3425-3430.
Eaker, D., and Porath, J. (1967), *Sep. Sci. 2,* 507-550.
Easley, C. W. (1965), *Biochim. Biophys. Acta 107,* 386-388.
Eylar, E. H., Brostoff, S., Hashim, G., Caccam, J., and Burnett, P. (1971), *J. Biol. Chem. 246,* 5770-5784.
Fontana, A. (1972), *Methods Enzymol. 25 B,* 419-423.
Fontana, A., Vita, C., and Toniolo, C. (1973), *FEBS Lett. 32,* 139-142.
Hinman, R. L., and Bauman, C. P. (1964), *J. Org. Chem. 29,* 2431-2437.
Lawson, W. B., Patchornik, A., and Witkop, B. (1960), *J. Am. Chem. Soc. 82,* 5918-5923.
le Cam, A. (1973), *J. Chromatogr. 77,* 450-454.
London, Y., Demel, R. A., Geurts van Kessel, W. S. M., Vossenberg, F. G. A., and van Deenen, L. L. M. (1973), *Biochim. Biophys Acta 331,* 520-530.
Martenson, R. E., and Deibler, G. E. (1975), *J. Neurochem. 24,* 79-88.
Martenson, R. E., Deibler, G. E., Kramer, A. J., and Levine, S. (1975a), *J. Neurochem. 24,* 173-182.
Martenson, R. E., Deibler, G. E., Kramer, A. J., and Levine, S. (1976a), *Trans. Am. Soc. Neurochem. 7,* 182.
Martenson, R. E., Kramer, A. J., and Deibler, G. E. (1975b), *Biochemistry 14,* 1067-1073.
Martenson, R. E., Kramer, A. J., and Deibler, G. E. (1976b), *J. Neurochem. 26,* 733-736.
Martenson, R. E., Levine, S., and Sowinski, R. (1975c), *J. Immunol. 114,* 592-596.
Mateu, L., Luzzati, V., London, Y., Gould, R. M., Vossenberg, F. G. A., and Olive, J. (1973), *J. Mol. Biol. 75,* 697-709.
Omenn, G. S., Fontana, A., and Anfinsen, C. B. (1970), *J. Biol. Chem. 245,* 1895-1902.
Patchornik, A., Lawson, W. B., Gross, E., and Witkop, B. (1960), *J. Am. Chem. Soc. 82,* 5923-5927.
Thornhill, D. P. (1972), *Biochim. Biophys. Acta 279,* 1-7.
Welinder, B. S. (1972), *Biochim, Biophys. Acta 279,* 491-497.
Whitaker, J. N., Chou, C.-H. J., Chou, F. C.-H., and Kibler, R. F. (1975), *J. Biol. Chem. 250,* 9106-9111.

Magnetic Resonance Studies of the Binding of ^{13}C-Labeled Carbon Monoxide to Myoglobins and Hemoglobins Containing Modified Hemes†

Richard B. Moon, Kilian Dill, and John H. Richards*

ABSTRACT: The effects of changes in the groups attached to the periphery of the porphyrin ring of the heme of various hemoglobins and myoglobins on the environment experienced by the ligand, carbon monoxide, have been studied by observation of the chemical shift of the bound ^{13}CO. The results indicate that the major interaction between bound ligand and substituents around the porphyrin is that transmitted electronically from substituent to ligand. The nature of the protein environment around the ligand and the interaction between the proximal histidine (F8) and the ligand (through the iron atom) impose differences between subunits of hemoglobin and between myoglobins and hemoglobins which are largely, but not entirely, independent of these substituent effects. To assess the influence of protein structure on the chemical shifts of bound ligand, the shifts of ^{13}CO bound to myoglobin and hemoglobins from a wide range of species have also been measured.

