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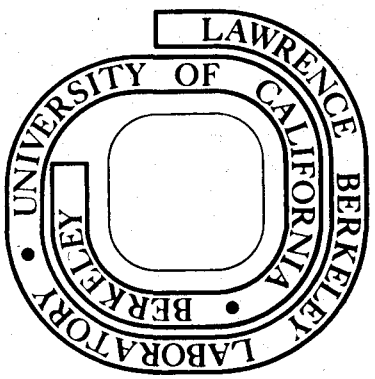

DISCLAIMER

Magnetic Circular Dichroism Studies of Myoglobin Complexes. Correlations with Heme Spin State and Axial Ligation[1]

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Running Title: MCD of Myoglobin Complexes

Abstract: Magnetic circular dichroism (MCD) spectra are reported for oxidized and reduced myoglobin complexes for the spectral region 300-650 nm from 22° to -196°C. For all paramagnetic derivatives, the effect of the heme iron atom on the MCD is reflected in the presence of Faraday C terms in the spectra. For the ferrimyoglobin derivatives investigated (cyanide, imidazole, azide, hydroxide, aquo and fluoride) the MCD intensity associated with the near UV Soret band was found to be correlated with the amount of low spin component present. While the MCD resembles the derivative of the absorption which is typical of Faraday A terms, the temperature dependence of the MCD of this band establishes that it is composed predominantly of C terms and is not a Faraday A term. The visible MCD spectra of the ferrimyoglobin complexes exhibit weaker A and C terms associated with the porphyrin π-π^* transitions and porphyrin-to-metal charge transfer bands. The shape of these bands is sensitive to the chemical nature of the sixth ligand even for similar spin states. In addition, the MCD spectra show the presence of previously unresolved absorption bands which may be charge transfer transitions in the 440-500 nm region. For ferromyoglobin the high spin aquo, or deoxy, form shows intense C terms in the region of the Soret band and weaker A and C terms associated with the visible bands. Only the low spin, diamagnetic oxygen and carbon monoxide complexes of reduced myoglobin exhibit the simple A terms associated with the porphyrin π-π^* transitions which are typical of other metalloporphyrins.

Introduction

Hemoproteins participate in a wide variety of biological processes, and a diversity of experimental approaches have been utilized in order to gain insight into their complex structure and functional capabilities. Recently a number of reports on the magneto-optical activity, both magnetic optical rotatory dispersion[2-8] (MORD) and magnetic circular dichroism[8-23] (MCD), have appeared. The results obtained thus far suggest that MORD and MCD spectra of hemoproteins, much like the absorption spectra, are quite sensitive to changes in the heme electronic structure. Many components of the absorption spectrum can be resolved by MCD, however, since the Faraday effect can have three different origins: (A) a Zeeman splitting of a degenerate ground or excited state; (B) mixing of the ground or excited state with other excited states by the magnetic field; and (C) a temperature-dependent population difference in ground state levels whose degeneracy has been removed by the magnetic field (see references 24 and 25 for recent reviews). While electron paramagnetic resonance (EPR) studies are also useful for investigating heme chemistry, they are restricted to Fe(III) complexes. In addition, low temperatures are required for EPR due to the rapid spin-lattice relaxation of the iron, whereas results at physiological temperatures and rapid kinetic measurements can be obtained by the MCD technique. MCD experiments can also be carried out with low, often less than micromolar, concentrations of heme.

The theory for the origin of the magnetically induced optical activity in hemes, however, is not well developed. Previous treatments[25] successfully applied to the MCD of non-iron metalloporphyrins[27] assumed a non-degenerate ground state for the system and predicted that no Faraday C

terms would exist for the predominantly π-π^* transitions of the porphyrin. While few investigators have measured the temperature dependence of the MCD of iron porphyrins, the results of Briat et al.[15] for ferricytochrome b_2 suggested that C terms could play an important role in the MCD of low spin oxidized heme even at room temperature. The results described herein demonstrate that this is also true for high spin and reduced heme complexes. This indicates that in the case of iron porphyrins the central metal must be considered explicitly in describing the MCD of the porphyrin system, which is not true for most other metalloporphyrins.[26] We have chosen to study ferri- (or met) and ferromyoglobin complexes rather than free heme as a model system for the MCD of heme derivatives, since the chemistry of this protein is well known and the polypeptide precludes aggregation of the heme. In addition, histidine, a common biological ligand, remains fixed in the fifth coordination position facilitating variation of a single sixth ligand. In a companion paper we will extend these studies to include two low spin cytochromes.[21] The correlation of the MCD spectra in the visible and near UV spectral regions with the heme redox state, the spin state and nature of the axial (5th and 6th) ligands illustrates the utility of MCD in probing the active center of hemoproteins.

Materials and Methods

Sperm whale met-myoglobin (A grade, purity > 98%) was obtained from Calbiochem. Complete formation of ferrimyoglobin was assured by oxidation with potassium ferricyanide and chromatography on Sephadex G-25. Reduction was carried out on deaerated solutions using a slight excess of sodium dithionite. Derivatives of aquo ferrimyoglobin were formed by the addition

of an excess of the sodium salt of the complexing anion; the hydroxide derivative was formed at pH 11.5. Oxy- and carbonmonoxymyoglobin were prepared by gentle bubbling of the purified gases thru solutions of ferromyoglobin. Titrations and concentrations were monitored spectrophotometrically using published extinction coefficients.[28,29]

Absorption spectra were recorded on a Cary Model 118C spectrophotometer. MCD measurements were obtained with a spectrometer designed in this laboratory and described elsewhere.[30] The instrument was calibrated using the natural CD of D-10-camphorsulfonic acid as a standard, with $\Delta\varepsilon_{290} = 2.20\ (cm \cdot M)^{-1}$. The MCD results are expressed as $\Delta\varepsilon/H$ $(cm \cdot M \cdot Tesla)^{-1}$, where 1 Tesla = 10,000 gauss. The magnetic field was determined with freshly prepared potassium ferricyanide, $\Delta\varepsilon_{422}/H = 3.0$ $(cm \cdot M \cdot T)^{-1}$, and was close to 1.4 T in ambient temperature (near 22°) experiments and approximately 0.9 T in experiments in which wider gap pole pieces were used to incorporate a dewar for low temperatures. Multiple scans were signal averaged, and natural circular dichroism was corrected for by reversal of the field direction.[30] Comparison of individual scans provided a check for time-dependent changes. All solid curves in Figures 1 and 3-9 represent computer plots of the raw data and illustrate the signal-to-noise levels obtained. Maximal absorbances of less than 1.5 were used. A 1-cm path cell was used for room temperature measurements; a 0.23-cm cell with a copper-constantan thermocouple was subjected to a stream of cold nitrogen gas or liquid nitrogen in a dewar for measurements to -196°. A constant slit width of 0.4 mm affording a spectral bandwidth of less than 2 nm was found sufficient to resolve all bands. Scan rates of 0.5 $nm\text{-}sec^{-1}$ for the Soret or near UV bands and 0.25 $nm\text{-}sec^{-1}$ for the visible bands were

routinely used in conjunction with a time constant of 0.3 sec. Noise levels for a single pass were generally $\Delta A \approx 2 \times 10^{-5}$.

Results

Ferrimyoglobin

Soret band. The room temperature MCD spectra in the region of the Soret band of several complexes of met or ferrimyoglobin are plotted in Figure 1. The wavelengths and ellipticities of the extrema and zero crossings are summarized in Table I. With the exception of the fluoride complex, the curves all have a similar shape which resembles the first derivative of the absorption spectrum. The shoulder to the blue (390-400 nm) of the main MCD feature corresponds to a shoulder also seen in the absorption spectra of these complexes and probably reflects a vibrational component of the main transition. The overlap of an "S-shaped" MCD associated with this band would be expected to decrease the intensity of the main positive peak and may account for some degree of the asymmetry observed in some of the spectra.

Magnetic susceptibility[31] and visible absorption[28] measurements have established that the binding of ligands to aquo ferrimyoglobin alters the high spin-low spin equilibrium present. This sequence is determined by both the spectrochemical and nephelauxetic series. The amount of low spin form present at room temperature decreases from approximately 100% to less than 3% in the order cyanide > imidazole > azide > hydroxide > thiocyanate > aquo > fluoride.[28,31] This same ordering is seen in the intensity of the Soret band MCD shown in Figure 1. The essentially pure low spin cyanide complex has the most intense MCD, while the almost completely high spin fluoride complex has only a very weak MCD and lacks the

characteristic "S-" or "derivative shaped" curve seen in complexes possessing significant amounts of low spin component.

