Biophys Struct Mech (1983) 10:47–59

Biophysics of Structure and Mechanism

Paramagnetic Effects in Magnetic Circular Dichroism Spectra of High-Spin Ferrous Hemoproteins in the Visible and Near Infrared Spectral Regions

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Abstract. Magnetic circular dichroism spectra of high-spin ferrous hemoproteins (deoxyhemoglobin, deoxymyoglobin, ferroperoxidase and reduced cytochrome *c* oxidase) are compared for spectral region 350–800 nm at temperatures 293 – 10 K. At the lowest temperatures the MCD spectra over the whole spectral region are shown to be composed predominantly of the temperature-dependent *C* terms. The clear evidence are given for paramagnetic origin of positive MCD bands at about 760 and 680 nm and negative MCD band at about 630 nm associated with CT or $d \rightarrow d$ transitions. The *C* terms associated with these bands as well as those associated with $\pi-\pi^*$ transitions in the visible *Q* band region are sensitive to the peculiarities in heme environment induced by protein conformation. In contrast, the Soret region MCD spectra of these proteins are very similar. The origin of *C* terms for porphyrin $\pi-\pi^*$ transitions is discussed in the framework of $\pi-d_\pi$ interaction suggested earlier for the explanation of *C* terms in the visible and Soret MCD of low-spin ferric hemoproteins [Mineyev AP, Sharonov YA (1978) Theor Chim Acta 49:295–307]. The results and discussion illustrate the capability of *C* terms in near infrared and visible MCD in probing the active center in high-spin ferrous hemoproteins.

Key words: Magnetic circular dichroism – Hemoproteins – Low temperatures

Introduction

The different hemoproteins perform different functions (e.g., oxygen storage and transport, electron transfer, detoxification of poisonous chemicals) though all of them contain the same molecular group, heme, in the active site. Thus the protein must suitably modify the heme to achieve the proper functions. A knowledge of the electronic structure of the iron-porphyrin-protein complex

provides a basis for the understanding of their functional capabilities. The magnetic circular dichroism (MCD) spectra have been found to be sensitive to the electronic state of the heme and to some of the effects of protein structure on it. The shape and intensity of the spectra can be correlated with the redox state, the spin state, the axial ligation of iron and with the features of an altered hemoprotein interaction (see reviews: Sharonov 1976; Hatano and Nozawa 1978; Holmquist 1978; Sutherland and Holmquist 1980).

The MCD effects have three different origins: a Zeemann splitting of either ground and/or excited states (*A* terms); a mixing of different unperturbed states by external magnetic field (*B* terms); the change in population of sublevels of the ground state (*C* terms) (Stephens 1974). The *A* and *B* terms are temperature independent, while the intensity of *C* terms depends on the temperature according to Boltzmann's low.

Briat et al. (1972) first have shown that temperature-dependent signals are present in the MCD spectrum of paramagnetic hemoprotein, ferricytochrome b_2. Later the *C* terms have been found in the region of the Soret band (*B* band) of paramagnetic ferric and ferrous hemoproteins (Livshitz et al. 1975, 1976; Treu and Hopfield 1975; Vickery et al. 1976a, b; Springall et al. 1976; Babcock et al. 1976; Thomson et al. 1976, 1977; Brittain et al. 1976; Mineyev and Sharonov 1978; Sharonov et al. 1978).

Two of us have explained the arising of *C* terms in the MCD spectra for the porphyrin $\pi-\pi^*$ transitions of low-spin ferric hemoproteins by intraconfigurational interaction between π-electrons of porphyrin and d_π-electrons of metal (Mineyev and Sharonov 1978). The mechanism of $\pi-d_\pi$ interaction suggested for the explanation of *C* terms in MCD of low-spin ferric hemoproteins can be used in principle for high-spin ferrous forms as well, provided the low-lying states of ferrous iron exhibit the orbital paramagnetism.

To clear the origin of the paramagnetic MCD effects and to get new information on the electronic state of heme within high-spin ferrous hemoproteins the temperature dependence of the multiband MCD in the visible (*Q* band) and near IR regions is to be studied. In the Soret region the temperature-dependent *C* terms dominate the temperature-independent terms even at room temperature. As a result the measurements from room to liquid nitrogen temperatures are sufficient for detecting the *C* terms in the *B* band. In the visible region the temperature-independent effects in MCD dominate the *C* terms at room temperature and even at 77 K. Therefore the measurements at the temperatures lower than 77 K are required for their undoubtful detection. Till now the reliable evidence for the presence of the temperature-dependent MCD signals in the *Q* absorption bands has been given only for the paramagnetic derivatives of myoglobin and cytochrome *c* oxidase (Springall et al. 1976; Thomson et al. 1976). However, the authors have not published the temperature dependences of the observed MCD effects.

