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CONFORMATIONAL CHANGES IN HEME PROTEINS AND MODEL COMPOUNDS

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Résumé. — On a mesuré des spectres Mössbauer de hémoglobine, myoglobine et quelques composés modèles de type hème à différentes températures. Chaque échantillon a donné un doublet quadrupolaire dont la largeur des raies et l'éclatement quadrupolaire dépend de la température. On a comparé les spectres des protéines naturelles avec les spectres d'un des composés modèles étudié en détail avec la spectroscopie Mössbauer et on conclut qu'on peut attribuer la variation en fonction de la température à la relaxation de l'oxygène parmi des conformations différentes.

Abstract. — Zero-field Mössbauer spectra of oxygenated hemoglobin, myoglobin and some of their model compounds were recorded at various temperatures. Each sample gave rise to quadrupole doublet with temperature-dependent linewidth and quadrupole splitting. We have compared the native protein spectra with the spectra from one of the model compounds extensively studied by Mössbauer spectroscopy, and we conclude that oxygen relaxation between conformational states is responsible for the temperature dependence in both cases.

1. Introduction. — The zero-field Mössbauer spectra of hemoglobin (Hb) and myoglobin (Mb) in their oxygenated form exhibit a characteristic temperature dependence, the essential features of which are a quadrupole splitting which decreases with increasing temperature and an effective linewidth which reaches a maximum at an intermediate temperature. The oxygenated complexes of these proteins are EPR-silent and they are found to be diamagnetic at all temperatures. This indicates that the heme iron is in the low-spin ferrous ($S = 0$) state with the three t_{2g} orbitals fully occupied. The temperature dependence of the zero-field spectra cannot then result from electronic excitation into unoccupied orbitals. An attractive explanation of this is conformational excitation, involving the motion or displacement of some part of the molecular structure in the neighborhood of the iron. This could redistribute charge without unpairing electrons. The relative flexibility of protein originally led to a suggestion that distortion of the peptide chain near the heme might be involved [1]. This explanation of the temperature dependence of ΔE was, however, almost certainly ruled out by the Mössbauer emission measurements of Münck *et al.* [2] who observed the transient oxygenated iron imidazole heme formed in the decay of the corresponding ^{57}Co complex, and found a quadrupole-split spectrum whose behavior closely followed that of oxyhemoglobin. This finding seems to implicate either the oxygen or the heme, probably the former since no other ligand shows such effects. The detailed nature of the implied oxygen motion is of interest because of the light it could throw on the electronic

structure of the complex and because of its possible relevance to biological function.

The study of relatively small non-protein models has traditionally been of importance in efforts to understand the behavior of the large biological molecules. A recent successful simulation of the oxygen binding sites of hemoglobin is described in reference [3], where the synthesis and characterization of a series of compounds of iron, meso-tetra ($\alpha, \alpha, \alpha, \alpha$ -o-pivalamidophenyl) porphyrin, are presented. With a variety of bases attached to the iron on the side opposite the *picket fence* structure, these are capable of binding O_2 reversibly as the sixth iron ligand. Our Mössbauer measurements of the oxygenated model indicate a close similarity to oxyhemoglobin with respect to isomer shift, quadrupole splitting, and lineshape over a range of temperature. X-ray studies of the N-Me-imid oxygenated complex show a statistical disorder in oxygen position at room temperature [4]. We believe this is a dynamic thermal distribution and have accounted for and analyzed the observed Mössbauer spectra in terms of it [5]. In the present paper we discuss the relevance of such an analysis to the oxygen binding sites in hemoglobin and myoglobin.

2. Experimental. — The preparation of samples is described elsewhere [3, 6]. The spectra at 4.2 K were recorded by keeping the sample immersed in liquid helium in a cryostat of a design described elsewhere [1]. The temperatures between 4.2 and 195 K were achieved with a variable temperature insert in which the sample was mounted inside a vacuum can immersed in the

helium bath. The temperature was maintained by an electronic controller and monitored with a thermocouple (0.03 at. % iron-gold vs. chromel).

The Mössbauer spectra were taken in horizontal transmission geometry using a constant acceleration spectrometer operated in connection with a 256 channel analyzer in the time scale mode. The source was kept at room temperature and consisted of 50 millicuries of ^{57}Co diffused in rhodium foil. The spectrometer was calibrated against metallic iron foil and zero velocity was taken as the centroid of its room temperature Mössbauer spectrum. In calibration spectra linewidths of about 0.23 mm/s were normally observed.

3. Results. — Figure 1 presents the zero-field Mössbauer spectra of the N-Me imid model compound (left)

FIG. 1. — Zero-field Mössbauer spectra of the FeO₂(N-Me imid) P model compound (left) and oxyhemoglobin (right) at temperatures as indicated. The horizontal scale is in units of mm.s⁻¹.

and oxyhemoglobin (right) at representative temperatures over the relevant range. Comparison between the two series of spectra yields similarities and differences upon examination of the quadrupole splitting and the lineshape.

The quadrupole splitting ΔE is temperature dependent and decreases with increasing temperature for both systems. However, although at 4.2 K the quadrupole splitting for the model compound and HbO₂ is nearly the same, the value at the high end of the temperature scale for the protein is considerably higher than the corresponding value for the model compound.

