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Investigation of Two Deoxygenated Haemoglobin-Haptoglobin Complexes by Mössbauer Spectroscopy*

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Abstract. Haemoglobin Haptoglobin complexes formed when $[Hp^+]/[Hb] = 1/1$ and $[Hp]/[Hb] = 2/1$ were investigated by ^{57}Fe Mössbauer spectroscopy. Both samples gave a spectrum consisting of a single quadrupole doublet. The temperature dependence of the quadrupole splitting was also identical for both samples. This proves that in both samples the nearest neighbour environment of the iron atom must be the same. A comparison with earlier investigations on myoglobin and haemoglobin indicates that the electronic structure of iron in the HbHp-complexes is similar to that in myoglobin.

Key words: Haemoglobin – Haptoglobin – Mössbauer spectroscopy

Introduction

Haptoglobin (Hp) is a serum protein with the basic property of binding haemoglobin (Hb). This property has given haptoglobin its name. In 1955, Smithies (Smithies, 1955a, 1955b) described three genetic types of haptoglobins called Hp 1-1, Hp 2-1 and Hp 2-2. This subdivision was based on differences in their polypeptide constituants. The hepatic origin of Hp was proposed after 1964 by different authors (Mouray et al., 1964; Krauss and Sarcione, 1964; Alper et al., 1965; Lombard et al., 1968; Kashiwagi et al., 1968; Merril et al., 1964; Neuhaus and Liu, 1964; Peters and Alper, 1966; Cheftel et al., 1960; Cloarec et al., 1963; Hermann-Boussier et al., 1960; Schultze et al., 1963; Malchy and Dixon, 1970; Chiancone et al., 1968; Lavialle et al., 1974; Shim and Bearn, 1964; Black and Dixon, 1968). The localization by immuno-fluorescence of cells which specifically produce the Hp has been demonstrated by Peters and Alper (1966). Until now it is rather difficult to attribute a precise biological function to Hp, but the marked changes in Hp level in haemolytic and inflammatory processes should be related to the functional properties of haptoglobins. However, its capacity of binding Hb attained its main role in the

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pathologically enhanced Hb release in haemolytic states as a defence against renal clearance. The chemical structure of Hp has been extensively studied (Cheftel et al., 1960; Cloarec et al., 1963; Hermann-Boussier et al., 1960; Schultze et al., 1963). About the biochemical approach of the Hp molecule one important work is constituted by the publication of Malchy and Dixon (1970) which described the antibody behaviour of Hp in its binding with Hb. The similarity with an antigen-antibody system is supported by the infinite value of the binding constant (Chiancone et al., 1968) and the value of the thermodynamic quantities (Lavialle et al., 1974) for the Hp-Hb association. The constraints on flexibility of bound antigen by antibody (Crumpton, 1972) are highly similar to the observation on a Hp-Hb system. Some authors (Malchy and Dixon, 1969; Makinen and Kon, 1971; Malchy et al., 1972) described conformational changes of Hb molecule bound to Hp which can be compared to the conformational difference between oxy and deoxy Hb. Kinetic study of the reaction between O_2 and Hb bound to Hp (Chiancone et al., 1973) suggests also that Hp may impose contraints on the conformational flexibility of Hb.

On the other hand it is known that among the normal mammalian Hb, the only form which does not bind Hp is the deoxy Hb (Nagel and Gibson, 1972; Alfsen et al., 1970). But the isolated deoxy α and β chains from human Hb do bind Hp. Furthermore, after binding of Hp to HbO_2, the deoxygenation of the complex does not lead to any dissociation. It is then possible to study a complex of deoxy Hb-Hp in order to compare the structure of the haem iron in the Hp bound – oxy and – deoxy Hb. Experiments on the functional properties of the Hb bound to Hp (Alfsen et al., 1970) have shown that depending of the [Hp]/[Hb] molar ratio, the bound Hb has lost partially or totally its cooperative characteristics.

In one case, when [Hp]/[Hb] = 1, only complexes with two ($\alpha\beta$)-dimers of Hb bound to one molecule of Hp 1-1 (MW = 85 000) are formed.