In the present paper we report and analyse the MCD spectra of high-spin ferrous forms of myoglobin, hemoglobin, peroxidase and cytochrome *c* oxidase over the spectral region 350–800 nm from 293 to 10 K. At the lowest temperatures the *C* terms were shown to dominate for all MCD bands. We compare also the temperature dependences of the peak intensities for the Soret

band. In the region of Q band the intensities and the shapes of C terms varied greatly in different proteins although in the Soret region the C terms are very similar. Three MCD bands of different signs associated with temperature-dependent C terms are observed in the spectral region 600–800 nm. The assignment of the temperature-dependent MCD terms is discussed.

Materials and Methods

Samples

Sperm whale myoglobin (Sigma, type II) and horseradish peroxidase (Boehringer I,R.Z. = 3) were used without further purification. Human hemoglobin was prepared using the method of Geraci and Li (1969). The concentration of proteins were assessed by using the published absorption coefficients (Antonini and Brunori 1971; Dunford and Stillman 1976). Cytochrome *c* oxidase (EC 1.9.3.1) was prepared from ox heart mitochondria according to Fowler et al. (1962) and then was purified by the method of MacLennon and Tzagoloff (1965). The final product contained about 8 nmol heme *a* per mg of protein. The protein concentration was determined by modified biuret method applied for membrane proteins (Watters 1978). The concentration of the reduced cytochrome *c* oxidase was established per enzyme functional unit by using $\Delta\varepsilon^{605-630\text{ nm}}_{\text{oxidized-reduced}} = 27\ \text{mM}^{-1} \cdot \text{cm}^{-1}$.

The proteins were reduced by a few grains of sodium dithionite (Merck). To obtain the transparent glasses for the low temperature measurements all proteins with the exception of cytochrome *c* oxidase were dissolved in a mixture of 0.2 M sodium phosphate buffer pH 7 with a pure glycerol (1 : 2 v/v). Cytochrome *c* oxidase was first dissolved in 0.05 M *Tris*-HCl buffer pH 7.4 containing 0.66 M sucrose and 1 mM histidine and then was mixed with glycerol.

MCD and Absorption Measurements

MCD spectra were recorded with an instrument constructed on the basis of a "Roussel-Jouan" dichrograph equipped with an electromagnet capable of generating a field of 1.8 T in a 2 cm pole gap (Figlovsky and Sharonov 1979). The MCD spectrometer has a powerful light source (1,000 W xenon lamp) and a grating monochromator with a significant light-gathering power (1 : 2.5) and spectral resolution of 2 nm/mm. The effective magnetic field was determined by Dratz (1966) method. For the same solution of ferrocytochrome *c* the magnetic rotatory dispersion was recorded with a magnetospectropolarimeter and the MCD spectra were obtained with a magnetic dichrograph. Then the observed magnetooptical rotatory dispersion was transformed to MCD using Kronig-Kramers relations and the calculated MCD spectra were compared with the experimental one. The magnetospectropolarimeter had a precise device to determine the angle of magnetic rotation. The electromagnet of the magnetospectropolarimeter was calibrated using a Verdet constant for water. The

measured MCD value was proportional to the dichrograph sensitivity and to the strength of a magnetic field. One of the advantages of the described method is that it takes into account an inaccuracy in dichrograph calibration in the units of optical density. After the calculation the MCD spectrum of Nd-doped glass filter has been recorded and then the dichrograph was systematically calibrated by using the MCD of this filter.

Contact type hermetically closed quartz cells with 0.5–2 mm path lengths were placed in a specially constructed cryostat. The temperature of the sample was monitored with thermocouple put into the sample and was varied by controlling the cold nitrogen or helium gas flow rate through the holes in the copper cell holder.

MCD spectra were obtained as a difference between the curves recorded at the opposite directions of a magnetic field to exclude the natural circular dichroism and linear birefringence.

A typical run at low temperatures was performed with a scan speed of 1 nm/s and time constant 0.25 s.

The absorption spectra were recorded with a Beckman-26 spectrophotometer using the same cells as in the MCD measurements.

Experimental Data Processing

The experimental MCD spectra were digitized in a computer Hewlett-Packard HP-9830 (100–150 points per 100 nm) and after appropriate transformation were plotted as $\Delta\varepsilon/H$ against wavelength where $\Delta\varepsilon$ was the difference in molar extinction coefficients for left and right circularly polarized light and H was an applied magnetic field in Tesla. The units of $\Delta\varepsilon/H$ were $(M \cdot cm \cdot T)^{-1}$ on the heme basis.

