Effect of Hydrostatic Pressure on Spectra of Heme Compounds*

(Received for publication, November 4, 1976)

QUENTIN H. GIBSON
From the Department of Biochemistry, Molecular & Cell Biology, Cornell University, Ithaca, New York 14853

FRANCIS G. CAREY
From the Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

Increase in hydrostatic pressure shifts the absorption bands of oxy-, carboxy-, and deoxyhemoglobin and myoglobin toward the red by 0.4 to 0.7 nm corresponding to a change in extinction coefficient of from 4 to 8% at the peak of the difference spectrum. The pressure difference spectrum for oxyhemoglobin closely resembles the difference spectrum described by Adams and Schuster ((1974) Biochem. Biophys. Res. Commun. 58, 528-533) following addition of inositol hexaphosphate to oxyhemoglobin.

A similar shift was observed for derivatives of dimethyldeuterohemedisulfonate in both Fe²⁺ and Fe³⁺ forms indicating that the protein is not required for this effect, in contrast to earlier reports of T. L. Fabry and J. W. Hunt ((1968) Arch. Biochem. Biophys. R3, 428-429) and Q. H. Gibson and F. G. Carey ((1975) Biochem. Biophys. Res. Commun. 67, 747-751) who were unable to observe changes in aqueous solutions of protoheme derivatives.

Many studies of hemoproteins have made use of their characteristic absorption spectra which are both intense and widely different for different hemoproteins and for various derivatives of the same hemoprotein. In most cases it has been assumed that the spectrum of a given compound is independent of solution conditions, such as pH, pressure, temperature, and buffer composition, and this is certainly a good first approximation. More detailed studies have shown, however, that some variation in the spectrum of individual derivatives may occur, and the two best known examples are deoxy- and oxyhemoglobins. Deoxyhemoglobin occurs in two forms, one of which has a stronger and sharper Soret band than the other. The stronger band, seen in normal mammalian deoxyhemoglobin, is associated with a low ligand affinity and may be interpreted as showing that the hemoglobin is in the T state of the Monod-Wyman-Changeux model (1). The form with the weaker Soret band is associated with high affinity and the R state of the model. This correlation is, so far, without exception and has been extended to many examples as discussed in the recent review of Baldwin (2), although this is not concerned with spectra as such.

The position for oxyhemoglobin is much less clear, but Adams and Schuster (3) have recently described a characteristic difference spectrum on adding inositol hexaphosphate to hemoglobin A, which they suggested might be due to an R to T transition of the liganded form. Since then, similar spectral changes have been observed as a result of change in pH with carp carboxyhemoglobin, and on changing the temperature of oxyhemoglobin A (4). One component of trout hemoglobin also responds similarly to pH change (5). While there is good reason to think that carp carboxyhemoglobin may change from the R to T conformation on lowering the pH (6,7), there is less reason to suppose that changing the temperature alters the conformation of a series of hemoglobin A derivatives, and the specificity of the Adams and Schuster spectrum is thus open to some question. Some years ago Fabry and Hunt (8) gave a preliminary account of studies of the effect of pressure on the absorption spectrum of several hemoproteins and heme derivatives, finding effects on the Soret but not on the visible spectrum of hemoglobin, or on protohematin. More recently Zipp and Kauzmann (9) have made detailed studies of the effect of pressure on the denaturation of metmyoglobin, and have reported the associated spectral changes, but in neither case was the work designed to explore a possible relation between the spectrum and the R-T transition.

This paper describes the effect of increased hydrostatic pressure on the spectrum of several hemoglobin derivatives. It appears that pressure will produce changes analogous to those observed by Adams and Schuster, and that the protein is not necessary for this to occur.

**EXPERIMENTAL PROCEDURES**

**Materials** — Human hemoglobin was from hemolyzed human blood obtained by capillary puncture. Menhaden (Brevoortia tyrannus) blood was obtained from the caudal vein of several fish. The red cells were washed twice with saline and stored in liquid nitrogen. This frozen material was thawed in 20 volumes of distilled water and the gelatinous precipitate centrifuged off before use.