In Figure 2 the absolute value of the MCD intensity at the trough to the long wavelength side of the Soret band (Figure 1 and Table I) is plotted against the percent of low spin form as determined by susceptibility[31] and absorption[28] methods. For mixed spin complexes which are predominantly high spin there is a linear rise in the MCD intensity as the amount of the low spin form increases. At higher proportions of low spin this relation does not appear to hold rigorously. The azide and imidazole complexes show weaker intensities than would be expected on the basis of the observed MCD arising solely and directly from the low spin form. While the extrema of such a derivative-shaped MCD curve would be expected to be quite sensitive to the intensity and bandwidth of the absorption spectrum, no significant change in the plot was obtained when these factors or integrated MCD areas and oscillator strengths were compared. Measurement of the MCD peak to the short wavelength side of the Soret band is complicated by overlap with the vibrational component. We have measured the MCD intensity of some other hemoproteins which are thought to be completely low spin at room temperature, including cytochrome b_5[21] (cyt b_5), cytochrome c[21] (cyt c) and proto- and deuteroheme hemopexin[23] (p- and dRHx), and these are also given in Figure 2 and compared with the ferrimyoglobin cyanide complex.

The effect of temperature on the MCD of the Soret band was first measured for cytochrome c,[14] but no significant changes were detected for temperatures down to -100°. Experiments in which MCD spectra were recorded for ferricytochromeme b_2 at 6°K and 11°K, however, clearly showed temperature

Table I. Absorption and Magnetic Circular Dichroism of the Soret Transition of Ferrimyoglobin Complexes

	Absorption		MCD	
Ligand	λ_{max}(nm)	$\varepsilon x10^{-3}$ $(cm \cdot M)^{-1}$	λ(nm)	$\Delta\varepsilon/H(cm \cdot M \cdot T)^{-1}$
Cyanide	423	110	415	72
			423	0
			431	-91
Imidazole	416	121	408	51
			415	0
			423	-63
Azide	421	112	415	39
			423	0
			430	-45
Hydroxide	414	97	412	25
			421	0
			428	-25
Thiocyanate	413	132	410	20
			419	0
			427	-18
Aquo	409	157	404	14
			413	0
			429	-13
Fluoride	416	133	403	-4
			410,419	0
			413	2
			426	-2

dependent increases in the MCD associated with the Soret band.[15] Our results showing the effect of liquid nitrogen temperature on the spectrum of ferrimyoglobin cyanide are given in Figure 3. A large difference in the MCD

intensity is observed between 22 and -196°. The increase in the intensity at the trough is approximately 5-fold while that at the peak is about 2.4-fold. On the basis of a Boltzman distribution (1/T dependence) one would expect a 3.8-fold change. The near agreement of these values together with the lack of any major change in band shape suggests that the spectrum observed at room temperature is composed primarily of C terms. The slightly larger increase for the trough may indicate the presence of a weaker, temperature independent, positive component (A or B term) at this wavelength. A slight sharpening of the absorption band and a decrease in the MCD peak-to-trough splitting were also observed, however, and the deviation may partially reflect band narrowing.

Low temperature MCD spectra were also recorded for ferrimyoglobin fluoride in the near UV region. For temperatures to -132° only small changes in both shape and intensity were observed (not shown) indicating the possible presence of weak C terms.

Visible region. For this spectral region the results have been grouped into the predominantly low and predominantly high spin cases and then further divided according to the type of ligand which has been bound to the sixth coordination position of the ferric iron.

The room temperature MCD spectra of the imidazole and azide complexes of ferrimyoglobin are shown in Figure 4. The curve of the latter derivative resembles that reported by Bolard and Garnier.[13] Both of these complexes are predominantly low spin and their absorption and MCD spectra are similar in their general features. Weak negative extrema with shapes resembling C terms are observed in the MCD spectra at 647 nm for the imidazole complex and at 643 nm for the azide complex; these correspond

to shoulders seen in the absorption spectra in this region. In the case of ferrimyoglobin azide this band is thought to arise from a ligand- or porphyrin-to-metal charge transfer transition.[32,33] Sharp A terms with crossovers at 564 and 575 nm correspond to the Q_0 or α absorption bands of the imidazole and azide complex, respectively. The greatest difference in the structure of the two MCD curves is seen in the 440-540 nm region, to the high energy side of the Q_v or β absorption bands which occur at 535 nm for the imidazole complex and at 540 nm for the azide complex. These bands may reflect charge transfer transitions located between the main Soret and visible π-π^* bands. The shape of these MCD bands resembles that expected for C terms.

MCD spectra in the same wavelength region are given in Figure 5 for the cyanide complex. The room temperature curve (- - - -) of this low spin derivative has the same general shape and origins of the preceding low spin forms (Figure 4) but differs in detail. No absorption peak or MCD trough appears in the 650 nm region. This band, which is expected to be quite sensitive to axial ligand effects on the metal d orbitals,[32-34] may be shifted in energy. The 582 nm trough, 567 nm zerocrossing, and 565 nm shoulder probably constitute the A term expected for the Q_0 band. This is weaker than the A terms observed for the imidazole and azide complexes, and probably results from the fact that the Q_0 absorption band is broader and less well resolved in the cyanide complex. The peaks to the blue of the band are also different from the nitrogen coordinated complexes, as would be expected for charge transfer transitions.

The effect of temperature on the MCD of ferrimyoglobin cyanide is also shown in Figure 5. Faraday C type terms are observed in the region

of both the π-π^* transitions and charge transfer bands. The peak at 487 nm shows a clear linear dependence upon the inverse of the absolute temperature (inset, Figure 5). The intercept extrapolated to infinite temperature is near zero, and thus there must be few other components present at this wavelength. The peak at 457 nm also shows a strong temperature dependence but overlaps with the negative MCD associated with the long wavelength tail of the Soret band. The low temperature behavior of the region near the Q_o band is more complicated. A peak rises near 600 nm and a trough begins to appear at 575 nm. The inset of Figure 5 shows that this increase is linear with 1/T. This behavior in the visible, an increased positive MCD at longer wavelengths and an increased negative MCD at shorter wavelengths, is just the reverse of that found for the near UV π-π^* band. The small changes observed and the overlap with the A term present, however, make the effects difficult to analyze.

The visible region MCD spectra of the predominantly high spin derivatives of ferrimyoglobin are shown in Figure 6. These ave been grouped into those low, fluoride and hydroxide, and those high, aquo and thiocyanate, in the spectrochemical series. Our results are qualitatively similar to thos which Bolard and Garnier[13] reported for the 450-620 nm region. These spectra are more complex than those of the low spin derivatives. The porphyrin π-π^*, Q_o and Q_v bands are thought to mix with charge transfer bands so that none of the bands in the 500-650 nm region can be considered pure transitions.[32-34] The long wavelength charge transfer bands occur near 600 nm in the hydroxide and fluoride complexes, and these derivatives exhibit similar spectra. In both the aquo and thiocyanate complexes the low energy charge transfer band is found near 640 nm. In these derivatives the spectra are also similar

to one another but differ from the fluoride and hydroxide complexes. This is probably because the charge transfer band, located at longer wavelength, interacts more weakly with the π-π^* transitions.

The MCD peaks observed from 440-480 nm in the hydroxide and fluoride complexes (Figure 6A) and from 460-490 nm in the aquo and thiocyanate complexes (Figure 6B) do not have obvious counterparts in the absorption spectra and would appear to lie at too high energy to correspond to part of the Q_v transition. They are similar to MCD peaks observed in the same region for the low spin complexes (Figures 4 and 5) and may also originate from heretofore unresolved charge transfer transitions. Low temperature MCD spectra to -147° were recorded for the aquo and fluoride complexes, bu these did not clarify the spectra. Some increase in the intensity, but little change in shape, of all bands was observed.

Ferromyoglobin

Deoxymyoglobin. The near UV and visible MCD spectra of reduced or ferromyoglobin obtained under anaerobic conditions are shown in Figure 7. In the Soret region the MCD shows an intense peak at 437 nm, a crossing at 427 nm, and a trough at 420 nm with a negative shoulder near 405 nm. In the absorption spectrum the Soret peak occurs at 434 nm and gives no evidence for the presence of a shoulder at higher energy. The small trough on the shoulder seen at 405 nm could, however, correspond to a vibrational component of the main band with the same shape of MCD since its spacing from the trough at 420 nm is about that expected, i.e., ca. 1000 cm^{-1}. This could account for the asymmetry of the MCD bands. The temperature dependence of the Soret MCD indicates that it is composed predominantly of C terms; the increase in intensity by a factor of 1.54 is exactly what is expected on the basis of a Boltzman distribution.

