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Magnetic Circular Dichroism Studies of Myoglobin, Hemoglobin and Peroxidase at Room and Low Temperatures. Ferrous High Spin Derivatives

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Abstract. The magnetic circular dichroism spectra (MCD) recorded for the visible and near-UV regions of high-spin ferrous derivatives of myoglobin, hemoglobin, hemoglobin dimers and isolated chains as well as of horseradish peroxidase at pH 6.8 and 11.4 have been compared at the room and liquid nitrogen temperatures. The MCD of the Q_{00}- and Q_v-bands have been shown to be sensitive to structural differences in the heme environment of these hemoproteins. The room temperature visible MCD of native hemoglobin differs from that of myoglobin, hemoglobin dimers and isolated chains as well as from that of model pentacoordinated complex. The MCD of hemoglobin is characterized by the greater value of the MCD intensity ratio of derivative shape A-term in the Q_{00}-band to the A-term in the Q_v-band. The evidences are presented for the existence of two pH-dependent forms of ferroperoxidase, the neutral peroxidase shows the "hemoglobin-like" MCD, while the alkaline ferroperoxidase is characterized by the "myoglobin-like" MCD spectrum in the visible region. The differences in the MCD of deoxyhemoglobin and neutral ferroperoxidase as compared with other high-spin ferrous hemoproteins are considered to result from the constraints on heme group imposed by quaternary and/or tertiary protein structure. The differences between hemoproteins which are seen at the room temperature become more pronounced at liquid nitrogen temperature. Except the peak at $\sim$ 580 nm in the MCD of deoxymyoglobin and reduced peroxidase at pH 11.4 the visible MCD does not show appreciable temperature dependent C-terms. The nature of the temperature dependent effect at $\sim$ 580 nm is not clear. The Soret MCD of all hemoproteins studied are similar and are predominantly composed of the derivative-shaped C-terms as revealed by the increase of the MCD peaks approximately in accordance with Boltzmann distribution. The interpretation of temperature-dependent MCD observed for the Soret band has been made in terms of porphyrin to Fe-ion charge-transfer electronic transition which may be assigned as $b(\pi) \rightarrow 3d$. This charge-transfer band is strongly overlapped with usual $B(\pi - \pi^*)$ band resulting in diffuse Soret band. Adopting that only two normal vibrations are sinphase with charge-transfer transition the extracted C-terms of the Soret MCD have been fitted by theoretical dispersion curves.

Key words: Hemoproteins – Magnetic circular dichroism – Heme environment – Transition assignment – Least squares fit.

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To answer the question why hemoproteins having the same active site (heme group) perform quite different functions one must know the details of heme coordinating and electronic states and their variations under different perturbations. Being determined by the nature of the ground and excited states of a molecule magnetic optical activity was expected to be a useful method in obtaining the parameters of heme electronic states. The last decade is characterized by the extensive studies of magnetooptical rotatory dispersion (MORD) and magnetic circular dichroism (MCD) of hemoproteins and their model compounds in solution as well as in multicomponent hemoprotein systems: submitochondrial and microsomal particles and chloroplasts. The data obtained show that MORD and MCD are very promising methods both in spectroscopical and analytical aspects (see review by Sharonov, 1976). Unlike the *A*-type and *B*-type effects *C*-type effects in MCD are temperature dependent (Buckingham and Stephens, 1966; Schatz and McCaffery, 1969; Stephens, 1974), that is why the low temperature MCD measurements are very useful in the interpretation of the nature of electronic transitions.

Most of the MCD data so far reported has been presented as indicative of high sensitivity of MCD method to axial ligation and oxidation and spin states of heme iron in heme proteins. In this paper to obtain more structural and spectroscopical information on the heme electronic state and on the influence of protein environment on this state we compare the MCD of ferrous forms of myoglobin, hemoglobin tetramers, dimers and isolated chains and peroxidase both at the room and liquid nitrogen temperature in the visible and near UV regions. In all these hemoproteins the protoheme group has been found to be in pentacoordinated high-spin ferrous state, therefore any difference in MCD is due to the protein parts of molecules. The observed differences have been ascribed to the constraints on heme group imposed by quaternary and/or tertiary protein structure.

The evidences are presented for the existence of two different pH-dependent structural forms of ferroperoxidase.

We also present here the theoretical treatment which shows that the similar temperature dependent *C*-type MCD Soret spectra of hemoproteins studied can be associated with the charge transfer transition from porphyrin $b(\pi)$ to iron d_π-orbitals. The parameters of the transitions and of spin-orbit coupling are estimated.

Experimental

Materials

Whale myoglobin (Calbiochem, A grade) and horseradish peroxidase (Worthington, R. Z. = 3.0) were used without further purification. Human and pig hemoglobins were prepared by the method of Geraci and Li (1969). Isolated α^{SH} and β^{SH} chains of human hemoglobin were prepared by Dr. K. Jinoria using the procedure of Geraci et al. (1969) and Waks et al. (1973). Nativity and homogeneity of chains were characterized as described in the paper of Abaturov et al. (1976). Dimers were obtained by pH increasing of deoxyhemoglobin solution up to 11.5. Deoxydimers of porcine hemoglobin being more stable than those of human hemoglobin (Sharonov and Sharonova, 1972) the former was used for MCD studies on dimers. The pH

dependences of tetramer-dimer dissociation constants are the same for porcine and human hemoglobins (Sharonov and Sharonova, 1972) and at pH 11.5 the fraction of dimers is about 0.9 at the used hemoglobin concentration (Sharonova et al., 1972).

All solutions were prepared in a mixture of pure glycerol and 0.2 M buffer (1 : 1 v/v). The buffers used were: sodium phosphate at pH 6.8 and phosphate/glycine at alkaline pH. The control test has shown that glycerol has no effect on the spectral characteristics of hemoproteins. Ferroforms of proteins were prepared by adding a small excess of sodium dithionite (Merck).

Concentrations of the proteins were determined according to the published extinction coefficients (Antonini and Brunori, 1971; Blumberg et al., 1968).

Methods

MCD was measured with "Roussel-Jouan, A" dichrograph equipped with an electromagnet which had a 30-mm pole gap and produced a magnetic field of 13.5 kG. To exclude the contribution of natural CD the measurements were carried out at the two opposite directions of the magnetic field. The signs of the MCD effects correspond to the direction of the magnetic field when it coincides to the direction of the propagation of light. The magnitude of the magnetic field was determined by the method of Dratz (1966). In this method the observed MCD of a suitable sample is mathematically transformed to MORD curve using the Kronig-Kramers relations and the calculated MORD is compared to the measured MORD. For calibration we used the solution of ferrocytochrome *c*. The MORD was recorded by use of magnetospectropolarimeter (Sharonov, 1968) equipped with the limb to read out the angle of rotation with the accuracy up to $5 \cdot 10^{-4}$ degree. The average field strength of the MORD magnet was determined by measuring of the magnetooptical rotation of water. Such calibration involves automatically the correction for possible inaccuracy in the CD instrument calibration (Dratz, 1966).

The low temperature MCD measurements were carried out by using a specially constructed cryostat immediately after the room temperature measurements using the same solution in the same 1.5-mm cell.

The absorption spectra were obtained by use of a Specord UV-VIS (G.D.R.). For measurements of absorption spectra at low temperatures a sample tube from ESP-spectrometer filled with a solution was placed in a quartz Dewar which was put to the compartment of the spectrophotometer for the light scattering samples. To compensate a scattering, the tube filled with latex suspension was placed in the reference beam. The magnitude of absorption is expressed as molar extinction coefficient ε in $(M \cdot cm)^{-1}$ on heme basis.