Results

Visible and near IR MCD spectra of deoxymyoglobin, deoxyhemoglobin, ferroperoxidase, and reduced cytochrome *c* oxidase at different temperatures are shown in Figs. 1–4. The temperature dependences of MCD at several wavelengths are plotted in the insets in the figures. These dependences are usually composed of two linear parts with the slope change near 77 K. In principle, the complex character of the graphs may be explained by sharpening of the bands on lowering the temperature down to 77 K. Such a sharpening is often observed in the MCD spectrum of diamagnetic molecules. The sign of the slope change depends on the relative values of temperature-dependent and temperature independent effects in MCD. If at a given wavelength A terms (and/or B) and C terms possess the same sign the band narrowing will result in an increase of a line slope in respect to slope value caused by the presence of C terms only. If the temperature-dependent and temperature-independent effects have the opposite signs the band narrowing leads to the slope decrease and even to the change of its sign. The latter situation occurs for the MCD of

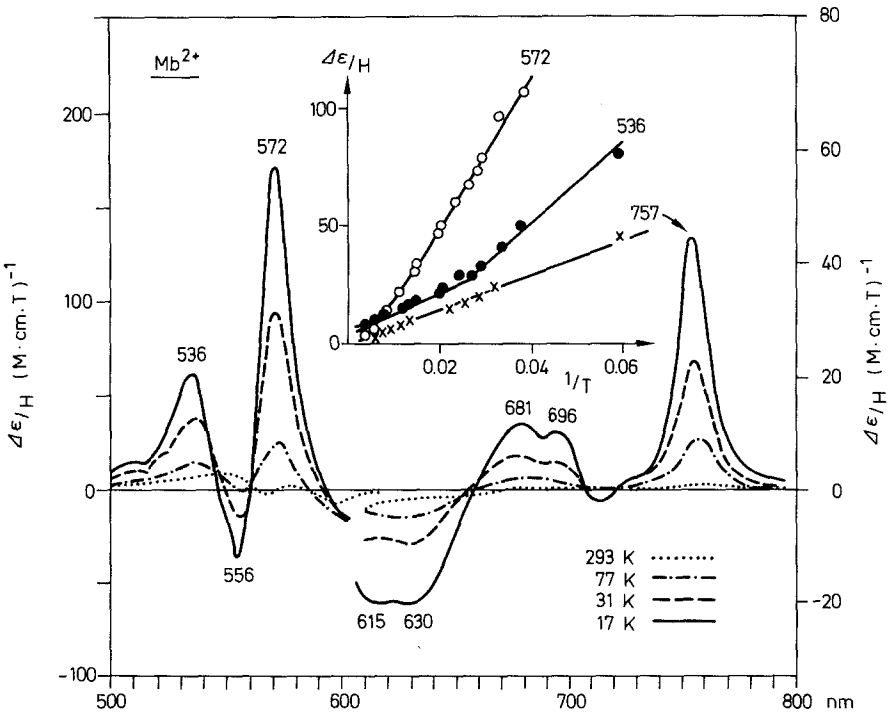

Fig. 1. Temperature dependence of the MCD of deoxymyoglobin. Concentration 1 mM in a mixture of 0.2 M sodium phosphate buffer at pH 7 with glycerol (1 : 2 v/v); path = 1 mm, field = 1.56 T. The inset is the plot of the value of the MCD intensity at the indicated wavelengths against the reciprocal of the absolute temperature

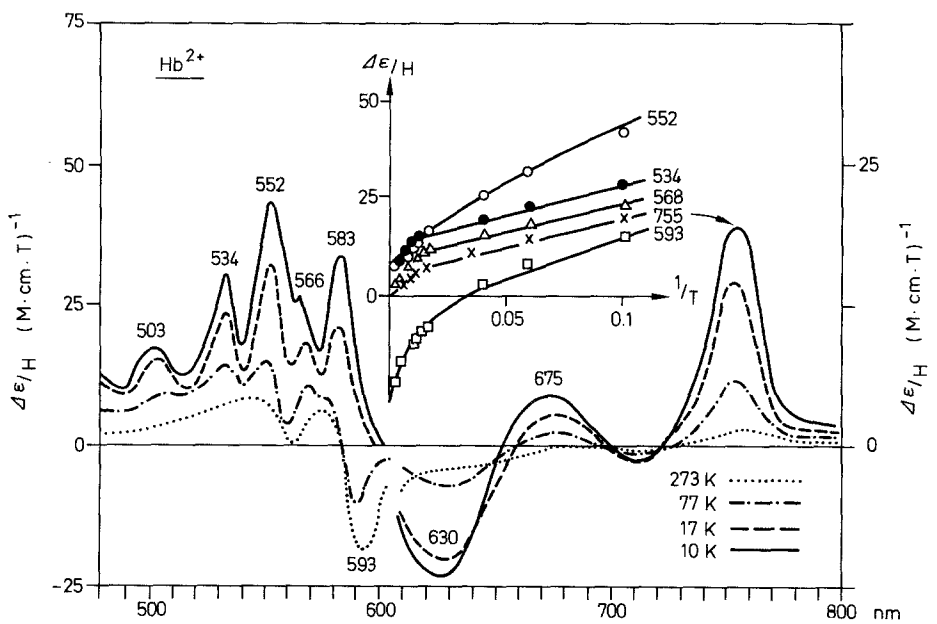

Fig. 2. Temperature dependence of the MCD of deoxyhemoglobin. Concentration 1.7 mM in a mixture of 0.2 M sodium phosphate buffer at pH 7 with glycerol (1 : 2 v/v); path = 0.5 mm; field = 1.56 T. The inset is the plot of the value of the MCD intensity at the indicated wavelengths against the reciprocal of the absolute temperature