The room temperature visible region MCD spectrum of deoxymyoglobin is similar to that reported by Bolard and Garnier[13] and shows more structure than the MCD in the near UV. A negative MCD appears near 630 nm, where a broad band is seen in the absorption spectrum. This trough shows a strong temperature dependence indicating the presence of C terms. A peak occurs at 573 nm which becomes more intense and shifts to 571 nm as the temperature is lowered. This is in the region where the Q_o or α absorption band is expected. It is not clear whether the temperature dependence reflects C terms or is due to a sharpening or narrowing of the A term expected for the Q_o band, but the effect is almost linear from 26° to -158° (insert Figure 7B). The fine structure in the 500-560 nm region of the MCD is probably associated with the Q_v bands and shows some temperature dependence which may also arise from band narrowing.

Oxymyoglobin. The binding of oxygen to high spin ferromyoglobin converts the iron to the low spin diamagnetic form. The MCD spectrum of the hemochrome formed is shown in Figure 8. In the near UV a simple S-shaped MCD is observed with a negative extremum at 427 nm, a crossover at 418 nm and a peak at 408 nm. This curve closely resembles the first derivative of the absorption spectrum whose maximum occurs at 418 nm. On lowering the temperature to -107° the MCD intensity increased by about 10-20% and shifted to the blue 2 nm. These changes were accompanied by an increase in the intensity of the absorption and CD spectra, also suggesting that the changes reflect band narrowing phenomena rather than the presence of C terms. Thus we assign the Soret MCD as the expected Faraday A term.

The results from 500-620 nm are in general agreement with those of Bolard and Garnier.[13] There is a large A term associated with the Q_o

absorption band at 581 nm. The wavelengths of the MCD trough, crossover and peak are 584, 578 and 572 nm, respectively. An additional A term centered about 540 nm is associated with the Q_v band. The small trough near 645 nm and the peak near 545 nm probably arise from a small amount of ferrimyoglobin; evidence for the presence of 5-10% oxidized myoglobin was obtained from the absorption spectrum. No temperature dependent MCD measurements were carried out in view of the extreme sharpening of the visible absorption bands of oxymyoglobin at low temperatures.[35] Although no C terms are expected, narrowing would give rise to a strong temperature dependence of the MCD A terms.[36]

Carbonmonoxymyoglobin. The MCD spectrum of the low spin carbon monoxide adduct shown in Figure 9 is very similar to that of oxymyoglobin. The Soret band exhibits an A term with trough, crossover and peak wavelength values of 427, 422 and 417 nm, respectively. This corresponds to the absorption maximum which occurs at 422 nm; the shoulder at 396 nm is probably an A term associated with the vibrational component seen at 400 nm in the absorption spectrum. The twofold decrease in Soret bandwidth of carbonmonoxy- relative to oxymyoglobin results in a fivefold, or approximate inverse square, increase in the MCD intensity.

In the visible region, on the other hand, the A term associated with the Q_0 band is broader for carbonmonoxy- than for oxymyoglobin. Tne trough, zero crossing and peak wavelengths for the carbon monoxide derivative are 581, 572 and 565 nm, respectively, and the increase of 4 nm in the peak-to-trough splitting is reflected in a lowered intensity relative to the oxygenated form.

The liquid nitrogen temperature MCD spectrum of carbonmonoxymyoglobin is also shown in Figure 9. A slight sharpening and an increase in intensity

of the visible and Soret A terms are evident, but the most striking feature is the appearance of a new peak at 441 nm. A porphyrin-to-iron charge transfer band was predicted for this region,[32] but the origin of the temperature dependence of the MCD is not clear. There was no indication of a new band in the low temperature absorption spectrum.

Discussion

The heme chromophore of myoglobin has approximately D_{4h} symmetry; thus the porphyrin π^* excited state is nearly X-Y degenerate. Transitions to this state should therefore give rise to Faraday A terms when a magnetic field is used to remove the excited state orbital degeneracy. These should dominate the B terms which appear more intense in the D_{2h} case. This is what is observed for the visible (Q) and near UV (Soret) porphyrin π-π^* bands in the cases where a diamagnetic (S=0) complex is investigated. Thus the MCD spectra of oxy- and carbonmonoxymyoglobins resemble those of other reduced, low spin hemoproteins such as cytochrome c,[9,10,14,17,19,21] cytochrome b_5,[17,18,21] cytochrome b_2,[15,19] hemo$_r$exin[23] oxy- and carbonmonoxy-hemoglobins,[8-11] and carbonmonoxy cytochrome P-450.[18,22] In fact, the spectra are quite similar to the MCD spectra of other metalloporphyrins[25,37] and appear to be relatively insensitive to the chemistry of the iron. An exception to this appears to be the temperature dependent MCD band near 441 nm in carbonmonoxymyoglobin.

In the cases of the high spin (S=2) ferrous, and low (S=1/2) and high spin (S=5/2) ferric hemoproteins, however, the spectra are more complex. This is due both to the presence of new transitions, charge transfer bands (and possibly weaker d-d transitions), and to the unpaired electrons on the iron which produce a spin degeneracy for the heme system. The charge

transfer transitions are sensitive to the energy levels of the iron d orbitals and hence reflect directly any changes in the environment of the iron. The ground state spin degeneracy gives rise to two effects: first, a paramagnetic or C term MCD is produced, and second, the spin angular momentum of the iron couples with the orbital angular momentum of the porphyrin to split the porphyrin energy levels.[19,20] Thus the C terms present in the MCD associated with the porphyrin π-π^* transitions are also likely to be quite sensitive to changes in the iron electronic state. The spin-orbit coupling which gives rise to the temperature dependent MCD in the heme group does not appear to be a strong interaction in the cases of other metalloporphyrins, since the MCD spectra of some paramagnetic copper and cobalt complexes do not show C terms even at 8°K.[37] This is probably due to the fact that in these cases the unpaired spin is in a metal d_z2 or $d_{x^2-y^2}$ orbital which is farther removed in energy from the filled porphyrin π orbitals than the d_{xz} and d_{yz} orbitals of the iron complexes and would not be expected to mix so well.[38]

Deoxymyoglobin is the only high spin ferrous complex investigated in this report. Other examples of reduced hemoproteins which are high spin are deoxyhemoglobin, ferroperoxidase and ferrocytochrome P-450. The Soret MCD spectra of the myoglobin, hemoglobin[20] and peroxidase[39] derivatives are all very similar and seem to be consistent with the spin-orbit coupling theory advanced by Treu and Hopfield[20] to account for the origin of the C terms. These proteins all have Soret absorption maxima in the 430-437 nm region and exhibit an intense, positive MCD slightly to the red of the absorption peak and a weaker, negative MCD to the blue. This "plus-to-minus" pattern with decreasing wavelength is reversed from that seen in the low spin ferric hemoproteins and corresponds to a spin-orbit coupling constant

of opposite sign. The shape of the MCD for these proteins, however, is quite asymmetric and the zero crossing is considerably blue shifted from the absorption maximum. Reduced cytochrome P-450, on the other hand, has a Soret maximum near 410 nm and a nearly symmetric MCD spectrum approximating the derivative of this band.[22] In addition, the sign of the Soret MCD is opposite to that of the other ferrohemoproteins, i.e., it exhibits a minus-to-plus pattern through the absorption band. These results can be explained by assuming that either 1) the theory and the sign of the spin-orbit coupling constant used by Treu and Hopfield[20] is correct but does not apply to cytochrome P-450; or 2) the intense positive MCD band near 440 nm is not due to the Soret transition, but arises from another absorption band and tends to obscure the Soret MCD, and the sign of the spin-orbit coupling constant used by Treu and Hopfield should be reversed. In either case these studies show that differences are seen in the MCD spectra of high spin reduced hemoproteins which carry out different biochemical functions.

The clearest correlation between the heme electronic state and the MCD is seen in the Soret spectra of the ferrimyoglobin complexes. An intense derivative-shaped MCD curve is associated with the low spin forms, while high spin forms show a much weaker MCD. A dependence of the Soret MORD intensity upon spin state has also been observed with ferrihemoglobin derivatives.[7a] Other completely low spin ferrihemoproteins such as cytochrome b_2,[15,17] cytochrome b_5,[17,18,21,22] cytochrome c,[9,10,14,17,21] cytochrome P-450,[18,22] hemopexin[23] and hemoglobin cyanide[20] exhibit Soret MCD spectra similar in shape and intensity to myoglobin cyanide. This suggests the utility of this band as a marker for the low spin state in oxidized hemoproteins and for monitoring changes in spin state equilibria.

