CBPF-NF-015/95

Contribution of Electron Paramagnetic Resonance to the Studies of Hemoglobin: The Nitrosylhemoglobin System

G. Bemski

Centro Brasileiro de Pesquisas Físicas - CBPF
Rua Dr. Xavier Sigaud, 150
22290-180 - Rio de Janeiro-RJ, Brasil

ABSTRACT

Since the initial work of Ingram (8,10) Electron Paramagnetic Resonance contributed considerably to research in hemoglobins. Now, 40 years later we review some of the results of the application of EPR to nitrosylhemoglobin (HbNO), as an example of the diversity of information which this technique can provide.

Key-words: Hemoglobin; Paramagnetic resonance.

Research in heme proteins constitutes one of the most voluminous chapters of studies which use physical concepts and techniques in biology and is responsible for awakening interest of many physicists in biophysics.

Hemoglobin is one of several hemoproteins, so called because heme is their central part. Other hemoproteins are myoglobin, cytochrome c, etc. Let us take a closer look at hemoglobin with which we will be mainly concerned.

Hemoglobin is a protein whose physiological function is to transport oxygen in blood. This transport is quite efficient. Hemoglobin in 100ml of human blood is capable of binding 20ml of O_2, while if the transport consisted of dissolved O_2 in plasma, 100ml of plasma would transport only 0.3ml of O_2.

This system of transport has been used during about 500 million years of the evolutionary development, without undergoing substantial changes. It is another proof that when nature "invents" a succesfull molecule it sticks to it!

Hemoglobin consists of 574 amino acids and has a molecular weight of 64650 daltons fig. 1 [1]. It is composed of four polypeptide chains, each one containing a unit called heme, which consists of an iron ion surrounded by five nitrogen atoms; the sixth position can be occupied by O_2, marked by a sphere in fig. 2. This description is actually oversimplified, since the four nitrogens with iron in their center form part of pyrrole groups, as seen in fig. 2. The nitrogen in the fifth coordination position (N_ϵ) belongs to an amino acid, histidine. The surrounding of the heme is shown in fig. 3. The oxygen molecule can easily be replaced by many other ligands, such as water, CN, N_3, NO, etc. Each of these ligands determines the magnetic properties of the protein. In the case of the physiologically important deoxyhemoglobin, the sixth coordination position is vacant.

The unusual magnetic properties of heme proteins have been noticed already by Faraday [2], who however did not pursue this line of research. It was left to Pauling to measure the magnetic susceptibility of hemoglobins with various ligands and to derive the first conclusions concerning the electronic state of iron in the heme proteins [3, 4, 5]. Without iron the heme proteins, similarly to most other proteins, are diamagnetic. Iron is not only the source of magnetism, but also is the reaction site, the heart of the protein which performs the task for which it is designed. Thus hemoglobin transports oxygen, myoglobin stores oxygen, and cytochromes are involved in electron transfer.

Many questions come to mind in as far as functioning of hemoglobin. How is the oxygen molecule transported from blood to the iron? How is it released from the molecule as blood travels through different parts of the body? Why does it require much more energy to bind the first oxygen in each molecule, than the third or the fourth one? We will not answer these questions here, but the research that will be discussed gradually provides the answers.

The first step in the physical studies of such a complicated molecule is to obtain a three dimensional map with the positions of all atoms. Using x-ray diffraction method, this was first accomplished for the smaller molecule myoglobin (17815 daltons, single polypeptide chain) by Kendrew and by Perutz for hemoglobin [6, 7]

To the delight of the physicists the magnetic susceptibility measurements disclosed that nature rarely provides such a laboratory of varied possibilities. While the physiologically important form of iron is Fe^{2+} which in oxyhemoglobin is diamagnetic (S=0), and in deoxyhemoglobin is paramagnetic (S=2), other ligands are responsible for the Fe^{3+} state.

The first Electron Paramagnetic Resonant (EPR) experiments in heme proteins appeared in 1955 [8], not long after the discovery of the EPR spectroscopy by Zavoisky in 1945 [9] . These early experiments lead to a detailed description of the geometry of the heme plane in the hemoglobin and myoglobin unit cells [10] (fig. 4). Single crystals were necessary for that work, but the know-how of their preparation existed since the x-ray pioneering studies. Since the resolution of the early x-ray diffraction work was not sufficient for such a description, the EPR results constituted a triumphant entry of this technique into biology.

Bennett et al [8] were the first to observe an EPR signal in nitrosylhemoglobin (HbNO). NO was introduced to substitute for O_2, and it was verified that it binds to iron at the same sixth coordination position (fig. 2) at which oxygen is normally bound.

The interest in these studies resulted primarily from the fact the HbNO is paramagnetic while HbO_2 is not, and that NO is electronically very similar to O_2. It also became evident that HbNO spectrum reflects not only the local properties of the heme unit, but also the general (quaternary) structure of the protein. Since 1962 a stream of papers described various aspects of HbNO research.