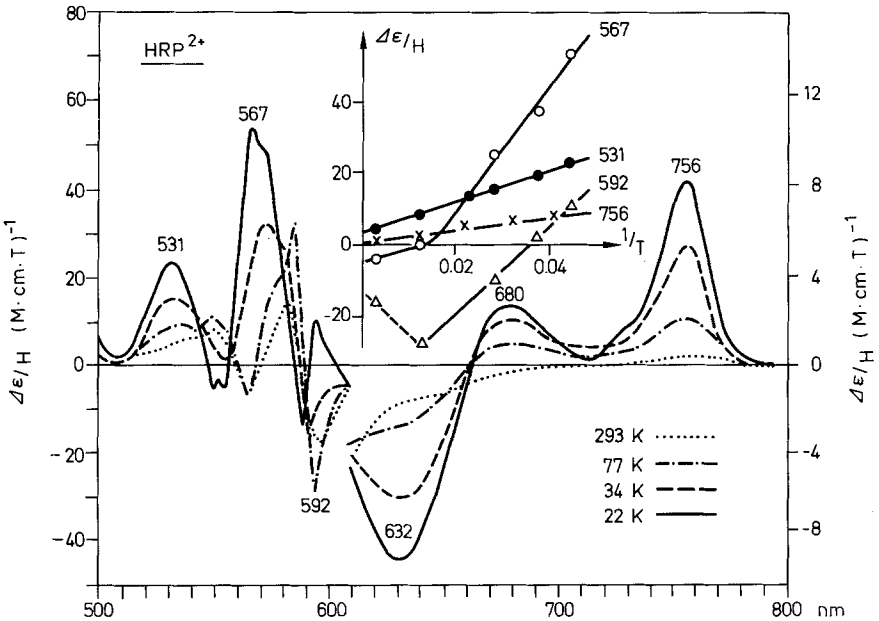

Fig. 3. Temperature dependence of the MCD of ferroperoxidase. Concentration 1.1 mM in a mixture of 0.2 M sodium phosphate buffer at pH 7 with glycerol (1 : 2 v/v); path = 1 mm; field = 1.56 T. The inset is the plot of the value of the MCD intensity at the indicated wavelengths against the reciprocal of the absolute temperature

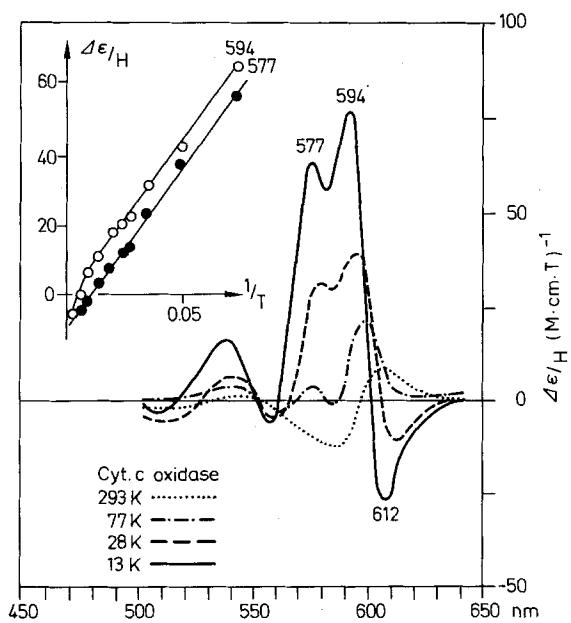

Fig. 4. Temperature dependence of the MCD of reduced cytochrome *c* oxidase. Concentration 0.34 mM in a mixture of 0.05 M Tris-HCl buffer at pH 7.4 containing 0.66 M sucrose and 1 mM histidine with glycerol (1 : 2 v/v); path = 1 mm; field = 1.55 T. The inset is the plot of the MCD intensity at the indicated wavelengths against the reciprocal of the absolute temperature

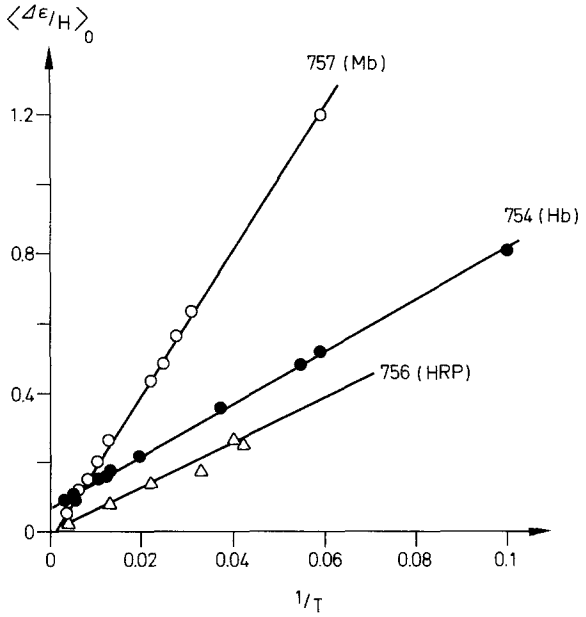

Fig. 5. The plot of zeroth moment of the MCD spectra of deoxymyoglobin (○), deoxyhemoglobin (●), and ferroperoxidase (△) in the near infrared region against the reciprocal of the absolute temperature

ferroperoxidase at 590 nm (Fig. 3, inset). The sign change of temperature dependence at about 77 K is due to sharpening of the *A* term by a factor of 2.3. The sharpening is larger than the increase of the positive *C* term at these temperatures.