The temperature dependence of Soret MCD of the low spin forms of ferrimyoglobin described here and of cytochrome b_2[15] clearly establish that the spectrum is composed predominantly of C terms. The derivative-like shape results from the overlap of two C terms of opposite sign displaced in energy about the absorption maximum. This splitting is expected when the ground state is spin degenerate and the excited state is orbitally degenerate and when these are mixed by spin-orbit coupling.[19,20] The intensity of the observed MCD thus depends upon the strength of the spin-orbit interaction which gives rise to the splitting. This in turn is a function of the overlap or mixing of the iron d and porphyrin π orbitals. The fact that the Soret MCD of low spin heme is much stronger than that of high spin heme suggests that the degree of iron electron delocalization is considerably greater for the low spin complexes. This is expected from the stereochemical structures of the two forms, which show that the iron atom is out of the porphyrin plane in high spin complexes.[40]

The visible region porphyrin π-π^* transitions in the low spin complexes exhibit the A term expected for the Q_o b nd. Evidence for some C term contribution in this region is obtained in the low temperature spectra, but these effects are much weaker than those observed for the Soret band and appear to be reversed in sign. MCD spectra at lower temperatures, such as those reported by Briat et al.,[15] would assist in the assignment of Faraday parameters for this spectral region, since further changes in shape may occur between liquid nitrogen and liquid helium temperatures. A molecular orbital description including the iron would also be of value in interpreting the visible and Soret spectra. While the Q_v bands of the low spin derivatives show a greater absorption intensity than the Q_o bands, the MCD of the Q_v bands is much weaker. This is due to the fact that vibrational

components of different symmetry can have A terms of opposite sign which will tend partially to cancel one another.[10]

In addition to the effects of unpaired spins in the metal on the porphyrin π-π^* transitions, iron d orbitals lying close in energy to the porphyrin orbitals allow for relatively low energy charge transfer transitions not present in most other transition metal porphyrins.[32,38] Stephens et al.[16,19] investigated the MCD of the near infrared charge transfer bands of several ferrihemoglobin derivatives and found a dependence of the shape of the MCD upon the spin state which could be explained by a spin-orbit coupling model. In the 500-650 nm region, our results for predominantly high spin derivatives show that mixing of the charge transfer bands with the Q bands precludes any simple interpretation, although a correlation between the general shape of the MCD spectrum and the position of the sixth ligand in the spectrochemical series is noted. Between the main visible bands and the Soret band, however, the MCD spectra provide evidence for transitions not resolved in the absorption spectra. These occur at 440-480 nm in the hydroxide and fluoride complexes, 460-490 nm in the aquo and thiocyanate complexes and 440-500 nm in the low spin complexes. Because of the overlap with the Soret and visible bands it is difficult to analyze their shape, but the temperature dependence of the intensity of the MCD of the ferrimyoglobin cyanide complex clearly suggests that the bands are C terms. The exact shape and position of the MCD of these bands is sensitive to the nature of the sixth ligand. This behavior is expected for charge transfer transitions, since the band positions will reflect the effects of the ligand field strength on the iron d orbital energy levels. Thus, this spectral region may provide

a means of monitoring changes in axial coordination in hemoproteins. An empirical study of the shape of the MCD in this region as well as an extension of the conclusions reached from the other spectral regions of the myoglobin complexes to low spin cytochromes is considered in the following paper.[21]

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Figure Legends

Figure 1. MCD spectra of the Soret band of ferrimyoglobin complexes. Concentrations: approximately 10^{-5} M; solvent: 0.1 M sodium phosphate, pH 6.8, except hydroxide pH 11.5; pathlength: 1 cm; magnetic field: 1.4 Tesla; 8 to 10 passes were signal averaged, 4 to 5 for each field direction, to improve the signal-to-noise ratio by a factor of about 3; temperature: ambient (near 22°). Note that the right-hand ordinate is an expanded scale.

Figure 2. Correlation of the Soret MCD intensity of ferrimyoglobin complexes with low spin content. The strength of the negative MCD extremum to long wavelength of the Soret band is from Figure 1; the percentage low spin form is taken from references 28 and 31.

Figure 3. The effect of temperature on the Soret MCD of ferrimyoglobin cyanide. The sample was 6 x 10^{-5} M in a solvent of potassium glycerophosphate, glycerol, 0.1 M sodium phosphate (1:1:1 :: v/v/v) at pH 6.8; path = 0.23 cm; field = 0.9 T; 4 passes averaged. Note that the abscissa is linear in energy ($\tilde{\nu}$).

Figure 4. MCD spectra of the imidazole and azide complexes of ferrimyoglobin. Protein approximately 6 x 10^{-5} M; in 0.1 M sodium phosphate, pH 6.8, with 0.5 M imidazole (Im ——) or 10^{-2} M azide (N_3 ---); path = 1 cm; field = 1.4 T; 6 passes averaged; near 22°.

Figure 5. MCD spectra of ferrimyoglobin cyanide as a function of temperature. Protein 2.7 x 10^{-4} M; in a 3:1 v/v mixture of glycerol:0.1 M

Figure Legends (Cont.)

sodium phosphate, pH 6.8 with 5 x 10^{-3} M cyanide; path = 0.23 cm; field = 0.9 T; 4 passes averaged. Room temperature spectra in buffer alone were indistinguishable from those in the glycerol system. The inset shows a plot of the MCD intensity of the peaks near 487 and 600 nm vs. the reciprocal of the absolute temperature.

Figure 6. MCD spectra of the visible bands of high spin complexes of ferrimyoglobin. Concentrations approximately 6 x 10^{-5} M in 0.1 M sodium phosphate, pH 6.8, with (A) 1.5 M fluoride (F^- ——) or pH 11.5 (OH^- ----), (B) 1 M thiocyanate (SCN^- ----) or no addition (H_2O ——); path = 1 cm; field = 1.4 T; 6 passes averaged; near 22°.

Figure 7. MCD spectra of deoxymyoglobin as a function of temperature. Protein concentration 6.7 x 10^{-5} M in the near UV (A) and 3.3 x 10^{-4} M in the visible (B); solvent equal volumes of potassium glycerophate, glycerol, 0.1 M sodium phosphate at pH 6.8; path = 0.23 cm; field = 0.9 T; 2 passes averaged in (A), 4 in (B). No effect of the solvent relative to spectra recorded in buffer alone were observed. The inset shows the dependence of the extremum near 571 nm upon the reciprocal of the absolute temperature.

Figure 8. MCD spectrum of oxymyoglobin. Protein concentration 8.5 x 10^{-6} M in the near UV and 6.2 x 10^{-5} M in the visible; 0.1 M sodium phosphate, pH 6.8; path = 1 cm; field = 1.4 T; 12 passes were averaged in the near UV, 4 in the visible.

Figure Legends (Cont.)

Figure 9. MCD spectra of carbonmonoxymyoglobin as a function of temperature. Protein concentration 2.8×10^{-5} M in an equal volumes mixture of potassium glycerophosphate, glycerol and 0.1 M sodium phosphate, pH 6.8; path = 0.23 cm; field = 0.9 T; 4 passes averaged. Essentially identical spectra were recorded in buffer alone at 22°.