Hemoglobin's four polypeptide chains are divided into two α and two β ($\alpha_2\beta_2$). During the early years of these studies it was realized that the HbNO spectra are a composite of absorption in the α and β chains. X-ray diffraction has shown that there is no equivalence in terms of the geometrical surrouding of the α and β hemes [11]. α hemes have more freedom of motion than β hemes. Hence it was not surprising that their EPR spectra differed. The identification of the spectra was made possible separating the two types of chains. It was observed that α_{NO} spectra have axial symmetry at room temperature, while β_{NO} deviate from that symmetry; furthermore the spectrum of the tetramer (HbNO) turned out to be a sum of the isolated chain spectra. This is understendable since the distances between the individual hemes are larger than 20A, making any direct magnetic interaction between them highly improbable. Myoglobin which consists of one polypeptide chain with a single iron is a convenient control for these experiments.

Similarly to the research in solid state physics important progress was made using crystals instead of solutions. The difficulty with single crystals in heme proteins is their small size. While the necessary crystals for x-ray diffraction can be of minute size, EPR requires larger crystals because of the unfavorable ratio of iron atoms to the "background" of the remaining atoms. Single crystal measurements [12, 13] in MbNO and HbNO have provided information on the bent angle Fe-N-O which turned out to be 105^0 in β hemes and 165^0 in α hemes. The g and hyperfine tensors have also been obtained. Furthermore, the electron density of 44% on Fe and 55% on the nitrogen of NO was obtained, showing that practically no electron density is located on the four pyrrole nitrogens and that iron is not really a pure Fe^{2+} ion. These experiments relied on substitution of nitrogen with N^{15} and of iron with Fe^{57}.

Another useful technique is the preparation of hybrids [14, 15] (fig. 5). A hybrid is a tetrameric hemoglobin whose α hemes have a different ligand than the β hemes. Many mixed state hybrids of the type $\alpha_{NO}\beta_x$ and $\alpha_x\beta_{NO}$ where x stands for another ligand such as CO, CN, O_2, H_2O, etc. have been examined. The interesting conclusions of these studies are:

1) at both room temperature and at 77K the spectrum of HbNO is the sum of the spectra

of $\alpha_{NO} + \beta_{NO}$;

2) α_{NO} has rhombic symmetry with hyperfine structure originating from two non-equivalent nitrogens: NO and N_ϵ of the proximal histidine;

3) at room temperature the spectrum of MbNO is similar to β_{NO}, of the hemoglobin;

4) α_{NO} spectrum in the hybrids depends greatly on the β heme ligands, while β_{NO} spectrum does not depend on α ligands;

5) there is a splitting of about 6G due to N_ϵ visible only in some cases.

Since α, β and MbNO, all with the same ligand in the 6th position present a variety of EPR spectra, these differences must be due to minute changes in the structure of the chains. These conclusions remain valid and have been reinforced by Frauenfelder's studies of protein dynamics [16].

All the previously described studies dealt with fully NO saturated hemoglobin. It is however known that hemoglobin without any oxygens attached has a quaternary structure, called T (tense). At the other extreme, when fully oxygenated (with oxygen attached to all four hemes) the structure is different and called R (relaxed). These different structures have been seen in diffraction maps and correspond to considerable displacements of different amino acids. Such allosteric transitions are now well documented and described by a theoretical model [18]. It has also been known that organic phosphates, such as IHP (inositol hexaphosphate) also induce such conformational changes [1, 6]. Since HbNO also exhibits transitions from T to R structure, EPR turned out to be useful in studying the effect of T to R transition on the heme region.

The main conclusion emerging so far from these studies is that in the T conformation α_{NO} is 5-coordinated, while β_{NO} is 6-coordinated; in the R conformation both types of chains are 6-coordinated. This implies that the distance between Fe and N_ϵ increases considerably in the α chains in T conformation [19, 20]. Such an increase in distance leads to an extension, or even breakage of the bond. The 3 line hyperfine structure due to N_ϵ disappears, while the remaining 3 line structure is due to the nitrogen of NO and becomes very pronounced in T conformation. Hence the bond between N_ϵ and Fe of the α chains is fundamental in the structure-function relation in hemoglobin, since the motion of this nitrogen trigers the vast transformation which occurs in the whole molecule on attachment of NO (and, by analogy of O_2) (see ref. 1 for illustrations and discussion).

These studies were, as we have seen based on single crystals, hybrids and one ought to add, mutants [19, 21]. In discussing mutants we deal with a specifically biological phenomenon with no parallel in solid state physics. Until recently mutant hemoglobins-with an amino acid exchanged by another one, due to an error in the DNA gene, responsible for the amino acid sequence in this protein-could only be obtained from the blood of persons with such a mutation. Today mutations can be tailored at will. Mutants were very useful in complementing the work done with hybrids, since certain mutations in the neighborhood of the heme change the characteristics of that heme, leaving it, for instance in the ferric state, useless for ligation with O_2, or NO. Since mutational events are very rare, and the α and β chains are coded by different genes, humans do not usually exhibit

more than one mutation. Mutants thus provide an opportunity to observe characteristics of either α or β chains without having to temper with the hemoglobin.

