Catalytic Antibodies. Circular Dichroism and UV–Vis Studies of Antibody–Metalloporphyrin Interactions

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Circular dichroism and UV–vis measurements were used to study the interaction between several water-soluble metalloporphyrins and monoclonal antibodies (MABs) that were elicited against a structurally related dihydroxytin(IV) porphyrin, **1a**. Some of the MAB–metalloporphyrin complexes studied were previously shown to mimic hemoprotein-like activity, such as catalytic epoxidation of styrene by iodosobenzene. MAB–metalloporphyrin complex formation is usually accompanied by significant bathochromic shift and hypo-/hyperchromicity changes of the absorption maxima in the porphyrin Soret band region. Induced CD spectra in the same region (350–450 nm) are detected upon complex formation. They follow Beer's law, exhibit isosbestic behavior, and show no significant change in the general shape of the Cotton effect as a function of the antibody:porphyrin ratio. Signal intensity (ellipticity) increases with increasing antibody:metalloporphyrin ratio, reaching an upper limit that represents saturation complexation, all indicating a specific 1:1 binding phenomenon. Strong dependence of both signal shape and signal intensity upon the specific metal and antibody were observed, suggesting that induced CD may serve as a powerful tool for the unequivocal differentiation of two seemingly identical MAB clones. In cases of intense induced CD signals, it is possible to obtain MAB–metalloporphyrin dissociation constants using a titration curve where ellipticity is measured as a function of the antibody:porphyrin ratio and presented as a Scatchard plot. Both the UV–vis absorbance and induced CD spectrum of the metalloporphyrin–MAB complex remain unchanged over a broad pH range between 6 and 11, indicating remarkable stability of these complexes and reflecting the dominant role of electrostatic interactions between the hapten carboxylate groups and the antibody combining site.

Introduction

Circular dichroism (CD) measurements have been proposed as a unique tool for the analysis of the structure of proteins in solution because they are highly sensitive to variations in tertiary structure and are capable of detecting small differences in stereochemical relationships.[1] Induced CD bands may arise from the noncovalent association of a symmetric haptenic chromophore with a specific antibody. This induced optical activity results from the binding of an optically inactive hapten in the asymmetric environment of the antibody's combining site, leading to an extrinsic Cotton effect in the absorption region of the haptenic chromophore. This phenomenon, first observed in the early 1970's, has been considered to be a potent means for investigating the nature of the immediate environment of the bound ligand. It has been shown that extrinsic Cotton effects discriminate strongly between antibody-combining sites of similar specificity and can be used to detect small conformational differences in the complementarity-determining region (CDR).[2]

In our attempts to mimic cytochrome P-450-like activity using antibodies elicited against metalloporphyrins[3] we have raised monoclonal antibodies (MABs) against a water-soluble porphyrin complex $Sn(OH)_2$(TCP), **1a** (Chart I). These MABs display a high affinity for a variety of metalloporphyrins, including the (μ-oxo)iron(III) porphyrin dimer $[(TCP)Fe]_2O$, **1b**, as well as chloromanganese porphyrin ClMn(TCP), **1c**, and several other metalloporphyrins. Complexes of **1c** with several MABs displayed catalytic activity in the epoxidation of styrene by iodosobenzene.[3] More recently we observed hemoprotein-like activity with a MAB–Fe^{III}(TCP) complex.[4]

In order better to understand the factors governing the catalytic and other activities of the MAB–metalloporphyrin complex (e.g. reversible oxygen binding) it is important to obtain structural information about the antibody–porphyrin interaction. The intense Soret absorption of metalloporphyrins in the 400-nm region suggests that induced CD spectroscopy may be a useful tool to study the interactions of porphyrins and macromolecules. We have therefore investigated the induced CD as well as UV–vis spectra of a number of complexes formed between each of the metalloporphyrins shown in Chart I and the previously studied anti-metalloporphyrin MABs: BEH135.2, BEH76.1, BEH26.2, and BEH104.1.[5]

Results and Discussion

UV–Vis Studies. The effect of each of the MABs on the characteristics of the Soret absorption bands of the metalloporphyrins was studied at pH 8.05 using a 10^{-6} M solution of the appropriate porphyrin in the presence of excess MAB (8-fold excess of binding sites). Results are presented in Table I.

As seen from the table, formation of an antibody–porphyrin complex is usually accompanied by changes in the UV spectrum

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(5) An independent study currently underway attacks the problem using X-ray crystallography. The Fab fragment of BEH135.2 is being crystallized in the presence of several metalloporphyrins. These crystallographic studies will be reported in due time.

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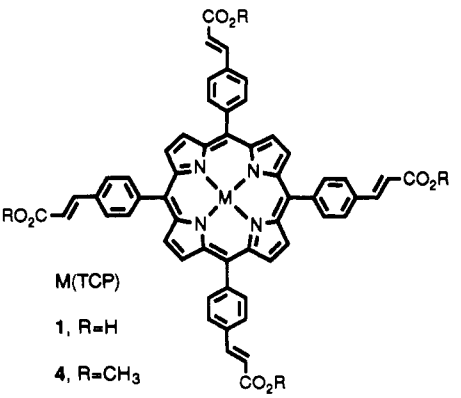
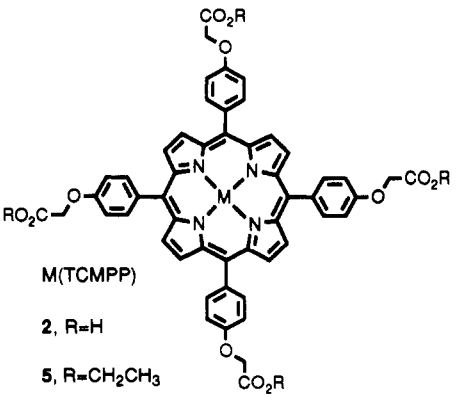
Chart I. Substituted Metalloporphyrins





a) M=Sn(OH)$_2$

b) M=1/2(FeOFe)

c) M=MnCl

d) M=Ni

e) M=H$_2$ (free base)