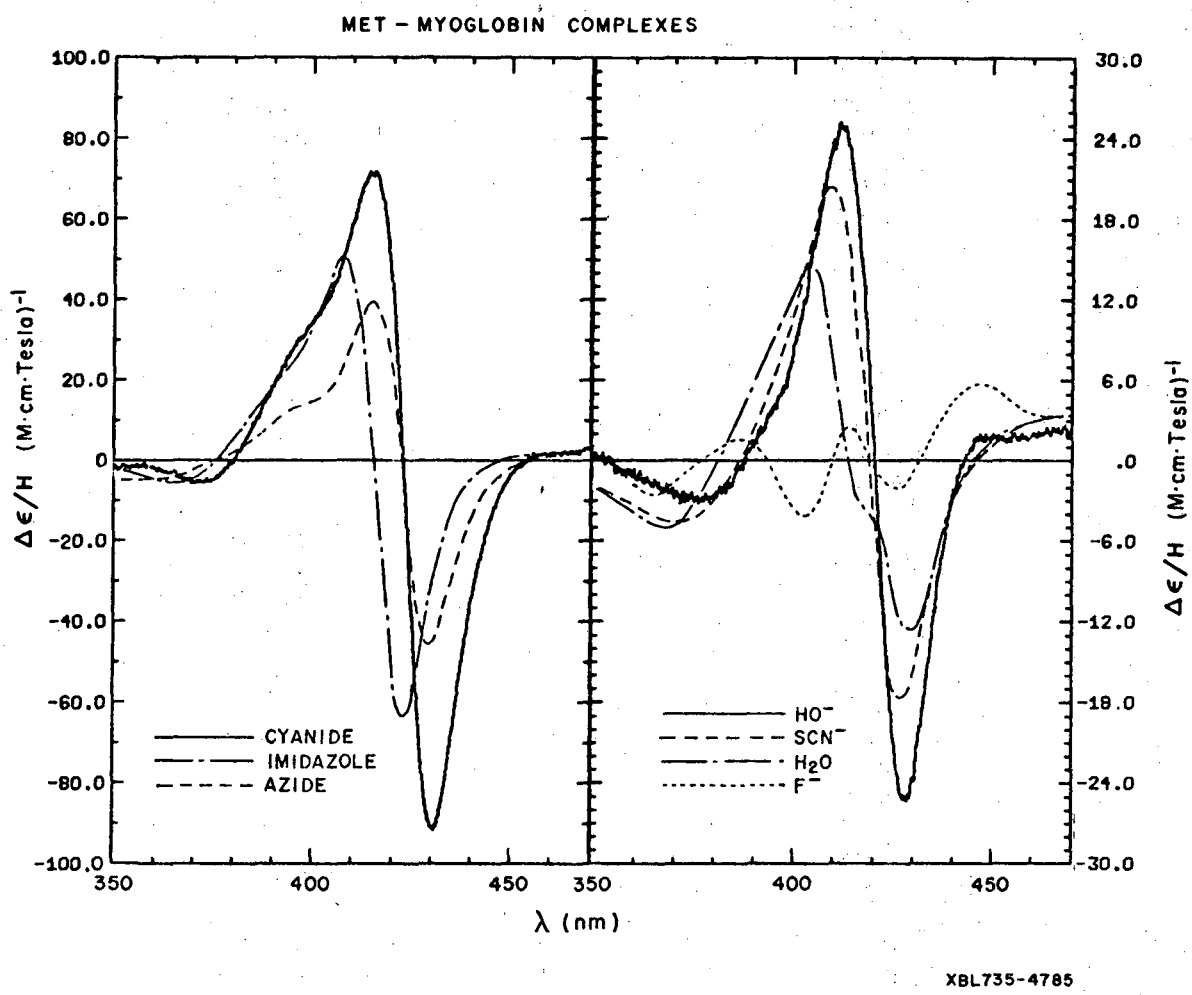
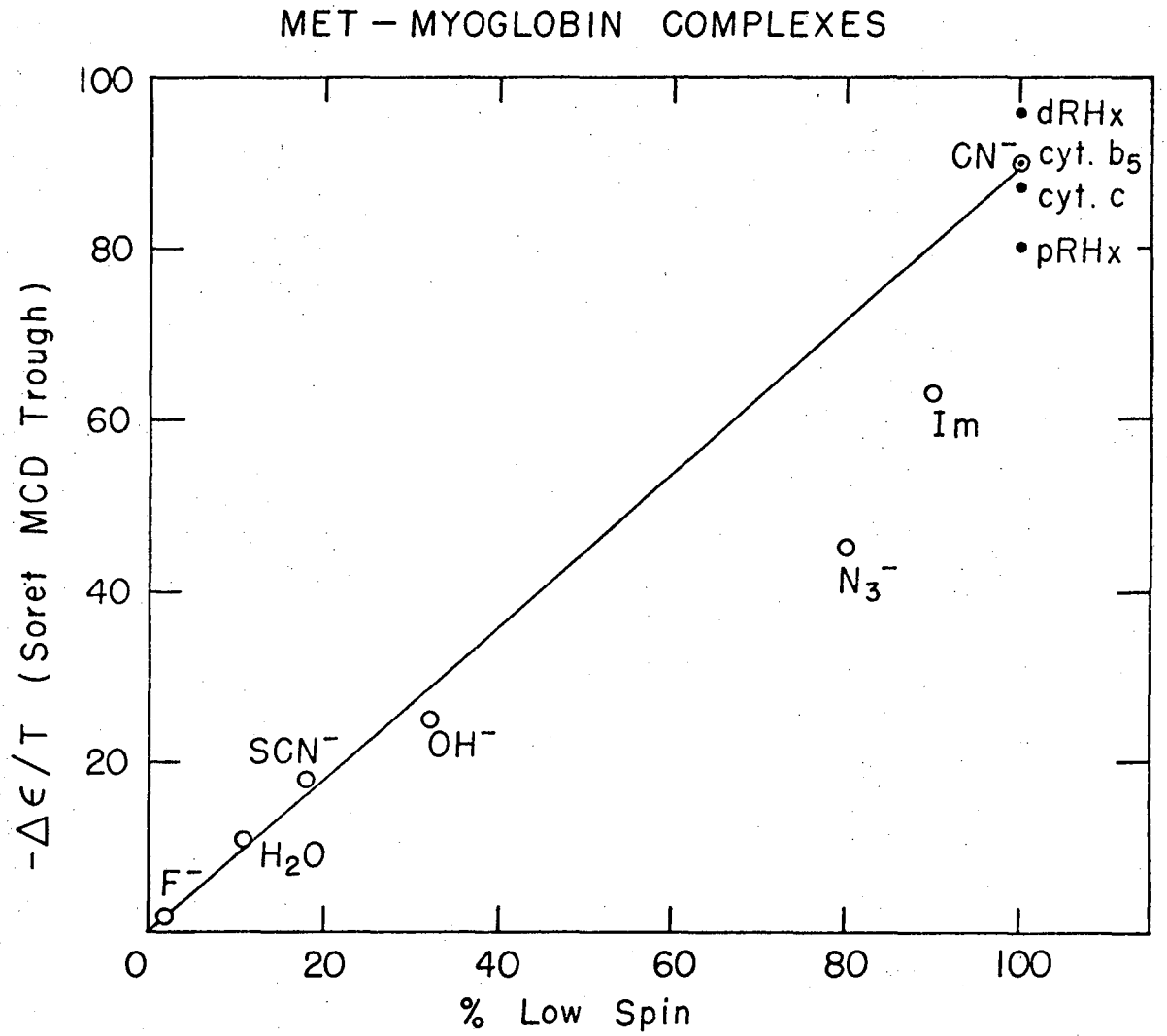


Fig. 1.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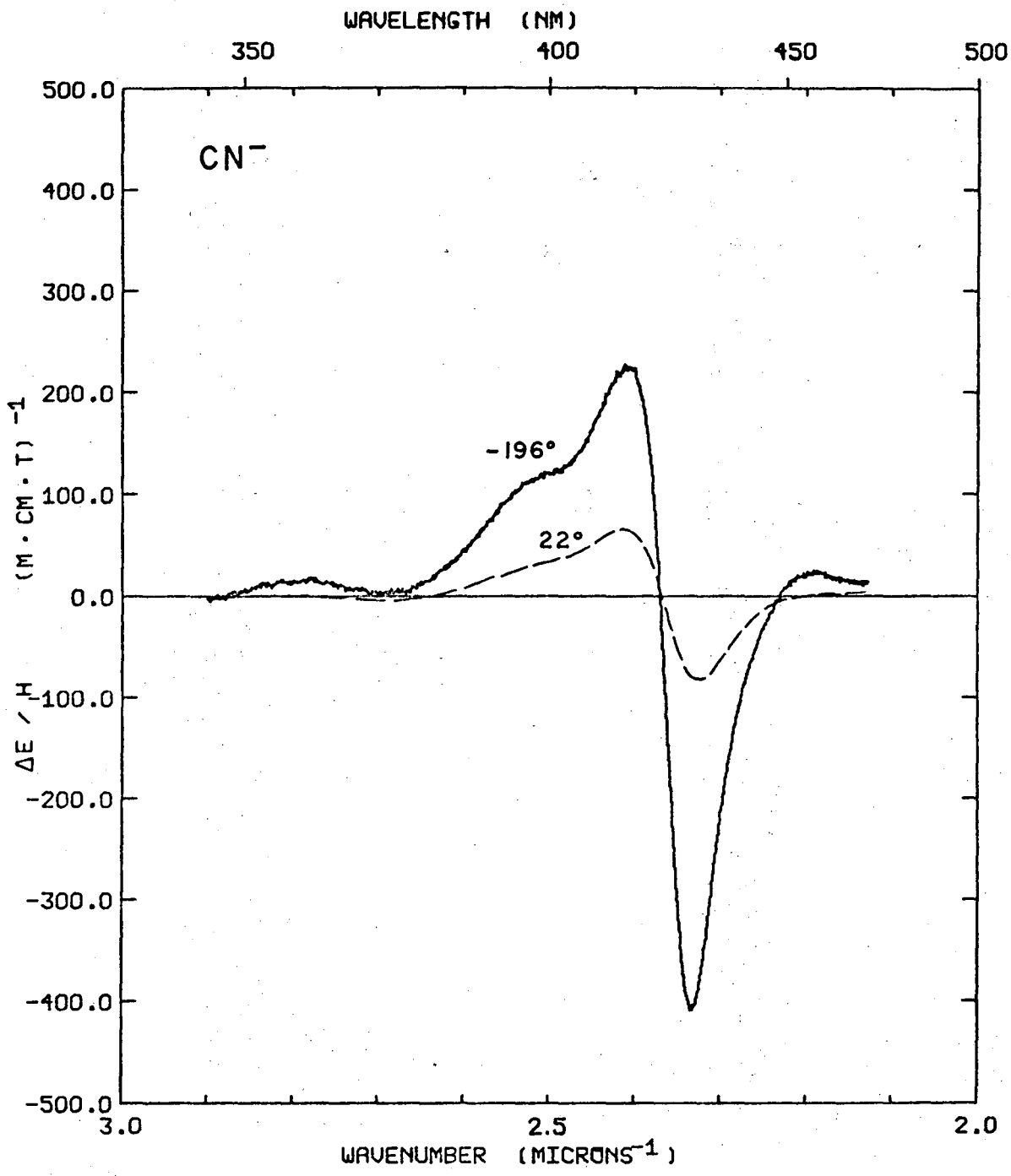
60
40
20
0
20
40
60
80
100
-Δε/T (Soret MCD Trough)
% Low Spin
dRHx
CN⁻
cyt. b5
cyt. c
pRHx
Im
N3⁻
OH⁻
SCN⁻
H2O
F⁻
XBL747-5273