We have discussed so far aspects relating to the individual chains, their transformations and the effect they have on the EPR spectra. It became however apparent that these spectra contain additional informations beyond the ones discussed. They relate to other conformational aspects of the hemoglobins.

Morse [22] observed that the spectra of MbNO, as well as of the cytochrome c, protoporphyrins, etc. exhibit a temperature dependent spectrum which can be decomposed into two. He interprets this as arising from two six-coordinated conformers, differing in the position of Fe with respect to the ligands and to the heme plane (fig. 6). The predominantly low temperature species (30K) corresponds to fig. 6a and the high temperature one (180K) to fig. 6b. Similarly, Neto [23] has shown the existence of two species in MbNO between 111K and 267K, whose spectra taken in different proportions at different temperatures, add to yield the observed spectra. Sanches [24] in her studies of the effect of hydration on HbNO showed also the existence of two species: one corresponding to normal hydration, and the other one to dehydrated condition. When summed in different proportions they yield the experimental spectra corresponding to intermediate hydrations. The interpretation is that there exist two configurations of the heme pocket in the region of the 6th ligand, with varying ratio of occupation, depending on hydration.

Low temperature experiments (7.5K-104K) in HbNO have similarly indicated a superposition of spectra [25]. In these case three spectra add in different proportion, depending on the temperature to produce the observed spectra, with the added feature that their relaxation times differ. Difference in the relaxation rates produces spectra whose amplitude decreases and even disappears at high microwave power for a long relaxation time component, and increases continuously with increasing power, for a short relaxation one.

These results are indicative of phenomena described in the series of papers by Frauenfelder and his group [16] which deal with the dynamics of the proteins. Their studies on MbCO have conclusively shown that the ligand can assume, at low temperatures, various orientations with respect to the heme plane, producing conformational substates. Three substates have been determined and correspond to three different groups of CO bound at different angles to iron in myoglobin (fig. 7). There exists no detailed description of heterogeneity of NO bound to hemoglobin, but it is almost certain that the substate model applies also in this case, giving rise to the observed phenomena [26].

This model has been studies mainly by optical spectrocopy in experiments involving photodissociation and the subsequent reassociation of the ligand. Similarly photodissociation observed in HbNO by EPR has shown that, at low temperatures NO escapes to a site very close to the liganded position, from which it can reassociate with iron by tunneling [27].

One should mention also the theoretical effort to obtain a coherent description of the electronic diagram of HbNO. There exists a consensus that the unpaired electron is located in an orbital formed by the π^* orbital of bound NO and d orbitals of iron (28). An interesting study by Waleh [29] shows that the spin density of this electron can concentrate at Fe or at NO depending on very slight changes in the Fe-N_ϵ distance and in the bond angles.

It is probably fair to say that one is close to the limit of the information which EPR can

provide about HbNO. This is due, in part to the limits on the resolution of the observed lines. Their line widths is determined principally by hyperfine interactions of the electron not only with the various nitrogens, but also with the large number of protons in the close neighborhood of iron. For that reason it is of great interest to see that progress is being made in a variant of EPR tecnhique-ENDOR [30, 31]. This technique combines the electron paramagnetic resonance and nuclear magnetic resonance with a very large improvement in resolution, allowing so to say to look inside of the EPR spectra. Since ENDOR is not a simple technique, it has so far not been used extensively. Recent papers show a further and still more detailed elucidation of the events occurring around the heme iron in HbNO [32, 34]. In principle the distances between iron and the ligand, as well as between iron and other nitrogens and protons can be obtained and very small changes in these quantities can be observed.

We have restricted ourselves in this paper exclusively to provide some high lights of the EPR research in HbNO which occurred over the years. This work required always results of other investigations, using other techniques which there was no room to mention. In effect the research in biophysics requires an ever increasing cooperation between different specialists dominating various techniques.

Figure Captions

Fig. 1 - Quaternary structure of hemoglobin. Four polypeptide chains and four hemes can be seen (from ref. 1, fig. 1.15).

Fig. 2 - The heme group with a ligand (sphere) and proximal and distal histidines.

Fig. 3 - The heme environment. W represents the ligand. Various amino acids are indicated. This section corresponds to a helical portion of myoglobin (from ref. 1, fig. 2.9).

Fig. 4 - The location of the heme planes in hemoglobin with respect to the crystallographic axes, (from ref. 8, fig. 10).

Fig. 5 - The EPR spectra of the hybrids $\alpha^{NO}\beta^{x}$ and $\alpha^{x}\beta^{NO}$ (from ref. 15, fig. 3).