Dimethyldeuterohemedisulfonate was the gift of Dr. R. L. J. Lyster, National Institute for Research in Dairying, Reading, England. Compressed gasses were from Matheson Co. Other chemicals were reagent grade.

**Pressure Chamber** — The pressure chamber was bored from a 7.5 cm high, 5.6 cm long, 4 cm wide block of 303 high tensile strength

*This research was generously supported by National Science Foundation Grants BMS 74-083233 and GB 3872 and United States Public Health Service Grant GM 14276. Contribution 3903 and 3904 of the Woods Hole Oceanographic Institution.
stainless steel, and had a 1-cm light path and a 2.5-ml volume. The windows were 1 cm thick fused silica with a thickness to unsupported diameter ratio of 1.3. They were held in stainless steel plugs which screwed into the body of the chamber. "O" ring seals were used throughout. Pressure was applied with a hand-operated hydraulic pump (Blackhawk model P228, 40,000 p.s.i.) through 1/4 inch outer diameter, 3/8th inch wall stainless tubing connected with swagelock fittings. A vertically mounted steel cylinder, 1.3 cm inner diameter, 15 cm long, was placed in line between the pump and the pressure chamber to serve as an oil-water separator, the system distal to this being filled with distilled water. A free piston in the pressure chamber separated the hydraulic system from the experimental solution and a separate filling port provided ready access for changing the solution.

Spectrophotometry — With the Cary 118C, groups of spectra which were to be compared with one another were recorded without releasing the drive gear train to preserve wavelength calibration. Absolute spectra were recorded in every case. These were measured at intervals of 0.2 nm; difference spectra were generated, and corrections for path length and concentration change were made after transforming the readings representing the spectra to an 8/1 computer (Digital Equipment Corp.). In addition to the spectra of the hemoglobin derivatives themselves, base-lines for solvent at 1, 100, and 1000 atm were recorded. These reflect changes in quantities such as stress-induced birefringence in the windows rather than changes in the solvent itself. Analysis of the data suggested that absorbance values had an error of about ±0.005.

Direct digital recording of spectra was performed using a laboratory-constructed instrument which has been described in detail elsewhere (Knowles and Gibson (10)). This is a simple split-beam spectrophotometer based on a Bausch and Lomb 500 mm f.1 monochromator. The position of the monochromator, the selection of the beam to be sampled, and the intensity of the reference beam were all controlled by a PDP 8/E minicomputer, which also applied the required base-line corrections automatically. In replicate runs on the same solution a photometric reproducibility of ±0.0002 and a wavelength reproducibility of ±0.03 Å were regularly attained. In practice, the limits of our accuracy were entirely determined by slight changes in turbidity or in concentration, as the solutions were loaded into the cell. The standard error of the absorbance value was ±0.001. Although any convenient separation between data points could be selected, this was usually 1 nm, with a Soret range of 380 to 459 nm and a visible range of 460 to 619 nm.

RESULTS

Effect of Pressure on Spectrum of Oxyhemoglobin — Experiments were performed in which the spectrum was recorded, usually at 1, 100, 1000 atm, and again at 1 atm. The data at 100 atm served to give an estimate of effects due to settling of the windows of the cell on their seats, and of strain-induced changes in the windows, while the repeat scan at 1 atm gave a check on the reversibility of the changes observed under pressure. The details of one experiment performed with oxyhemoglobin A are given in Figs. 1 and 2. Fig. 1 shows the absolute spectrum recorded for oxyhemoglobin over the range 400 to 460 nm, and Fig. 2 represents the same data expanded for the range 410 to 420 nm only. It is clear from Fig. 2 that a small but definite shift in the position of the band toward the red has occurred on raising the pressure to 1000 atm. The 1000 atm data in this figure were corrected by subtracting out the blank due to pressure on the cell, a uniform change of 0.004 in absorbance, and by multiplying each point by the factor 0.955 to allow for the increase in concentration due to compression of water (11). To allow an economical presentation of the results and to give an approximate idea of the size of the band shift, the difference spectrum between the 1 atm and 1000 atm spectra was generated and compared with the arbitrary difference spectrum obtained by shifting the 1 atm spectrum 1 nm toward the red and subtracting the unshifted 1 atm spectrum from it. This procedure gives a difference spectrum which, when suitably scaled, agrees quite closely with the observed pressure difference spectrum. An example is given in Fig. 3. This agreement suggests that the effect of pressure is, indeed, primarily to produce a shift in the position of the absorption bands. This is true both of the Soret and visible regions of the spectrum. In the visible region the resulting spectrum is virtually identical in form with that reported by Adams and Schuster (3) on adding inositol hexaphosphate to hemoglobin A and with the spectra reported by Knowles et al. (4). In using data from plots such as that of Fig. 3 it was assumed that the peak amplitude of the difference spectrum is proportional to
Fig. 3. A comparison of the difference spectrum produced by 1000 atm hydrostatic pressure (●) and an arbitrary difference spectrum generated by a numerical shifting of the wavelength by 0.46 nm (▲).

