SPIN STATES OF DIVALENT IRON IN ANHYDROHEMOGLOBIN

R. W. GRANT, J. A. CAPE, U. GONSER, L. E. TOPOL, *and* P. SALTMAN

From the North American Aviation Science Center, Thousand Oaks, California 91360; and the Department of Biological Sciences and The Graduate Program in Biochemistry, University of Southern California, Los Angeles, California 92007. Dr. Saltman's present address is the Department of Biology, University of California at San Diego, La Jolla, California 92038.

ABSTRACT Mössbauer spectra of anhydrohemoglobin establish the existence of two ferrous iron spin states in anhydrohemoglobin. Magnetic susceptibility measurements show that anhydrohemoglobin is paramagnetic and has an effective magnetic moment per iron atom of 3.5 ± 0.1 μ_B at low temperatures. In conjunction with the susceptibility results, the Mössbauer spectra indicate that the iron in anhydrohemoglobin is distributed between high and low spin states in roughly equal amounts.

INTRODUCTION

Mössbauer spectroscopy provides an interesting tool for studying biological molecules which contain iron. The main advantage of this type of spectroscopy is that it singles out the iron atoms and avoids complications often caused by overlapping spectra from the rest of the molecule.

In the present article we have used Mössbauer spectroscopy and magnetic susceptibility measurements to investigate dehydrated deoxyhemoglobin (anhydrohemoglobin). The dehydration of deoxyhemoglobin has been studied previously using conventional spectroscopic techniques (1, 2). Drying of the deoxyhemoglobin produces a marked and reversible change in the optical spectra which was interpreted as being associated with the removal of a bound water molecule coordinated in the sixth position of the heme iron. However, recent x-ray studies (3) of deoxymyoglobin indicate that no water molecule or other group is attached to iron in the sixth coordination position. Since deoxyhemoglobin is expected to be quite similar to deoxymyoglobin, the change in the optical spectra is apparently an indirect result of the dehydration process. To investigate this question further, the present study was undertaken. The results presented here indicate that two ferrous iron spin states exist in anhydrohemoglobin.

EXPERIMENTAL

The main difficulty in using Mössbauer spectroscopy to study hemoglobin derivatives is the extreme dilution of the resonant ^{57}Fe atoms (2.2% natural abundance) in naturally occurring specimens. We have employed the fructose chelate of Charley et al (4) to enrich the iron in rat hemoglobin to 15–20% ^{57}Fe as estimated from the intensity of the Mössbauer absorption lines. The method involves injection anemic rats intramuscularly with solutions of the trivalent iron (^{57}Fe) fructose compound for a month. Aliquots of blood were obtained when needed by cannulating the optic sinus; the red cells were separated by centrifuging, and were then hemolyzed in hypotonic solution. The cell wall debris was precipitated by centrifugation at 10,000 g for 15 min, and the salts were removed by dialysis against distilled water.

Deoxyhemoglobin, Hb, was first prepared by repeated flushing of a hemoglobin solution with nitrogen. Later samples were prepared by also adding small amounts of sodium hydrosulfite, $Na_2S_2O_4$, to the solution in an oxygen-free atmosphere. Samples prepared by both techniques yielded identical Mössbauer spectra.

The anhydrohemoglobin, AHb, was prepared by vacuum distillation of the above solution (containing small amounts of $Na_2S_2O_4$) at room temperature in a sealed evacuated U-tube, one end of which was immersed in liquid nitrogen. The $Na_2S_2O_4$ present prevented the formation of Fe^{3+} which occurs readily at low oxygen pressures. To be sure that the dehydration of AHb was complete, we have varied the length of time for the dehydration process from 1 to 7 days and observed no change in the Mössbauer spectrum. When dehydration was complete, as determined by visual inspection, the tube containing the hemoglobin was sealed off from the frozen water and opened in a nitrogen gas atmosphere dry box.

The samples of both Hb and AHb were placed into airtight copper holders before removal from the dry box. The Mössbauer sample holders had beryllium end windows. The temperature of the samples was measured with Cu–(Au–2% Co) thermocouples attached to the sample holders. The samples were allowed to equilibrate for several hours before measurements were made and the constancy of low temperature susceptibility and Mössbauer measurements after several hours indicated that thermal equilibrium was established. Mössbauer and susceptibility measurements were made on identical samples. The Mössbauer spectra were obtained with a constant velocity mechanical spectrometer described elsewhere (5). The source for all Mössbauer measurements was ^{57}Co in Pt at 295°K. The isomer shift of this source relative to a room temperature metallic iron absorber is −0.343 mm/sec. The magnetic susceptibility was determined from force measurements on a sample in a constant gradient magnetic field (6).