According to the quantitative analysis the narrowing of the MCD band is not the only reason for the inflection of the plots of the MCD intensities against 1/T. For example, the slope changes of the temperature dependences of MCD peaks at 595 nm for deoxyhemoglobin, MCD peaks at 536 and 572 nm for deoxymyoglobin and that at 592 nm for reduced cytochrome *c* oxidase are not explicable only by a band narrowing in the course of the temperature lowering from room to liquid nitrogen temperature. Apart from the narrowing of the MCD bands a nonlinear character of the temperature dependences of MCD peaks may be due to the conformational transitions in a protein and/or to Boltzmann redistribution among the ground and closely spaced excited states available for population at room temperature.

One can exclude the effect of narrowing on the MCD temperature dependence by using a method of moment analysis (Stephens 1974). The moments of the MCD spectra are independent on the band shape. The method is applicable only to isolated bands. The MCD bands located near 760 nm are well separated from the other bands (Figs. 1–3), their zeroth moments are plotted against 1/T in Fig. 5. The linear temperature dependence of the zeroth moment for deoxyhemoglobin MCD band at 755 nm suggests that the nonlinear dependence of the intensity of this band against 1/T (Fig. 2, inset) is due to the

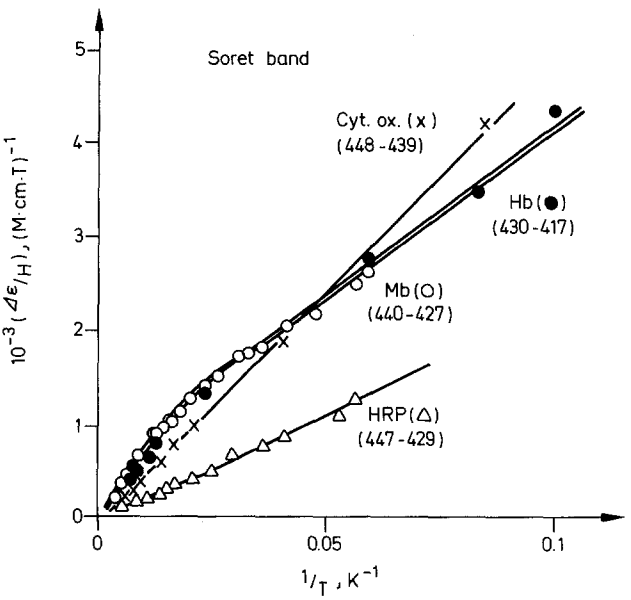

Fig. 6. The plot of the value of the amplitude between the positive and negative peaks of the Soret MCD spectrum of deoxymyoglobin, deoxyhemoglobin, ferroperoxidase and cytochrome *c* oxidase

band narrowing when the temperature decreases down to liquid nitrogen temperature.

The intercepts of the extrapolations of high temperature and low temperature linear parts of the MCD amplitude plots against 1/T are the magnitudes of temperature independent effects for the corresponding temperature region. As it follows from the comparison of the intercepts value with a whole MCD amplitude at a given temperature the *C* terms dominate in a visible region at the lowest temperatures while at room temperature *A* and/or *B* terms overwhelm *C* terms (Figs. 1–4, insets). In the near infrared spectral region the MCD spectra are composed predominantly of the paramagnetic *C* terms even at room temperature.

The temperature dependences of the Soret MCD spectra of the high-spin ferrohemoproteins have also been measured. Figure 6 shows the temperature dependences of the amplitude between the positive and negative MCD peaks. In the Soret band the temperature-dependent terms predominate over the temperature-independent terms even at room temperature. That is why at the lowest temperatures the shapes of the MCD Soret spectra are almost identical to those obtained previously at room and liquid nitrogen temperatures.

Discussion

At the temperatures below 77 K the amplitudes of all MCD peaks show a linear dependence on the inverse of the absolute temperature providing clear evidence for a presence of *C* terms for all the bands observed in the region 400–800 nm.

The magnitudes of the Q band MCD peaks at room and low temperatures given in the papers of Springall et al. (1976) and Thomson et al. (1976) differ from those observed by us and by the same authors (Brittain et al. 1976). Our MCD spectra coincide with the previously reported Soret and visible MCD spectra of deoxymyoglobin (Vickery et al. 1976a; Sharonov et al. 1978), deoxyhemoglobin (Sharonov et al. 1978), ferroperoxidase (Sharonov et al. 1978) and reduced cytochrome c oxidase (Babcock et al. 1976) obtained at liquid nitrogen and/or room temperatures and with room temperature near IR MCD spectrum of deoxyhemoglobin (Eaton et al. 1978).