Fig. 2.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-100.0
-200.0
-300.0
-400.0
-500.0
CN⁻
-196°
22°
(M · CM · T) -1
ΔE / H
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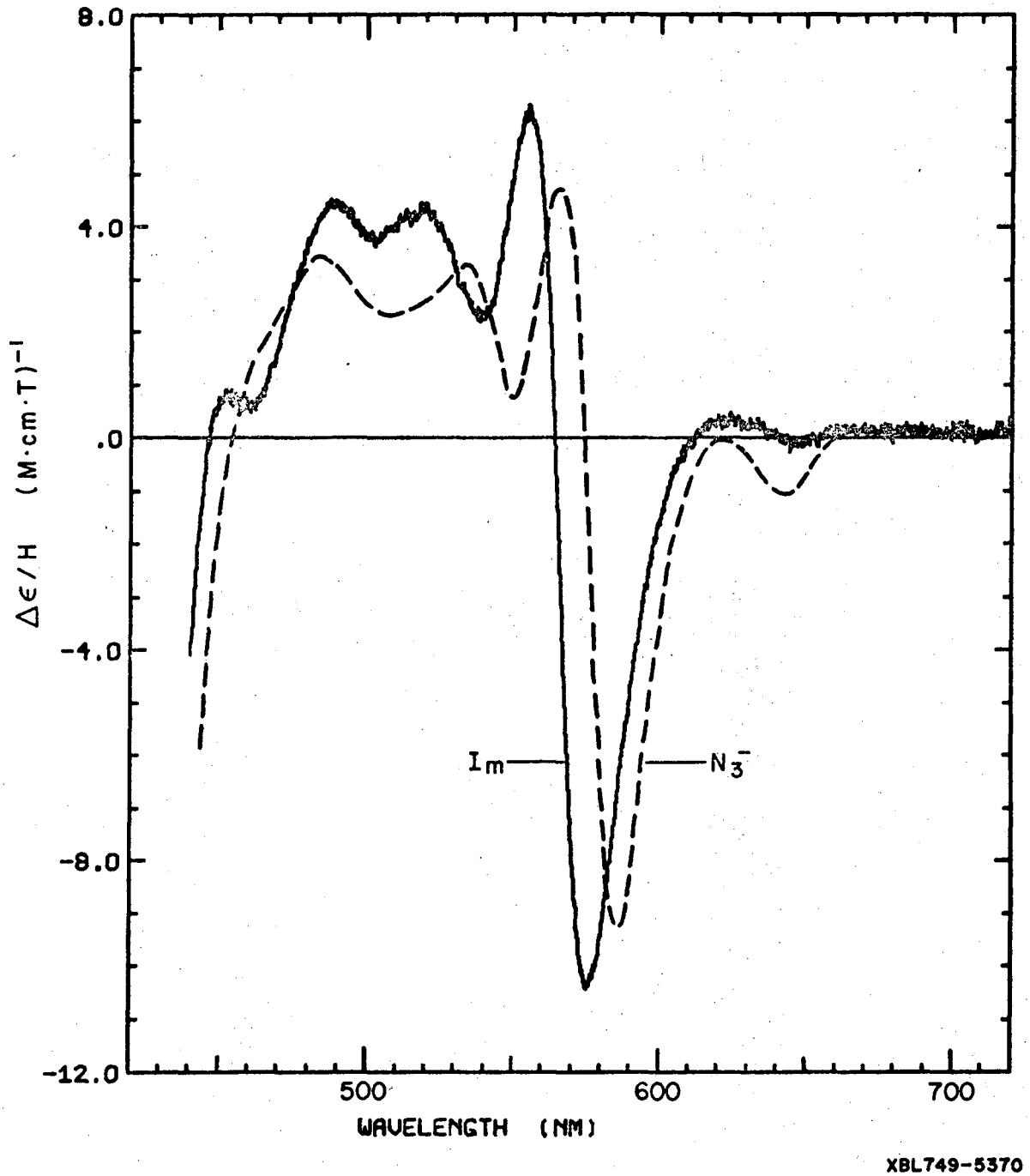
2.0
WAVENUMBER (MICRONS-1)
XBL742-5067

Fig. 3.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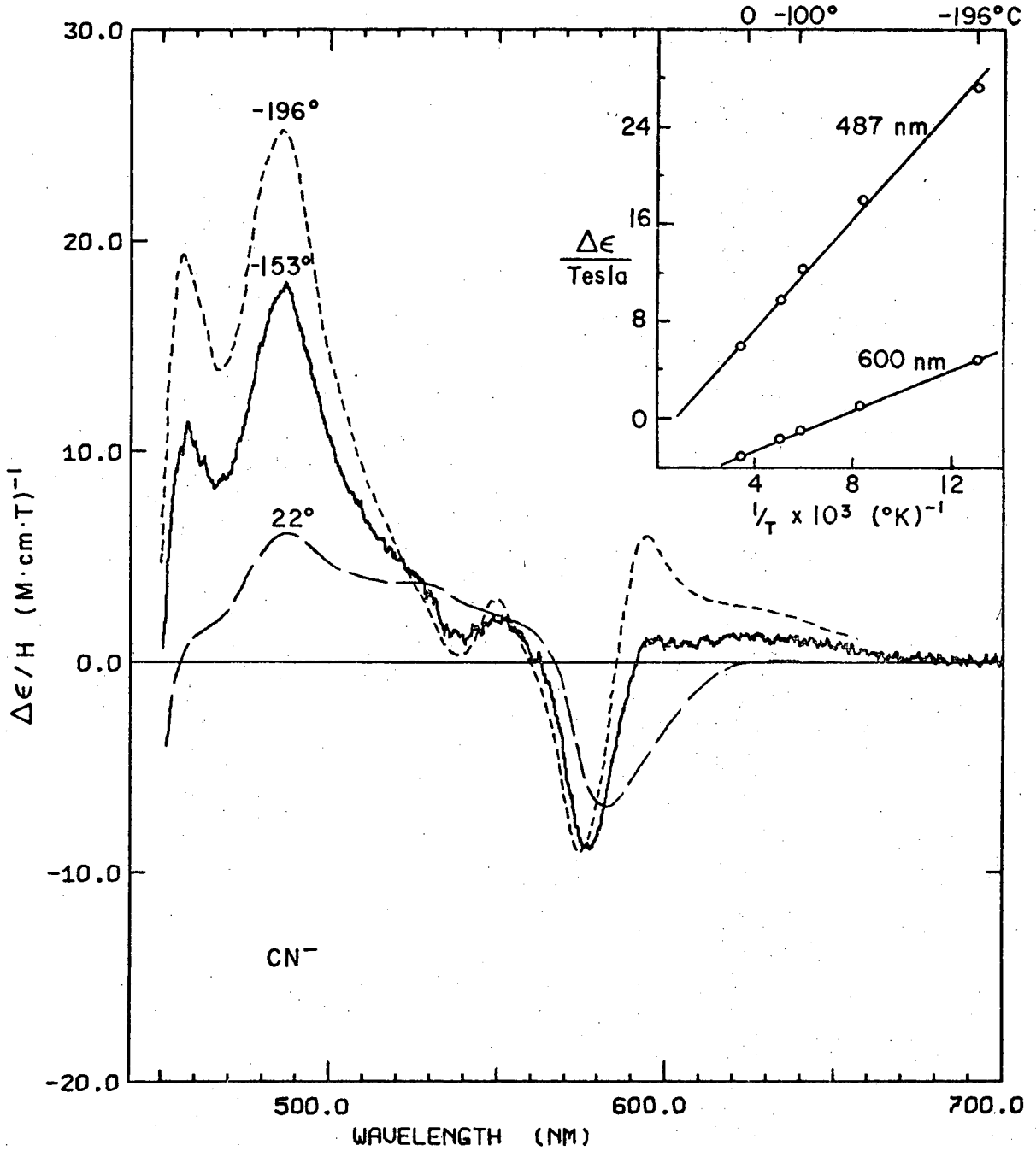
WAVELENGTH (NM)
Im
N3⁻
XBL749-5370

Fig. 4.