RESULTS

In the absence of a paramagnetic hyperfine interaction, the Mössbauer spectrum of an atom in a unique chemical site is usually characterized by only two parameters, the isomer shift, δ, and the quadrupole splitting (peak separation), ΔE_Q. (The origin of these parameters is discussed in many references, e.g. references 7 and 8, or for particular emphasis on iron-containing biological compounds see references 9 and 10.) The presence of an electric field gradient at the ^{57}Fe nucleus splits the absorption line into two components with line positions at $\delta \pm \Delta E_Q/2$.

The Mössbauer spectrum of rat deoxyhemoglobin at 5°K is shown in Fig. 1. The deoxyhemoglobin absorber was in the form of a frozen aqueous solution. This spectrum, which essentially consists of one quadrupole split doublet with $\Delta E_Q =$

2.38 ± 0.03 mm/sec and δ = 0.60 ± 0.02 mm/sec, is in good agreement with previous studies (10, 11) of this molecule and indicates the chemical similarity of the four iron sites in Hb. Mössbauer spectra of rat anhydrohemoglobin at several temperatures are shown in Fig. 2. The absorber in this case was a fine powdered specimen. The most obvious difference between the spectra of Hb and AHb is the appearance of the very pronounced absorption line at ~ +0.65 mm/sec in the spectra of the latter. The spectrum of AHb consists of two quadrupole split doublets with a ratio of peak separations ~ 2:1. We will show that these two sets of lines correspond to iron in high and low spin states. The two lower energy lines (smaller Doppler velocity) accidentally overlap as can be seen from the relative line intensities. At 16°K the experimental values for the isomer shift and quadrupole splitting associated with the spectra of high and low spin ferrous iron in AHb are ΔE_Q = 2.2 ±

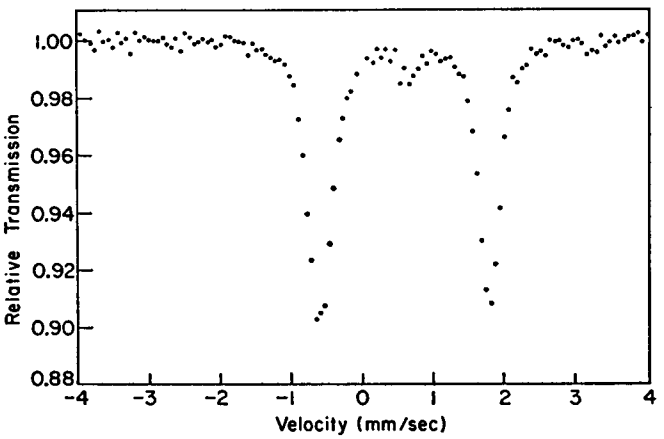

FIGURE 1 Mössbauer absorption spectrum of deoxygenated rat hemoglobin at 5°K.

0.1 mm/sec, δ = +0.61 ± 0.06 mm/sec and ΔE_Q = 1.1 ± 0.1 mm/sec, δ = +0.08 ± 0.06 mm/sec, respectively. At room temperature the quadrupole splittings for the two spectra decrease to 1.6 ± 0.1 mm/sec and 1.0 ± 0.1 mm/sec, respectively. Several spectra at temperatures intermediate to those shown in Fig. 2 were taken but showed no additional interesting features. Samples of human anhydrohemoglobin were also prepared and, aside from a large decrease in absorption intensity (because of the much lower ^{57}Fe concentration), they were similar within experimental error to the spectra in Fig. 2. The reversibility of the reaction in both rat and human hemoglobin was checked by rehydrating the AHb and reproducing the spectra in Fig. 1. Subsequent dehydration again produced the spectra in Fig. 2. The reproducibility of the Mössbauer spectra at ~85°K was also checked after cycling the AHb absorber between room temperature and 16°K.