The experimental data are taken from Fig. 1.

the shift in the position of a peak in the absolute spectrum, which is closely true for small shifts such as those considered here. The shift in the wavelength of maximum absorbance on applying increased hydrostatic pressure was calculated by comparing the effect of a 1-nm shift with that actually observed. Marked asymmetry of the difference spectrum is an indication of a change in intensity of the heme absorbance, and in the few cases where this occurred a note has been added to the data collected in Table I.

Table I also includes the peak to peak amplitude of the pressure difference spectrum expressed as a percentage of peak amplitude. This figure obviously depends upon the shape of the absorption bands and is included only to give a concrete idea of the meaning of the figures for the band shift included in Table I.

Effect of Pressure on Spectrum of Several Heme Derivatives—The compounds listed in Table I include several derivatives of human and menhaden hemoglobin and of dimethyldeuterohemedisulfonate. The data for these compounds were collected in a form which allowed their transfer to the computer and analysis as described in the previous paragraph. In addition, CO myoglobin, deoxymyoglobin, and n-butylisocyanide hemoglobin were examined using the Cary 118C spectrophotometer; the CO compound showed a clear shift similar to that for human hemoglobin, but no effect on deoxymyoglobin was seen in the visible region. The effect of pressure on the spectrum of n-butylisocyanide hemoglobin and myoglobin is also to produce a shift to the red of about 0.5 nm. A number of experiments were also performed using several protoheme derivatives. These were technically much less satisfactory than those with hemoproteins, with a tendency for pressure to produce slowly reversible shifts and changes in intensity of the spectrum. Shack and Clark (12) long ago established that several hematin compounds tend to be polydisperse in aqueous media, and these difficulties were attributed to relatively slow changes in aggregation on change in hydrostatic pressure. A fresh series of pressure experiments was therefore performed using dimethyldeuterohemisulfonate. This compound is freely soluble in water at neutral pH, and has sharper and better defined bands than protoheme. With it, clear shifts were easily demonstrated on increasing the hydrostatic pressure of about the same amplitude as shown by the hemoproteins. A single experiment with reduced cytochrome c showed no effect of pressure on the α band.

DISCUSSION

The results described here confirm the report of Fabry and Hunt (8) that increased hydrostatic pressure shifts the Soret absorption bands of several heme derivatives toward the red. In addition, we have been able to observe similar shifts in the position of the visible absorption bands except for deoxyhemoglobin. In our first experiments (13), like Fabry and Hunt, we were unable to find a clear effect of pressure on protohematin, and so thought that the protein was necessary to obtain the effect. In experiments with the freely water-soluble dimethyldeuterohemisulfonate, however, shifts of about the same extent were observed with increased pressure for a series of derivatives both ferrous and ferric, showing clearly that the effect is upon the heme, not upon the protein. In fact, the only compounds which failed to show an appreciable effect were deoxyhemoglobin and reduced cytochrome c. It is tempting to speculate that the effects are really due to changes in the character of water which, on increase in pressure, loses partially ordered elements to less bulky random arrangements.
and so increases in effective polarity as a solvent. The effect of
pressure would then be a solvent effect exerted on the \( \pi - \pi^* \)
transition which is associated with the principal absorption
bands of heme compounds. However this may be, the experi-
ments described here seem to establish that the Adams and
Schuster difference spectrum or an analogue of it may be
obtained from almost all the heme compounds tested by the
application of hydrostatic pressure. The spectrum is closely
imitated in every case by a synthetic spectrum formed by
numerical differentiation of the spectrum of the appropriate
compound at 1 atm pressure. The main exception was deoxy-
hemoglobin where no shift could be seen in the visible region,
although a normal shift was observed in the Soret. Numerical
experiment suggests that our methods should have detected a
shift in the visible band, and we have no explanation for our
failure.