The MCD results are expressed as $\Delta\varepsilon_M$ in $(M \cdot cm \cdot T)^{-1}$, where 1 T = 10^4 Gauss.

Experimental Data Processing

The experimental MCD and absorption spectra were digitized (100—150 points per 100 nm) in the HP-9830 calculator and after appropriate transformation were output to the plotter in a scale linear either by energy (kK) or wavelength (nm).

The MCD curve-fitting was carried out on the Nova-2 computer in two steps. First the theoretical curves parameters were varied manually by rotating potentiometers connected with the computer via analog-to-digital convertor. It was possible to vary up to six parameters simultaneously. The fitting process was visualized on the graphic display where experimental and summary theoretical curves were drawn and the component varying at the moment as well. All the curves were calculated and output at the same frequences taken in unequal intervals, the points were chosen mostly in the characteristic parts of the spectrum. The fitting could be controlled by the magnitude of the least-square error also drawn on the screen. The error was defined as $F = [\sum (f_e - f_t)^2 / \sum f_e^2]^{1/2}$ where f_e and f_t are the meanings of experimental and theoretical curves respectively at the chosen frequences. On the second stage the set of parameters obtained manually served as the initial point for the minimization procedure (a somewhat modified method of Rosenbrock, 1960). The process of minimization was visualized on the display in the same way.

Results and Discussion

Visible Region

Room Temperature. MCD and absorption spectra at the room and low temperatures of high spin ferrous derivatives of myoglobin[1], hemoglobin tetramers, dimers and chains, peroxidase at pH 6.8 and 11.4 are shown in Figures 1–4. The room temperature visible absorption spectra of all these hemoproteins are very similar, the shortwavelength shoulder is better resolved in myoglobin spectrum (Fig. 1b) while the longwavelength one at 590 nm is more pronounced in peroxidase spectrum (Fig. 2a).

The visible MCD at room temperature shows much more details than the absorption spectra and there are some characteristic features which can be correlated with the peculiarities of heme state. Taking into account that heme iron in all hemoproteins is assigned to be pentacoordinated and is in the high spin state, the observed differences in their MCD reflect the influence of protein environment on the heme group.

The largest effect in the MCD is observed in the Q_{00} or α-band near 590 nm. This band is very weak in absorption and is masked by more intense Q_v- or β-band centered at 555–560 nm. In the Q_{00}-band all hemoproteins studied show a derivative shape A-type effect of normal sign, the crossover coinciding with the absorption band. The shortwavelength lobe of the A-term associated with pure electronic transi-

[1] The positions and the relative intensities of peaks observed in the room temperature MCD of deoxymyoglobin (Fig. 1b) are in agreement with the previous data (Bolard and Garnier, 1966; Vickery et al., 1976a). However, the absolute intensities of the MCD effects coincide with those reported by Bolard and Garnier but are 1.5 times as great as those reported by Vickery et al. All extrema in our MCD spectrum of ferroperoxidase are blue shifted by 2–3 nm and their intensities are 1.5 times as great as those reported by Nozawa et al. (1976). We have recorded the MCD of myoglobin and peroxidase during the same date. Therefore, the coincidence of the peak positions in our MCD spectrum of myoglobin with those reported by other authors (Bolard and Garnier, 1966; Vickery et al., 1976a) excludes any possible error in wavelength calibration of our dichrograph

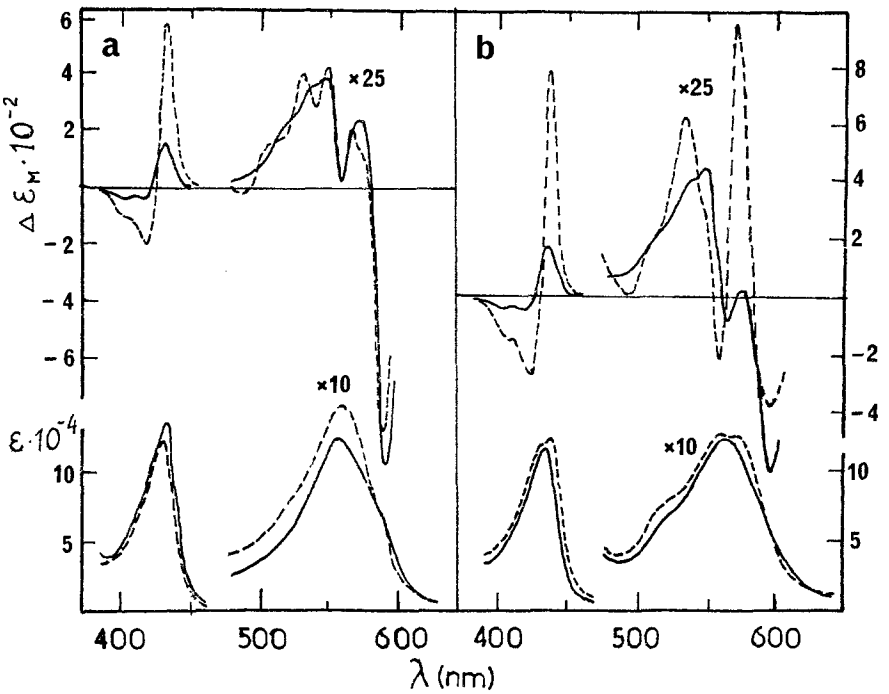

Fig. 1. Magnetic circular dichroism and absorption spectra of deoxyhemoglobin and deoxymyoglobin at the room (——) and liquid nitrogen (– – –) temperatures. (**a**) Deoxyhemoglobin. Protein concentration $7.2 \cdot 10^{-5}$ M in near-UV and $7.2 \cdot 10^{-4}$ M in the visible regions. (**b**) Deoxymyoglobin. Protein concentration $5.4 \cdot 10^{-5}$ M in near-UV and $5.4 \cdot 10^{-4}$ M in the visible regions. In an equal volume mixture of glycerol and 0.2 M sodium phosphate buffer at pH 6.8; pathlength = 1.5 mm; field = 1.35 T

tion is disturbed by overlapping with the effects arising from Q_v absorption bands and possibly from other transitions occured in this region.

In the 500–560 nm region the overall MCD curve is due to the contribution of MCD signals of opposite sign from a number of superimposed vibronic Q_v-bands. As it follows from theoretical arguments the modes of symmetry representation a_g exhibit MCD A-terms of the same sign as the electronic state alone, while the b_g modes are characterized by the A-terms reversed in sign from the pure electronic state. One of these vibronic A-terms of normal sign is resolved in the Q_v-band region, being coincided with the maximum of the overall absorption band.

The hemoproteins show different ratio of peak-to-peak intensity of A-term for Q_{00}-transition to the peak-to-peak intensity of derivative MCD feature centered at 555–560 nm in the Q_v-bands region. This ratio given in parenthesis changes in the order myoglobin (1.3) < dimers (1.7) ≈ pH 11.4-peroxidase (1.7) ≈ β-chains (1.8) ≈ α-chains (1.9) < pH 6.8 peroxidase (2.5) < hemoglobin (3.1). By this criterion the proteins may be divided into two groups, the proteins exhibiting the ratio between 1.4 and 1.8 and ones characterizing by the ratio > 2 (hemoglobin and pH 6.8 peroxidase). Pentacoordinated complex of free deuteroheme with 2-methylimidazole (Fig. 6) which can be considered as an active site model of high-spin ferrous hemoproteins has to be included into the first group (the ratio is equal to 1.4).