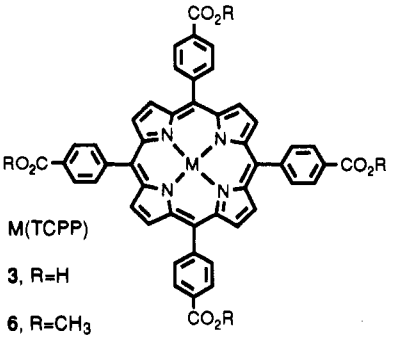


Table I. Effect of Antibodies on Wavelength Maximum and Intensity of the Soret Bands[a]

	absorption				
		MAB			
metallo-porphyrin	λ_{max} ($10^{-5}\epsilon$)	BEH135.2 $\Delta\lambda_{max}$ ($\Delta\epsilon$)	BEH76.1 $\Delta\lambda_{max}$ ($\Delta\epsilon$)	BEH26.2 $\Delta\lambda_{max}$ ($\Delta\epsilon$)	BEH104.1 $\Delta\lambda_{max}$ ($\Delta\epsilon$)
1a	426 (3.19)	+2 (−11.5)	+4 (+13.6)	0 (−4.3)	0 (+13.3)
1b	412 (2.28)	+6 (+5.1)	+8 (+5.3)	+2 (−16.9)	+6 (−8.6)
1c	402 (1.6)	0 (0)	0 (−20.8)	0 (−22.8)	0 (−16.4)
1c	424 (1.29)	0 (0)	0 (−18.7)	0 (−4.7)	0 (−15.3)
1c	468 (2.49)[b]	+6 (−1.2)	+8 (−6.0)	+2 (−4.6)	0 (−15.3)
1d	408 (5.05)	+8 (+21.7)	+8 (+14.1)	+4 (−8.8)	0 (−7.6)
1e	412 (4.49)	+2 (+6.3)	0 (+10.5)	+4 (−8.8)	0 (−5.7)
2d	414 (6.23)	+8 (+4.0)	+6 (+22.3)	0 (−1.9)	0 (−18.2)
3a	420 (5.75)	+4 (−8.9)	+4 (−10.3)	+2 (+2.0)	0 (−8.0)
3c	400 (2.38)	0 (−2.4)	0 (+6.4)	0 (+25.4)	0 (−16.4)
3c	420 (2.32)	0 (−4.0)	0 (+3.0)	0 (+22.9)	0 (−15.3)
3c	468 (3.28)[b]	+2 (−6.2)	+2 (−3.4)	0 (+11.6)	0 (−15.3)
3d	410 (7.45)	+2 (+20.1)	+6 (−6.9)	+6 (−5.1)	0 (−9.1)
3e	412 (3.93)	0 (+17.9)	0 (+5.4)	0 (+5.0)	0 (−10.2)

[a] Antibody solution (8 equiv) was added to a 10^{-6} M solution of the appropriate porphyrin. All solutions were buffered with ammonium carbonate (pH 8.05). Changes in wavelength maximum ($\Delta\lambda_{max}$) are given in nm (+ indicates red shift). Changes in absorption intensity ($\Delta\epsilon$) are given in % (+ indicates hyperchromicity). Values are corrected for concentration changes upon mixing. [b] This absorption is not a Soret band.

of the latter. The most significant changes occur in the Soret band region, where the absorption maximum is generally red-shifted ($\Delta\lambda_{max}$ between 0 and 8 nm). An interesting consistency can be observed in the case of the MAB BEH104.1, where no changes in λ_{max} could be detected (except with the μ-oxo dimer, **1b**). The consistent bathochromic shift may reflect more rigid, restricted conformations of the cinnamate substituents upon antibody binding, thereby extending the chromophore. A representative example of these changes is given in Figure 1.

With respect to changes of intensity, no particular consistency can be seen. Although BEH104.1 shows a consistent hypochromicity trend (with the sole exception of the original hapten **1a**), all other MABs show widely varying patterns of behavior.

The study of a family of MABs is advantageous in preventing erroneous conclusions regarding generalities of behavior that might result from examining a narrow sample. For example, with BEH104.1 and BEH26.2 there is no change in λ_{max} of the original hapten **1a**, while with BEH76.1 and BEH135.2 **1a** exhibits significant bathochromic shifts. It is hoped that more detailed structural information regarding the MAB–porphyrin complexes will allow a better understanding of the UV absorption differences.[5]

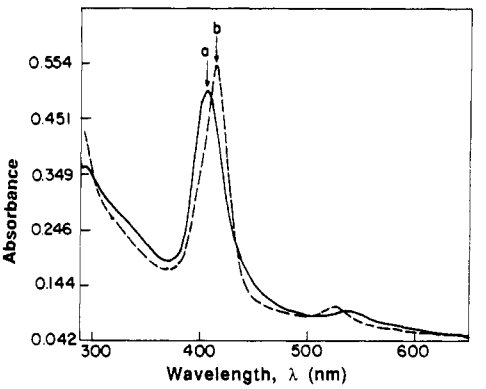


Figure 1. UV–vis changes accompanying binding of **1d** to BEH135.2: (a) UV–vis spectrum of **1d** (10^{-6} M) in phosphate buffer solution, pH 7.5 at 23 °C; (b) spectrum of **1d** taken under the same conditions, in the presence of BEH135.2 (2.5×10^{-6} M).

