abbreviations: IAA, N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-iodoacetamide.

Reprint requests to Prof. E. v. Goldammer.

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genity in order to mount the pressure vessel, which needs more space than a common cavity.

The core of the cavity is a rectangular quartz block with $\varepsilon_r = 3.8$. It is covered by a silvered bronze frame and two silvered wall plates, which are mounted with 15 screws each onto the frame to deliver good conductivity. The front side of the frame has got a drill with a cylindrical flange to hold the pressure resistant microwave transmission line, which is a Wittaker stainless steel cable of 2.3 mm in diameter. The overhanging inner conductor of the cable is bent to form a loop, which is placed inside a slot of the quartz block perpendicular to the planes of the wall plates to deliver inductive coupling of the transmission line to the cavity. For coupling adjustments it is possible to vary the depth of the loop position. At the basis of the loop the high frequency conductor is sealed with a drop of epoxide resin to avoid pouring out of the dielectric medium (pressed quartz sand) at the atmospheric pressure side of the transmission line.

The quartz block and also the frame have got a drill at maximum magnetic field position due to the TE_{103}-mode for taking up the teflon sample tube. On both wall plates a cylindrical holder corresponding to the sample tube position is mounted to carry the modulation coils. Together they have an inductivity of 40 mH. For the TE_{103}-mode this setup has a resonant frequency of 9.2523 GHz (X-band).

For pressure varying measurements the cavity is placed inside a cylindrical copper-beryllium (CuBe) block which also allows variations of temperature by a tempered water flow through a copper pipeline wired around the vessel. The pressure vessel is closed by two (upper and lower) sealing systems which work by a locking principle. The basis of these systems is a solid carrier cylinder holding the sealing package. Mainly it consists of a set of crushing rings with an indium ring in between. The soft indium ring is pressed against the carrier and the inner wall of the vessel by tightening the lock nuts. Both carriers have got a pipeline inside, the lower one contains the microwave transmission line, which is fixed to the carrier by hard soldering, the upper one functions as pressure inlet and also contains the modulation transmission line. Further technical details and drawings may be obtained from the authors.

Outside the vessel the pressure resistant high frequency cable is connected with the X-band waveguide of the microwave power bridge belonging to the Varian E-209 spectrometer system. Because of unequaled characteristic impedances of waveguide and cable there is a nonavoidable power attenuation of 3 dB at the coupling unit. So at sample tube position the temporal and spatial averaged magnetic flux density is about 1.8×10^{-5} T by 50 mW microwave power inside the waveguide. The induction loop inside the quartz block delivers undercritical coupling (1.24), so that the system is less sensitive for frequency noise from the clystron. The filling factor (magnetic field average weighed ratio of sample volume and cavity volume) is 9.41×10^{-4}. Another problem was the choice of an adequate pressure transmission medium, because between the walls and the frame and also at the sample tube drill it penetrates into the cavity. Common pressure oil has a high dielectric constant, which causes both lowering of Q and shifts of resonant frequency. A few experiments lead to a 2/1 mixture of silicone oil and petroleum ether showing low compressibility and very low dielectric constant. But still a slight nonlinear decrease of resonant frequency of 0.05 MHz/MPa in average exists.

Experimental Section

Sample preparation

The hemoglobin derivatives metHb, DeoxyHb and OxyHb were prepared according to the method described by Benesch *et al.* [1]. The spin label used was N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-iodoacetamide (IAA), whose EPR-spectra slopes show a slight dependence upon the quarternary structure of the Hb-tetramer, when the nitroxide moiety is bound to the SH-group of the β-93-cysteine [2, 3]. The potassium phosphate buffer solution has a pH of 6.8. Hb concentrations were measured by optical extinction and their values lie between 5×10^{-4} mol/l and 10^{-3} mol/l. Addition of KCN to the metHb solution yields the $HbCN^-$-derivative.

Detection of second derivative absorption spectra

To minimize the S/N-ratio one usually detects the first derivative of the EPR-absorption signal by static magnetic field modulation and phase sensitive detection at the same frequency. But if these frequencies have the ratio detection/modulation = 2/1, one detects the second harmonic of the absorption signal, which in general can be described as a superposition of all even numbered derivatives of the signal [4]. This method allows higher modulation amplitudes yielding better S/N-ratios. The admixture of higher

derivatives is proportional to the modulation amplitude. If it is chosen not too high, the detected EPR lines are represented to good approximation by the second derivative of the absorption signal. This can be easily verified by comparing the measured second derivative with a computed one obtained from the standard EPR (first) derivative.

Measurements

Fig. 1 shows both a typical second derivative absorption spectrum of an IAA-labeled hemoglobin and also gives the spectral parameters relevant for analysis. In the case of a free radical the three spectral lines will nearly have the same amplitude, so that the ratios of the line amplitudes, which are h_0/h_{+1}, h_0/h_{-1} and h_{+1}/h_{-1}, can be taken as a qualitative measure for the radical's mobility.