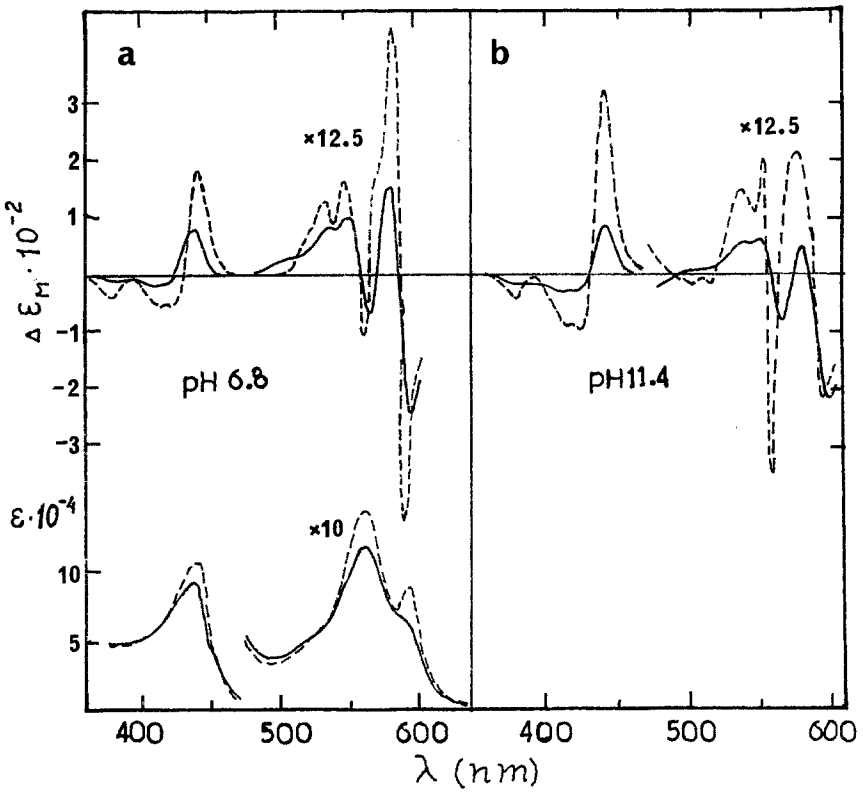

Fig. 2. Magnetic circular dichroism and absorption spectra of reduced horseradish peroxidase at the room (——) and liquid nitrogen (– – –) temperatures. (**a**) Protein concentration $1.45 \cdot 10^{-4}$ M; in an equal volume mixture of glycerol and 0.2 M sodium phosphate buffer at pH 6.8. (**b**) Protein concentration $1.45 \cdot 10^{-4}$ M; in an equal volume mixture of glycerol and 0.2 M sodium phosphate-glycine buffer at pH 11.4. Pathlength = 1.5 mm; field = 1.35 T

The above difference in the ratio of the MCD intensities correlates with the previously observed difference in the ratio of two corresponding minima in magnetic optical rotatory dispersion curve not only between deoxyhemoglobin tetramers and isolated subunits but also between native deoxyhemoglobin in "tensed" or *T*-state (in terms of Monod et al., 1965) with low ligand affinity and chemically modified or mutant deoxyhemoglobins in "relaxed" or *R*-state with high ligand affinity (Sharonov et al., 1976). The sensitivity of the visible rotatory dispersion to the heme electronic state has been used to identify the quaternary structure of mutant and modified hemoglobins in solution (Sharonov et al., 1976), to study tetramers dissociation into dimers (Sharonova et al., 1972) and to investigate the allosteric transition between the *T*- and *R*-structures in carp hemoglobin (Sharonov and Sharonova, 1976). The magnetic optical rotatory dispersion effects exhibited by deoxytetramers in the *R*-state as well as those exhibited by its isolated chains and myoglobin resembles also the effects revealed by the pentacoordinated complex of free heme (Sharonov et al., 1976).

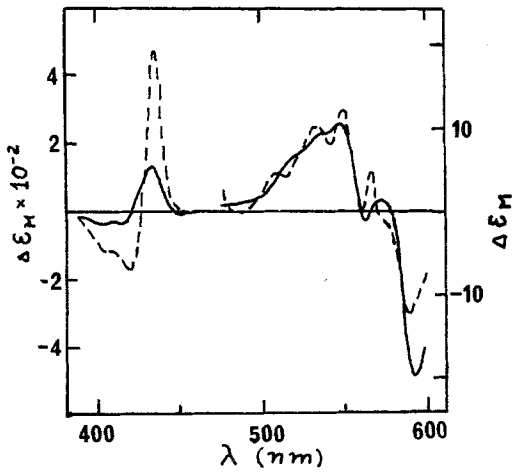

Fig. 3. Magnetic circular dichroism of deoxyhemoglobin dimers at the room (——) and liquid nitrogen (– – –) temperatures. Protein concentration $7.2 \cdot 10^{-5}$ M in near-UV and $7.2 \cdot 10^{-4}$ M in the visible regions; in an equal volume mixture of glycerol and 0.2 M sodium phosphate-glycine buffer at pH 11.5; pathlength = 1.5 mm; field = 1.35 T

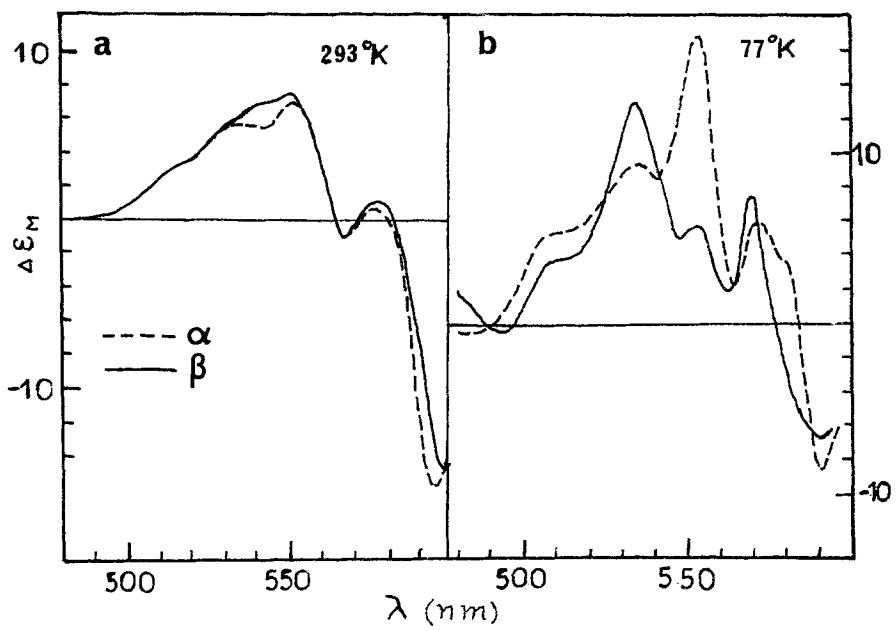

Fig. 4. Magnetic circular dichroism of isolated α (– – –) and β (——) chains. (**a**) Room temperature. (**b**) Liquid nitrogen temperature. In an equal volume mixture of glycerol and 0.1 M phosphate buffer at pH 7.0 (α-chains) and pH 7.5 (β-chains). Pathlength = 1.5 mm; field = 1.35 T

The visible room temperature MCD spectra of isolated α- and β-chains are nearly identical (Fig. 4a). The half-sum of the MCD effects of α- and β-subunits coincides practically with MCD of $\alpha\beta$-dimers (Fig. 3) but differ from that of deoxytetramers (Fig. 1a). Furthermore the MCD spectrum exhibited by equimolar mixture of subunits is identical to that of native deoxytetramers. Therefore the increased

ratio of MCD A-type effects for Q_{00}- and Q_v-bands in deoxyhemoglobin may be considered as an indicator of constraints on heme group imposed by protein-heme interaction which are induced by the native hemoglobin quaternary structure.

