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Effects of pressure on visible spectra of complexes of myoglobin, hemoglobin, cytochrome *c*, and horse radish peroxidase

(crevice structure/pressure denaturation)

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ABSTRACT **The spectra of the ferric form of most heme proteins [metmyoglobin, methemoglobin, horse radish peroxidase (EC 1.11.1.7), and ferricytochrome *c* at pH 1.5] are converted from high-spin (open crevice) structure to low-spin (closed crevice) form under pressure. Pressures up to 8000 kg/cm² (780 MPa) have no effect on the spectra of high-spin ferro- and ferricytochrome *c*, which have a closed crevice structure at pH 7.0. Spectra of deoxy-ferromyoglobin and deoxy-ferrohemoglobin are reduced in intensity, but pressure does not change the positions of the absorption maxima. Cyanide ion prevents pressure-induced spectral changes in metmyoglobin and methemoglobin up to 8000 kg/cm². Carbon monoxide (with a high affinity for the ferro heme iron) has a similar effect on ferromyoglobin and ferrohemoglobin. The pressure required to cause spectral changes in the heme proteins falls in the order, cytochrome *c* (pH 7.0) > horse radish peroxidase > myoglobin > hemoglobin. We have calculated a volume change of −50 cm³/mol associated with the configurational change accompanying the reformation of the iron-methionine bond in cytochrome *c* at low pH.**

The effect of pressure on the complexes of hemoglobin (1–4) and myoglobin (5–7) have been the subject of considerable investigation over the last few years. These investigations have, however, been largely concerned with changes in the functional properties (1, 2), the thermodynamic properties accompanying the binding of ligands (6), and the denaturation of the protein (5). However, the Soret and visible spectra of hemoglobin and hemoglobin complexes (3, 4) and the visible spectra of metmyoglobin fluoride (7) show interesting changes when these molecules are subjected to hydrostatic pressure. We have extended these high pressure studies on metmyoglobin and methemoglobin and have investigated, in addition, the spectral changes in the visible region accompanying the pressurization of two other hemoproteins, cytochrome *c* and horse radish peroxidase (donor:hydrogen-peroxide oxidoreductase; EC 1.11.1.7).

MATERIALS AND METHODS

Human hemoglobin was prepared by the usual method (8) and oxidized to methemoglobin with 2-fold excess of potassium ferricyanide. Ferrocyanide and excess ferricyanide were removed by dialysis against 0.05 M sodium chloride at pH 6.0. Sperm whale metmyoglobin and horse heart (type III) cytochrome *c* were obtained from Sigma Chemical Co. and were used without further purification. Buffers used were cacodylate and Tris. Tris buffer was prepared from Trizma base (Sigma) and Trizma·HCl (Sigma) that had been dried under reduced pressure before use. All methemoglobin and metmyoglobin solutions were 0.05 M in buffer ions. Hemoglobin and myoglobin were deoxygenated by addition of sodium dithionite in a glove box previously filled with dry nitrogen.

High Pressure Studies. The high pressure optical bomb used was designed by Dr. W. B. Daniels and has been described elsewhere (9).

At each increment of pressure, the system was allowed 5 min to attain temperature re-equilibration. Measurements were made at both increasing and decreasing pressures. For each test, the reversibility of the spectrum was checked by measurement of the absorbance of the sample at 1 atm pressure (100 kPa) after it had gone through a cycle of pressure changes. The spectrum at 1 atm remains unchanged in most cases. The experiments were carried out at 20° ± 0.05°.

RESULTS AND DISCUSSION

Complexes of myoglobin

In addition to the spectral changes accompanying the pressurization of metmyoglobin (5) and metmyoglobin fluoride (7), which have been reported elsewhere, changes in the visible region of the spectrum (450–700 nm) accompanying the application of pressure to metmyoglobin azide, metmyoglobin cyanide, as well as to oxymyoglobin and deoxymyoglobin, have now been examined. For metmyoglobin azide and oxymyoglobin, the visible absorption spectra at high pressure are essentially the same as those obtained for metmyoglobin or metmyoglobin fluoride under similar conditions of temperature and pH. That is, the high pressure spectra are characteristic of a hemoprotein with the ferric heme iron atom in a low spin configuration. These spectra, with maxima at about 540 nm, resemble (*i*) a hemochromogen spectrum (10), (*ii*) the spectrum of the imidazole complex of methemoglobin at 1 atm (11), and (*iii*) the spectrum of acid-denatured myoglobin at 20° (5). It is well known that on denaturation of oxyhemoglobin with urea, the iron is irreversibly oxidized to the ferric state (12). If pressure does denature oxymyoglobin, this would account for the observed spectral change in oxymyoglobin under pressure. The mechanism of the pressure-induced spectral change in the visible region of the spectrum (450–700 nm) for the aquo, fluoride, and azide complexes of metmyoglobin is not known with certainty. It presumably involves the replacement of the ligand in the sixth coordination position with an imidazole group (most likely the distal imidazole). This is suggested by the similarity of the high pressure spectrum to that of the imidazole complex of metmyoglobin at 1 atm. The resemblance of the high pressure spectra of these complexes to those of hemochromogen suggests that the heme iron in the high pressure form is coordinated to six nitrogen-containing ligands. This argument would support the earlier suggestion that under pressure the distal imidazole complex displaces the ligands OH_2, F^-, and N_3^- in