Pressure dependent optical extinction measurements at 402 nm on aqueous metHb solutions show a significant change in extinction in the region between 80 MPa and 120 MPa. The reason is a shift of the Soret to higher wavelengths due to a pressure induced change of thermal equilibrium of the spin-states towards the low-spin state [5]. This can be seen in Fig. 2 and the upper part of Fig. 3.

The lower part of Fig. 3 shows the pressure dependent behavior of the amplitude ratios for the second derivative EPR absorption spectrum. The ratio h_0/h_{+1} decreases slightly by increasing pressure. The ratios h_0/h_{-1} and h_{+1}/h_{-1} behave very similar pointing

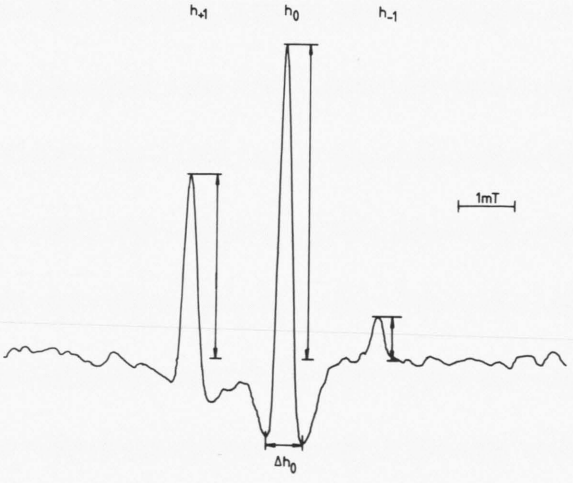

Fig. 1. Second derivative of the EPR absorption of spin labeled hemoglobin.

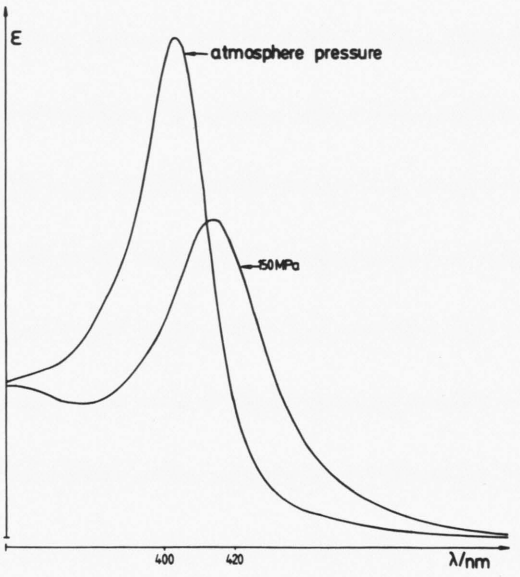

Fig. 2. Pressure induced frequency shift of the Soret band of metHb.

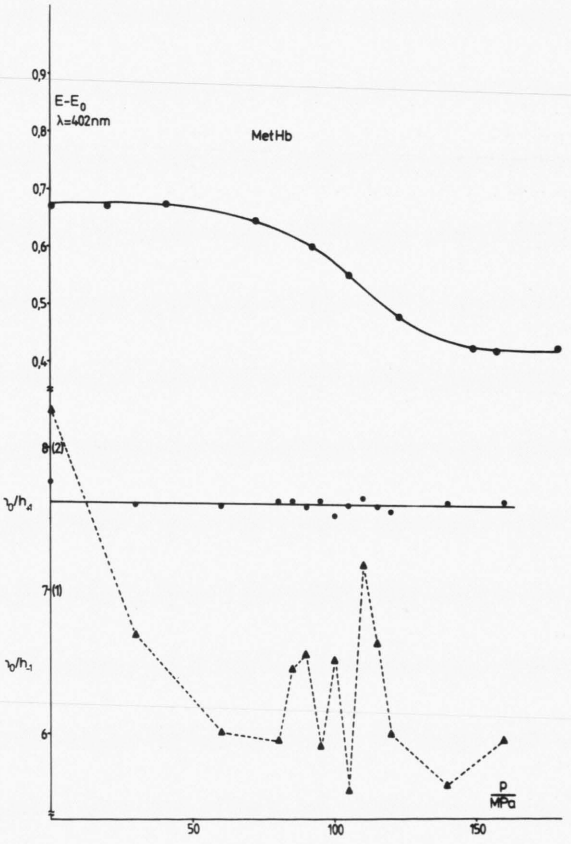

Fig. 3. Pressure dependence of the Soret maximum (upper curve) and the EPR spectrum of metHb (lower curve).

out an essential change of the high field line amplitude. These ratios also decrease with increasing pressure, but in the region of the optical transition between 80 MPa and 120 MPa oscillations could be observed due to oscillations of the mobility of the nitroxide radical. To verify whether the changes in EPR-spectra are correlated with the optical detectable thermal equilibrium shift of the spin states of the Fe-porphyrin complex, $HbCN^-$ was taken as a reference system. $HbCN^-$, in which Fe also is in the ferric state of oxidation, shows no pressure dependent optical absorption spectrum due to the fact, that the CN^--ligand causes the Fe-porphyrin complex to stay in the low-spin state [6]. But on the other hand its EPR spectra also reveals different oscillations of the line amplitude ratios in the pressure region discussed above.