In the second case – when [Hp]/[Hb] > 1 – two complexes of different stoechiometry named *Cx* and *Cd* exist as reaction products. In *Cx*, two dimers of Hb are bound on one molecule of Hp whereas *Cd* is the result of the association of one dimer of Hb with one molecule of Hp. The properties of Hb bound to Hp in *Cx* and *Cd* are those of isolated Hb chains.

Calorimetric studies (Lavialle et al., 1974), electrophoresis experiments (Lavialle et al., 1976) and solvent and perturbation difference spectra (De Foresta et al., 1975) indicate the existence of different conformational states of native molecules Hp_x and Hp^+.

From these data the Hp-Hb interactions can be schematically described by the following scheme.

$$\text{when } \frac{[Hp]}{[Hb]} = 1: (Hp^+ + Hp_x) + Hb \rightarrow Hp^+\text{-}Hb + Cx \tag{1a}$$

$$\text{when } \frac{[Hp]}{[Hb]} > 1: (Hp^+ + Hp_x) + Hb \rightarrow Cx + Cd + Hp^+ \tag{1b}$$

Hp^+ and Hp_x characterized by their Hb affinity give rise respectively to Hp^+-Hb (dissociable by an addition of Hp) and to *Cx* and *Cd* described as undissociable complexes.

The hydrophobic interactions largely involved in the Hp^+-Hb association for temperatures above 20° C (Lavialle et al., 1974) are hindered at low temperatures; the Hp^+-Hb is probably no more present below 0° C (Lavialle, 1975).

Although the binding between Hp and Hb takes place through the globin part of Hb, there is a change in haem iron properties induced by the Hp-Hb interactions as shown by absorption spectrosopic data. Thus Mössbauer spectroscopy can shed light on such modifications by direct study of the iron electronic environment.

In the present study we measured the temperature dependence of the quadrupole splitting of two deoxygenated Hp-Hb complexes with high accuracy. From these data the low electronic term scheme of the ferrous iron is determined in analogy to previous investigations on myoglobin and human haemoglobin (Eicher and Trautwein, 1969; Eicher and Trautwein, 1970; Eicher et al., 1974; Eicher et al., 1976).

Material and Methods

$Hb^{57}Fe$ was prepared as described in (Parak and Formanek, 1971). Haptoglobin of the genetic type 1-1 was prepared from ascitic fluids obtained from cancer patients by chromatography on D.E.A.E. cellulose (D.E.A.E. 23) as previously described (Waks and Alfsen, 1966).

The fraction of Hp^+ was obtained (cf. reactional scheme in previous paragraph) by mixing Hp 1-1 and horse Hb in a molar ratio Hp/Hb = 3.5. The mixture composed of $Cx + Cd + Hp^+$ — (cf. Eq. 1b) — was layered on a column of D.E.A.E. cellulose equilibrated with an acetate buffer pH 4.7, 0.01 M in acetate. All the steps (washing and elution) were identical to those described for the Hp 1-1 preparation. The complexes formed (*Cx* and *Cd*) are discarded and the remaining free Hp called "Hp^+" is used for experiments. Two types of experiments have been performed: the first one with one sample containing 50 mg of Hp 1-1 and 18 mg of human $Hb^{57}Fe$ (i.e. a molar ratio [Hp]/[Hb] = 2/1) and the second one with one sample containing 12 mg of Hp^+ and 9.1 mg of $Hb^{57}Fe$ (i.e. a molar ratio $[Hp^+]$/[Hb] = 1/1). Before mixing, the Hp and Hb samples were separately dialysed over night against a common phosphate buffer (KH_2PO_4, Na_2HPO_4) 0.1 M, pH = 7.4.

The cylindrical sample container was filled with 0.3 ml protein solution in the case of the ($[Hp^+]$/[Hb] = 1/1)-sample, and with 0.6 ml protein solution in the case of the ([Hp]/[Hb] = 2/1)-sample. The sample containers were then tightly closed by hostaphan windows with an area of 1 cm^2. The samples were frozen in liquid nitrogen immediately after the preparation procedure.