Nozawa et al. (1976) have studied the temperature dependence of the MCD band of deoxyhemoglobin at 760 nm down to 77 K. On the basis of the observed weak nonlinear dependence of the zeroth moment against 1/T the authors have concluded that MCD at 760 nm is a B term. However our present data down to 10 K show clear linear dependences of the $\langle \Delta\varepsilon/H \rangle_0$ versus 1/T for the MCD associated with the absorption band of deoxymyoglobin, deoxyhemoglobin, and reduced cytochrome c oxidase at about 760 nm (Fig. 5). This fact provides evidence for the paramagnetic origin of the MCD bands in the near IR regions.

Except cytochrome c oxidase where the structure of active site is yet unknown in all hemoproteins studied an iron possesses the same spin state, fifth coordination position is occupied by imidazole of histidine and the sixth ligand is absent. All differences in MCD of deoxymyoglobin, deoxyhemoglobin and ferroperoxidase are to be ascribed to the peculiarities in a geometry of iron coordination produced by a different protein surrounding of heme. Low temperature visible MCD of deoxyhemoglobin, deoxymyoglobin, and ferroperoxidase show the marked qualitative and quantitative differences. It is the C type effects that are responsible for the observed differences between low temperature MCD spectra of high-spin ferrous hemoproteins. Previously we have demonstrated (Sharonov et al. 1978) that the differences in room temperature MCD spectra of deoxymyoglobin, deoxyhemoglobin and ferroperoxidase are mainly due to temperature-independent A and/or B terms. However the differences in MCD caused by temperature-independent effects are not so striking as those caused by the temperature-dependent C terms.

What is the origin of such a high sensitivity of paramagnetic effects to the changes in an active center of hemoproteins? The C terms arise from a temperature-dependent population difference in the ground state sublevels. However, paramagnetism of a ground state is a necessary but not sufficient condition for C terms occuring. If there is a pure spin paramagnetism of a ground state a relative population of Zeemann sublevels per se is not able to affect a circular dichroism because a polarization of the transitions is determined by orbital but not by spin properties of the states participating in the transition. Therefore one should reveal the mechanisms which correlate the orbital and spin properties of a system and involve them in any interpretation of temperature-dependent C terms.

The spectral properties of heme and other metalloporphyrins in near UV and visible regions are due to the $\pi-\pi^*$ transitions of the porphyrin while paramagnetic properties of a ground molecule state are associated with metal.

Thus to explain C terms in the visible and near UV regions it is necessary to take into consideration the interaction between electrons of porphyrin ring and of metal. The paramagnetic effects may be considered as a measure of such interaction and hence they may be correlated with heme electronic state and with the peculiarities in iron coordination sphere associated with a protein conformation. These arguments permit to explain qualitatively a high sensitivity of C terms to the protein environment of the heme center in paramagnetic hemoproteins.

The influence of interactions between the porphyrin and metal subsystems of heme has been previously theoretically analyzed for low-spin ferric hemoproteins (Mineyev and Sharonov 1978). Using both the free electron model and the more sophisticated "four-orbital-like" consideration the authors have shown that $\pi-d_\pi$ interactions split the excited multiplet into Kramers doublets so that the selection rules for right and left circularly polarized light upon excitation on the sublevels of a given doublet are connected rigorously with a z-component of spin on metal. The excitation into each doublet is accompanied by generating the C term in MCD. A sign and value of the C term depend both on the signs and the values of the parameters of $\pi-d_\pi$ interaction and of spin-orbit coupling of metal.

The observed low temperature MCD spectrum is a sum of the C terms of the opposite signs while the experimental absorption spectrum is a sum of the bands of the same signs. In this sense MCD is a differential characteristic and absorption is an integral one. For this reason MCD happens to be very sensitive to the variation of the parameters of both $\pi-d_\pi$ and spin-orbit interactions while absorption is practically independent on these parameters. Multiplet splitting leads to some broadening of the absorption bands. As a rule the paramagnetic hemoproteins exhibits more broad absorption bands than diamagnetic ones.

In principle the interpretation of temperature-dependent MCD for $\pi-\pi^*$ transitions in terms of $\pi-d_\pi$ interaction (Mineyev and Sharonov 1978) may be applied not only to low-spin ferric hemoproteins but also to other paramagnetic complexes providing their ground state exhibits the orbital paramagnetism. Seno et al. (1980b) have recently used the mechanism of $\pi-d_\pi$ interactions for the explanation of C terms in the MCD associated with $\pi-\pi^*$ transitions in the high-spin ferrous hemoproteins. On the basis of the ASMO-CI calculation MCD curves have been computed at different temperatures in the range 350–1,000 nm involving not only $\pi-\pi^*$ transitions but also $d-d$ transitions of metal and metal-to-porphyrin charge-transfer transitions (Seno et al. 1980a). By varying both the energies of the predicted transitions and the parameters of $\pi-\pi$ and $\pi-d_\pi$ interactions Seno et al. have explained the sign and the shape of low temperature MCD spectrum of deoxymyoglobin in the Q and B absorption bands with the exception of the short wavelength part of the Q band. The calculation predicts the negative C term at 500–530 nm whereas our experimental MCD spectra of all studied hemoproteins show a positive MCD band in this region. However, the theoretical analysis of MCD in this spectral region is complicated by the presence of vibronic bands.