Considerable work has been devoted to gaining an understanding of the various molecular interactions between the heme and the protein which modulate the kinetic and thermodynamic aspects of heme-ligand binding in hemoglobins and myoglobins. An unquestionably crucial role in the ligand affinity of hemoglobin arises from the conformational changes which occur on ligation of this tetrasubunited protein and which account in large measure for the positive allosteric cooperativity shown for hemoglobin ligation by such ligands as oxygen and carbon monoxide. Thus steric constraints operate to exclude ligands from the heme pocket when the protein is in one conformation; in the other conformation there is essentially unobstructed approach to the heme available to ligand (Perutz, 1970). Moreover, the ease of moving the iron atom into the plane of the porphyrin ring (for ligand binding) from a position out of that plane on the proximal face of the porphyrin probably also contributes importantly; the ease of this movement depends on steric factors which are ultimately transferred to the iron through the proximal histidine (F8) (Perutz, 1970).

To what degree do the electronic characteristics of the hemes influence the process of ligation and the nature of the bound ligand? Considerable work has been devoted to this question by studies of reconstituted myoglobins and hemoglobins containing hemes modified at the 2 and 4 positions of the porphyrin ring from the vinyl groups normally present (protoheme). In principle these studies can be of three general types: (i) observations of the effects of heme modification on the kinetic or thermodynamic aspects of ligation, including the effect of heme modification on the allosteric behavior of hemoglobins; (ii) observations on the nature of the modified heme within the globin protein; and (iii) the effect of heme modification on the

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nature of the bound ligand. The first of these approaches depends on energy differences between unliganded and liganded hemoglobins (thermodynamic) or differences in activation energies for ligation (kinetic) and has been extensively employed. For example, early studies of reconstituted hemoglobins containing heme modified at the 2 and 4 positions of the porphyrin ring clearly demonstrated that such substitutions can have pronounced effects on ligand affinity and the reactivity of the heme (Rossi-Fanelli et al., 1959; Antonini et al., 1964). Later studies (Sugita and Yoneyama, 1971) suggested that the oxygen affinities of reconstituted hemoglobins containing modified hemes reflected largely the inductive electronic nature of the modifying substituent. More recent studies on cobalt-containing hemoglobins and hybrid hemoglobins (in which two kinds of subunits have different types of hemes) suggest, however, that these apparent electronic substituent effects are coincidental and that the observed alterations in the oxygen affinities of these reconstituted hemoglobins arise from steric perturbations induced in the globin structure by the modifying substituents which cause anomalous allosteric behavior (Yonetani et al., 1974; Yamamoto and Yonetani, 1974).

Studies of type ii have shown shifts in the electronic spectra for the pyridine hemochrome derivatives which correlate well with the inductive effects of the substituent groups about the porphyrin ring (Falk, 1964). When incorporated into myoglobin, however, these modified hemes give electronic spectra which do not correlate in a simple linear fashion with their behavior in the model hemochromes (Rossi-Fanelli and Antonini, 1957). These different behaviors of the hemochrome systems as compared with those of the reconstituted myoglobins suggest that interactions between the protein and the heme, particularly its substituent groups, can significantly affect the electronic energy levels of the modified hemes.

Work of type iii on model hemochrome systems with carbon monoxide as a ligand has been reported for the carbon–oxygen stretching frequencies as a function both of the electronic nature of substituents about the periphery of the porphyrin ring and of the trans ligand (Caughey, 1967).

This paper is concerned with studies of the third type in which the environment experienced by the bound ligand is observed as a function of the changing character of the substituent group around the porphyrin ring. In this case, the ligand is carbon monoxide and the environment is probed by observation of the ^{13}C chemical shift of the ligand bound to reconstituted hemoglobins and myoglobins. These studies should be free of complications produced by effects which modify the cooperativity of ligation; they might accordingly assist in understanding the behaviors observed in more complex processes, such as ligation, and also assist in dissecting more rigorously previously observed effects into their electronic and steric components. In this work we have also varied the globin by examining the nature of carbon monoxide bound to modified hemes in different protein environments, specifically those reconstituted with dolphin apomyoglobin and human and rabbit apohemoglobins. These results are further compared with those for ^{13}CO bound to a variety of native myoglobins and hemoglobins from various animal species.