Measurements were performed in a liquid He cryostat at 4.2 K and in a vapour cryostat operated either with liquid He or N_2 in the temperature range between 10 K and 220 K. The temperature control was calibrated with the aid of a Pt calibration resistor yielding an absolute temperature accuracy of ±2 K.

Results

Both samples showed only a single quadrupole doublet with a quadrupole splitting

$$1.9 \leq \tfrac{1}{2}\, e^2qQ \leq 2.5 \text{ mm/s}$$

between 4.2 K and 220 K.

Table 1. Experimental quadrupole splitting data. Total errors including temperature calibration and measurement are smaller then 1%

$[Hp^+]/[Hb] = 1/1$		$[Hp]/[Hb] = 2/1$	
T[K]	$\frac{1}{2} e^2qQ$ [mm/s]	T[K]	$\frac{1}{2} e^2qQ$ [mm/s]
4.2	2.495	4.2	2.506
10.0	2.496	10.0	2.503
20.0	2.483	15.0	2.495
30.0	2.462	19.0	2.483
32.0	2.465	20.0	2.496
36.0	2.458	30.0	2.480
43.0	2.460	39.0	2.466
50.0	2.437	45.0	2.459
55.0	2.450	52.0	2.450
60.0	2.449	60.0	2.442
62.0	2.429	70.0	2.424
63.0	2.440	77.0	2.414
70.0	2.420	85.0	2.386
79.0	2.405	86.0	2.407
82.0	2.418	95.0	2.384
88.0	2.385	99.0	2.380
93.0	2.384	105.0	2.351
99.0	2.370	112.0	2.343
105.0	2.348	115.0	2.317
109.0	2.330	125.0	2.304
119.0	2.304	136.0	2.258
130.0	2.300	140.0	2.260
135.0	2.271	145.0	2.244
140.0	2.270	155.0	2.204
150.0	2.228	160.0	2.166
156.0	2.191	167.0	2.154
159.0	2.200	180.0	2.088
169.0	2.140	185.0	2.080
178.0	2.112	200.0	1.999
186.0	2.085	203.0	2.008
195.0	2.053	220.0	1.933
208.0	2.006	221.0	1.926
		225.0	1.910
		230.0	1.882

The linewidth Γ_{exp} was 0.30 mm/s, and the energy shift relative to metallic iron varied between 0.91 mm/s and 0.84 mm/s for $T = 4.2$ K and $T = 220$ K, respectively. The source was always kept at room temperature, and no correction for the second order Doppler shift was done. Table 1 gives the experimental quadrupole splitting data, and Figure 1 shows two typical Mössbauer spectra. Practically no contamination of the samples with low spin haemochrom [$\frac{1}{2} e^2qQ = 1.35$ mm/s, see for example (Eicher et al., 1976)] is seen. Figures 2 and 3 show the temperature dependence of the quadrupole splitting of $[Hp]/[Hb] = 2/1$ and $[Hp^+]/[Hb] = 1/1$, respectively.

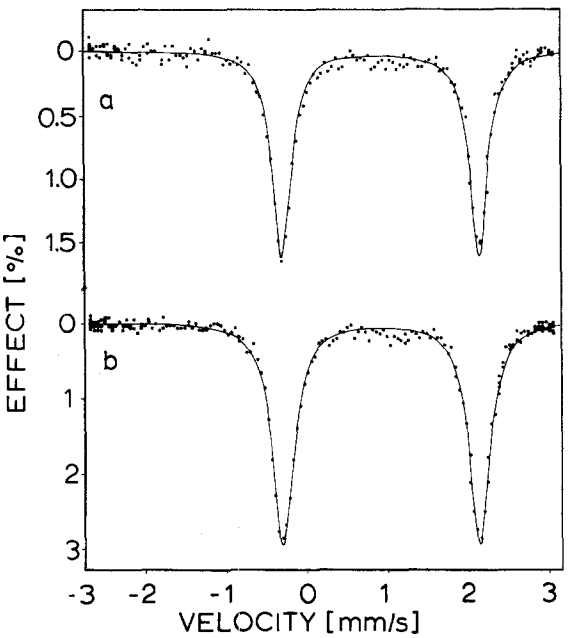

Fig. 1. Mössbauer spectrum of deoxy HpHb. a) $[Hp^+]/[Hb] = 1/1$ at 82 K. b) $[Hp]/[Hb] = 2/1$ at 88 K