Induced CD. The generally intense Soret band absorption of porphyrins (in the region of 350–450 nm) has proven useful for CD studies of the interactions between metalloporphyrins and macromolecules.[6] In order to measure the induced CD spectra of our metalloporphyrins in the presence of the appropriate MABs,

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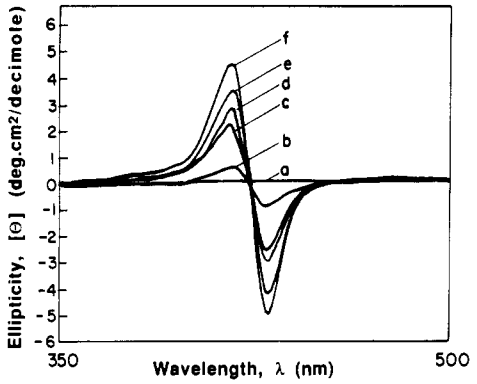

Figure 2. Induced CD spectra of **1d** and BEH135.2. Various amounts of BEH135.2 were added to a solution of **1d** (10^{-6} M) in ammonium carbonate buffer, pH 8.0 at 23 °C. CD spectra were recorded after 1 min of mixing. The following MAB:**1d** equivalent ratios were used: (a) 0; (b) 0.5; (c) 2; (d) 3; (e) 4; (f) 5. No further changes in the spectrum were observed at higher ratios.

we prepared 10^{-6} M buffered solutions (pH 8.05) of a given metalloporphyrin, added increasing amounts of MAB, and recorded the CD spectra in the Soret region. Signal intensity (ellipticity) increases with increasing antibody:porphyrin ratio, reaching an upper limit that represents saturation complexation of all porphyrin molecules. A representative set of such measurements, using **1d** and BEH135.2, is shown in Figure 2. Other metalloporphyrins behave similarly in the presence of the four relevant MABs (vide infra).

As seen from Figure 2, the spectra follow Beer's law, exhibit isosbestic behavior, and show no significant change in the general shape of the Cotton effect as a function of the antibody:porphyrin ratio. This indicates that a single hapten–MAB complex has formed[2b] and that the spectral changes do not result from aggregation phenomena.[7] Control experiments using a large excess of a nonrelevant MAB (elicited against dinitrophenol) did not exhibit any detectable CD spectra in this region. One conclusion from these data is that the observed induced CD signal reflects a specific binding phenomenon. This provides us with unique opportunities to study antibody–porphyrin interactions. These induced CD spectra may be a particularly convenient research tool, as no correction or substraction of spectra is required, because neither the free metalloporphyrins nor the MABs show any measurable CD spectrum in the Soret region.

Determination of the Binding Constant with CD. We have previously[3] estimated MAB–metalloporphyrin binding constants using a competition ELISA (enzyme-linked immunosorbent assay) technique to measure IC_{50} values. In principle, the same information may be obtained with sensitive spectroscopic techniques, such as UV–vis, CD, and fluorescence spectroscopy, by means of binding isotherms. However, for the observed IC_{50} values it is necessary to work with hapten concentrations of the order of 10^{-9} M. Such levels are below the sensitivity thresholds of UV and CD spectrometers. Nevertheless, qualitative estimates of the binding constants may be obtained by CD spectroscopy. We measured the induced CD, using solutions of **1d** (ranging from 10^{-6} to 10^{-7} M) and variable amounts of BEH135.2 (up to 10^{-5} M) and obtained a titration curve where ellipticity is measured as a function of the antibody:porphyrin ratio and presented as a Scatchard plot.[8] The dissociation constant obtained from that plot was in the range of 10^{-6}. This value is close to the IC_{50} value obtained from competition ELISA measurements ($10^{-5.3}$).[3]

CD Spectral Variations. The induced CD spectra of the metalloporphyrin complexes with MABs were recorded using 10^{-6} M buffered solutions (pH 8.05) of the given metalloporphyrin

(7) Pasternack, R. F.; Brigandi, R. A.; Abrams, M. J.; *Williams*, A. P.; Gibbs, E. J. *Inorg. Chem.* **1990**, *29*, 4483.
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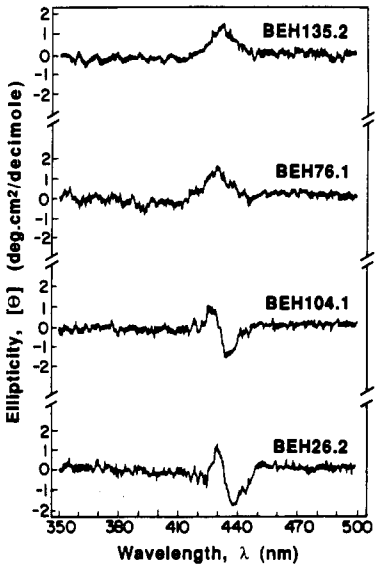

Figure 3. Induced CD spectra of **1a** with four different MABs. All spectroscopic experiments were conducted at 23 °C. Stock solutions containing metalloporphyrins (10^{-6} M) in ammonium carbonate buffer at pH 8.05 were prepared. All experiments with MAB–porphyrin complexes were performed after addition of the appropriate amount of MAB stock solution to 800 μL of stock solution of the porphyrin with vigorous shaking for 1 min. Each experiment lasted for approximately 10 min, including the time required for mixing and measurement.