Experimental Section

Myoglobins. Sperm whale and horse heart myoglobins were obtained from Sigma.

Dolphin myoglobin, used in all reconstitution studies, was prepared from frozen Wright's whale dolphin muscle obtained from Marineland of the Pacific in Palos Verdes, Calif. The isolation procedures used were essentially those of Huh and Gurd (1970).

Hemoglobins. Hemoglobins were prepared from freshly drawn, citrated whole blood obtained from a wide variety of animal sources by the methods described previously (Moon and Richards, 1974). The various animal sources included human, bovine, mouse, guinea pig, rabbit, opossum, chick, pigeon, bullfrog, carp, gar, and torpedo ray. The monomeric hemoglobin from the blood worm, *Glycera dibranchiata,* was prepared from worms obtained through Pacific Biomarine Supply Co. The worms were washed and bled into cold artificial sea water. The red cells were collected by centrifugation at 500*g* and washed several times with sea water. The packed red cells (about 15 ml) were then lysed hypotonically with 3 volumes of distilled water at room temperature for 30 min. The stromata were removed by centrifugation at 30 000*g* and the resulting hemolysate was dialyzed exhaustively against 0.15 M sodium chloride (three 4-l. changes) at 4 °C. The crude *Glycera* hemoglobin was then concentrated by ultrafiltration (Amicon UM-10 membrane), centrifuged to remove traces of precipitated material, and applied to a 5 × 100 cm column of G-75 Sephadex. The column was eluted with 0.15 M sodium chloride and the slow moving band of monomeric *Glycera* hemoglobin was collected and concentrated by ultrafiltration.

Modified Hemes. Crystalline protohemin chloride was obtained from Sigma.

Deuterohemin chloride was prepared from protohemin chloride by methods essentially like those of Fischer and Orth (1937a) and similar to those of Caughey et al. (1966) used for the synthesis of deuteroporphyrin IX dimethyl ester. The yield of the deuteroporphyrin dimethyl ester was 58.2%. A portion of this product (1.2 g) was saved for the synthesis of 2,4-diacetyldeuterohemin chloride. The remainder of the deuterohemin dimethyl ester was saponified in 1% methanolic potassium hydroxide. The methanolic solution was extracted with chloroform to remove unreacted ester and then acidified with dilute hydrochloric acid to about pH 4. The resulting precipitate of deuterohemin chloride was collected in a sintered-glass funnel, washed with water, and dried. Elemental analysis of deuterohemin chloride gave: C, 59.71; H, 5.23; N, 8.22.

2,4-Diacetyldeuterohemin chloride was prepared by a method adapted from that of Fischer and Orth (1937b) and is similar to the method of Brockman et al. (1968). Deuterohemin dimethyl ester (1.17 g, prepared above) was dissolved in 150 ml of chloroform. To this mixture were added 150 ml of acetic anhydride and 15 ml of anhydrous stannic chloride. The reaction mixture was stirred for 1 h and then poured into a mixture of ice and ammonium hydroxide. After standing overnight, the methylene chloride layer was washed several times with water, dried over anhydrous sodium sulfate, and concentrated on a rotary evaporator. The concentrate was then applied to a column of neutral alumina (activity V) and chromatographed with methylene chloride to remove impurities. After all contaminating bands had been eluted, leaving only the green product band, the solvent was changed to chloroform, and the 2,4-diacetyldeuterohemin dimethyl ester was immediately eluted. The dimethyl ester was hydrolyzed in 1% methanolic potassium hydroxide and acidified with dilute hydrochloric acid. The precipitate of 2,4-diacetyldeuterohemin dimethyl ester was immediately eluted. The dimethyl ester was hydrolyzed in 1% methanolic potassium hydroxide and acidified with dilute hydrochloric acid. The precipitate of 2,4-diacetyldeuterohemin chloride was collected in a sintered-glass funnel, washed several times with water, and dried. The yield