The spectra in Fig. 2 permit a rough estimate to be made regarding the relative amounts of iron in the two ferrous iron spin states which are observed. If the recoil-

free fraction is the same for both states and no preferential orientation exists in the absorber, the relative absorption areas of the two quadrupole split doublets would directly measure the relative amounts of the two states (in the thin absorber approximation). The small temperature dependence of the relative absorption areas which

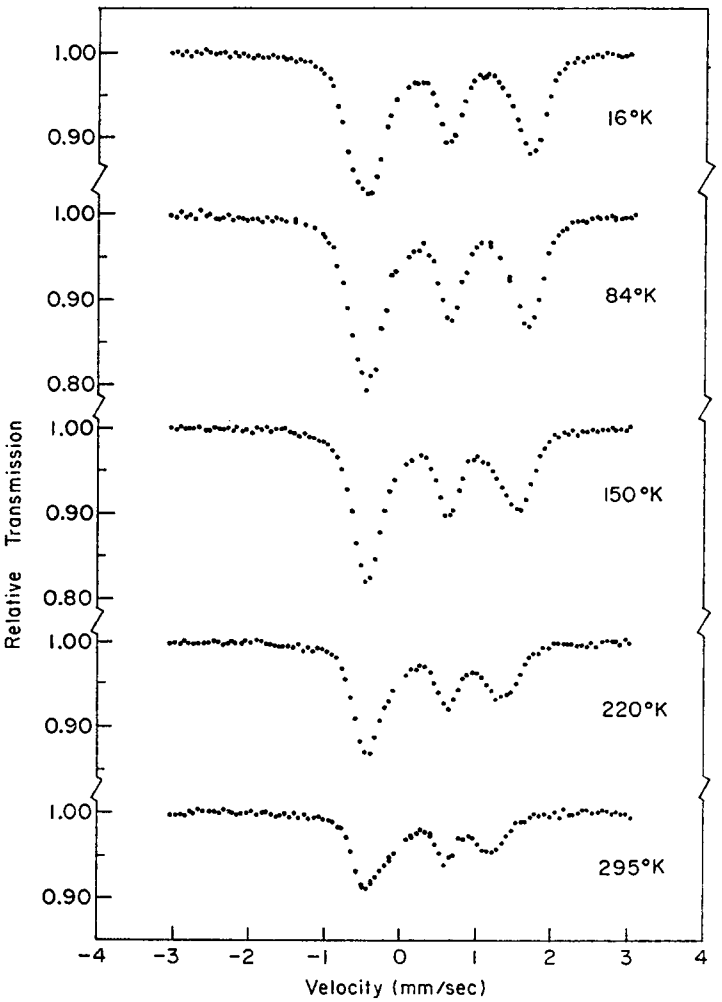

FIGURE 2 Mössbauer absorption spectra of dehydrated deoxygenated rat hemoglobin at several absorber temperatures.

correspond to the two states in AHb suggests that the recoil-free fractions of these two states cannot be greatly different unless the distribution of iron between the high and low spin states is changing considerably. The absorption line, centered at about −0.45 mm/sec, consists of two overlapping components and has only a slightly larger line width below ~220°K than the two resolved absorption lines at

higher energies. Thus these two overlapping lines must have very nearly the same position.

Since we are unable to resolve the two lines centered at ~ -0.45 mm/sec, we have used only the absorption areas of the two higher energy lines to estimate the relative amounts of the two iron states. The spectra in Fig. 2 indicate that to within $\pm 10\%$ the iron is present to an equal extent in both states. The absorption area of the highest energy line ($\sim$1.5 mm/sec) is somewhat larger at low temperatures than the absorption area of the line at $\sim$ 0.65 mm/sec, but this may be due to preferential orientation effects.

To further characterize the nature of the iron in AHb, the magnetic susceptibility was measured from liquid helium to liquid nitrogen temperature. The molar para-

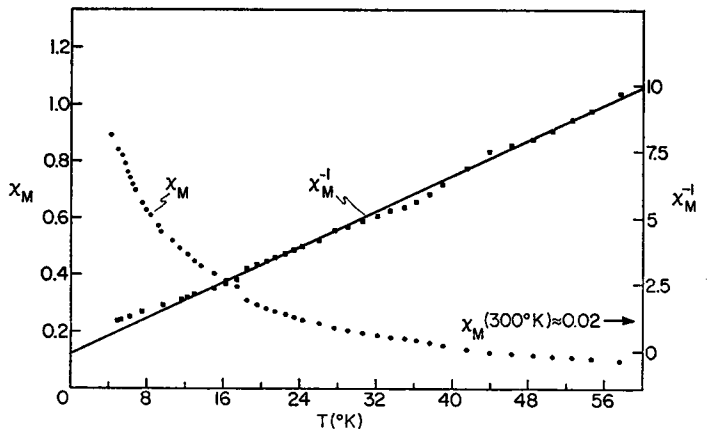

FIGURE 3 The measured molar magnetic susceptibility of anhydrohemoglobin. Data were taken in an inhomogeneous magnetic field $H \simeq 4.4$ kG, $dH/dx \simeq 680$ G/cm. Note that at 300°K χ_M is negligibly small relative to the low temperature values.