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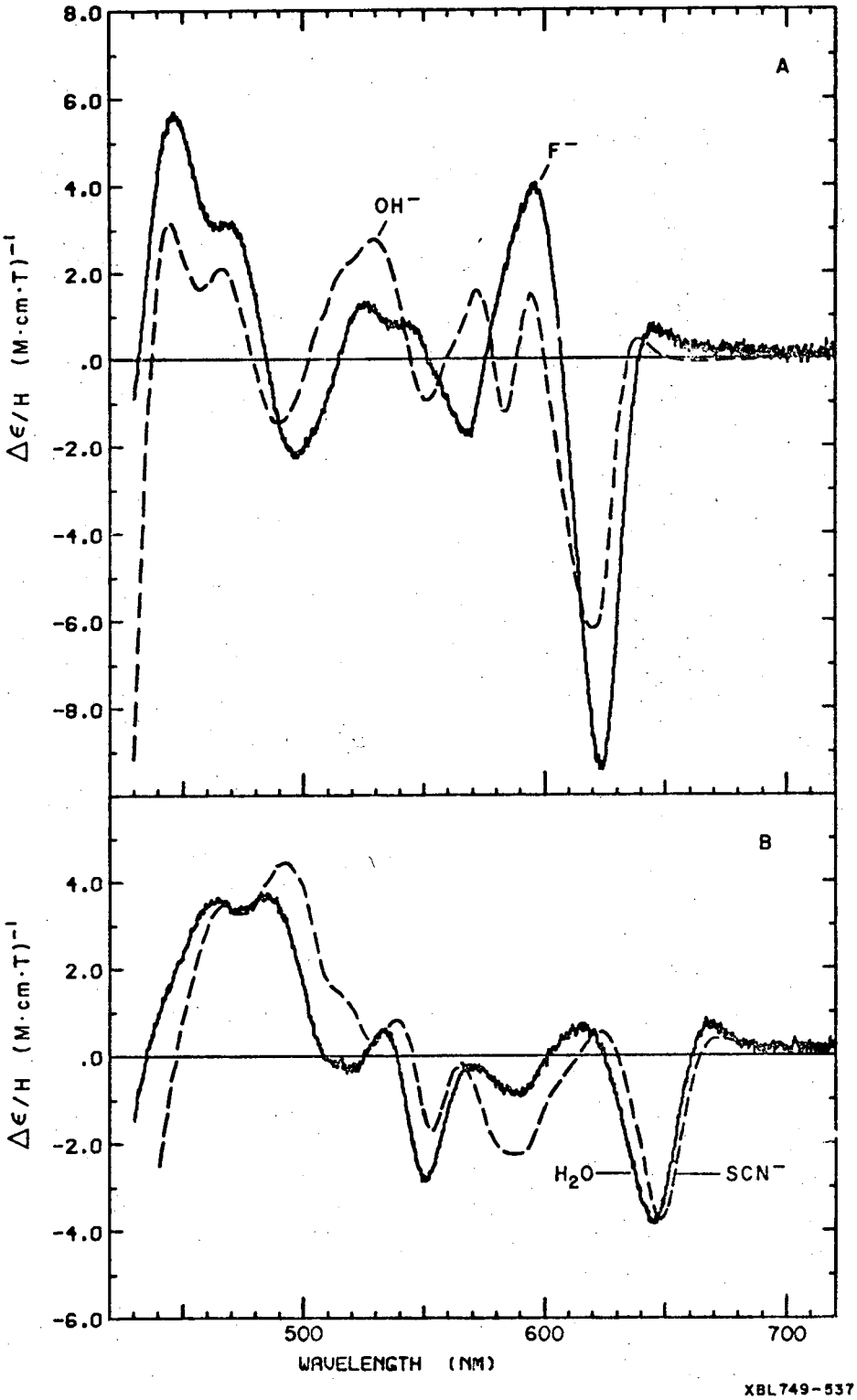
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F⁻
OH⁻
Δε/H (M·cm·T)⁻¹
4.0
2.0
.0
-2.0
-4.0
-6.0
B
H₂O
SCN⁻
Δε/H (M·cm·T)⁻¹
500
600
700
WAVELENGTH (NM)
XBL749-5371

Fig. 6.