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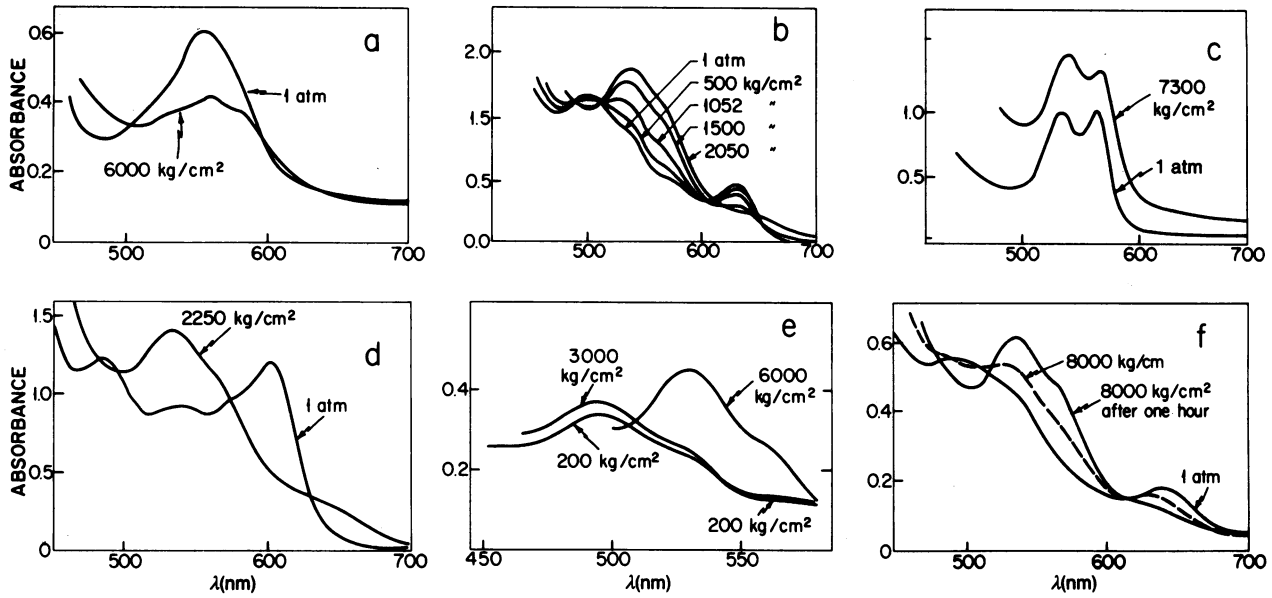

FIG. 1. Pressure dependence of the visible absorption spectrum of hemoproteins at 20°. Spectra are not corrected for solvent compression. (a) Deoxymyoglobin in 0.05 M phosphate buffer (pH 6 at 1 atm). (b) Methemoglobin in 0.05 M phosphate buffer (pH 6 at 1 atm). (c) CO-hemoglobin in 0.05 M phosphate buffer (pH 6 at 1 atm). (d) Methemoglobin fluoride in 0.05 M phosphate buffer (pH 6 at 1 atm). (e) Horse heart ferricytochrome *c* at pH 1.5 (0.032 M HCl, no added salt). (f) Horse radish ferriperoxidase (aquo complex) in 0.05 M cacodylate buffer (pH 6.55 at 1 atm).

the aquo, fluoride, and azide complexes of metmyoglobin, respectively (7).

The visible spectrum of metmyoglobin cyanide at pH 6.0 and 20° shows no change on pressurization to 8000 kg/cm² (780 MPa), except for an increase in absorbance due to compression of the solvent. It is surprising that the cyanide ion so efficiently stabilizes metmyoglobin to denaturation by pressure. The failure of the visible spectrum of metmyoglobin cyanide to change with increasing pressure could be a result of (*i*) a close similarity in the spectra of pressure-denatured metmyoglobin cyanide and native metmyoglobin cyanide at 1 atm; (*ii*) the instability of the denatured metmyoglobin cyanide complex at high pressure; or (*iii*) a much larger binding constant of cyanide ion than imidazole to the heme iron so that metmyoglobin is unable to form a hemochromogen in the presence of cyanide ion at high pressure.