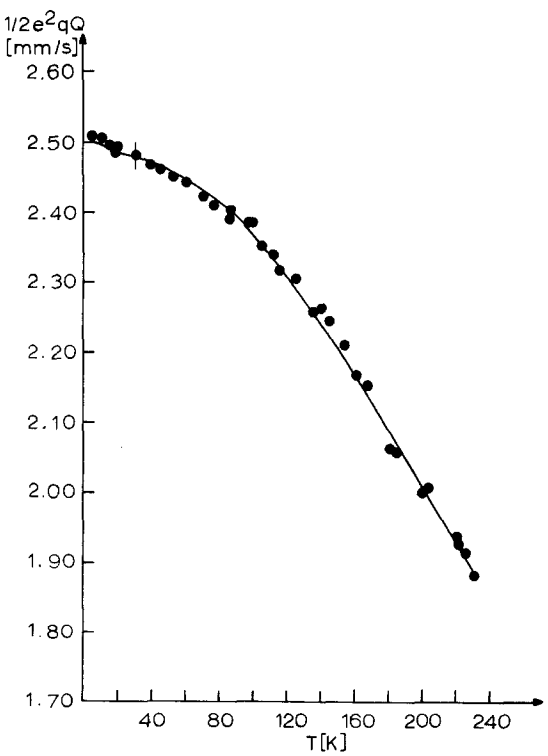

Fig. 2. Temperature dependence of the quadrupole splitting of HpHb 2/1. The solid line is obtained by a least squares fit procedure

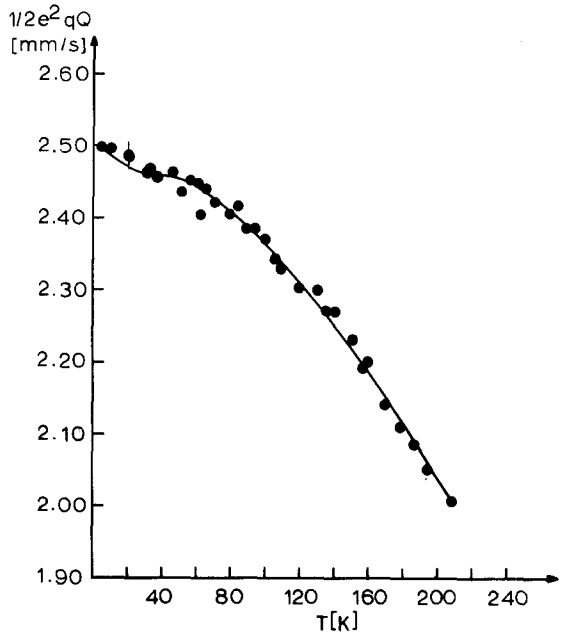

Fig. 3. Temperature dependence of the quadrupole splitting of Hp⁺Hb 1/1. The solid line is obtained by a least squares fit procedure

The determination of the electronic level scheme of the Fe was performed according to the procedure introduced by Eicher et al. (Eicher and Trautwein, 1969; Eicher and Trautwein, 1970; Eicher et al., 1974; Eicher et al., 1976). In this scheme the electronic energy levels of the $3d^6$ configuration are evaluated using a Hamiltonian which contains the Coulomb repulsion of the $3d$ electrons as well as their interaction with the N-ligands of the Fe, arranged in C_{4v} point symmetry. The four lowest levels ($^5B_2$, ^3E, $^1A_1$, ^5E) are used as the basis for a perturbation Hamiltonian taking into account a small rhombic distortion of the C_{4v} point symmetry and the spin orbit coupling which yields 22 levels. Their occupation according to Boltzmann statistics gives rise to the temperature dependence of the electric field gradient. The measured temperature dependence of the quadrupole splitting of the 14.4 keV state of ^{57}Fe is least squares fitted to the theory just described with seven adjustable parameters. These are: E_1, E_2, E_3, D, V_{zz} (lat), α^2 and λ. The energies E_1 to E_3 are defined as:

$$E_1 = E(^5E) - E(^5B_2)$$
$$E_2 = E(^3E) - E(^5B_2)$$
$$E_3 = E(^1A_1) - E(^5B_2)$$

$2D$ is the splitting of the ^5E level due to the rhombic point symmetry. The parameter V_{zz} (lat) takes into account a temperature independent contribution to the electric field gradient, α^2 is the covalency factor and λ the spin orbit coupling constant of Fe^{2+} porphyrin compounds. A detailed discussion of the parameters is given in (Eicher et al., 1976).