Further measurements on Hb-derivatives with Fe in ferrous state of oxidation also deliver oscillations of the amplitude ratios h_0/h_{-1} and h_{+1}/h_{-1} which again lie within the pressure region of interest.

The high-field line h_{-1} of the deoxyHb EPR-spectrum is much more defined than the h_{-1}-lines of all other derivatives, indicating the radical to be more movable. This may be rationalized from stereochemistry. While for deoxyHb the so-called "tyrosine pocket" is occupied by the tyrosine molecule causing the radical to move relatively free at the surface of the β-Hb-subunit, the tyrosine is twisted out and the radical penetrates the tyrosine pocket in all cases where the Fe-ion is six-coordinated [2]. Optical spectra of deoxyHb (high-spin) and oxyHb (low-spin) only reveal very slight shifts of the Soret (below 0.5 nm) but no changes in extinction. It follows that there is no pressure induced change of thermal equilibrium between the spin states [7].

Discussion

In summary the measurements described above yield two results:

a) The pressure dependent behavior of the amplitude ratios h_0,h_{+1}/h_{-1} seems to be characteristic for hemoglobin derivatives, and

b) the oscillations of the ratios, which can be understood as oscillations of the radical's mobility, exclusively occur in the pressure range between 80 MPa and 120 MPa, where methemoglobin reveals its characteristic shift of spin state equilibrium.

According to these summaries several possible effects and their superpositions have to be considered:

1) Changes of the electron configuration, *i.e.*, high-low spin transitions in the Fe-porphyrin system, and/or

2) ligand exchange at the position of the Fe-porphyrin accompanied with a change of the oxidation state of iron (see point 1),
and/or

3) decreasing mobility of the spin label molecule with increasing pressure, resulting from pressure induced structural changes within the protein matrix, which may cause a compression of the tyrosine pocket,
and/or

4) increasing mobility of the radical, caused by changes within the protein matrix twisting the label out of the tyrosine pocket.

The continuous decrease of the amplitude ratios of the methemoglobin EPR-spectra is understandable by point 4); otherwise, in the transition region between 80 MPa and 120 MPa point 3) and also, because of the optical measurements, point 1) has to be considered.

For CN^--methemoglobin the situation is similar, but point 1) can be excluded with assurance, because there is no pressure dependence of the optical spectra, *i.e.*, the Fe-porphyrin electron configuration is low-spin fixed by the ligand CN^-.

For deoxyhemoglobin point 1), *i.e.*, changes of the spin state, can be excluded from corresponding results of optical measurements. At a pH of 6.8, on the other hand, one can assume an unchangeable tensed quarternary (*T*)-conformation, which points out deoxyhemoglobin to be in a state of low O_2-affinity. An increase of pH favors the relaxed (*R*)-conformation, but the potassium phosphate buffer shows increasing dissociation with increasing pressure [8], so the (*R*)-conformation can be excluded. Further, there is no significant pressure induced change of the amplitude ratios outside the pressure region of interest.

For oxyhemoglobin there is also no significant change of the amplitude ratios, except for the mentioned pressure region where point 3) and 4) have to be considered. The small shift of the Soret allows exclusion of point 1) and 2).

Finally one can conclude that there are no correlations between the pressure dependent mobilities of

the spin label molecule and the spin state of the Fe-porphyrin ring system. However, the actual spin state of the Fe-porphyrin influences these mobility changes, as can be observed in the case of methemoglobin. This indicates that pressure dependent fluctuations only occur inside the globin protein matrix affecting the tertiary structure of the Hb-subunits (tyrosine pocket) as well as the quarternary structure of the whole tetramer.

By pressure it is not possible to induce *T-R*-transitions inside the Hb-derivates HbO_2, $HbCN^-$, and deoxyHb. This corresponds well with the results of pressure dependent NMR measurements on Hb-derivates reported by Morishima and Hara [9]. They discovered a pressure dependence of the resonance lines of the hydrogen bonds between the Hb-subunits. The characteristic hydrogen bonds associated with the (*T*)- or (*R*)-conformation of the tetramer are partly weakened or disrupted by increasing pressure, but without a collaborating spin state transition of the Fe-porphyrin systems as well as a change of the quarternary conformation in general. So we conclude that the pressure dependent fluctuations of the inter subunit hydrogen bonds affect the mobility of the nitroxide radical.

Conclusions

Further development of the method and measurements on other biological systems are in progress. In concrete, the construction of a modified cavity is envisaged with a quartz block directly coated with gold, in order to avoid penetration of the cavity by the pressure transmission medium, which will result in an estimated Q-factor one order of magnitude higher than the old one. Such an equipment allows both, saturation transfer measurements and pulsed EPR under high pressure.

Measurements on chymotrypsin, an enzyme, where the spin label molecule may be fixed very close to the active center are in progress.

Acknowledgement

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