TABLE I: Electronic Spectra of Pyridine Hemochromes.[a]

Heme	α (nm)		β (nm)		Soret (nm)	
	Obsd	Lit.	Obsd	Lit.	Obsd	Lit.
Meso	546	547	517	518	407	407
Deutero	545	545	516	515	406	406
Hemato	553	549	522	519	415	
2,4-Diacetyl-deutero	574	573	538.5	539	439	439

[a] Literature values were taken from Falk (1964, p 240).

was 38.8%. Elemental analysis gave: C, 60.20; H, 5.03; N, 8.50.

Mesohemin chloride was prepared by a method similar to that of Caughey et al. (1966) for the synthesis of mesoporphyrin IX dimethyl ester from protohemin chloride. The iron was then reintroduced into the porphyrin ring (Falk, 1964) and the crude mesohemin product was chromatographed on neutral alumina with 1,2-dichloroethane. The second band (red-brown) was collected. The methyl ester was hydrolyzed in 1% methanolic potassium hydroxide and acidified with dilute hydrochloric acid. The mesohemin chloride precipitate was collected in a sintered-glass funnel, washed with water, and dried. The yield was 34.9%. Elemental analysis gave: C, 61.62; H, 5.49; N, 8.55.

Hematoporphyrin IX was obtained from Sigma and converted to hematohemin by introduction of iron into the porphyrin ring (Falk, 1964). The yield of hematohemin was 42%.

The electronic spectral data of the pyridine hemochromes synthesized are collected in Table I. The hemochromes were prepared by dissolving a small amount of the hemin in an aqueous solution of 0.1 M potassium hydroxide and 3 M pyridine. The solutions were then reduced with a small amount of sodium dithionite. Pyridine hemochrome spectra were recorded on a Cary 14 spectrophotometer.

Infrared analysis of the carbonyl absorptions of 2,4-diacetyldeuterohemin dimethyl ester was also carried out. The methyl ester carbonyl stretching frequency was observed at 1750 cm^{-1}. The ratio of the two absorption bands, $A_{acetylCO}/A_{esterCO} = 0.75$, is similar to the value obtained by Caughey et al. (1966).

Apomyoglobin. Apomyoglobin was prepared by the method of Hapner et al. (1968). Yields of the lyophilized product ranged between 2.0 and 2.9% apomyoglobin per unit wet weight of muscle.

Apohemoglobin. Human and rabbit apohemoglobins were prepared by the method of Winterhalter and Huehns (1964).

Reconstitution of Myoglobins and Hemoglobins. The procedure used for reconstitution of dolphin apomyoglobin with modified hemes was an adaptation of the method described by Atassi and Caruso (1968). Hemin solutions were prepared in 0.1 M sodium phosphate (dibasic) with 1% sodium cyanide. About a twofold excess of hemin solution was added dropwise to a stirred apomyoglobin solution in distilled water at 0 °C. After stirring at 0–4 °C for about 2 h, the reconstituted myoglobin was applied to a column of G-15 Sephadex and eluted with 0.01 M dibasic phosphate. The samples were then concentrated by ultrafiltration.

Human and rabbit apohemoglobins were reconstituted with modified hemes by a method essentially like that of Winterhalter and Huehns (1964). About 1.0 g of the lyophilized apohemoglobin powder was dissolved in 100–120 ml of water and was dialyzed against 4 l. of phosphate buffer, pH 6.8. The protein was then dialyzed against two 1-l. changes of pH 7.5 phosphate buffer for 1.5 h each. Precipitated material amounting to about 10–15% of the total apohemoglobins was removed by centrifugation at 4 °C. About a 1.5-fold excess of hemin was dissolved in a minimal amount of 0.1 N sodium hydroxide (~1 ml) and was diluted to 25 ml with 0.01 M sodium cyanide–0.01 M phosphate buffer, pH 7.5. The hemin solution was added dropwise to the apohemoglobin solution and stirred for several hours at 4 °C. The resulting cyanmethemoglobin solutions were dialyzed against cyanide–phosphate, pH 7.5, in an ultrafiltration cell (Amicon PM-10 membrane) to remove excess hemin. The solutions were then dialyzed against 0.15 M sodium chloride and concentrated by ultrafiltration.