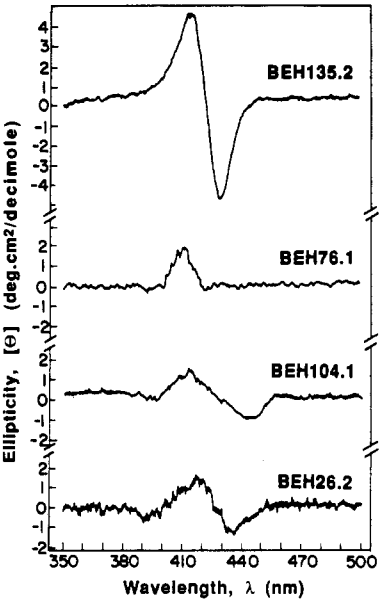

Figure 4. Induced CD spectra of **1d** with four different MABs. For experimental details, see Figure 3.

and an excess of MAB (the concentration of MAB which caused maximal ellipticity values). Strong dependence of both signal shape and signal intensity upon the specific metal and antibody were observed. Figures 3 and 4 show the observed induced CD spectra of **1a** and **1d**, respectively, with all four MABs. Figure 5 presents the spectra observed with five different metalloporphyrins (**1a,b,d, 2d, 3d**) using one MAB (BEH135.2). The other metalloporphyrins mentioned in Chart I did not show significant CD spectra with any of the four MABs examined.

The induced CD effect is highly dependent on the relative disposition and degree of immobilization of both the porphyrin system and the protein chromophores (e.g. aromatic residues and carbonyl groups). Consequently, the observed signal reflects an overall balance of many factors, some of them may be quite subtle. Indeed, at first glance, the CD spectra of various metalloporphyrins with different MABs appear to be a collection of conservative,

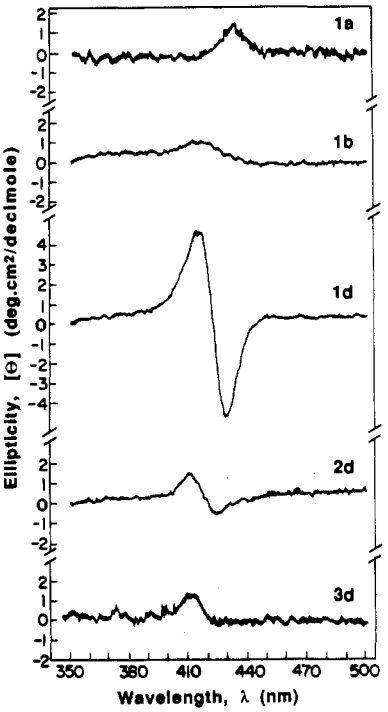

Figure 5. Induced CD spectra of BEH135.2 with five different metalloporphyrins. For experimental details, see Figure 3.

nonconservative, negative, and positive signals of various intensities (Figures 3–5). Nevertheless, looking at the CD spectra in more detail, one can observe some regularities in the shape of the curves. Complexes **1a**–BEH135.2 and **1a**–BEH76.1 exhibit positive CD curves with maxima approximately coincident with the absorption maxima (Figure 3). Conversely, complexes **1a**–BEH104.1 and **1a**–BEH26.2 and most of the **1d**–MAB complexes shown in Figure 4 exhibit CD spectra with two-signed curves in the Soret region. The occurrence of these "doublets" is not unexpected: the Soret band encompasses two degenerate or almost degenerate transitions. This behavior is well documented in the porphyrin literature.[9] The observed similar CD doublets may imply the same identity and overall dominant conformational disposition of the protein chromophores responsible for interaction with the porphyrin. The oddity in the behavior of **1a**–BEH135.2 and **1a**–BEH76.1 may reflect a situation where the interacting protein chromophores are different or they are differently orientated with respect to the metalloporphyrin chromophore. Thus, as seen from the examples shown in Figures 3 and 4, induced CD may serve as a powerful tool for the unequivocal differentiation of two seemingly identical MAB clones which display the same IC_{50} values for a given hapten.

The shape and the intensity of the signal are difficult to interpret in terms of binding mode and binding efficiency. For example, it might have been thought that a given antibody would interact similarly with related metalloporphyrins. This however, is not reflected in the shapes and intensities of the induced CD signals. Moreover, the intensity of the signal is unrelated to the magnitude of the binding constant. For example, the tin porphyrin **1a**, which has the largest binding constants (according to ELISA), exhibits much smaller signals than the Ni analog (**1d**), whose binding constants are much smaller. One may speculate on the reason for the intense CD spectra of the Ni complexes **1d**, **2d**, and **3d** (complexes **3a**–**c**,**e** did not show significant CD spectra with any of the MABs examined). Considering the fact that the latter have no axial ligands, and assuming some conformational flexibility of the antibody combining site, it is conceivable that the Ni porphyrin chromophore lies closer to the protein surface

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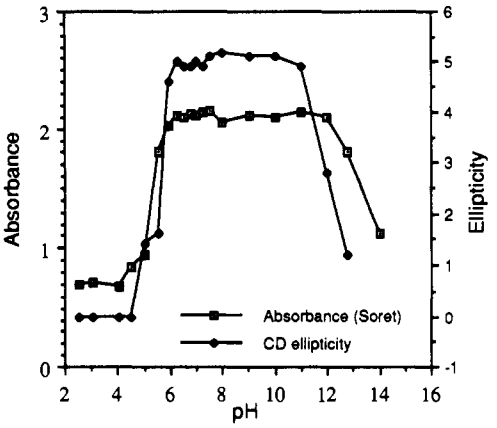

Figure 6. Dependence of UV and CD intensities on pH. Stock solutions containing **1d** (10^{-6} M) in phosphate buffer solution, pH 7.00 at 23 °C, were prepared. The pH was changed by addition of a minimum amount of aqueous NaOH or HCl. The final pH value was measured by a pH-meter.

than the six-coordinate Sn complex, maximizing the induced CD effect. In line with this is the observation that the induced CD for the bulky Fe porphyrin dimer **1b** is weak. The pentacoordinated Mn complex **1c** represents an interesting case, as it has two unequal faces. Its weak CD signal may reflect a selective binding mode, where the protein prefers the face containing the axial ligand (a situation that mimics the interaction mode of the hapten **1a**). Although the weak signal exhibited by the metal-free porphyrin **1e** does not correlate with the above analysis, one should recall that the case of **1e** may involve additional factors, such as potential nonplanarity of the porphyrin ring and new interactions of its nitrogen atoms with the protein residues.