Fig. 6 - Model of the equilibrium of two species (from ref. 22, fig. 7).

Fig. 7 - Structure and conformational energy landscape of MbCO. Observe the three substates A_0, A_1,A_3 corresponding to three orientation of CO with respect to the heme (from ref. 16, fig. 1)

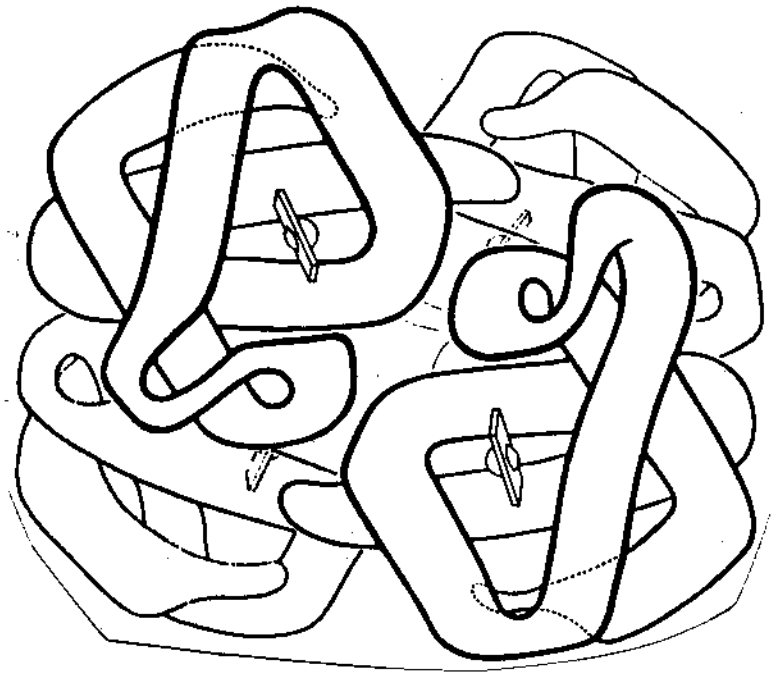

Fig. 1

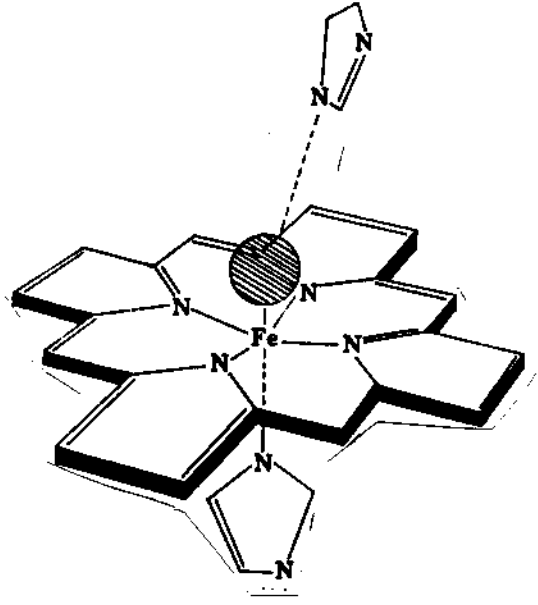

Fig. 2

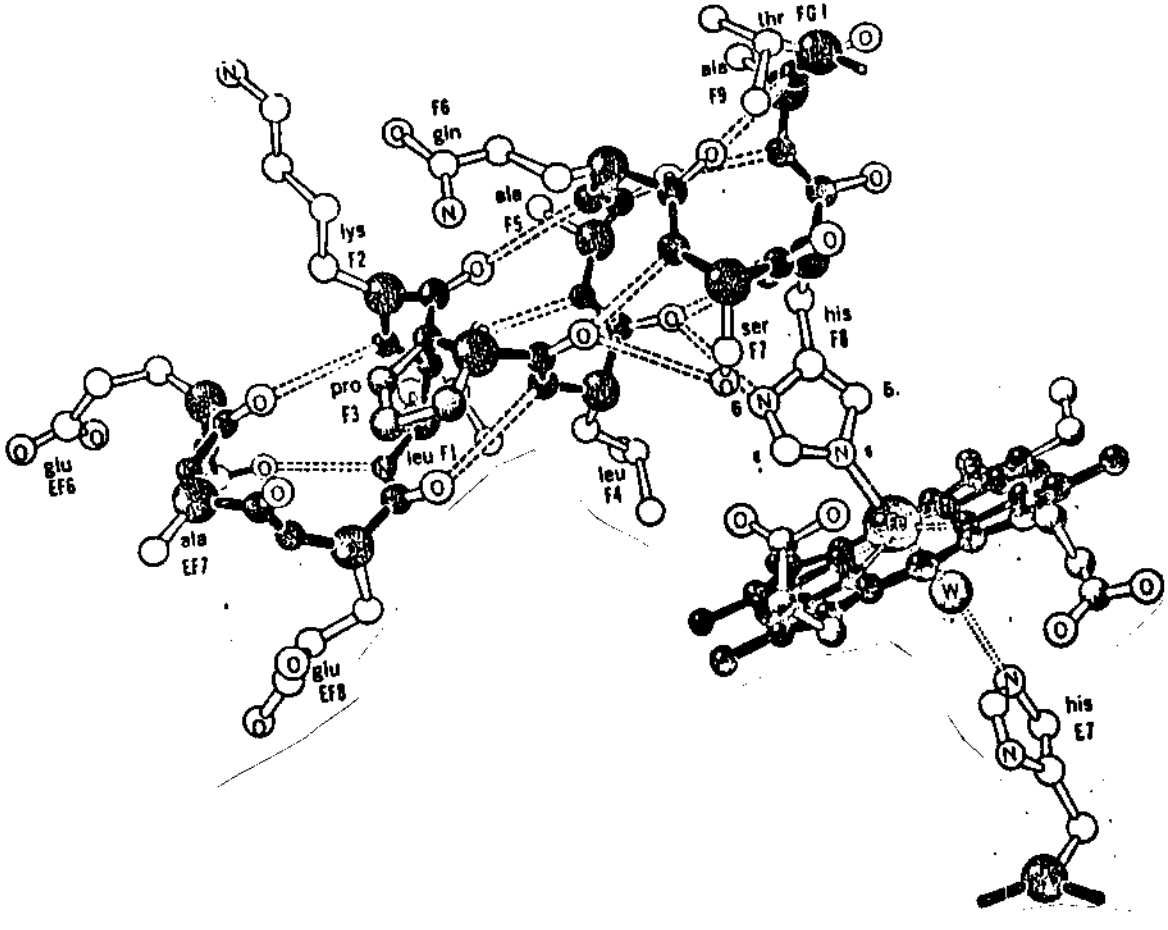