magnetic susceptibility, χ_M, of AHb was assumed to obey the Curie law

$$\chi_M = \chi_\infty + 0.496\, \mu_{EFF}^2/T \qquad (1)$$

where T is the absolute temperature, χ_∞ the temperature-independent susceptibility, and μ_{EFF} is the effective magnetic moment per iron atom. The numerical factor 0.496 incorporates the assumption that the temperature-dependent susceptibility results from each of four independent and equivalent Fe atoms per formula unit. Fig. 3 shows the temperature dependence of the measured molar susceptibility and its reciprocal, χ_M^{-1}, in the temperature range 4°–60°K. From the slope of the straight line drawn through the reciprocal susceptibility data, we obtain the value $\mu_{EFF} = 3.5 \pm 0.1\ \mu_B$ (Bohr magnetons) per Fe atom in AHb. By determining μ_{EFF} in this way we have neglected χ_∞ as being negligibly small relative to χ_M in this temperature range. This is justified by the room temperature value of χ_M (300°K) = 0.02 (see Fig. 3).

DISCUSSION

The isomer shift observed in Hb is just slightly less than is usually observed in most high spin ionic ferrous compounds (12, 13) in agreement with the conclusion, based on the observed large effective magnetic moment (14) that the iron atoms in Hb are in the high spin divalent state. The isomer shift of the widely split quadrupole pattern in the spectrum of AHb also is characteristic of high spin divalent iron. However, δ for the pattern with the smaller ΔE_Q in AHb is much too small for a high spin state and falls in the range usually observed for low spin divalent iron (12) For comparison the isomer shifts of O_2–Hb and CO-Hb at 5°K and relative to the same source are -0.08 and -0.07 mm/sec respectively (10). This indicates the existence of two ferrous iron spin states in AHb.

The ferrous atom has six electrons to fill the 3*d* shell. The high spin state is associated with four unpaired electrons and the low spin state with no unpaired electrons. There also exists the possibility of an intermediate spin state with 2 unpaired electrons. Energetic arguments suggest that the coexistence of all three spin states in a hemoprotein is unlikely (15). Also the spectra in Fig. 2 do not seem to require more than two sets of lines for an explanation although the absorption lines are about twice natural width. Therefore, three possible mixtures of spin states exist to account for the iron in AHb: high-intermediate, high-low, or intermediate-low.

In most 3*d* transition metal compounds the orbital contribution to the magnetic moment is quenched and one observes moments close to the spin only value. The spin contribution to the magnetic moment for n unpaired electron spins is

$$\mu_{spin} = [n(n + 2)]^{1/2}. \tag{2}$$

Therefore, for 0, 2, and 4 unpaired electrons, effective magnetic moments of 0, 2.83, and 4.90 μ_B, respectively, should result. In hemoglobin compounds one may also observe an orbital contribution to the moment, but this contribution is usually small (16). We can, therefore, distinguish between the three possible mixtures of spin states mentioned above. The value of μ_{EFF} should be related to the magnetic moments of the two states, μ_1 and μ_2, by

$$\mu^2_{EFF} = f_1\mu_1^2 + (1 - f_1)\mu_2^2 \tag{3}$$

where f_1 is the fraction of iron with moment μ_1. A mixture of intermediate-low spin states would require that virtually all the iron is in the intermediate spin state to produce an effective moment of 3.5 μ_B, even considering a sizable orbital contribution to the moment. Similarly, a mixture of high-intermediate spin states would have to be more than 73% intermediate spin to produce the observed moment. Thus, to be consistent with the data in Fig. 2, anhydrohemoglobin must be a mixture of high and low spin states. If the spin only values for the magnetic moments of iron in the high and low spin states are assumed, the observed magnetic moment $\mu_{EFF} = 3.5 \pm 0.1$ μ_B would indicate approximately 50% high spin and 50% low spin ferrous iron in AHb in reasonable agreement with the spectra of Fig. 2.

At least two explanations seem possible for the coexistence of high and low spin ferrous iron in AHb. The fact that the iron atoms are distributed almost equally between the two spin states may reflect a difference in the ligand field at the iron atoms associated with the α- and β-globin chains in the anhydrohemoglobin molecule. On the other hand, if the energy difference between the high and low spin states is very small, a thermal equilibrium between the two states in AHb of the type discussed by George et al (16) may exist. Similar high and low spin states have been observed in hydrated and dehydrated (ferric) metmyoglobin by several techniques (16–18).