The range of compounds showing the effect is such as to
make it clear that the R-T transition of the Monod-Wyman-
Changeux model (1) is in no way involved, and with the
menhaden hemoglobin it was possible to observe quite similar
effects of pressure on both R and T forms of deoxy and liganded
hemoglobins. The population of the R and T forms was judged
by functional criteria in ligand binding.1

Although our observations do not explain the origin of the
changes seen by Adams and Schuster (3), they do show that
these changes cannot be regarded by themselves as diagnostic
of the occurrence of an R-T transition in solution.

The effects on the spectrum are not large enough to make it
easy to establish an accurate relation between pressure and
spectral shift, and it is not possible to say if we are observing a
large effect on a few molecules or a small effect on most of the
molecules in solution. So far as our observations permit an
estimate, it seems that the effect increases in proportion with
pressure. Since it is possible to obtain effects with soluble
heme derivatives it might be of interest to an investigator with
apparatus able to operate at higher pressures to see to what
point the effects will continue to increase, since with these
compounds no limitation due to protein denaturation will
arise.

There is, finally, the matter of the practical significance of
these shifts in absorption spectrum. Although the shifts are
not very large, the error introduced in the determination of a
ligand binding curve at high pressure could be very considera-
ble, and would vary significantly with the observing wave-
length used. The effect under ordinary conditions is not appreci-
able.

REFERENCES

1. Monod, J., Wyman, J., and Changeux, J. P. (1965) J. Mol. Biol.
   12, 88-118
2. Baldwin, J. M. (1975) Prog. Biophys. Mol. Biol. 29, 225-320
3. Adams, M. L., and Schuster, T. M. (1974) Biochem. Biophys.
   Res. Commun. 58, 525-533
4. Knowles, F. G., MacDonald, M. J., and Gibson, Q. H. (1975)
   Biochem. Biophys. Res. Commun. 66, 550-563
5. Giardina, B., Ascoli, F., and Brunori, M. (1975) Nature 256, 761-
   762
6. Noble, R. W., Parkhurst, L. J., and Gibson, Q. H. (1970) J. Biol.
   Chem. 245, 6628-6633
7. Tan, A. L., Noble, R. W., and Gibson, Q. H. (1973) J. Biol.
   Chem. 248, 2600-2608
8. Fabry, T. L., and Hunt, J. W. (1968) Arch. Biochem. Biophys.
   R3, 428-429
9. Zipp, A., and Kauzmann, W. (1973) Biochemistry 12, 4217-4228
10. Knowles, F. G., and Gibson, Q. H. (1976) Anal. Biochem. 76,
    458-486
11. Amagat 1893 from Handbook of Chemistry & Physics (1956) 36th
    Ed, Chemical Rubber Publishing Co., Cleveland
12. Shack, J., and Clark, W. M. (1947) J. Biol. Chem. 171, 143-168
13. Gibson, Q. H., and Carey, F. G. (1975) Biochem. Biophys. Res.
    Commun. 67, 747-751

F. G. Carey and Q. H. Gibson, unpublished observations.
Effect of hydrostatic pressure on spectra of heme compounds.
Q H Gibson and F G Carey

J. Biol. Chem. 1977, 252:4098-4101.

Access the most updated version of this article at http://www.jbc.org/content/252/12/4098

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/252/12/4098.full.html#ref-list-1