Table 2. Results of the least square fitting procedure of the parameters E_1, E_2, E_3, D and V_{zz} (lat) in HpHb 2/1 and Hp^+Hb 1/1 and the corresponding values of ε_1, ε_2, and ε_3. For a comparison, the ε-values of Mb and HbA have been added (taken from Eicher et al., 1976)

	[Hp]/[Hb] = 2/1	$[Hp^+]$/[Hb] = 1/1	Mb	HbA
E_1 (cm^{-1})	− 58 ± 30	− 10 ± 42	121 ± 35	28 ± 50
E_2 (cm^{-1})	966 ± 47	933 ± 106	760 ± 150	1992 ± 550
E_3 (cm^{-1})	− 139 ± 17	− 108 ± 26	+ 61 ± 60	− 198 ± 36
D (cm^{-1})	− 243 ± 13	− 290 ± 25	− 310 ± 33	− 336 ± 32
$\left(\frac{V_{zz}\,(\text{lat})\,(1-\gamma_\infty)}{(1-R)\,e\,\langle r^{-3}\rangle}\right)$	0.035 ± 0.018	0.079 ± 0.02	0.144 ± 0.015	0.0883 ± 0.014
ε_1 (cm^{-1})	− 58 ± 30	− 10 ± 42	121 ± 35	28 ± 50
ε_2 (cm^{-1})	8170 ± 58	8148 ± 117	7907 ± 166	9442 ± 553
ε_3 (cm^{-1})	16868 ± 56	16953 ± 114	17269 ± 154	15881 ± 552

For a comparison of the different electronic level schemes of various Fe^{2+} haemoglobins only the parameters $E_{1\ldots3}$ and D are of interest. To a good approximation the parameters α^2 and λ should have the same values in these compounds since the arrangement of nearest neighbours of the iron is fairly similar. We first produced least squares fits to the measured temperature dependence of the quadrupole interaction in the following deoxygenated samples: Sperm whale myoglobin (Mb) and human haemoglobin (HbA) (Eicher et al., 1976), β_{PMB}-chain of human haemoglobin and Chironomushaemoglobin (to be published elsewhere) and the two haemoglobin haptoglobin complexes described in this paper leaving all seven parameters as free variables. For all substances mentioned best fits were obtained with $\lambda = 69\ cm^{-1}$ and $\alpha^2 = 0.89$. In the next step $\lambda = 69\ cm^{-1}$ and $\alpha^2 = 0.89$ were kept constant and only the remaining five parameters were allowed to vary during the fit procedure. The results of these fits are listed in Table 2 together with the values ε_1, ε_2 and ε_3 which are derived from the energies $E_{1\ldots3}$ according to Equation (2) of (Eicher et al., 1976). ε_1 is the energy of the $3d_{xz}$ and the $3d_{yz}$ single electron level, ε_2 the energy of the $3d_{z^2}$ level and ε_3 the energy of the $3d_{x^2-y^2}$ level to $3d_{xy}$.

Discussion

As mentioned earlier both HpHb samples show essentially only a single quadrupole doublet. The splitting is similar in order of magnitude to that of deoxygenated HbA and Mb samples and thus typical for a high spin state of Fe^{2+} in haemoglobin. A more detailed discussion requires a comparison of the values of ε_1, ε_2 and ε_3 for the two HpHb complexes and for Mb and HbA (see Eicher et al., 1976). From this one deduces the following results:

1. The values of ε_1, ε_2 and ε_3 for the two complexes $[Hp^+]$/[Hb] = 1/1 and [Hp]/[Hb] = 2/1 are identical within the limit of error.