Although the basic mechanism of generating C terms suggested by Seno et al. seems to be correct there are some theoretical limitations which do not allow

to understand the striking qualitative differences between low-temperature visible MCD spectra of deoxymyoglobin and deoxyhemoglobin. One can not explain these differences by the heterogeneity of α- and β-chains in hemoglobin tetramers.

According to Seno et al. (1980b) the MCD signs and shapes for B and Q bands should be the same. This contradicts our experimental results. The Soret band MCD spectra of all high-spin ferrous hemoproteins are very similar (Sharonov et al. 1978) whereas their Q band MCD spectra are quite different. In accordance with our theoretical analysis (Mineyev et al. 1983) we believe that the observed difference can be understood by taking into account not only $\pi-d$ interaction within B (or Q) states (Seno et al. 1980b) but also the borrowing of intensity by weak Q band from very strong B band through $\pi-d_\pi$ interaction.

In the spectral range 600–800 nm all studied proteins (except reduced cytochrome c oxidase) show three MCD bands, i.e., a negative band at 630 nm and positive bands at 680–690 nm and at 754–757 nm. The bands at about 630 and 690 nm are complex (see for example the MCD of deoxymyoglobin). The intensities of all MCD bands increase on lowering the temperature (Figs. 1–3). The extrapolation of the temperature dependences to $1/T = 0$ shows that the main contribution to MCD is made by the C terms even at room temperature. The intensities and positions of the MCD bands are sensitive to the nature of protein. At low temperatures the MCD peaks of deoxyhemoglobin at 755 nm, of deoxymyoglobin at 757 nm and of ferroperoxidase at 756 nm are smaller in intensity only in ~ 2, 4, and 7 times than the peaks in the visible spectra of these proteins. The intensity of the absorption band at ~ 760 nm is about 100 times smaller than that in the visible region, while the absorption bands at ~ 630 nm and at 680 nm are seen only as shoulders on the "tail" of the Q bands. Thus the study of the near IR MCD bands and their temperature dependences seems to be very promising both in understanding of the origin of near IR transitions and in monitoring the changes in the protein conformation.

On the basis of quantum mechanical calculations and the analysis of the signs of MCD bands Seno et al. (1980b) have assigned the positive MCD bands of deoxymyoglobin at about 760 nm to $d \rightarrow d$ ($\bar{d}_{xz} \rightarrow d_{z2}$) transition of iron and the MCD band at 630 nm to a transition with a charge transfer from metal to mainly porphyrin orbital ($\bar{d}_{xz} - 3\ b_{2u}$). In accordance with the experimental data the computational and analytical methods predict that temperature-dependent effects are predominant even at room temperature. Complex positive band at 680–690 nm may be explained by two charge-transfer transitions $d \rightarrow 4\ e_g$ (mainly $d_{x2-y2} \rightarrow 4\ e_g$) which have been calculated to be located in the visible region with relative separation about 10 nm.

The obtained data show the high sensitivity of temperature-dependent paramagnetic MCD effects in the visible and near IR regions to variations in the active sites of high-spin ferrous hemoproteins and to the peculiarities of protein structure. It is promising to use low temperature MCD measurements for the study of electronic-conformational interactions in proteins as well as for a further development of theoretical and conformational analysis in order to connect the experimentally observed differences in MCD spectra of various proteins with

their electronic and structural parameters. One of the possible steps in this direction is a comparison of equilibrium and photodissociated hemoproteins (Sharonov et al. 1982).

Acknowledgements. We would like to thank Prof. M. Volkenstein for his encouragement during the course of this work.

References

Antonini E, Brunori M (1971) Hemoglobin and myoglobin in their reactions with ligands. North-Holland, Amsterdam

Babcock GT, Vickery LE, Palmer G (1976) Electronic state of heme in cytochrome oxidase. I MCD of the isolated enzyme and its derivatives. J Biol Chem 251: 7907–7919

Briat B, Berger D, Leliboux M (1972) Low-temperature magnetic circular dichroism of oxydized cytochromes. J Chem Phys 57: 5606–5607

Brittain T, Springall J, Greenwood C, Thomson AJ (1976) Low temperature studies on mixed valence cytochrome oxidase by using MCD. Biochem J 159: 811–813

Brittain T, Greenwood C, Springall JP, Thomson AJ (1978) MCD studies of heme *a* and its derivatives. Biochem J 173: 411–417

Dratz EA (1966) The geometry and electronic structure of biologically significant molecules as observed by natural and magnetic optical activity. Ph.D. Thesis, University of California, Berkeley, California

Dunford HB, Stillman JS (1976) On the function and mechanism of action of peroxidases. Coord Chem Rev 19: 187–251