NMR Samples. Heme-modified carboxymyoglobin and carboxyhemoglobin samples were prepared by shaking the corresponding cyanmet derivative with 90% ^{13}C-enriched carbon monoxide (Prochem or Analytical Supplied Development Corp.) and a 1.5-fold excess of sodium dithionite in a syringe. The ^{13}CO derivatives were then transferred to an ultrafiltration cell, dialyzed against buffer (0.01 M phosphate for myoglobins; 0.15 M sodium chloride for hemoglobins), and concentrated. Sample concentrations were 1–3 mM myoglobin monomer or hemoglobin tetramer as determined spectrophotometrically from the Soret maxima using an approximate extinction coefficient of 154 mM for the carbon monoxide derivatives. Sample pH was determined on a Radiometer Model 26 pH meter.

NMR Studies. ^{13}C magnetic resonance spectra were obtained using the pulse Fourier transform technique on a Varian Associates XL-100-15 spectrometer operating at 25.17 MHz. The spectrometer was interfaced with a Varian 620i computer (16K memory) for data accumulation and Fourier transformation. A sensitivity enhancement of 0.10 s was applied to the free induction decay before the Fourier transformation was carried out. Samples were contained in a 12-mm tube with a 5-mm tube containing D_2O inserted concentrically to serve as a field frequency lock. All spectra were obtained at a probe temperature of 32 °C using a 90° pulse of 150 μs duration and an acquisition time of 0.8 s. Proton noise decoupling with a band width of 1.5 kHz centered on water was used throughout.

Results

Table II collects the chemical shift data for ^{13}CO bound to native myoglobins and hemoglobins from a number of animal sources. Native myoglobins all show a single sharp resonance about 207.8 ppm downfield of external tetramethylsilane. Native hemoglobins generally exhibit two distinct resonances which can be assigned to ^{13}CO bound to α or β subunits; these assignments are based on studies of the individual *p*-mercuribenzoate (PMB[1]) derivatized subunits and by analogies between different species (Moon and Richards, 1974). The chemical shifts for ^{13}CO bound to β subunits occur 206.2 ppm downfield of tetramethylsilane with the one major exception of the abnormally high-field resonance (205.21 ppm) observed in torpedo ray hemoglobin which we have tentatively assigned to ^{13}CO bound to the β subunit. Minor differences are observed in mouse and, perhaps, carp hemoglobins where the $\beta_{^{13}CO}$ resonances appear slightly upfield of the others. Resonances of ^{13}CO bound to α subunits show somewhat more variability.

[1] Abbreviations used: PMB, *p*-mercuribenzoate; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance.

TABLE II: ^{13}C Chemical Shifts for ^{13}CO Bound to Native Myoglobins and Hemoglobins.[a]

Source	δ α Subunit	δ β Subunit
Myoglobins		
Sperm whale	207.93	
Dolphin	207.70	
Horse	207.80	
Hemoglobins		
Opossum	207.42	206.13
Carp[b]	206.62	206.07
Gar[b]	206.48	206.19
Torpedo ray[b]	206.96	206.21
Blood worm (monomer)	205.45	
^{13}CO (solution)	184.60	

[a] Chemical shifts in ppm from external $Me_4Si \pm 0.05$. [b] Chemical shifts based on a single sample of blood.