thr FG1
ala F9
F6 gln
ala F5
lys F2
ser F7
his F8
pro F3
leu F1
leu F4
glu EF6
ala EF7
glu EF8
his E7

Fig. 3

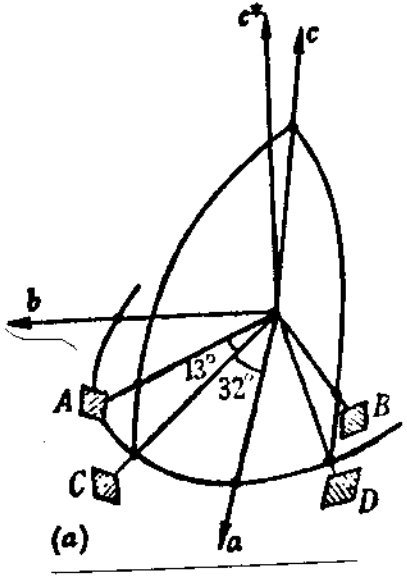

Fig. 4

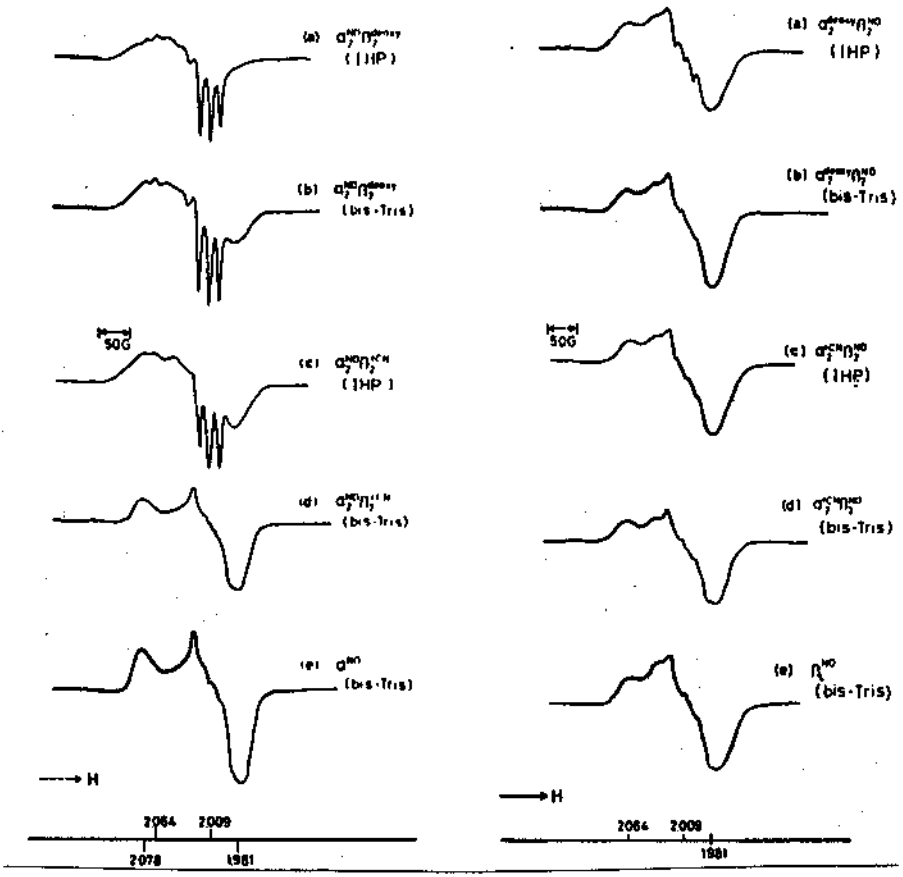
(a) (IHP)
(b) (bis-Tris)
(c) (IHP)
(d) (bis-Tris)
(e) (bis-Tris)
(a) (IHP)
(b) (bis-Tris)
(c) (IHP)
(d) (bis-Tris)
(e) (bis-Tris)
50G
H
2064
2009
2078
1981
2064
2009
1981