Eaton WA, Hanson LK, Stephens PJ, Sutherland JC, Dunn JBR (1978) Optical spectra of oxy- and deoxyhemoglobin. J Am Chem Soc 100: 4991–5003

Figlovsky VA, Sharonov YA (1979) Magnetic dichrograph. Prib Teckh Exp (USSR) 4: 220–222

Fowler LR, Richardson SH, Hatefy Y (1962) A rapid method for the preparation of highly purified cytochrome oxidase. Biochim Biophys Acta 64: 170–173

Geraci G, Li T-K (1969) Circular dichroism of isolated and recombined hemoglobin chains. Biochemistry 8: 1849–1855

Hatano M, Nozawa T (1978) Magnetic circular dichroism approach to hemoglobin analysis. Adv Biophys 11: 95–149

Holmquist B (1978) The magnetic optical activity of hemoproteins. In: Dolphyn D (ed) The porphyrins, vol 3. Academic Press, New York, pp 249–270

Livshitz MA, Arutjunjan AM, Sharonov YA (1975) Multiplet structure of hemoproteins excited states. Dokl Akad Nauk USSR 223: 487–490

Livshitz MA, Arutjunjan AM, Sharonov YA (1976) Spin-orbit coupling in the excited states of ferricytochrome *c* and deoxyhemoglobin studied by magnetic circular dichroism. J Chem Phys 64: 1276–1280

MacLennon DH, Tzagoloff A (1965) The isolation of a copper protein from cytochrome oxidase. Biochim Biophys Acta 96: 166–168

Mineyev AP, Sharonov YA (1978) Orbit-orbit interaction between iron (III) Kramers doublet and porphyrin π-orbitals studied by magnetic circular dichroism of low-spin ferriheme compounds. Theor Chim Acta 49: 295–307

Mineyev AP, Sharonov YA, Sharonova NA, Figlovsky VA, Lysov YP (1983) $\pi-d$ interaction and the temperature dependent magnetic circular dichroism spectra of low spin Fe(III)-heme compounds. Theor Chim Acta (in press)

Nozawa T, Yamamoto T, Hatano M (1976) Infrared MCD of myoglobin derivatives. Biochim Biophys Acta 427: 28–37

Sharonov YA (1976) Magnetic optical activity of biologically significant molecules. Molek Biol Moscow Institute of Scientific Information 8: 70–161

Sharonov YA, Mineyev AP, Livshitz MA, Sharonova NA, Zhurkin VB, Lysov YP (1978) Magnetic circular dichroism studies of myoglobin, hemoglobin and peroxidase at room and low temperatures. Biophys Struct Mech 4: 139–158

Sharonov YA, Sharonova NA, Figlovsky VA, Grigorjev VA (1982) A comparison of the heme electronic states in equilibrium and nonequilibrium protein conformations of high-spin ferrous hemoproteins. Low temperature magnetic circular dichroism studies. Biochim Biophys Acta 709: 332–341

Seno Y, Kameda N, Otsuka J (1980a) The electronic structure of ferrous heme imidasole as the fifth ligand. I SCF LCAO-ASMO and CI calculation on the low-lying multiplets and optically allowed exited states. J Chem Phys 72: 6048–6058

Seno Y, Kameda N, Otsuka J (1980b) The electronic structure of ferrous heme with imidazole as the fifth ligand. II Explanation of MCD spectra from the near IR region to the Soret region. J Chem Phys 72: 6060–6069

Springall J, Stillman MJ, Thomson AJ (1976) Low temperature circular dichroism spectra of met- and myoglobin derivatives. Biochim Biophys Acta 453: 494–501

Stephens PJ (1974) Magnetic circular dichroism. Annu Rev Phys Chem 25: 201–232

Sutherland JC, Holmquist B (1980) Magnetic circular dichroism of biological molecules. Annu Rev Biophys Bioeng 9: 293–326

Thomson AJ, Brittain T, Greenwood C, Springall J (1976) Determination of the heme spin states in cytochrome *c* oxidase using MCD. FEBS Lett 67: 94–98

Thomson AJ, Brittain T, Greenwood C, Springall JP (1977) Variable temperature magnetic circular dichroism spectra of cytochrome *c* oxidase and its derivatives. Biochem J 165: 327–336

Treu JI, Hopfield JV (1975) Magnetic circular dichroism in hemoglobin. J Chem Phys 63: 613–623

Vickery L, Nozawa T, Sauer K (1976a) Magnetic circular dichroism studies of myoglobin complexes. Correlation with heme spin state and axial ligation. J Am Chem Soc 98: 343–350

Vickery L, Nozawa T, Sauer K (1976b) Magnetic circular dichroism studies of low-spin cytochromes. Temperature dependence and effects of axial coordination on the spectra of cytochrome *c* and cytochrome b_5. J Am Chem Soc 98: 351–357

Watters C (1978) A one-step biuret assay for protein in the presence of detergent. Anal Biochem 88: 695–698

Received July 12, 1982/Accepted January 21, 1983