TABLE III: ^{13}CO Chemical Shifts for Dolphin Myoglobin and Adult and Rabbit Hemoglobin Containing Unnatural Hemes.[a]

	CH_3CH_2-	H-	CH_2=CH-	CH_3CO-
Myoglobin	207.91	207.71	207.70	207.19
Adult α	207.04	206.65	206.79	205.85
Adult β	206.78	206.27	206.16	205.28
Rabbit α	208.61	208.45	208.21	Not obsd
Rabbit β	206.69	206.30	206.15	205.22

[a] Chemical shifts in ppm from external $Me_4Si \pm 0.08$.

Most species exhibit an $\alpha_{^{13}CO}$ resonance at 206.7 ppm, whereas those of rabbit (208.18 ppm), opossum (207.42 ppm), and torpedo ray (206.96 ppm) come at unusually low fields. Minor differences in the $\alpha_{^{13}CO}$ resonances of guinea pig (206.87 ppm) and gar (206.48 ppm) may also be significant. ^{13}CO-pigeon hemoglobin, in contrast, shows only a single rather broad resonance at 206.36 ppm (presumably due to an unusual high field $\alpha_{^{13}CO}$ resonance). The ^{13}CO adduct of the hemoglobin of the blood worm, *Glycera dibranchiata,* expectedly has a single absorption at 205.45 ppm for both the purified monomer and for the high molecular weight oligomeric fraction.

Table III collects data for the chemical shifts for ^{13}CO bound to modified hemes in reconstituted dolphin myoglobin, human adult hemoglobin, and rabbit hemoglobin. If the apoproteins were reconstituted with protoheme, the resulting ^{13}CO hemoglobin exhibited shifts virtually identical with those of native proteins; the reconstituted proteins also had excellent stability with no significant precipitation after several hours of NMR study at 32 °C. The effect of pH on reconstituted myoglobin, as shown in Figure 1, was essentially the same as that observed for native dolphin myoglobin. In contrast, globins reconstituted with modified hemes showed reduced stability toward denaturation and precipitation. This was especially true of ^{13}CO-hematomyoglobin (α-hydroxyethyl groups in place of vinyl groups on the porphyrin ring) which is not listed in Table II and which precipitated rapidly at 32 °C but gave a consistent shift of 207.7 ppm in three separate samples. No hematohemoglobin samples were prepared. While all reconstituted myoglobin samples were insensitive to pH above 7, myoglobins reconstituted with unnatural hemes showed increased susceptibility to acid denaturation. Even in the case of native ^{13}CO-myoglobin (see Figure 1) the ^{13}C resonance shifts upfield below pH 6.5 and disappears below pH 5. At

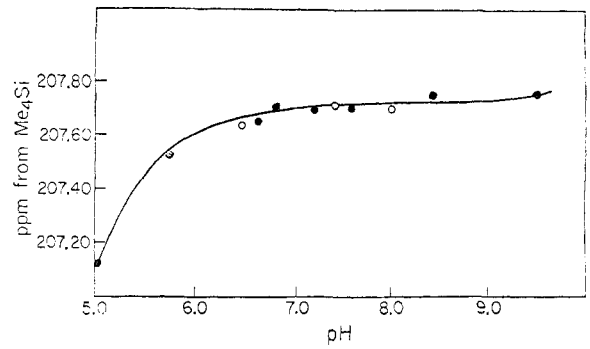

FIGURE 1: The pH dependence for the ^{13}CO chemical shifts of native (●) and reconstituted (○) protomyoglobin.

lower pH there is also an increased rate of sample precipitation. For ^{13}CO-myoglobin with modified hemes, this instability at lower pH is much more pronounced; such samples had good stability at high pH but precipitated rapidly much below pH 7.2. The native and reconstituted ^{13}CO-hemoglobins were not affected by acidity in the range pH 6.5-7.5; indeed no pH dependent shifts have been observed for any of the native tetrameric hemoglobins even as low as pH 5. The samples of hemoglobins reconstituted with deuterohemin were somewhat less stable toward precipitation than those containing mesoheme or 2,4-diacetyldeuteroheme.