Comparison of the visible MCD effects of peroxidase at pH 6.8 (Fig. 2a) with those of other hemoproteins and model compound shows that at neutral pH heme electronic state in ferroperoxidase is also tensed as it is seen from relatively high value of A-terms ratio. Unlike the deoxyhemoglobin where the heme constraints are induced by protein quaternary structure the tensed heme state in peroxidase must be due to its tertiary structure because of the lack of quaternary structure in this protein.

Heme constraints arised in peroxidase from the protein folding are pH-dependent. On increasing of pH the characteristic ratio of the MCD effects decreases in 1.5 times whild the positive peak at 580 nm shows about three-fold decrease (Fig. 2b). At pH 11.4 MCD of ferroperoxidase are characterized by the ratio equal to 1.7 which is specific for the relaxed heme state. The further evidence that the two forms of ferroperoxidase exist at neutral and alkaline pH follows from the low temperature visible and Soret measurements given below.

Low Temperature

The visible absorption spectra show only slight temperature effects (Figs. 1–4). The shortwavelength shoulders in liquid nitrogen temperature absorption spectra become more evident in all hemoproteins, the longwavelength shoulder in peroxidase spectrum resolves into separate maxima (Fig. 2a). At 80 K myoglobin shows nearly resolved additional maximum at 570 nm which is associated with the temperature dependent effect in the MCD (Fig. 1b).

The low temperature measurements permit further differentiation between the electronic states in high-spin ferrous hemoproteins via MCD spectra and to reveal the differences which are not seen at room temperature. At liquid nitrogen temperature the overall positive MCD effect in the Q_v-region becomes well resolved into three separate maxima at $\sim$ 505, $\sim$ 530 and $\sim$ 550 nm. In this region the significant MCD differences between α- and β-chains arise (Fig. 4b) in the contrast to the similarity of their MCD spectra at room temperature (Fig. 4a). The positive MCD peak at 552 nm of α-chains is about three times as great as that of β-chains, while the intensity of 534 nm peak of α-chains is smaller than that of β-chains. The MCD intensities distribution in the Q_v-bands region of β-chains resembles that of myoglobin. The half-sum of the low temperature visible MCD of α- and β-chains is very similar to the MCD spectra of hemoglobin and its dimers (Figs. 1b, 3). Thus at low temperature one can see the difference in the intensities distribution of vibronic MCD components which is undoubtedly due to the influence of protein environment.

We have not observed so drastic changes in the MCD of isolated chains on freezing the sample as were described by Treu and Hopfield (1975). We believe that the chains we dealt with were native even after freezing because the half-sum of their MCD effects repeats the shape of the MCD of hemoglobin reconstructed from these chains, the latter in turn coincides with the curve for native hemoglobin. Moreover

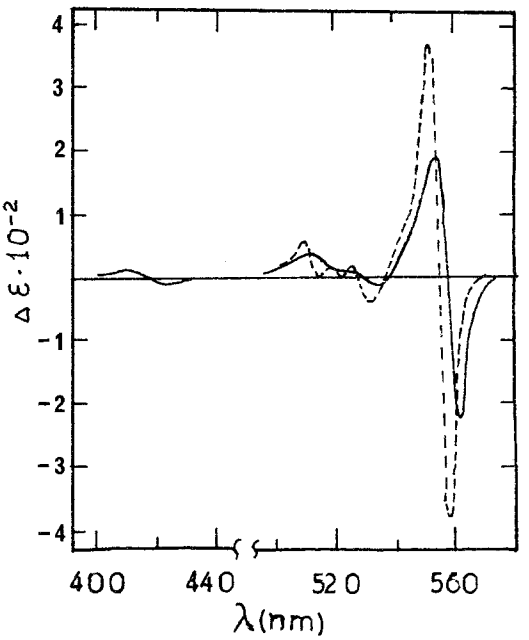

Fig. 5. Magnetic circular dichroism of hemoglobin after alkaline denaturation at the room (——) and liquid nitrogen (– – –) temperatures. Protein concentration $4 \cdot 10^{-5}$ M in near-UV and $4 \cdot 10^{-4}$ M in the visible regions; in an equal volume mixture of glycerol and 1 M NaOH; pathlength = 1.5 mm; field = 1.35 T

the temperature induced changes in MCD of α and β isolated subunits and hemoglobin are completely reversible.

The temperature effects observed by Treu and Hopfield can be explained by the appearance of hemochrome rather than by intrinsic features of high-affinity subunits. In Figure 5 the MCD of alkali denatured deoxyhemoglobin at room and liquid nitrogen temperatures is shown. The MCD is characterized by very intense A-term for the Q_{00}-band usual for the low spin ferrous derivatives of heme (Atanasov et al., 1967; Sutherland and Klein, 1972; Sharonov and Lampe, 1976) and by very weak effect in the B or Soret band region. When the temperature is lowered the A-term at 555 nm is shifted to 552 nm and its intensity is increased by a factor of 1.8. Such an increase was shown also for ferrocytochrome c (Vickery et al., 1976b) and is certainly due to a band narrowing. The effect observed by Treu and Hopfield at low temperature is very similar to that of denatured hemoglobin and can be assigned to the presence of hemochrome since the isolated hemoglobin chains are known to be very unstable.

It is interesting that the visible region MCD of denatured hemoglobin exactly follows the MCD of reduced cytochrome b_5 (Dolinger et al., 1974; Vickery et al., 1975). As evidenced by X-ray studies (Mathews et al., 1971) the heme iron in oxidized cytochrome b_5 is axially coordinated to two imidazole side chains from histidine residues. Since in denatured hemoglobin the sixth iron ligand is thought to be distal histidine the similarity of the visible MCD of denatured deoxyhemoglobin and of reduced cytochrome b_5 suggests that in the latter the axial heme sites are also occupied by two histidines. The observed differences in the Soret region MCD of two proteins may reflect the presence of a very small amount of a high spin form in reduced cytochrome b_5. Indeed our alkali titration of deoxyhemoglobin has shown that when a small amount of denatured hemoglobin occurs the Soret region MCD

resembles the reported MCD (Dolinger et al., 1974; Vickery et al., 1975) of reduced cytochrome b_5.