As both UV and CD effects arise from a perturbation of the porphyrin chromophore by the protein residues, a correlation between the two phenomena may be expected. Indeed, a correlation between the intensity of the induced CD and the magnitude of the UV absorption changes (both λ_{max} and ϵ) is generally observed. This correlation is best illustrated by the case of the nickel porphyrins **1d**, **2d**, and **3d**. Relative to other metalloporphyrins they exhibit the most intense CD signals (Figures 4 and 5), and also display some of the largest changes in the UV spectra (Table I).

Dependence on pH. In order to study the dependence of the induced CD spectrum on pH, we prepared solutions of the nickel porphyrin **1d** with excess antibody BEH135.2 at several pH values and measured both their UV absorbance (at the Soret maximum) and CD ellipticity (Figure 6). The combination of **1d** with BEH135.2 was chosen because of the relatively intense CD signal exhibited. As may be seen from Figure 6, the UV absorbance remains constant over a broad pH range, viz. between 6 and 12. At pH values below 5 absorbance is reduced to approximately one-third of its former value and the signals are significantly broadened. This decrease and line broadening may reflect, in part, a change in the porphyrin solubility at low pH, where the carboxylates are neutralized.

The CD spectrum provides useful information concerning the antibody combining site. It is unchanged (in both intensity and shape) over a range of five pH units (between 6 and 11). This remarkable stability of the spectrum probably reflects the dominant role of electrostatic interactions between the hapten carboxylate groups and the antibody combining site. Apparently, there is no change in the relative orientation of the porphyrin in the antibody combining site and the distances between the porphyrin and the CDR (complementarity-determining region) residues responsible for the induced CD effect. It is interesting to compare our results with two reported cases, in which the induced CD spectra of achiral haptens were measured in the presence of specific antibodies at various pH values and a dramatic

dependence of the CD spectra on pH was found.[10] From the results in Figure 6 we conclude that the disappearance of induced CD signal below pH 6 does not result from poor hapten solubility alone. The similar behavior of both UV and CD spectra indicates that solubility as well as binding phenomena are highly dependent on the p*K*a of the cinnamic acid residues (~4.4[11]) in the hapten and perhaps also on the p*K*a values of the CDR residues. This view is consistent with the following experiments. A solution containing both **1d** and BEH135.2 at pH 8.0 (showing an intense CD signal) was acidified to pH 3, and its CD spectrum was immediately measured. A similar experiment was carried out with the starting pH being 6.5. No CD spectrum was detected in either case, suggesting that neutralization of the hapten caused its immediate rejection from the antibody's combining site.

Conclusion

Induced CD is a useful tool to study the interaction between metalloporphyrins and their complementary MABs. MAB–metalloporphyrin complex formation is usually accompanied by significant bathochromic shift and hypo-/hyperchromicity changes of the absorption maxima in the porphyrin Soret band region. Induced CD spectra in the same region (350–450 mm) follow Beer's law, exhibit isosbestic behavior, and show no significant change in the general shape of the Cotton effect as a function of the antibody:porphyrin ratio. Signal intensity (ellipticity) increases with increasing antibody:metalloporphyrin ratio, reaching an upper limit that represents saturation complexation, all indicating a specific 1:1 binding phenomenon. Strong dependence of both signal shape and signal intensity upon the specific metal and antibody were observed, suggesting that induced CD may serve as a powerful tool for the unequivocal differentiation of two seemingly identical MAB clones. In cases of intense induced CD signals, it is possible to obtain MAB–metalloporphyrin dissociation constants using a titration curve where ellipticity is measured as a function of the antibody:porphyrin ratio and presented as a Scatchard plot. Both the UV–vis absorbance and induced CD spectrum of the **1d**–BEH135.2 complex remain unchanged over a broad pH range between 6 and 11, indicating remarkable stability of the metalloporphyrin–MAB complex and reflecting the dominant role of electrostatic interactions between the hapten carboxylate groups and the antibody combining site.

Further studies on the scope and limitation of the oxidation reactions catalyzed by metalloporphyrin–MAB complexes, as well as reversible oxygen binding by these species, are currently underway in our laboratories.

Experimental Section

General Methods. Spectral measurements were carried out on a HP 8452A diode array spectrophotometer, in a 1-cm cell; circular dichroism spectra were taken on a JASCO J-500C spectropolarimeter, in a 1-cm cell. All spectroscopic experiments were conducted at 23 °C. Stock solutions containing metalloporphyrins (10^{-6} M) in ammonium carbonate buffer at pH 8.05 or phosphate-buffered saline solution (PBS) pH 7.00 were prepared. All experiments with MAB–porphyrin complex were performed after addition of the appropriate amount of MAB stock solution to 800-μL stock solution of the porphyrin with good mixing. Each experiment lasted for approximately 10 min, including the time required for mixing and measurement. As a negative control, all porphyrins were tested for CD activity when added to a nonrelevant monoclonal antibody (that was elicited against dinitrophenol); no detectable signal was observed using a MAB:porphyrin ratio of 20:1. In the pH dependence experiments, values were measured by a two-point calibrated pH-meter using a combined electrode. All UV and CD spectra were carried out in duplicates.