2. The values of ε_1, ε_2 and ε_3 for the HpHb complexes are rather similar to those of Mb but differ significantly from the HbA values.

In (Eicher et al., 1976) the distances of the iron atom from the haem plane and its distance from the N_ϵ-atom of the proximal Histidine F8 was calculated from the values of $\varepsilon_{1\ldots3}$ using a crystal field model. Even without such a detailed calculation, it is possible to deduce some basic conclusions on the geometrical arrangement of the iron from the numbers $\varepsilon_{1\ldots3}$. Since the *d*-orbitals have nonbonding character, the energy of an orbital will rise, if the overlap with its ligands increases. The ε_3 value gives the energy of the $d_{x^2-y^2}$-orbital with respect to the d_{xy}-orbital. In HpHb ε_3 is higher than in HbA which means that the overlap of the $d_{x^2-y^2}$-orbital with the four N-atoms of the haem group is larger and consequently the iron must be closer to the haem plane.

On the other hand, ε_2 gives the energy of the d_{z^2}-orbital with respect to the d_{xy}-orbital. In HpHb the value of ε_2 is significantly smaller than in HbA. Consequently, the overlap of the d_{z^2}-orbital with N_ϵ of His F8 is also smaller and the distance Fe—N_ϵ should be larger.

On the first view it is surprising that we did not observe any differences between the samples $[Hp^+]/[Hb] = 1/1$ and $[Hp]/[Hb] = 2/1$. As mentioned in the introduction the $[Hp^+]/[Hb] = 1/1$ sample should contain the complex Hp^+-Hb in which Hb has at least partially maintained its cooperativity. The Fe atoms in that complex should therefore have properties more similar to HbA than in Mb or isolated single chains. One would expect therefore a second quadrupole doublet in the Mössbauer spectrum of the $[Hp^+]/[Hb] = 1/1$ sample, however, in Figure 1 no indication for the presence of a second species is seen. We have to conclude therefore that the environment of Fe in Hp^+-Hb is very similar to *Cx* and *Cd*. A less probable explanation would be that our freezing procedure was not fast enough, giving the species Hp^+-Hb time to decay, so that it was not present in our sample. No differences in the Mössbauer spectra of *Cx* and *Cd* could be detected either.

As discussed in (Bade and Parak, 1976) level scheme differences of the haem iron in HbA and Mb can be correlated with different structural states of the whole molecule. From the similarity of the values of ε_2 and ε_3 between the HbHp-complexes and Mb one may conclude that deoxy haemoglobin bound to haptoglobin is in the relaxed high affinity state. In view of the energy change associated with the allosterical transition of Hb the evaluation of the energy terms of Fe in HbA and HbHp-complexes allows us to make an estimate on the contribution to this energy change coming from the iron atom itself. Our data indicate that the energy of the non-bonding *d*-orbitals is 1.2 kcal/mole Fe larger in HbA than in Hp-Hb. Of course, also the bonding orbitals may change their energy during the allosterical transition. However, we are not able to make a prediction for that contribution. Nevertheless, the indicated value may be compared with other contributions. From calorimetric measurements one may determine the energy of O_2 binding to haemoglobin. Such a measurement takes into account the heat of oxygen binding to the haem sites, the energy of the salt bridges, the rearrangement within the chains and the influence of the Bohr protons yielding a total of −14.2 kcal/mole Fe (Atha and Ackers, 1974). According to Perutz (1970) the energy difference due to disrupture of salt bridges during the oxigenation of Hb is about 2.5–3 kcal/mole Fe. In (Arata and Otsuka, 1975) the energy change between deoxy Hb and oxy Hb due to the conformational difference is estimated to be about 8 kcal/mole Fe.