Rabbit ^{13}CO-2,4-diacetyldeuterohemoglobin was unique in that only a single ^{13}CO resonance was observed despite thorough reduction of the hemes and extensive saturation with CO. The single observed resonance occurred at virtually the same position as that of ^{13}CO bound to the β subunit of human diacetyldeuterohemoglobin. The α subunit of native rabbit hemoglobin has a lower affinity for CO than the β subunit (Moon and Richards, 1974). However, all samples reconstituted with 2,4-diacetyldeuteroheme were difficult to saturate with CO and show a persistence of deoxy character in their electronic spectra. Accordingly, we conclude that the α subunit of rabbit 2,4-diacetyldeuterohemoglobin either does not bind ^{13}CO or that, if ^{13}CO is bound, it is in sufficiently rapid exchange with solution ^{13}CO that the resonance for the bound ^{13}CO is not observed. We consider very unlikely the possibility that the rabbit α-globin chains simply fail to bind 2,4-diacetyldeuteroheme since these subunits exhibit normal binding of other modified hemes and human α-globin chains bind 2,4-diacetyldeuteroheme readily. This possibility seems also unlikely in view of the results of Winterhalter et al. (1971), which suggest that α-globin chains actually have a greater affinity for hemes than do β-globins. Thus, we have assigned the single observed resonance for rabbit ^{13}CO-diacetyldeuterohemoglobin to ligand bound to β subunits.

The data in Table III suggest that the observed ^{13}CO chemical shifts are importantly influenced by the electronic nature of the substituent groups on the periphery of the porphyrin ring. Table IV emphasizes this point more clearly by listing the ^{13}CO chemical shifts observed for the modified myoglobins and the modified α and β subunits of human adult and rabbit hemoglobin relative to those for the respective native proteins. Both inductive and resonance effects should contribute to the overall electronic influence but this dissection is difficult. We have chosen the Hammett substituent constants σ_p as one of the simplest measures of the expected electronic influence of the substituent; Table IV includes these values. For further reference, the third ionization constants (pK_3) for neutral, 2,4-disubstituted deuteroporphyrins and the infrared

TABLE IV: Relative ^{13}CO Chemical Shifts for Globins Containing Modified Hemes.

	2,4 Substituent			
	CH_3CH_2-	CH_2=CH-	H-	CH_3CO-
Myoglobin	−5.2 Hz	0	−0.1 Hz	+12.8 Hz
Adult α	−6.2 Hz	0	+3.5 Hz	+23.7 Hz
Adult β	−15.6 Hz	0	−3.1 Hz	+22.3 Hz
Rabbit α	−10.2 Hz	0	−6.0 Hz	Not obsd
Rabbit β	−13.6 Hz	0	−4.0 Hz	+23.3 Hz
σ_p[a]	−0.15	−0.02	0	+0.46
pK_3[b]	5.8	4.8	5.5	3.3
ν_{CO}[c]	1973.0 cm^{-1}	1976.6 cm^{-1}	1975.0 cm^{-1}	1983.7 cm^{-1}

[a] σ_p values from McDaniel and Brown (1958) except for vinyl group σ_p which is calculated from the σ_I and σ_R values reported by Taft et al. (1963a,b). [b] pK_3 values for modified deuteroporphyrins IX from J. E. Falk (1964, p. 29). [c] Infrared carbonyl stretching frequencies for pyridine carbonyl hemochromes from Alben and Caughey (1968).