If a thermal equilibrium does exist, the spectra in Fig. 2 set a lower limit on the transition frequency between the high and low spin states. If this frequency were fast compared to the lifetime of the first excited nuclear state in ^{57}Fe, one would observe only a single quadrupole split doublet with a time-averaged value for ΔE_Q and δ. The presence of two well-resolved quadrupole split doublets indicates that this frequency must be smaller than 10^7 sec^{-1}.

To be consistent with the present results, the interpretation of Haurowitz's optical data (2) requires some modification. Anhydrohemoglobin was observed to have two relatively narrow absorption bands at 559 mμ and 530 mμ similar to the diamagnetic hemochromogens while Hb has a rather wide absorption band in this spectral region. Pauling and Coryell (19) have considered the origin of the characteristic hemochromogen spectrum and concluded that the spectrum is to be correlated with a structure in which the four porphyrin nitrogen atoms form covalent bonds with the central iron atom. Thus the large fraction of low spin iron in anhydrohemoglobin probably accounts for the two narrow absorption bands.

A careful inspection of the data in Fig. 1 for Hb suggests the presence of a very weak line at $\sim +0.65$ mm/sec. A suggestion of this line has appeared in all the spectra which were taken with sufficient statistics for the line to be observable; and it also is observable in the spectrum of Hb in reference 11. This line may indicate the presence of a few per cent of low spin iron and suggests that both high and low spin states may be present in Hb as well as in AHb.

We thank Drs. R. M. Housley and H. Wiedersich for helpful discussions and M. R. Bloombaum, F. W. Booth, D. H. Leslie, L. F. Nevarez, and K. G. Rasmussen for technical assistance.

Dr. Saltman thanks the John A. Hartford Foundation and the U. S. Public Health Service, Research Career Development award for partial support of this work.

Received for publication 5 May 1967.

REFERENCES

1. HAUROWITZ, F. 1949. *In* Hemoglobin. F. J. W. Roughton and J. C. Kendrew, editors. Butterworth & Co. Ltd., London. 53.
2. HAUROWITZ, F. 1951. *J. Biol. Chem.* **193**:443.
3. NOBBS, C. L., H. C. WATSON, and J. C. KENDREV. 1966. *Nature.* **209**:339.
4. CHARLEY, P. J., B. SARKAR, C. F. STITT, and P. SALTMAN. 1963. *Biochim. Biophys. Acta* **69**:313.
5. GONSER, U., R. W. GRANT, C. J. MEECHAN, A. H. MUIR, JR., and H. WIEDERSICH. 1965. *J. Appl. Phys.* **36**:2124.

6. BATES, L. F. 1961. Modern Magnetism. Cambridge University Press, London.
7. WERTHEIM, G. K. 1964. Mössbauer Effect: Principles and Applications. Academic Press, Inc., New York.
8. FRAUENFELDER, H. 1962. The Mössbauer Effect. W. A. Benjamin, Inc., New York.
9. GONSER, U., and R. W. GRANT. 1965. *In* Mössbauer Effect Methodology. I. J. GRUVERMAN, editor. Plenum Press, New York. **1**:21.
10. GONSER, U., and R. W. GRANT. 1965. *Biophys. J.* **5**:823.
11. LANG, G., and W. MARSHALL. 1966. *Proc. Phys. Soc. London* **87**:3.
12. MUIR, A. H., JR., K. J. ANDO, and H. M. COOGAN. Mössbauer Effect Data Index, 1958–1965. Interscience Publishers, Inc., New York. In press.
13. GRANT, R. W., H. WIEDERSICH, A. H. MUIR, JR., U. GONSER, and W. N. DELGASS. 1966. *J. Chem. Phys.* **45**:1015.
14. PAULING, L., and C. D. CORYELL. 1936. *Proc. Natl. Acad. Sci. U. S.* **22**:210.
15. GRIFFITH, J. S. 1956. *Proc. Roy. Soc. London Ser. A.* **235**:23.
16. GEORGE, P., J. BEETLESTONE, and J. S. GRIFFITH. 1964. *Rev. Mod. Phys.* **36**:441.
17. EHRENBERG, A. 1962. *Arkiv. Kemi.* **19**:119.
18. CAUGHEY, W. S., W. Y. FUJIMOTO, A. J. BEARDEN, and T. H. MOSS. 1966. *Biochemistry.* **5**:1255
19. PAULING, L. and C. D. CORYELL. 1936. *Proc. Natl. Acad. Sci. U. S.* **22**: 159.