Synthesis of Metalloporphyrins. Porphyrins were synthesized by conventional methods from the appropriate aldehyde. Methyl 4-(carboxyvinyl)benzaldehyde was prepared from 4-bromobenzaldehyde and methyl acrylate;[12] *p*-((ethylcarboxy)methoxy)benzaldehyde was prepared from *p*-hydroxybenzaldehyde and ethyl bromoacetate and methyl 4-carboxybenzaldehyde was purchased from Fluka. Reaction with pyrrole afforded the free-base porphyrin esters of **4e**, **5e**, and **6e**.[13] All metalation reactions were carried out by heating the porphyrin with either the metal chloride or metal acetate under basic conditions,[14] except for compound **1b** that was prepared under acidic condition.[15] Hydrolysis of the metalloporphyrin esters is described below. The structures of all the metalloporphyrins were confirmed by NMR, some structures were confirmed by microanalysis, and in the case of **4a**, the structure was confirmed by a single-crystal X-ray structure determination.[3]

***meso*-5,10,15,20-Tetrakis[4-(2-(methoxycarbonyl)vinyl)phenyl]porphyrin, 4e.** A solution of pyrrole (0.62 g, 9.2 mmol) and methyl 4-formylcinnamate (1.74 g, 9.2 mmol) was stirred at room temperature for 0.5 h. $BF_4{\cdot}Et_2O$ (0.08 mL) was added, and the mixture was kept in the dark for 1 h. DDQ (2,3-dichloro-5,6-dicyanobenzoquinone) (1.53 g, 6.75 mmol) was added, and the mixture was stirred at room temperature for 0.5 h and then at 45 °C for 1 h. Solvent was removed under reduced pressure, and the residue was passed through a florisil column using CH_2Cl_2. The crude product was recrystallized from CH_2Cl_2/MeOH, affording **4e** (0.7 g, 30%) in the form of purple needles.

^{1}H NMR ($CDCl_3$): δ 8.85 (s, 8H), 8.22 (d, J = 8 Hz, 8H), 8.03 (d, J = 16 Hz, 4H), 7.90 (d, J = 8 Hz, 8H), 6.74 (d, J = 16 Hz, 4H), 3.91 (s, 12H), –2.5 (br, 2H).

***meso*-5,10,15,20-Tetrakis[4-((ethoxycarbonyl)methoxy)phenyl]porphyrin, 5e.** Pyrrole (0.97 g, 14 mmol) was added to a hot (140 °C) solution of 4-((ethylcarboxy)methoxy)benzaldehyde (3.1 g, 8 mmol) in propionic acid, and the mixture was refluxed for 30 min. Solvent was removed under reduced pressure, affording purple powder. This crude product was dissolved in chloroform, washed with potassium carbonate and with water, and then subjected to column chromatography (silica gel, CH_2Cl_2), affording **5e** (1.17 g, 60%) in the form of a purple solid.

^{1}H NMR ($CDCl_3$): δ 8.83 (s, 8H), 8.08 (d, J = 10 Hz, 8H), 7.2 (d, J = 10 Hz, 8H), 4.88 (s, 8H), 4.35 (q, J = 20 Hz, 8H), 1.37 (t, J = 14 Hz, 12H), –2.7 (s, 2H).

***meso*-5,10,15,20-Tetrakis[4-(methoxycarbonyl)phenyl]porphyrin, 6e.** Synthesis was carried out as described above for **4e** using pyrrole (1.0 g, 15 mmol), methyl 4-carboxybenzaldehyde (2.46 g, 15 mmol), $BF_4{\cdot}Et_2O$ (0.15 mL), and DDQ (2.0 g, 9 mmol), affording **6e** (1.2 g, 38%) in the form of purple crystals.

^{1}H NMR ($CDCl_3$): δ 8.82 (s, 8H), 8.44 (d, J = 8 Hz, 8H), 8.29 (d, J = 8 Hz, 8H), 4.11 (s, 12H), –2.8 (br, 2H). Anal. Calc: C, 73.75; H, 4.52; N, 6.62. Found: C, 73.55; H, 4.82; N, 6.71.

[*meso*-5,10,15,20-Tetrakis[4-(2-(methoxycarbonyl)vinyl)phenyl]porphyrinato]tin(IV) Dichloride, 4a. A solution of **4e** (607 mg, 0.63 mmol) and $SnCl_2$ (610 mg, 2.5 mmol) in pyridine (30 mL) was heated at 80–90 °C for 2 h, cooled to room temperature, and poured into 100 mL of ether. The organic solution was washed with 3 N aqueous HCl (3 × 100 mL), solvent was removed under reduced pressure, and the resultant solid was dissolved in CH_2Cl_2, washed with water, and dried over Na_2SO_4. Solvent was removed again, and the crude product was recrystallized from CH_2Cl_2/MeOH, affording **4a** (450 mg, 63% based on porphyrin) in the form of bright purple needles.

^{1}H NMR ($CDCl_3$): δ 9.23 (s, 8H), 8.36 (d, J = 7.8 Hz, 8H), 8.06 (d, J = 16 Hz, 4H), 8.00 (d, J = 7.8 Hz, 8H), 6.69 (d, J = 16 Hz, 4H), 3.93 (s, 12H).

[*meso*-5,10,15,20-Tetrakis[4-(methoxycarbonyl)phenyl]porphyrinato]tin(IV) Dichloride, 6a. A solution of **6e** (500 mg, 0.59 mmol) and $SnCl_2$ (500 mg, 2.1 mmol) in pyridine (30 mL) was heated at 80–90 °C for 2 h, cooled to room temperature, and poured into 100 mL of ether. The mixture was worked up as described above for compound **4a**, affording **6a** (450 mg, 74% based on porphyrin) in the form of bright purple needles.

^{1}H NMR ($CDCl_3$): 9.13 (s, 8H), 8.47 (d, J = 8 Hz, 8H), 8.37 (d, J = 8 Hz, 8H), 4.10 (s, 12H).