Fig. 5

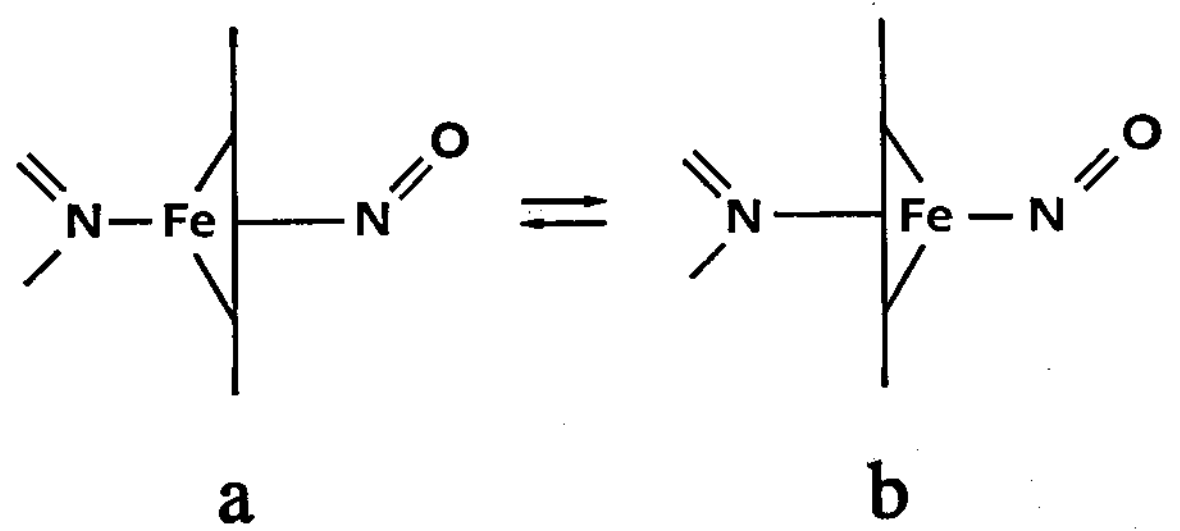

Fig. 6

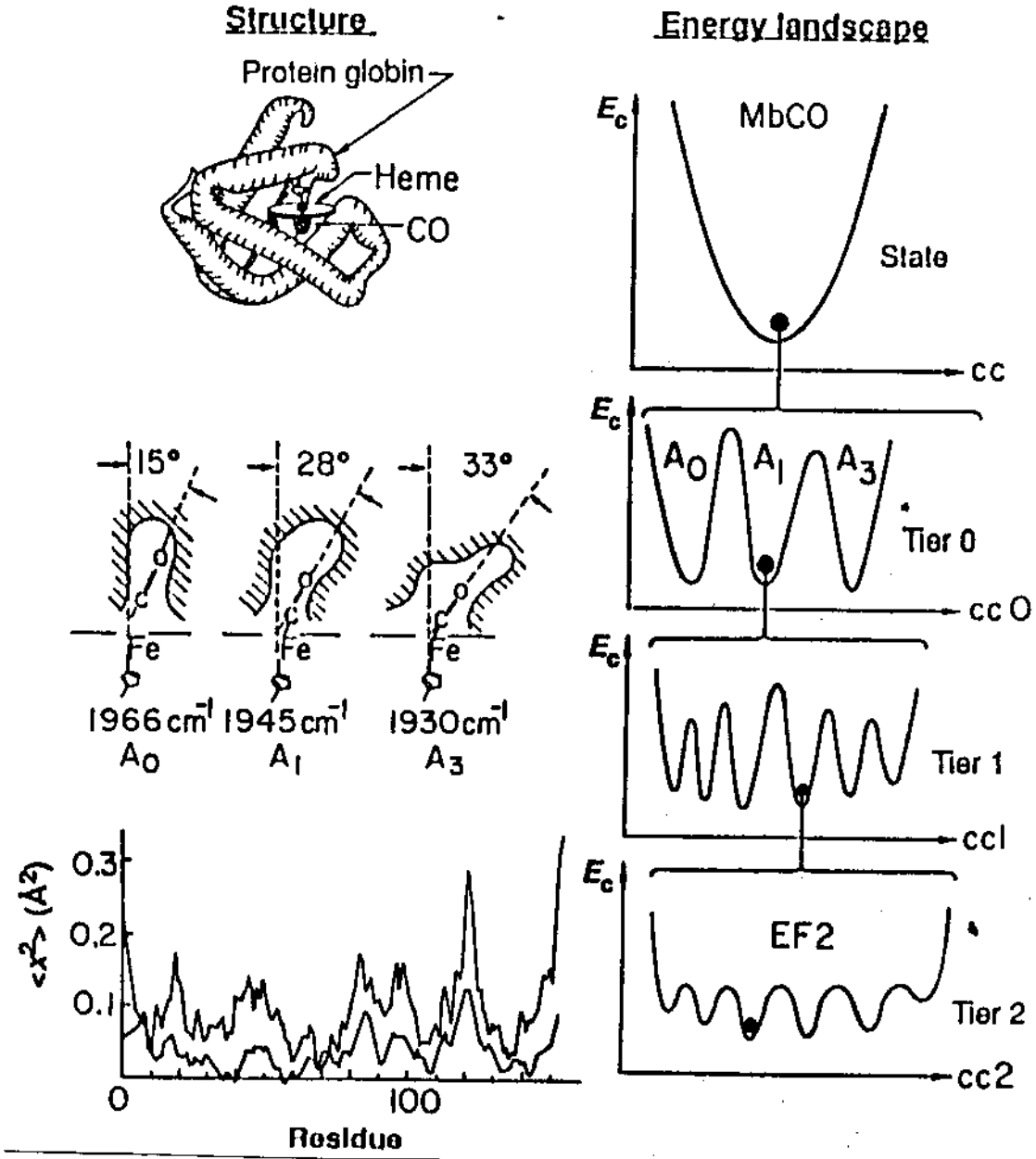
Structure
Protein globin
Heme
CO
Energy landscape
E_c
MbCO
State
cc
E_c
A_0
A_1
A_3
Tier 0
cc 0
15°
28°
33°
Fe
Fe
Fe
1966 cm⁻¹
1945 cm⁻¹
1930 cm⁻¹
A_0
A_1
A_3
E_c
Tier 1
cc1
E_c
EF2
Tier 2
cc2
$\langle x^2 \rangle$ (Å²)
0.3
0.2
0.1
0
100
Residue

Fig. 7

References

[1] R.E. Dickerson, I. Geis: "Hemoglobin: Structure, function, evolution and pathology"; The Benjamin/Cummins Publ. Co. 1983.

[2] As reported by L. Pauling in Proc. Natl. Ac. Sci. USA **22**, 210 (1936).

[3] L. Pauling, C.D. Coryell, "The magnetic properties and structure of the hemochromogens and related substances", Proc. Natl. Ac. Sci. USA **22**, 159 (1936).

[4] L. Pauling, C.D. Coryell, "The magnetic properties and structure of hemoglobin, oxyhemoglobin and carbonmonoxy hemoglobin", Proc. Natl. Ac. Sci. USA **22**, 210-216 (1936).

[5] C.D. Coryell, L. Pauling, R. Dodson, "The magnetic properties of intermediates in the reactions of hemoglobin", J. Phys. Chem. **43**, 825-839 (1939).