The most striking low temperature observation is the strong temperature dependence of the MCD of deoxymyoglobin in the region 560–580 nm which was first demonstrated by Vickery et al. (1976a). The small positive peak in the room temperature MCD of myoglobin shifts to 570 nm and grows about 30-fold in intensity on lowering the temperature up to 77 K (Fig. 3b). The temperature dependent effects in this region is absent in the MCD spectra of deoxyhemoglobin (Fig. 1a), its dimers (Fig. 3) and isolated subunits (Fig. 4) but it exists in the MCD of ferroperoxidase at pH 11.4 (Fig. 2b). The effect in the MCD of deoxymyoglobin and alkaline peroxidase can not be explained by narrowing of A-term for Q_{00}-band since on lowering the temperature no appreciable changes occur in the intensity of the longwavelength negative lobe of the A-type effect of peroxidase while in myoglobin this intensity even decreases. In addition the peak-to-peak distances for the derivative shape MCD effects under Q_{00}-band remain invariant upon freezing. The effect at $\sim$ 570 nm for deoxymyoglobin varies almost linearly with temperature over the rang of 26° to −158° C (Vickery et al., 1976). Such behavior is typical for the C-type MCD effects. However the corresponding temperature effect occurs also in the absorption spectrum of deoxymyoglobin (Fig. 1b). At 80 K this hemoprotein shows additional maximum at 570 nm not seen at the room temperature. The nature of the temperature dependent 570-nm MCD effects of myoglobin and peroxidase at pH 11.4 is still uncertain. Whatever its origin it may not be account for C-type effects associated with the porphyrin $\pi - \pi^*$ transition since this effect is absent in the MCD of hemoglobin as well as in its dimers and isolated chains.

The low temperature MCD spectra of peroxidase at pH 6.8 and 11.4 give further evidence for the existence of two protein forms at neutral and alkaline pH (Fig. 2). On lowering the temperature the significant increase of the A-type effect at 585 nm is observed in the MCD of peroxidase at pH 6.8. This two-fold increase is undoubtedly due to the narrowing of Q_{00}-band by a factor of 1.4 because the A-type effects are inversely proportional to a square of the band width (Buckingham and Stephens, 1966). As an indicator of the narrowing one can use the change in the distance between positive and negative peaks as this distance is proportional to the half-band width (Buckingham and Stephens, 1966). This distance is 14 nm at the room temperature and 10 nm at liquid nitrogen temperature. This sharpening of the Q_{00}-band A-term is not seen in the MCD of ferroperoxidase at pH 11.4 but there is a significant increase of positive peak in the 560–580 nm region analogous to that in the MCD of deoxymyoglobin. The appearance of a very narrow A-term at 554 nm during the freezing of the alkaline solution of ferroperoxidase is very likely due to the presence of a small amount of hemochrome during freezing of the sample. The formation of hemochrome is reversible, when the temperature increases up to the room one the A-term at 554 nm disappears. Assuming that 100% hemochrome observed in pH 11.4 peroxidase at liquid nitrogen temperature has the same intensity of A-term at 554 nm as denatured hemoglobin at 552 nm, the estimated content of hemochrome in peroxidase does not exceed 7%. Apart this effect the temperature dependence of the MCD exhibited by peroxidase at pH 11.4 is "myoglobin-like".

The low temperature visible MCD spectra of hemoproteins studied are very different. In attempt to know what spectrum resembles the MCD of high-spin penta-

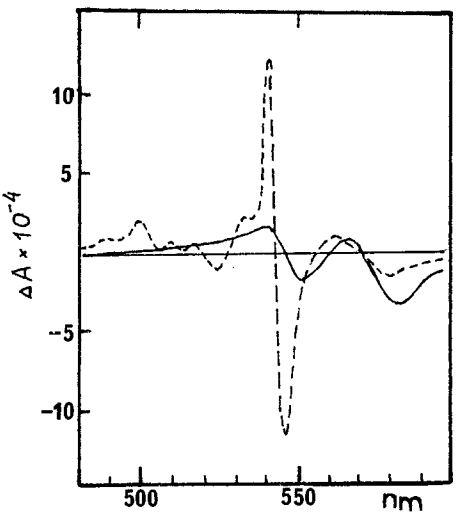

Fig. 6. Magnetic circular dichroism of the 2-methylimidazole complex of deuteroheme at the room (——) and liquid nitrogen (– – –) temperatures. The experimental values $\Delta A = A_L - A_R$ are shown. Deuteroheme $1.25 \cdot 10^{-3}$ M; 2-methyl-imidazole 0.5 M; in an equal volume mixture of glycerol and water; pathlength = 1.5 mm; field = 0.55 T

coordinated complex of free heme at liquid nitrogen temperature we have recorded the MCD of deuteroheme complex with 2-methyl-imidazole (Fig. 6). We were unable to compare its MCD with the MCD of hemoproteins in high-spin ferrous state because on lowering of the temperature the high-spin heme complex is reversibly converted into a low-spin one as it follows from our data (Fig. 6) and from the data on the temperature dependence of the absorption spectra (the appearance of hemochromogen-type spectrum; Wagner and Kassner, 1975a, b). On cooling up to the liquid nitrogen temperature, about 50% of the complex undergoes the transition to a low-spin form as evidenced by the two-fold decrease of the high-spin MCD effect at 582 nm and by the appearance of the MCD spectrum which is similar to that of denatured deoxyhemoglobin (Fig. 5).

From above results and discussion it could be concluded that the room and low temperature MCD spectra of hemoproteins serves as a very sensitive probe for studying the differences in heme state arising from peculiarities of protein environment. It should be noted that the attempts to detect any spectral differences between isolated deoxychains and deoxyhemoglobin using the resonance Raman spectroscopy (Sussner et al., 1974) and Mössbauer effect (Huynh et al., 1974) were unsuccessful. The distinctions between the absorption spectra of hemoglobin chains and tetramers are so small that they could be detected only by perturbation difference spectroscopy (Perutz et al., 1974a).

Soret Region

Unlike the visible MCD spectra the Soret MCD of hemoglobin, myoglobin, dimers and peroxidase are very similar both at the room and liquid nitrogen temperatures (Figs. 1–3). There are however some differences in positions, widths and vibronic features. The increase of the Soret MCD at liquid nitrogen temperature by a factor of 3.5–3.9 observed for hemoglobin, myoglobin, dimers and alkaline form of peroxi-

dase is in accordance with Boltzmann distribution. No significant effect of lowering of the temperature on the intensity or on the bandwidth of absorption spectra was observed. These facts indicate that near ultraviolet MCD spectrum is composed predominantly of *C*-terms. Earlier this conclusion has been made for hemoglobin and myoglobin (Vickery et al., 1976a; Livshitz et al., 1976; Treu and Hopfield, 1975). However for peroxidase at pH 6.8 the temperature dependence of MCD is weaker than that expected on the basis of Boltzmann distribution, the peak increases only by a factor of 2.1 (Fig. 2a). It suggests that contribution of *A*-term is comparable to that of *C*-terms. The difference in temperature behavior of the Soret MCD of peroxidase at two pH gives further evidence that there are two different pH-dependent protein forms.

Recently Springall et al. (1976) have reported the MCD spectra of deoxymyoglobin over the temperature range of 293 K to 20 K. They observed only 1.8-fold increase of the Soret MCD positive peak on cooling the sample from room temperature to 80 K and 3.2-fold increase when temperature was lowered to 20 K. This result is in disagreement both with Vickery et al. (1976a) and our present data. The increase in the Soret MCD intensity by a factor of 1.5 and of 3.9 was found on lowering the temperature to 185 K (Vickery et al., 1976a) and to 80 K (our data) respectively. This increase is to be expected on the basis of Boltzmann distribution.