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[*meso*-5,10,15,20-Tetrakis[4-(2-(methoxycarbonyl)vinyl)phenyl]porphyrinato]manganese(III) Chloride, 4c. A solution of **4e** (300 mg, 0.32 mmol) and manganese acetate (300 mg, 1.73 mmol) in DMF (25 mL) was refluxed for 2 h, cooled to room temperature, and poured into ice cold aqueous NaCl (6 g/20 mL). The resultant green solid (~300 mg) was filtered out, washed several times with H_2O, and air-dried for 30 min. It was then dissolved methanol (5 mL), and the solution was filtered and poured into an equal volume of 6 M aqueous HCl. The resultant green precipitate (**4c**) was collected by filtration, washed with H_2O, and air dried (250 mg, 85% based on porphyrin).

^{1}H NMR ($CDCl_3$): δ 8.80–8.30 (br, 24H), 8.11 (br, 4H), 7.46 (br, 4H), 3.90 (s, 12H).

[*meso*-5,10,15,20-Tetrakis[4-(2-(methoxycarbonyl)vinyl)phenyl]porphyrinato]nickel(II), 4d. A solution of **4e** (300 mg, 0.31 mmol) and nickel acetate (300 mg, 1.69 mmol) in DMF (25 mL) was refluxed for 2 h, cooled to room temperature, and poured into ice cold aqueous NaCl (6 g/20 mL). The resultant red solid was filtered out, washed several times with H_2O, and air-dried overnight, affording **4d** (300 mg, 94% based on porphyrin).

^{1}H NMR ($CDCl_3$): δ 8.72 (s, 8H), 8.0 (d, J = 7.7 Hz, 8H), 7.95 (d, J = 16 Hz, 4H), 7.82 (d, J = 7.7 Hz, 8H), 6.66 (d, J = 16 Hz, 4H) 3.87 (12H, s).

[*meso*-5,10,15,20-Tetrakis[4-((ethoxycarbonyl)methoxy)phenyl]porphyrinato]nickel(II), 5d. A solution of **5e** (1.17 g, 1.15 mmol) and nickel acetate (1.12 g, 6.3 mmol) in pyridine (100 mL) was heated to 100 °C for 12 h, cooled to room temperature, and worked up with chloroform and aqueous HCl (10%). Solvent was removed under reduced pressure and the residue was subjected to column chromatography (silica gel, CH_2Cl_2), affording **5d** (1.11 g, 94% based on porphyrin) in the form of a red powder.

^{1}H NMR ($CDCl_3$): δ 8.84 (s, 8H), 8.08 (d, J = 10 Hz, 8H), 7.2 (d, J = 10 Hz, 8H), 4.88 (s, 8H), 4.3 (q, J = 20 Hz, 8H), 1.24 (t, J = 14 Hz, 12H).

[*meso*-5,10,15,20-Tetrakis[4-(methoxycarbonyl)phenyl]porphyrinato]manganese(III) Chloride, 6c. A solution of **6e** (300 mg, 0.38 mmol) and manganese acetate (300 mg, 1.73 mmol) in DMF (25 mL) was refluxed for 2 h, cooled to room temperature, and poured into ice cold aqueous NaCl (6 g/20 mL). The resultant green solid (~300 mg) was filtered out, washed several times with H_2O, air dried for 30 min, and then dissolved in MeOH (5 mL). The methanolic solution was filtered and poured into 6 M aqueous HCl (5 mL), and the resultant green precipitate was collected by filtration, washed with H_2O, and air-dried, affording **6c** (250 mg, 76% based on porphyrin) in the form of a green solid.

^{1}H NMR ($CDCl_3$): 9.50–8.40 (br, 24H), 4.07 (s, 12H).

[*meso*-5,10,15,20-Tetrakis[4-(methoxycarbonyl)phenyl]porphyrinato]nickel(II) 6d. A solution of **6e** (300 mg, 0.35 mmol) and nickel acetate (300 mg, 1.7 mmol) in DMF (25 mL) was refluxed for 2 h, cooled, and worked up as described above for preparation of **4d**, affording **6d** (300 mg, 94% based on porphyrin).

^{1}H NMR ($CDCl_3$): 8.68 (s, 8H), 8.34 (d, J = 7.8 Hz, 8H), 8.08 (d, J = 7.8 Hz, 8H), 4.0 (s, 12H).

(μ-Oxo)bis[[*meso*-5,10,15,20-tetrakis[4-(2-carbonylvinyl)phenyl]porphyrinato]iron(III)], 1b. A solution of **4e** (100 mg, 0.105 mmol) and ferrous acetate (1.0 mmol, freshly prepared from iron powder and acetic acid) in acetic acid (50 mL) was refluxed for 5 h. The mixture was diluted with water and extracted with benzene. The organic layer was stirred overnight with 3 N aqueous NaOH, separated again, and mixed with silica gel (5 g). The resultant purple silica gel (impregnated with the crude product) was washed several times with CH_2Cl_2, and then the product was eluted with 5% MeOH in CH_2Cl_2. Solvent was removed under reduced pressure, and the residue was recrystallized from CH_2Cl_2–MeOH to give **4b** in the form of a dark-purple solid. The latter was mixed with 3 N methanolic NaOH and heated for 3 h at 40–50 °C, cooled to room temperature, and acidified with 1 N aqueous HCl. The mixture was basified again with triethylamine and extracted with EtOAc. Solvent was removed, and the residue was crystallized from MeOH–EtOAc, affording **1b** (88.8 mg, 91% based on porphyrin) in the form of a dark-purple powder.