[6] J.C. Kendrew, "The crystal structure of horse met-myoglobin. General features: the arrangement of the polypeptide chains", Proc. Roy. Soc. A **201**, 62-89 (1950).

[7] J. Boyes-Watson, E. Davidson, M.F. Perutz, "An x-ray study of horse methaemoglobin", Proc. Roy. Soc. A, **191**, 83-129 (1947).

[8] D.J.E. Ingram, J.E. Bennett, "Paramagnetic resonance in phtalocyanine, hemoglobin, and other organic derivatives", Disc. Faraday, Soc. **19**, 140-146 (1955).

[9] E. Zavoisky, "Paramagnetic relaxation of liquid solutions for perpendicular fields", J. Phys. USSR **9**, 211 (1945).

[10] J.E. Bennett, J.F. Gibson, D.J.E. Ingram, "Electron resonance studies of haemoglobin derivatives. I. Haem plane orientations", Proc. Roy. Soc. A **240**, 67-82 (1957).

[11] M.F. Perutz, "Mechanism of cooperativity and allosteric regulation in proteins "Quart. Rev. Bioph. **22**, 139-236 (1989).

[12] J.C.W. Chien, "EPR study of stereochemistry of Nitrosylhemoglobin", J. Chem. Phys. **51**, 4220-4227 (1969).