To extract pure *C*-effects from experimental data the following procedure has been performed. The relation between molecular Faraday parameters A_i, B_i, and C_i and experimental $\Delta\varepsilon_M$ is

$$\Delta\varepsilon_M(T) = \Delta\varepsilon_M^{A,B} + \Delta\varepsilon_M^C(T) = -64.7 \sum_i \left[A_i f_1^i + (B_i + \frac{1}{T} C_i) f_2^i\right]. \quad (1)$$

Here the summation is over all transitions, f_1 and f_2 define band shapes, the numerical coefficient originates from the choice of units.

The MCD measurements at two different temperatures allow to separate *C*-type effects as

$$\Delta\varepsilon_M^C(T_1) = -64.7 \frac{1}{T_1} \sum_i C_i f_2^i = \frac{T_2}{T_1 - T_2} [\Delta\varepsilon_M(T_2) - \Delta\varepsilon_M(T_1)]. \quad (2)$$

The extracted *C*-terms as well as the temperature independent effects in the MCD of deoxymyoglobin are shown as an example in Figure 7a. In the above mentioned procedure we have assumed that no changes in the band shape occured upon freezing. The lack of any appreciable temperature effects in the absorption spectra justifies this assumption.

We have previously discussed the temperature dependence of the Soret MCD of deoxyhemoglobin in terms of spin paramagnetism and spin-orbit coupling of the excited state (Livshitz et al., 1975, 1976). The same interpretation has been reported by Treu and Hopfield (1975). As generally accepted we have assumed the $\pi - \pi^*$ origin of the Soret band. However for the excited state of porphyrin ring the amount of spin-orbit splitting ζ (~ 400 cm^{-1}) estimated from the experimental data seems to be too large. Such value of splitting should require high degree of iron-to-porphyrin spin density delocalization as well as significant spin-orbit coupling in the π-conju-

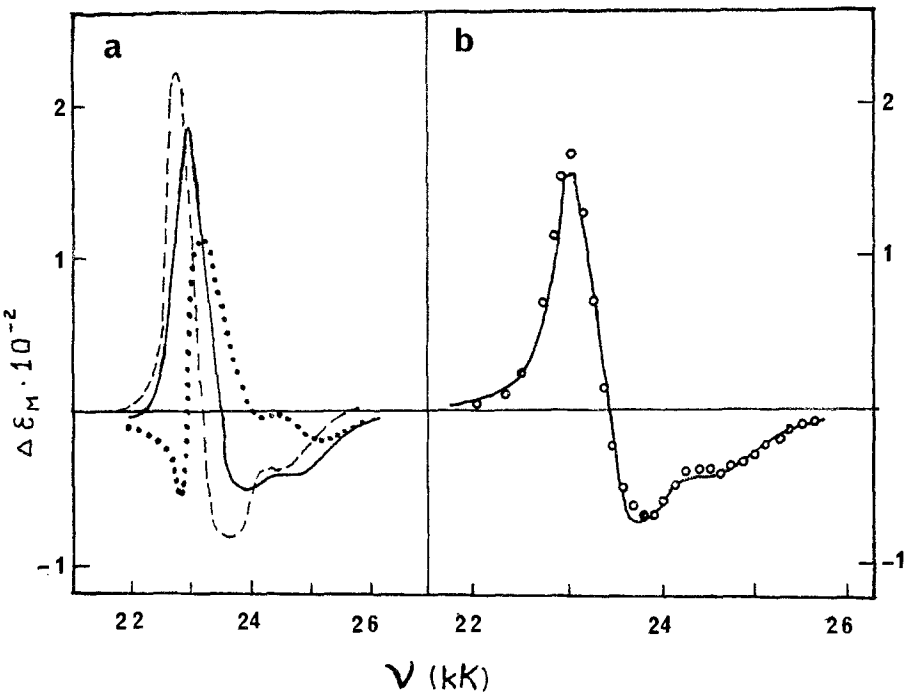

Fig. 7. (a) Extraction of the temperature-dependent part (*C*-part) of magnetic circular dichroism of deoxyhemoglobin. Magnetic circular dichroism observed at the room temperature (——); the temperature-independent part (· · · ·); the *C*-part at the room temperature (– – –). (b) The *C*-part of the magnetic circular dichroism of deoxyhemoglobin dimers at the room temperature extracted from the experimental curve (∘ ∘ ∘ ∘) and the theoretical magnetic circular dichroism (——)

gated system. As we believe now these requirements are both questioned. First, the exchange mechanism of the spin-density transfer from paramagnetic site to π-system seems to give small effects (Ake and Gouterman, 1969). Second, in such a sizable π-system as porphyrin the spin-orbit coupling is expected to be smaller than that in paramagnetic ion.

For the reasons stated we suggest now the charge-transfer ($\pi \rightarrow d$) transition strongly overlapped with the usual $B(\pi \rightarrow \pi^*)$ band. If this is valid, then it is clear why the constant of spin-orbit coupling, ζ, obtained in our previous work is in a good agreement with ζ for Fe^{2+} ion. The presence of charge-transfer band in this spectral region has been proposed to account for the anomalous width of Soret absorption band (Zerner et al., 1966). At low temperature the Soret band of myoglobin is seen to be complex (Fig. 1b). It gives further indirect evidence for the existence of two transitions in the near ultraviolet region. The blue shift of the Soret MCD at the low temperature with no appreciable changes in absorption (Figs. 1, 2) indicates also that there are at least two overlapping bands, one is the temperature dependent and the other does not depend on the temperature. Moreover, the shape and the sign of the temperature independent part of the Soret MCD (Fig. 7a) are very similar to those of usually observed in this region for diamagnetic metalloporphyrins.

We shall examine here the following model. Five components of the ground quintet can be written as Slater determinants:

$$
\begin{aligned}
{}^5\phi_2 &= |q\ \bar{q}\ d^+\ d^-\ d_1\ d_2\ A| \\
{}^5\phi_1 &= \tfrac{1}{2}\{|q\ \bar{q}\ d^+\ d^-\ d_1\ \bar{d}_2\ A| + |q\ \bar{q}\ d^+\ d^-\ \bar{d}_1\ d_2\ A| \\
&\quad + |q\ \bar{q}\ d^+\ \bar{d}^-\ d_1\ d_2\ A| + |q\ \bar{q}\ \bar{d}^+\ d^-\ d_1\ d_2\ A|\} \\
{}^5\phi_0 &= \frac{1}{\sqrt{6}}\{|q\ \bar{q}\ d^+\ d^-\ \bar{d}_1\ \bar{d}_2\ A| + |q\ \bar{q}\ d^+\ \bar{d}^-\ d_1\ d_2\ A| \\
&\quad + |q\ \bar{q}\ \bar{d}^+\ d^-\ d_1\ \bar{d}_2\ A| + |q\ \bar{q}\ d^+\ \bar{d}^-\ \bar{d}_1\ d_2\ A| \\
&\quad + |q\ \bar{q}\ \bar{d}^+\ d^-\ \bar{d}_1\ d_2\ A| + |q\ \bar{q}\ \bar{d}^+\ \bar{d}^-\ d_1\ d_2\ A| \\
{}^5\phi_{-1} &= \tfrac{1}{2}\{|q\ \bar{q}\ \bar{d}^+\ \bar{d}^-\ \bar{d}_1\ d_2\ A| + |q\ \bar{q}\ \bar{d}^+\ \bar{d}^-\ d_1\ \bar{d}_2\ A| \\
&\quad + |q\ \bar{q}\ \bar{d}^+\ d^-\ \bar{d}_1\ \bar{d}_2\ A| + |q\ \bar{q}\ d^+\ \bar{d}^-\ \bar{d}_1\ \bar{d}_2\ A|\} \\
{}^5\phi_{-2} &= |q\ \bar{q}\ \bar{d}^+\ \bar{d}^-\ \bar{d}_1\ \bar{d}_2\ A|.
\end{aligned}
\tag{3}
$$

Here subscripts in ϕ show the S_z values, q denotes one-electron porphyrin orbital leaved by electron during the exitation, d are $3d$ iron orbitals, A denotes all other closed-shell orbitals.