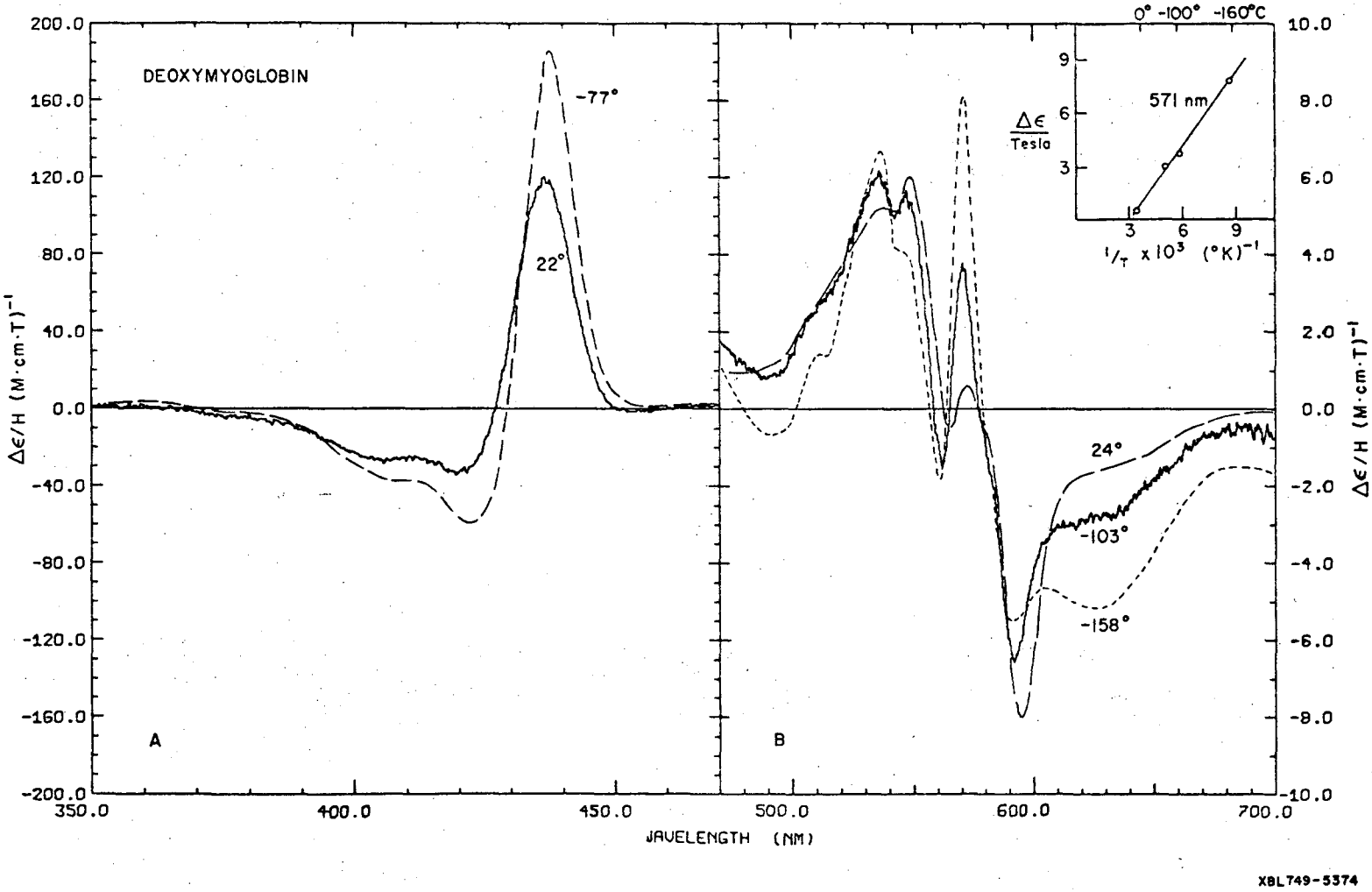
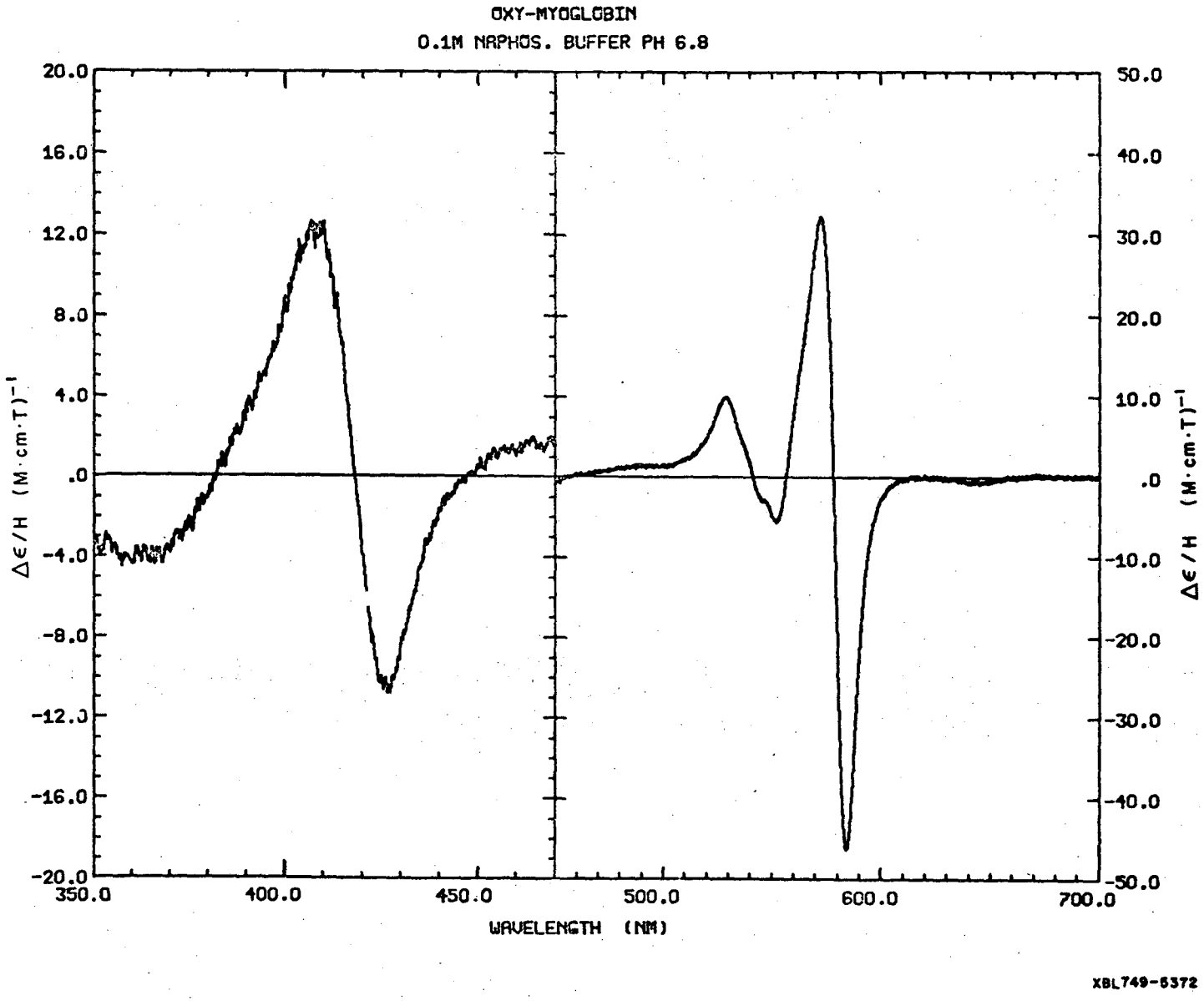


Fig. 7.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4.0
.0
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450.0
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600.0
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WAVELENGTH (NM)
Δε/H (M·cm·T)⁻¹
Δε/H (M·cm·T)⁻¹
XBL749-5372

Fig. 8.

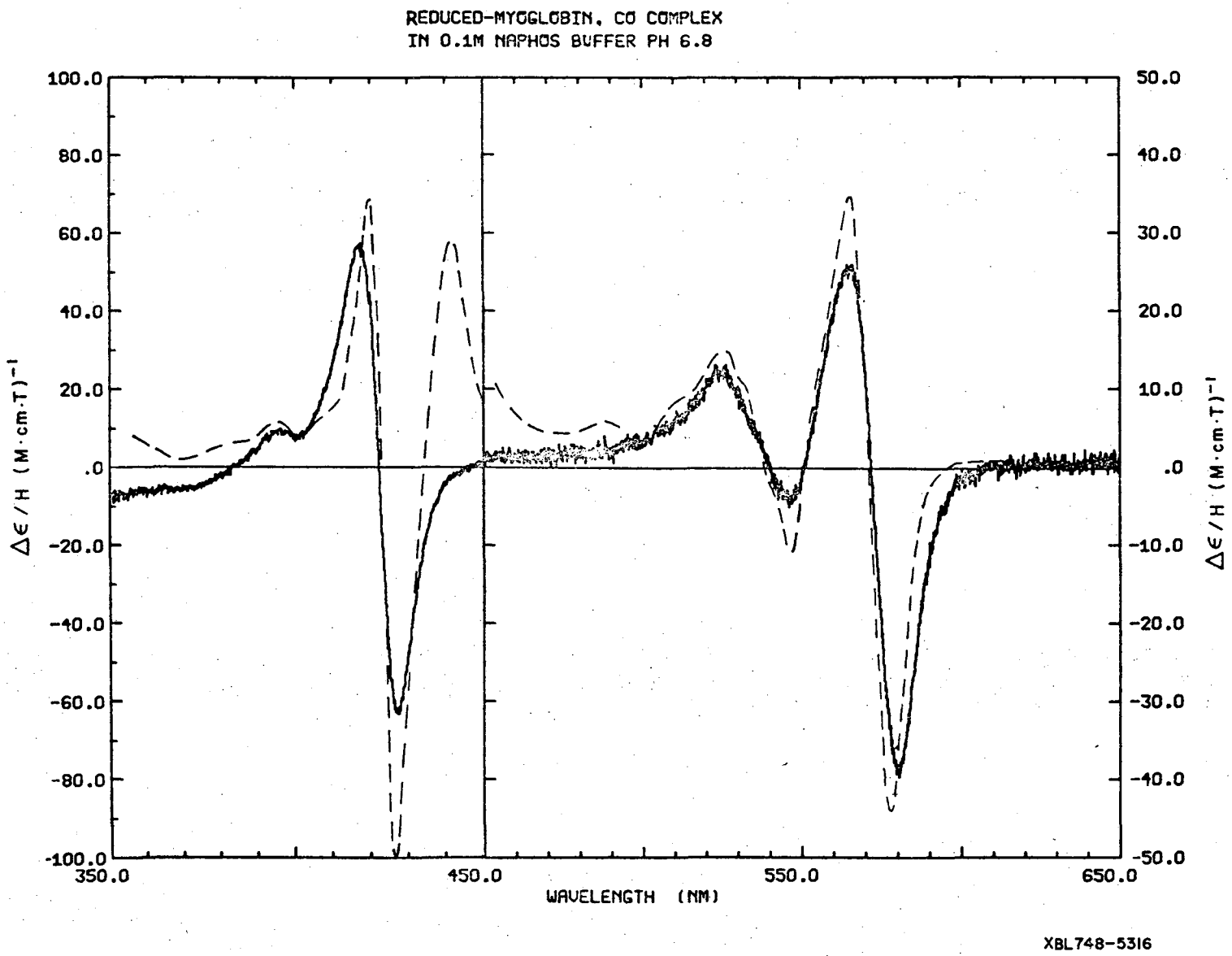

Fig. 9.

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