^{1}H NMR (DMSO-d_6): δ 81.5 (br, 8H), 13.41 (br, 8H), 8.44 (d, J = 14.3 Hz, 8H), 8.29 (d, J = 14.3 Hz, 8H), 7.59 (br, 8H).

Hydrolysis of Porphyrin Esters, General Procedure. The appropriate porphyrin ester (60 mg) was dissolved in MeOH (5 mL) together with 3 N aqueous KOH (5 mL) and stirred at 40 °C for 2 h. The mixture was cooled to room temperature, acidified with 3 N aqueous HCl and extracted with EtOAc. Removal of solvent under reduced pressure afforded the corresponding tetraacid in essentially quantitative yield. In the cases of dichlorotin porphyrins, the above procedure produced the corresponding dihydroxytin derivatives **1a** and **3a**.

Dihydroxy[*meso*-5,10,15,20-tetrakis[4-(2-carbonylvinyl)phenyl]porphyrinato]tin(IV), 1a. ^{1}H NMR (DMSO-d_6): δ 9.31 (s, 8H), 8.34 (d, J = 7.7 Hz, 8H), 8.23 (d, J = 7.7 Hz, 8H), 7.98 (d, J = 16 Hz, 4H), 6.89 (d, J = 16 Hz, 4H).

[*meso*-5,10,15,20-Tetrakis[4-(2-carbonylvinyl)phenyl]porphyrinato]manganese(III) Chloride, 1c. ^{1}H NMR (DMSO-d_6): δ 8.20–7.20 (br, 28H), 6.96 (br, 4H).

[*meso*-5,10,15,20-Tetrakis[4-(2-carbonylvinyl)phenyl]porphyrinato]nickel(II), 1d. ^{1}H NMR (DMSO-d_6): δ 8.76 (s, 8H), 8.04 (br, 16H), 7.87 (d, J = 16 Hz, 4H), 6.79 (d, J = 16 Hz, 4H).

***meso*-5,10,15,20-Tetrakis[4-(2-carbonylvinyl)phenyl]porphyrin, 1e.** ^{1}H NMR (DMSO-d_6): δ 9.31 (s, 8H), 8.34 (d, J = 8 Hz, 8H), 8.23 (d, J = 8 Hz, 8H), 7.98 (d, J = 16 Hz, 4H), 6.89 (d, J = 16 Hz, 4H).

[*meso*-5,10,15,20-Tetrakis[4-(carbonylmethoxy)phenyl]porphyrinato]nickel(II), 2d. ^{1}H NMR (DMSO-d_6): δ 8.71 (s, 8H), 7.87 (d, J = 8 Hz, 8H), 7.23 (d, J = 8 Hz, 8H), 4.88 (s, 8H).

[*meso*-5,10,15,20-Tetrakis(4-carboxyphenyl)porphyrinato]manganese(III) Chloride, 3c. ^{1}H NMR (DMSO-d_6): δ 8.80–7.20 (br, 24H).

[*meso*-5,10,15,20-Tetrakis(4-carboxyphenyl)porphyrinato]nickel(II), 3d. ^{1}H NMR (DMSO-d_6): δ 8.73 (s, 8H), 8.29 (d, J = 7.8 Hz, 8H), 8.09 (d, J = 7.8 Hz, 8H).

***meso*-5,10,15,20-Tetrakis(4-carboxyphenyl)porphyrin, 3e.** ^{1}H NMR (DMSO-d_6): δ 8.80 (s, 8H), 8.26 (d, J = 7.6 Hz, 8H), 7.98 (d, J = 7.6 Hz, 8H), –2.81 (br, 2H).

Preparation of Protein Conjugates. The metalloporphyrin hapten, **1a** (5 mg), was dissolved in 0.5 mL of dry DMF containing 0.8 mg of carbonyldiimidazole. The activation reaction was allowed to proceed at room temperature for 30 min, and the reaction mixture was then added dropwise to a solution of BSA (bovine serum albumin) (10 mg) or KLH (kehole limpet hemocyanin) (10 mg) in 0.5 mL of 0.01 M Na_2CO_3. The mixture was stirred for 30 min at 0 °C and then for 30 min at room temperature. The hapten protein conjugate was separated from unreacted hapten and side products by chromatography on a G50 gel permeation column. Fractions were collected and the o.d. was monitored at 427 nm; the first peak to elute was the conjugate. Hapten density was determined by the trinitrobenzenesulfonic acid (TNBS) procedure,[16] for KLH this value was 16, and for BSA it was 15.

Immunization, Immunoassays, and Monoclonal Antibody Production. Five BALB/c mice were each immunized (foot pad injection) with the KLH conjugate of **1a**, 100 μg, emulsified in complete Freunds's adjuvant. After 17 days a boost (50 μg in incomplete Freunds's adjuvant) was administered, and 1 day later a similar boost was given. On the 20th day the draining lymph node cells were fused with NSO myeloma cells using procedures described before.[17] The hybridoma supernatants were screened by an ELISA technique for binding to the hapten–BSA conjugate, using peroxidase-linked, goat antimouse antibodies as previously described. In the competitive inhibition assays (CIEIA) the final inhibitor concentration was 1 mM.

Out of the 1880 independent hybridoma wells tested in the first screening, 136 hybridomas (7.2%) produced antibodies with appreciable binding to the hapten **1a** (IC_{50} values smaller than 10^{-4} M). After further screening against both **1a** and **1b** the four best clones were selected.[3]

Antibodies were obtained by propagating selected clones as ascites in BALB/c mice, purified by protein A (Pharmacia) affinity chromatography[18] and dialyzed against 30 mM PBS, pH 8. Protein concentration was determined by measuring optical density at 280 nm. Homogeneity of antibody was judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), which yielded only heavy and light chains under reducing conditions, using Coomassie blue staining.

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