Iron $3d$ orbitals are transformed according to the following irreducible representations of C_{4v}-point group:

$$
\begin{aligned}
d^{\pm} &\equiv \mp \frac{1}{\sqrt{2}}(d_{xz} \pm i d_{yz}) \to e \\
d_1 &\equiv d_{x^2-y^2} \to b_1 \\
d_2 &\equiv d_{z^2} \to a_1
\end{aligned}
\tag{4}
$$

and thus the ground state in all high spin ferrous hemoproteins can be described by ${}^5B_2$-term. The excited states ($q \to d^{\pm}$) are

$$
\begin{aligned}
{}^5\chi^{\pm}_2 &= |q\ \bar{d}^{\pm}\ d^+\ d^-\ d_1\ d_2\ A| \\
{}^5\chi^{\pm}_1 &= \tfrac{1}{2}\{|q\ \bar{d}^{\pm}\ d^+\ d^-\ d_1\ \bar{d}_2\ A| + |q\ \bar{d}^{\pm}\ d^+\ d^-\ \bar{d}_1\ d_2\ A| \\
&\quad + |q\ \bar{d}^+\ d^{\pm}\ \bar{d}^-\ d_1\ d_2\ A| + |d^+\ \bar{q}\ \bar{d}^{\pm}\ d^-\ d_1\ d_2\ A|\} \\
{}^5\chi^{\pm}_0 &= \frac{1}{\sqrt{6}}\{|q\ \bar{d}^{\pm}\ d^+\ d^-\ \bar{d}_1\ \bar{d}_2\ A| + |q\ \bar{d}^+\ d^{\pm}\ \bar{d}^-\ d_1\ \bar{d}_2\ A| \\
&\quad + |q\ \bar{d}^+\ d^{\pm}\ \bar{d}^-\ \bar{d}_1\ d_2\ A| + |d^+\ \bar{q}\ \bar{d}^{\pm}\ d^-\ d_1\ \bar{d}_2\ A| \\
&\quad + |d^+\ \bar{q}\ \bar{d}^{\pm}\ d^-\ \bar{d}_1\ d_2\ A| + |d^{\pm}\ \bar{q}\ \bar{d}^+\ \bar{d}^-\ d_1\ d_2\ A|\}
\end{aligned}
\tag{5}
$$

$$
\begin{aligned}
{}^5\chi^{\pm}_{\pm 1} &= \tfrac{1}{2}\{|q\,\bar{d}^{+}\,d^{\pm}\,\bar{d}^{-}\,\bar{d}_1\,\bar{d}_2\,A| + |d^{+}\,\bar{q}\,\bar{d}^{\pm}\,d^{-}\,\bar{d}_1\,\bar{d}_2\,A| \\
&\quad + |d^{\pm}\,\bar{q}\,\bar{d}^{+}\,\bar{d}^{-}\,d_1\,\bar{d}_2\,A| + |d^{\pm}\,\bar{q}\,\bar{d}^{+}\,\bar{d}^{-}\,\bar{d}_1\,d_2\,A|\} \qquad (5)\\
{}^5\chi^{\pm}_{\mp 2} &= |d^{\pm}\,\bar{q}\,\bar{d}^{+}\,\bar{d}^{-}\,\bar{d}_1\,\bar{d}_2\,A|.
\end{aligned}
$$

The excited states are described by orbital degenerate ${}^5E$-term. This degeneracy is partially lifted by spin-orbit coupling. Spin-orbit operator can be written in the one-electron form

$$V_{so} = \sum_i \zeta_i\,\vec{l}_i\,\vec{s}_i \qquad (\zeta_i > 0). \qquad (6)$$

Here ζ_i is totally symmetric function of i-th electron's coordinates. Then it is easy to calculate the matrix elements of V_{so} between χ functions. The resulting spin-levels diagramm is shown in Figure 8c. One can now calculate the MCD which should be observed near the ν_{00}-frequency. The resulting calculated values for A and C parameters associated with $q \rightarrow d^{\pm}$ transition are given in Table 1. The $B(q \rightarrow d^{\pm})$ parameters equal to zero for symmetry reasons. The value Q in Table 1 is obtained according to the relation

$$Q = \frac{1}{10}\{|\langle q\,|m_+|\,d^{+}\rangle|^2 - |\langle q\,|m_-|\,d^{+}\rangle|^2 - |\langle q\,|m_+|\,d^{-}\rangle|^2 + |\langle q\,|m_-|\,d^{-}\rangle|^2\}. \qquad (7)$$

Expression (7) is adequate to known relationships for Faraday parameters (Buckingham and Stephens, 1966) written in circular coordinates $m_{\pm} = \mp\,(m_x \pm im_y)/\sqrt{2}$.

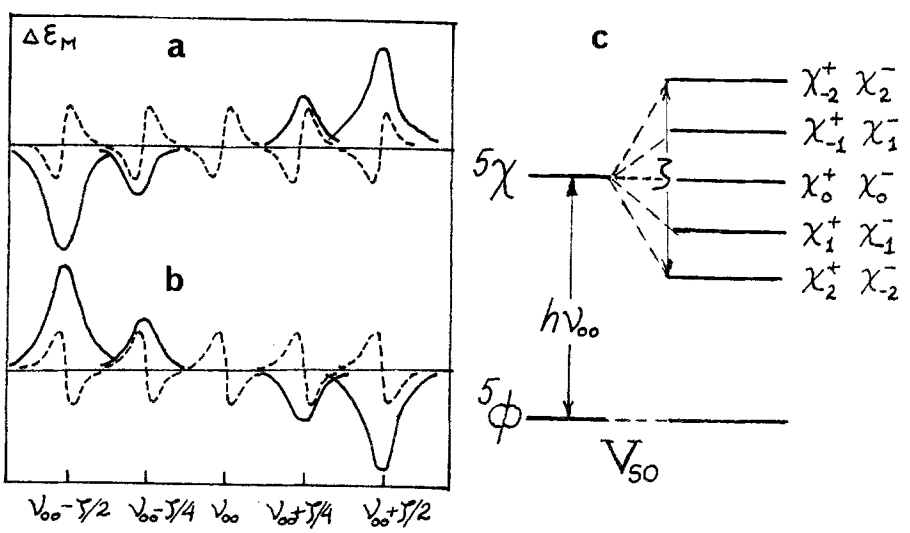

Fig. 8. The theoretically predicted charge-transfer magnetic circular dichroism: (**a**) magnetic circular dichroism for $a(\pi) \rightarrow 3d$ transition; (**b**) magnetic circular dichroism for $b(\pi) \rightarrow 3d$ transition; (**c**) diagram of the spin-orbit splitting of the charge-transfer excited state

Table 1. Faraday parameters for $\phi \rightarrow \chi$ transitions (see text and Fig. 8)