Fig. 1a gives the visible spectra of deoxymyoglobin at 1 atm and at 6000 kg/cm². The spectrum shows a reversible decrease in intensity relative to that at 1 atm between 500 and 575 nm. It is significantly different from that observed for oxymyoglobin or the various complexes of metmyoglobin under similar conditions of temperature and pH. Presumably, it represents a reversibly denatured deoxymyoglobin.

Complexes of hemoglobin

Fig. 1b shows the spectra of methemoglobin at various pressures. The spectral changes in the wavelength range 450–700 nm that accompany the pressurization of hemoglobin and its derivatives are similar to those obtained for metmyoglobin and its derivatives (7). For the aquo, fluoro, azide, and hydroxyl methemoglobin complexes, as well as for oxyhemoglobin, the visible spectrum at high pressure is characteristic of a completely low-spin hemochromogen at high pH at 20° and acid-denatured hemoglobin at low pH at 20°. The visible spectrum of methemoglobin cyanide at pH 6, 1 atm, and 20°, which is 100% low spin, shows no change on pressurization up to a pressure of 8000 kg/cm². The visible spectrum of CO-hemoglobin in Fig. 1c, while changing slightly in intensity on pressurization to 7300 kg/cm², does not change its general shape, suggesting either that carbon monoxide inhibits hemoglobin denaturation or that carbon monoxide is still bound to the iron atom of the denatured hemoglobin at high pressure. The visible spectrum of deoxyhemoglobin at 5000 kg/cm² (pH 6 at 1 atm, 20°) shows a decrease in intensity relative to that at 1 atm similar to that found for deoxymyoglobin (Fig. 1a). Fig. 1d shows the spectra of methemoglobin fluoride at 1 atm and 2250 kg/cm². The spectral change at high pressure is very similar to that of metmyoglobin fluoride, which has been reported (7).

The behavior of methemoglobin under pressure differs from that of metmyoglobin in two ways: first, the pressure needed to effect the spectral change from the native protein to a hemochromogen-type spectrum for a given complex is much less for methemoglobin than is required for the corresponding complex of metmyoglobin. For example, at pH 6.0 and 20°, a pressure of about 6000 kg/cm² is needed for complete conversion of the visible spectrum of native metmyoglobin fluoride to the high pressure type spectrum (7), whereas for methemoglobin fluoride under similar conditions of temperature and pH, only about 2000 kg/cm² is required for complete conversion (see Fig. 1d). Second, upon return of the pressure to 1 atm, hemoglobin does not display the same degree of reversibility as does metmyoglobin and its complexes (the reversibility for methemoglobin fluoride is 50% compared to about 95% reversibility for metmyoglobin fluoride). The above two differences between hemoglobin and myoglobin reflect the well-known greater stability toward denaturation of myoglobin than hemoglobin.

Although only the initial and final states for the pressure-induced changes of methemoglobin fluoride are shown in Fig. 1d, it should be mentioned that, like metmyoglobin fluoride, spectral changes at intermediate pressures were time-dependent. Unlike metmyoglobin fluoride, however, kinetic plots showed significant deviation from first-order behavior.

Cytochrome *c*

The effects of pressure on the visible absorption spectrum in the wavelength range 450–700 nm of both ferric- and ferro-

cytochrome *c* were examined in cacodylate buffer, at pH 6 and 20°. No change in either of these spectra was observed up to 8000 kg/cm^2. The failure of the visible spectrum to change with pressure probably reflects the covalent nature of the heme-protein linkage as well as the fact that the ligand occupying the sixth coordinating position, the sulfur of methionine-80, is endogenous to the protein and has a relatively high binding constant for the heme iron.

Spectral measurements under pressure were also carried out at low pH on ferricytochrome *c*, and here the results are much more interesting. The work of Theorell and Akesson (13) has demonstrated that as the pH of an aqueous solution of ferricytochrome *c* is lowered below approximately 3, the visible spectrum (450–700 nm) changes from that characteristic of low-spin hemoprotein (maximum near 540 nm) to a high-spin type spectrum. Three forms, differing in the protonation state of the heme group, are believed to exist at very low pH (13). The percentage of each form present depends on the pH.

The existence of various high-spin forms of ferricytochrome *c* at low pH presented another opportunity to study spectroscopically the pressure dependence of the high-spin to low-spin transition. At pH 1.5 and 1 atm, only one high-spin form of ferricytochrome *c* predominates (95% high spin) and the visible spectrum closely resembles that of metmyoglobin at pH 6. The form present at this pH, at least as regards the heme group, is believed to differ from ferricytochrome *c* at neutral pH in that the bonds between the iron atom and the proximal histidine and distal methionine groups are ruptured at pH 2.5 (14).