References

Alfsen, A., Chiancone, E., Antonini, E., Waks, M., Wyman, J.: Studies on the reaction of haptoglobin with hemoglobin and hemoglobin chains. III. Observations on the kinetics of the reaction of the haptoglobin-hemoglobin complexes with carbon monoxide. Biochim. Biophys. Acta **207,** 395-403 (1970)

Alper, C. A., Peters, J. H., Birtch, A. G., Gardner, F. H.: Haptoglobin synthesis I. In vivo studies of the production of haptoglobin, fibrinogen, and γ-globulin by the canine liver. J. Clin. Invest. **44,** 574–581 (1965)

Arata, Y., Otsuka, J.: Calculation of the energy difference between the quarterny structures of deoxy and oxyhemoglobin, Biochim. Biophys. Acta **405,** 388–403 (1975)

Atha, D. H., Ackers, G. K.: Calorimetric determination of the heat of oxygenation of the human hemoglobin as a function of p_H and the extent of reaction. Biochemistry **13,** 2376–2381 (1974)

Bade, D., Parak, F.: Investigation of the electronic term scheme of deoxygenated human hemoglobin by a least squares fit procedure using simultaneously magnetic suszeptibility and Mößbauer data. Biophys. Struct. Mechanism. **2,** 219–231 (1976)

Black, J. A., Dixon, G. H.: Amino acid sequence of alpha chains of human haptoglobins. Nature (Lond.) **218,** 736–741 (1968)

Cheftel, R. I., Cloarec, L., Moretti, J., Rafelson, M., Jayle, M. F.: Etude de l'haptoglobine III. Composition chimique de la partie glucidique. Bull. Soc. Chim. Biol. **42,** 993–1004 (1960)

Chiancone, E., Alfsen, A., Ioppolo, C., Vecchini, P., Finanzzi-Agro, A., Wyman, J., Antonini, E.: Studies on the reaction of haptoglobin with haemoglobin and haemoglobin chains. 1. Stoichiometry and affinity. J. Mol. Biol. **34,** 347–356 (1968)

Chiancone, E., Antonini, E., Brunori, M., Alfsen, A., Lavialle, F.: Kinetics of the reaction between oxygen and haemoglobin bound to haptoglobin. Biochem. J. **133,** 205–207 (1973)

Cloarec, L., Moretti, J., Rafelson, M.: Composition chimique de l'haptoglobine humaine. C.R. Acad. Sci. (Paris) **257,** 983–985 (1963)

Crumpton, M. J.: Conformational changes in protein antigens induced by specific antibodies: sperm whale myoglobin. In: Protein-Protein Interactions (ed. R. Jaenicke, E. Helmreich), pp. 395–408. Berlin-Heidelberg-New York: Springer 1972

Eicher, H., Trautwein, A.: Electronic structure and quadrupole splittings of ferrous iron in haemoglobin. J. Chem. Phys. **50,** 2540–2551 (1969)

Eicher, H., Trautwein, A.: Electronic structure of ferrous iron in haemoglobin inferred from Mössbauer measurements. J. Chem. Phys. **52,** 932–934 (1970)

Eicher, H., Parak, F., Bade, D., Tejada, J.: Electronic structure of the iron in deoxygenated myoglobin from Mössbauer spectroscopy. J. Physique (Paris) **35,** C6, 363–366 (1974)

Eicher, H., Bade, D., Parak, F.: Theoretical determination of the electronic structure and the spatial arrangement of ferrous iron in deoxygenated sperm whale myoglobin and human haemoglobin from Mössbauer experiments. J. Chem. Phys. **64,** 1446–1455 (1976)

De Foresta, E., Lavialle, F., Alfsen, A.: Thermal and solvent perturbation as probes of conformational heterogeneity of haptoglobin 1-1. Europ. J. Biochem. **60,** 35–39 (1975)

Hermann-Boussier, G., Moretti, J., Jayle, M. F.: Etude de l'haptoglobine II. Propriétés physiques et chimiques des haptoglobines des types I et II et de leurs complexes avec l'hemoglobine. Bull. Soc. Chim. Biol. **42,** 837–852 (1960)

Kashiwagi, N., Groth, C. G., Starzl, E.: Changes in serum haptoglobin and group specific components after orthotopic liver homotransplantation in humans. Proc. Soc. Exp. Biol. **128,** 247–250 (1968)

Krauss, S., Sarcione, E. I.: Synthesis of serum haptoglobin by the isolated perfused rat liver. Biochim. Biophys. Acta **90,** 301–308 (1964)