ν	A ($\phi \rightarrow \chi$)	C ($\phi \rightarrow \chi$)
$\nu_0 + \zeta/2$	$-\beta Q$	$+4\beta Q$
$\nu_0 + \zeta/4$	$-\beta Q$	$+2\beta Q$
ν_0	$-\beta Q$	0
$\nu_0 - \zeta/4$	$-\beta Q$	$-2\beta Q$
$\nu_0 - \zeta/2$	$-\beta Q$	$-4\beta Q$

β = Bohr magneton

Angular momentum associated with degenerate pair of $d^{\pm}$-orbitals is assumed to be equal to unity:

$$\langle d^{+} | l_z | d^{+} \rangle = - \langle d^{-} | l_z | d^{-} \rangle = 1 . \tag{8}$$

One can see now that the sign of Q and hence the signs of $A(q \rightarrow d)$ and $C(q \rightarrow d)$ depend on rotational symmetry of $|q\rangle$. If $|q\rangle$ transforms according to a-type representation in point group C_{4v} then the only nonzero matrix elements in expression (7) will be

$$\langle q | m_{\pm} | d^{\mp} \rangle \neq 0$$

and therefore $Q < 0$. If on the contrary $\Gamma(|q\rangle) = b$ then only

$$\langle q | m_{\pm} | d^{\pm} \rangle \neq 0$$

and $Q > 0$. Consequently the sign of the MCD in the absorption band is determined by the symmetry properties of one-electron orbitals participating in a given transition but not by those of many electron vectors as Stillman and Thomson (1974) have declared.

The A-type and C-type MCD components for $q \rightarrow d$ transition are shown in Figures 8a, b for $a \rightarrow d$ and $b \rightarrow d$ transitions respectively. It should be noted that the sign of $\Delta\varepsilon^C$ is opposite to that of C-parameters (see Eq. 2). By comparing the signs of the extracted (Fig. 7a) and predicted (Fig. 8a, b) MCD effects one can conclude that the C-part of the Soret band arises from $b(\pi) \rightarrow 3d$ transition. So we assume that there are two unresolved electronic bands in near ultraviolet spectral region exhibited by high spin ferrous hemoproteins. One of them is usual for this region $B(\pi \rightarrow \pi^*)$-band and the another one is associated with $b(\pi) \rightarrow 3d$ charge-transfer transition.

Having suggested the temperature-dependent effects in near ultraviolet region d c to charge-transfer transition and its vibronic satellites, we have tried to fit the extracted C-parts of the MCD (Fig. 7b) by a sum of theoretical curves. The latter were arranged in accordance with the following assumptions:

(a) all vibronic components consist of four lines as well as a pure electronic band (see Fig. 8b);

(b) each vibronic "quaternary" differs from a pure electronic one only by its amplitude but not by its shape, i.e. the values of half-width and ζ are the same for all vibronic bands;

Table 2. Parameters obtained by fitting procedure

Compound	C^a $(\nu_{00} + \zeta/2)$ $(D^2 \cdot \beta)$	ν_{00} (cm^{-1})	Γ (cm^{-1})	ζ (cm^{-1})	ν_1 (cm^{-1})	ν_2 (cm^{-1})	y_1	y_2	F^b
Hemoglobin	5.67	23,321	412	380	298	986	1.079	0.449	0.125
	$\binom{0.27}{-0.32}$ [c]	$\binom{18}{-14}$	$\binom{43}{-37}$	$\binom{24}{-20}$	$\binom{9}{-12}$	$\binom{73}{-83}$	$\binom{0.024}{-0.024}$	$\binom{0.047}{-0.051}$	
Dimers	5.01	23,124	450	365	293	982	1.141	0.470	0.101
	$\binom{0.13}{-0.15}$	$\binom{17}{-15}$	$\binom{35}{-43}$	$\binom{18}{-18}$	$\binom{12}{-10}$	$\binom{57}{-69}$	$\binom{0.019}{-0.022}$	$\binom{0.044}{-0.039}$	
Myoglobin	6.95	22,987	399	401	297	1,012	1.084	0.419	0.155
	$\binom{0.06}{-0.06}$	$\binom{16}{-23}$	$\binom{47}{-44}$	$\binom{28}{-24}$	$\binom{13}{-17}$	$\binom{75}{-143}$	$\binom{0.022}{-0.036}$	$\binom{0.063}{-0.061}$	
Peroxidase pH 6.8	5.01	22,896	877	356	382	1,128	0.878	0.473	0.096
	$\binom{0.15}{-0.13}$	$\binom{20}{-21}$	$\binom{71}{-69}$	$\binom{19}{-16}$	$\binom{20}{-22}$	$\binom{69}{-71}$	$\binom{0.026}{-0.026}$	$\binom{0.041}{-0.047}$	

[a] See Figure 8

[b] See "Experimental Data Processing"

[c] Deviations in parenthesis correspond to $\Delta F = 0.01$ obtained for given parameters with simultaneous fixation of all other parameters

(c) only Frank-Condon mechanism contributes to the spectrum. Adopting also the harmonic oscillator model one can rewrite the expression for the absorption vibronic intensities (Lukashin et al., 1968) as following:

$$C_0, \mathrm{v}_1, \ldots \mathrm{v}_N = C_{00} \prod_k^N \frac{y_k^{2\mathrm{v}_k}}{\mathrm{v}_k!},$$

where C_{00} denotes one of the pure electronic C-parameters. $C_0, \mathrm{v}, \ldots \mathrm{v}_N$ is a corresponding C-parameter in the vibronic quaternary whose midpoint is situated at the frequency $\nu = \nu_{00} + \sum_k^N \nu_k \mathrm{v}_k$; ν_k and v_k are the vibrational quantum number and the frequency of the k-th totally symmetric normal vibration respectively, y_k is the Poisson distribution parameter proportional to the displacement of a nuclear equilibrium position along the k-th normal coordinate during the electronic excitation. It is also assumed here that only the zero vibrational level of the ground electronic state is populated, i.e. $h\nu_i^0 \gg kT$;

(d) only two normal vibrations are active being the most "sinphase" (see Lukashin et al., 1968) with the charge-transfer transition;

(e) the band shape is chosen in the form of a damped oscillator dispersion curve: $f_2(\nu) = \nu^3\Gamma/[(\nu_0^2 - \nu^2)^2 + \nu^2\Gamma^2]$.

The resulting fit-parameters are given in Table 2 and the calculated MCD for hemoglobin dimers is shown in Figure 7b.

The most reliable values in Table 2 are ν_{00}. The increase of ν_{00} in the order myoglobin < dimers < hemoglobin could be interpreted as the increase of crystal field splitting and moreover as the decrease of the displacement of Fe from heme plane provided the iron-histidine bond is invariable (Perutz et al., 1974b). The ζ-values for all compounds are about 400 cm^{-1}, i.e. in a good agreement with a ζ-value for Fe^{2+}-ion. The frequences of the most sinphase vibrations, ν_1, ν_2, may be assigned to the totally symmetric Fe-heme and pyrrole vibrations respectively. This assignment may be correlated with the Raman data (Brunner and Sussner, 1973).

The more detailed discussion of fitting parameters seems to be meaningless because the model used is sufficiently crude. However the results of fitting would be considered as the illustration of the main hypothesis on the charge-transfer nature of the Soret region low temperature MCD of the high-spin ferrous hemoproteins.

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