[13] L.C. Dickinson, J.C.W. Chien, "Electron Paramagnetic Resonance of single crystal ^{15}N-Nitrosyl-^{57} Fe myoglobin", Biochem. Biophys. Res. Comm." **59**, 1292-1297 (1979).

[14] Y. Sugita, "Difference in spectra of α and β chains of hemoglobin between isolated state and in tetramer", Jour. Biol. Chem. **250**, 1251-1256 (1975).

[15] K. Nagai, H. Hori, S. Yoshida, H. Sakamoto, H. Morimoto, "The effect of quaternary structure on the state of the α and β subunits within nitrosyl haemoglobin", Biophys. Acta **532**, 17-28 (1978).

[16] H. Frauenfelder, S. Sligar, P. Wolynes, "The energy landscape and motions of proteins", Science **254**, 1598-1603 (1991).

[17] R. Benesch, R.E. Benesch, "The effect of organic phosphates from human erythrocyte on the allosteric properties of hemoglobin", Biochem. Biophys. Res. Comm. **26**, 162-167 (1967).

[18] J. Monod, J. Wyman, J.P. Changeux, "On the nature of allosteric transitions: a plausible model", J. Mol. Biol. **12**, 88-1 (1965).

[19] E. Trittelvitz, K. Gersonde, K. Winterhalter, "ESR of Nitrosyl Haemoglobins: Normal α and β chains and mutants Hb M Iwate and Hb Zurich", Eur. J. Biochem. **51**, 33-42 (1975).

[20] A. Szabo, M.F. Perutz, "Equilibrium between six and five-coordinated hemes in nitrosylhemoglobin: interpretation of electron spin resonance spectra", Biochem. **15**, 4427-4428 (1976).

[21] H. Twilfer, K. Gersonde, "Non-equivalence and inverse allosteric response of the α and β chains in hemoglobins. An electron spin resonance study of NO-ligated Hb Kansas", Naturforsch. **31C**, 661-674 (1976).

[22] R. Morse, S. Chan, "EPR studies of nitrosyl ferrous heme complexes. Determination of an equilibrium between two conformations", Jour. Biol. Chem. **255**, 7876-7882 (1980).

[23] L.M. Neto, O. Nascimento, M. Tabak, I. Caracelli, "The mechanism of reaction of nitrosyl with met and oxy myoglobin: an EPR study", Biochem. Biophys. Acta, **956**, 189-196 (1988).

[24] R. Sanches, "Dehydration effects on the heme environment of nitric oxide hemoglobin", Biochem. Biophys. Acta **955**, 310-314 (1988).

[25] E. Wajnberg, L. El-Jaick, M. Linhares, G. Bemski, "Nitrosyl hemoglobin: EPR components at low temperature, "Eur. Biophys. J. **21**, 57-61 (1992).

[26] P. Steinbach, A. Ansari, J. Berendzen, D. Braunstein, K. Chu, B. Cowen, D. Ehrenstein, H. Frauenfelder, J.B. Johnson, D. Lamb, S. Luck, J. Mourant, U. Nienhaus, P. Ormos, R. Philipp, A. Xie, R. Young, "Ligand binding to heme proteins: connection between dynamics and function", Biochem. **30**, 3988-4001 (1991).

[27] M. Linhares, L. El-Jaick, G. Bemski, E. Wajnberg, "EPR studies of photolysis of nitrosyl hemoglobin at low temperature", Int. J. Biol. Macromol. **12**, 59-63 (1990).

[28] D. Doetschman, S. Schwartz, S. Utterback, "The electron spin distribution in nitrosyl hemoglobin", Chem. Phys. J. **49**, 1-8 (1980).

[29] A. Waleh, N. Ho, L. Chantranupong, G.H. Loew, "Electron structure of NO-ferrous heme complexes", J. Am. Chem. Soc. **111**, 2767-2772 (1989).

[30] G. Feher, "Observation of nuclear magnetic resonances via the electron spin resonance line", Phys. Rev. **103**, 834-835 (1956).

[31] G. Feher, R.A. Isaacson, C.P. Scholes, R. Nagel, "Electron-nuclear double resonance investigation on myoglobin and hemoglobin", Ann. N.Y. Acad. Sci. **222**, 86-101 (1973).

[32] M. Höhn, J. Hütterman, J.C.W. Chien, L.C. Dickinson, "^{14}N and ^{1}H ENDOR of nitrosylhemoglobin", J. Am. Chem. Soc. **105**, 109-115 (1983).

[33] R. Kappl, J. Hütterman in "Advanced EPR application in biology and biochemistry", ed. A.J. Hoff, Elsevier (1989).

[34] J. Hütterman, C. Burgard, R. Kappl, "Proton ENDOR from randomly orientated NO-ligated haemoglobin: approaching the structural basis for the R-T transition", J. Chem. Soc. Faraday Trans. **90**, 3077-3087 (1994).