The lineshape for the two systems exhibits the same behavior at low and intermediate temperature ($T \lesssim 160$ K); the lines are relatively narrow and Lorentzian-like at low temperature while at intermediate temperature ($70 \lesssim T \lesssim 160$ K) they become

broad and asymmetric in the same fashion. At the high end of the temperature scale the asymmetry in the lines disappears, the lines in the model compound spectra tend to become narrow again, almost approaching their natural width, while those in the HbO₂ spectra remain broad at about twice the natural linewidth.

The results of Lorentzian fits to a variety of related compounds are shown in figure 2, where the quadru-

FIG. 2. — Quadrupole splitting as a function of temperature for the heme proteins and model compounds mentioned in the text. The data for cytochrome P 450 were taken from reference [9].

pole splittings are plotted as a function of temperature. All samples show approximately equal low-temperature quadrupole splittings and similar decreases in this quantity with increasing temperature. In particular, oxygenated cytochrome P450 which, like the tetrahydrothiophene (THT) compound, probably has a sulfur atom adjacent to the iron, exhibits a temperature dependence of ΔE characteristic of the group. At this point the reader must be cautioned that the results shown in figure 2 were derived from least-squares Lorentzian fits to lines which, in some instances, were patently non-Lorentzian. This was done as an approximation in order to obtain a unified picture of the quadrupole splittings.

4. Discussion. — The zero-field spectra from the N-Me imid model compound have been successfully analyzed and accounted for in terms of a dynamic relaxation model [5] based on the published crystal structure [4] of this compound. Briefly, this model is as follows. The dioxygen molecule occupying the sixth ligand position above the heme plane may make transitions between four possible orientations or conformational states as observed in the crystallographic studies. These orientations are equivalent in opposing pairs that are separated in energy by an amount E_0 ; each pair produces a different electric field gradient (efg) at the site of the nucleus. As the temperature is varied, the oxygen molecule relaxes between orientations with a characteristic relaxation rate that depends

on temperature. Furthermore, the contribution of each orientation to the efg at a given temperature must be weighted by the appropriate Boltzmann factor.

The above model was mathematically formulated by adapting relaxation theories found in the literature [7] and the experimental spectra were least-squares fitted by computer to obtain the pertinent parameters. The solid lines in figure 2 are the results of such fits. The details of the calculation can be found in reference [5]. It suffices to mention here that the implicit assumptions in the above model are that (a) the observed negative principal component of the low temperature efg lies in the heme plane, (b) the component normal to the heme plane, along which the z -direction is defined, is the same for both conformational states, (c) the efg at the nucleus for each conformation is different, i. e. the efg is not bodily rotated about the z axis as the oxygen relaxes between the two conformations.

Since the temperature behavior of ΔE and the line-shape in the HbO_2 spectra follow qualitatively the behavior of the model compound, it is safe to conclude that oxygen motion between conformations also occurs in the protein. On the other hand, there is no need to conclude that the detailed nature of the conformational excitation must be similar. In fact, a straightforward application of the above model to the HbO_2 problem fails to yield a satisfactory set of parameters and thus implies that the nature of the excitation is somehow different. This should hardly be surprising in view of the fact that x-ray results [8] indicate that the O_2 molecule in oxyhemoglobin is sterically hindered from completing a full circle about the Fe-O axis; instead it may wobble within an angle of 45° .

Attempts to account for the HbO_2 zero-field spectra in terms of a model whereby the efg bodily rotates with the oxygen within 45° about an axis perpendicular to the heme plane were unsuccessful mainly because the calculated quadrupole separation ΔE at high temperature was far less than the observed value. If the *bodily* rotation of the efg requirement is relaxed and we assume instead that each O_2 orientation results to a different efg at the nucleus, then it becomes possible to construct a large number of models for oxygen relaxation, all agreeing with the zero-field spectra. In short, we believe that the mathematical formulation of a

relaxation model for the zero-field spectra of HbO_2 is a risky endeavor if additional information about the possible O_2 orientations is not independently provided. It is, however, reasonable to claim that the sterically hindered O_2 motion is responsible for the relatively high value of ΔE at high temperature.

We now turn our attention to the other, less extensively studied, systems whose quadrupole splittings are also shown in figure 2. A striking deviation from the norm is the oxygenated complex of cytochrome P450 studied by Sharrock *et al.* [9] which shows little temperature dependence of ΔE . In this enzyme the axial ligand is assumed to be sulfur and it might be argued that it is the sulfur which somehow impedes the oxygen motion. In seeming contradiction to this is the temperature dependence of ΔE of the thioether model compound in which the axial ligand is *known* to be sulfur. In fact, the latter model compound seems to mimic the ΔE temperature dependence of HbO_2 and MbO_2 much closer than the N-Me imid model compound. This agreement may be fortuitous or it may show that indeed the sulfur ligand hinders the oxygen motion but not altogether. Then, one may further speculate, in the case of P450 there may be additional steric hindrances, possibly atoms neighboring the sixth ligand position, that define a unique oxygen orientation.

A final note may be added concerning lineshapes. It is certainly plausible to link the temperature dependence of the lineshape with oxygen relaxation. In cytochrome P450 there is no significant variation of the linewidth with temperature [9] as expected. The difference in linewidth between the model compound and HbO_2 at high temperature (cf. Fig. 1) may be an artifact introduced by the state of the samples. The protein was in frozen solution and therefore susceptible to solvent phase-transition effects that may broaden the lines. Such effects are of course non-existent in the powder sample of the model compound.

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