Fig. 1e shows the pressure dependence of the visible spectrum of ferricytochrome *c* at pH 1.5. As was observed for metmyoglobin and methemoglobin at pH 6 and 20°, the high pressure spectrum of ferricytochrome *c* at pH 1.5 is of the low-spin type. The spectrum observed at 6000 kg/cm^2 is virtually identical with the spectrum of ferricytochrome *c* at pH 7 and 1 atm. This result may reflect a pressure-induced reformation of the iron-methionine linkage ruptured at low pH. The reformation of this bond is not time-dependent. The spectral change was found to be completely reversible to the spectrum at 1 atm on depressurization.

The results presented in Fig. 1e, together with spectra obtained at additional pressures, allow us to calculate a volume change of about -50 cm^3/mol for the configurational change accompanying the reformation of the iron-methionine bond.

Horse radish peroxidase

Fig. 1f shows the pressure dependence of the visible spectrum of horse radish ferriperoxidase. Like the other heme proteins considered in this report, the high-spin spectrum at 1 atm converts to a low-spin spectrum at high pressure. However, for horse radish peroxidase much higher pressures are needed to shift the equilibrium from the high-spin to the low-spin form than those required for metmyoglobin under similar conditions of pH and temperature. Indeed, even at 8000 kg/cm^2, the spectral change is not complete until after 1 hr. Since the pressure was not raised above 8000 kg/cm^2, it is not known if the spectrum at this pressure represents complete conversion to the low-spin state.

Pressure and heme crevice equilibria

Evidence from spectroscopic (13, 15), magnetic (16), and reactivity studies (17–19) suggests that methemoglobin and metmyoglobin may be an equilibrium mixture of two forms. Electron paramagnetic resonance studies at low temperature have shown that there may be a form of methemoglobin that is stabilized in frozen solutions having a characteristic spectrum of a hemichromogen (20, 21). Such a structure may arise if the heme-linked ligand at the sixth coordination position is replaced by the distal histidine. Beetlestone and Irvine (11), in accounting for the abnormal spectrum of acid methemoglobin Norfolk, postulated such a situation in which the imidazole ring of the distal histidine is directly bonded to the iron atom by displacement of the water molecule at the sixth coordination position in the abnormal chain.

George and Lyster (22) have suggested that heme proteins can exist in either of two configurations, corresponding to an open crevice and a closed crevice heme structure. The normal heme environment in hemoproteins may therefore exist in dynamic equilibrium between these two crevice structures, and there are various experimental situations in which one or the other of the two structures may be stabilized (11, 18, 20, 21). Our results show that pressure is one of the experimental conditions that affects the position of this equilibrium.

The high pressure spectra of methemoglobin, methemoglobin azide, methemoglobin fluoride, metmyoglobin, metmyoglobin azide, metmyoglobin fluoride, horse radish peroxidase, and cytochrome *c* at low pH have been discussed earlier in this paper. They seem to be similar to the imidazole complex, having the closed crevice structure. It is obvious, therefore, that pressure stabilizes the closed crevice configuration not only in methemoglobin and metmyoglobin, but also in their complexes and in those hemoproteins that exist in an open crevice structure at 1 atm. In these hemoproteins, when the affinity of a ligand for the heme iron is quite high (as in the cyanide complexes of methemoglobin and metmyoglobin), the open crevice structure is stabilized even at high pressures. The high affinity of carbon monoxide for deoxyhemoglobin and deoxymyoglobin would similarly stabilize the open crevice structure at high pressures. This explanation would account for the absence of a pressure effect on the spectra of carbon monoxide complexes of hemoglobin and myoglobin. Cytochrome *c* at pH 7.0 has a closed crevice structure at all pressures because of the covalent bond between the heme iron and the methionine-80 at neutral pH. It is thus no surprise that pressure has no effect on the spectrum of cytochrome *c* at neutral pH. It is only when the cytochrome *c* acquires an open crevice structure at acid pH that its spectrum is affected by pressure. Pressure causes a reformation of the iron-methionine-80 bond at acid pH, and a significant contraction in volume occurs during the reformation of this bond.

In summary, we can say that while pressure may cause denaturation of hemoproteins, the primary effect of pressure on proteins with open crevice structure, such as metmyoglobin (5), is to shift the equilibrium in favor of the closed crevice structure. Ligands with high affinity for the heme iron will stabilize the open crevice configuration and prevent pressure denaturation. The primary event of pressure denaturation of open crevice hemoproteins may be the formation of a closed crevice structure.

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