Lavialle, F., Rogard, M., Alfsen, A.: Calorimetric determination of the enthalpy and heat capacity for the association of haptoglobin with hemoglobin. I. Demonstration of two interacting systems. Biochemistry **13,** 2231–2234 (1974)

Lavialle, F.: Des interactions entre l'haptoglobine et l'hemoglobine – ou comment se découvrent les potentialités d'une molécule. Thèse de doctorat d'état, Paris, Juillet 1975

Lavialle, F., Rogard, M., Alfsen, A.: Haptoglobin-haemoglobin interaction. Heterogeneity of the haptoglobin 1-1 molecule in its binding affinity. Europ. J. Biochem. **64,** 287–293 (1976)

Lombard, C., Nebut, M., Ollier, M. P., Jayle, M. F., Hartmann, L.: Synthese hépathique de l'haptoglobine. Rev. Franç. Etud. Clin. Biol. **13,** 258–265 (1968)

Makinen, M. W., Kon, H.: Circular dichroism and electron paramagnetic resonance of the haptoglobine-hemoglobin complex. Biochemistry **10,** 43–52 (1971)

Malchy, B., Dixon, G. H.: The influence of haptoglobin on the reactivity of the SH-groups of hemoglobin. Canad. J. Biochem. **47,** 1205–1208 (1969)

Malchy, B., Dixon, G. H.: The resemblance of the combination of human haptoglobin and double hemoglobin molecules to an antigen-antibody reaction. Canad. J. Biochem. **48,** 192–197 (1970)

Malchy, B., Dugas, H., Ofosu, F., Smith, I. P. C.: A spin lable study of hemoglobin-haptoglobin complexes. Biochemistry **11,** 1669–1672 (1972)

Merril, D. A., Kirkpatrick, C. M., Wilson, W. E. C., Riley, C. M.: Change in serum haptoglobine type following human liver transplantation. Proc. Soc. Exp. Biol. **116,** 748–751 (1964)

Mouray, H., Moretti, J., Jayle, M. F.: Le rôle du foie dans la métabolisme de l'haptoglobine. C.R. Acad. Sci. (Paris) **258,** 5095–5098 (1964)

Nagel, R. L., Gibson, Q. H.: The hemoglobin-haptoglobin reaction as a probe of hemoglobin conformation. Biochem. Biophys. Res. Commun. **48,** 959–966 (1972)

Neuhaus, O. W., Liu, A.: Biochemical significance of serum glycoproteins. III. Hepatic production of $\alpha 1$ and $\alpha 2$ globulins responding to injury. Proc. Soc. Exp. Biol. **117,** 244–248 (1964)

Parak, F., Formanek, H.: Untersuchung des Schwingungsanteils und des Kristallgitterfehleranteils des Temperaturfaktors in Myoglobin durch Vergleich von Mössbauerabsorptionsmessungen mit Röntgenstrukturdaten. Acta Cryst. **A27,** 573–578 (1971)

Perutz, M. F.: Stereochemistry and cooperative effects in haemoglobin. Nature (Lond.) **228,** 726–739 (1970)

Peters, J. H., Alper, C. A.: Haptoglobin synthesis. II. Cellular localisation studies. J. Clin. Invest. **45,** 314–320 (1966)

Schultze, H. E., Haupt, H., Heyde, K., Heimburger, N.: Über die Chemie des Haptoglobin-Polymorphismus. Clin. Chim. Acta **8,** 207–214 (1963)

Shim, B. S., Bearn, A. G.: Immunological and biochemical studies on serum haptoglobin. J. Exp. Med. **120,** 611–628 (1964)

Smithies, O.: Grouped variations in the occurrence of new protein components in normal human serum. Nature (Lond.) **175,** 307–308 (1955a)

Smithies, O.: Zone electrophoresis in starch gels: Group variations in the serum proteins of normal human adults. Biochem. J. **61,** 629–641 (1955b)

Waks, M., Alfsen, A.: Structural studies of haptoglobins. I. Hydrogen ion equilibria and optical rotatory dispersion of Hp 1-1 and Hp 2-2. Arch. Biochem. Biophys. **113,** 304–314 (1966)

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