carbonyl stretching frequencies for the corresponding pyridine carbonyl hemochromes are also included in the table. Figure 2 shows a plot of chemical shift, $\delta_{^{13}CO}$ vs. σ_p, for reconstituted myoglobins and hemoglobins and demonstrates the good correlation that exists between the electronic nature of the substituent and the magnetic environment of the ligand. The slopes of these correlations ($\delta_{^{13}CO}/\sigma_p$) are a measure of the efficiency with which the electronic nature of the substituent is transferred to the ligand. For the subunits of rabbit and human hemoglobins, slopes near −2 are observed (the differences between −2.26 for adult β subunits, −2.28 for rabbit β subunits, and 1.86 for human α subunits are probably within the experimental limits of the data), whereas myoglobins have a slope of −1.12. This difference (between hemoglobins and myoglobins) indicates that the ligand is about twice as effectively influenced by electron substituent effects in hemoglobins as in myoglobins. In contrast to this result, studies of the electronic spectra of myoglobins and hemoglobins with modified hemes by Antonini et al. (1964) showed that shifts in the Soret bands induced by a particular heme are nearly identical for both proteins.

The electronic effects experienced by ^{13}CO as a ligand can be further demonstrated by comparisons between $\delta_{^{13}CO}$ of the various ^{13}CO-hemeproteins and the chemical properties of the porphyrins themselves outside the protein environment. Such correlations exist between $\delta_{^{13}CO}$ and pK_3 values for the ionization of the appropriate porphyrins and between $\delta_{^{13}CO}$ and the infrared carbonyl stretching frequencies of the appropriate pyridine carbonyl hemochromes (see Figure 3). These correlations show that those substituent effects observed for simple porphyrins are manifest in the magnetic environment experienced by ^{13}CO bound to the corresponding hemes within a myoglobin or hemoglobin environment. Moreover, there are essentially linear relationships between the electronic behavior in the simple chemical systems and in the intact proteins, with these electronic effects being more pronounced in hemoglobins than in myoglobins.

Discussion

In broad terms two types of effects on the binding of a ligand by a hemoglobin may be caused by changes in the substituents about the porphyrin ring: steric and electronic. For electronic effects in model systems, the electron withdrawing or donating ability of substituents at the periphery of the porphyrin ring

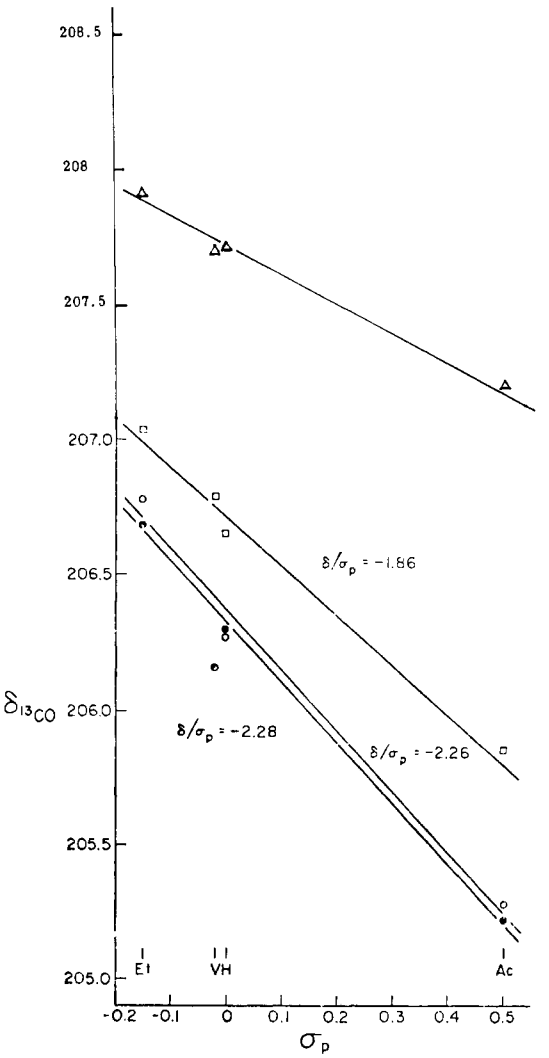

FIGURE 2: The correlation between the ^{13}CO chemical shifts of heme-modified myoglobins and hemoglobin and the substituent constants, σ_p, of the ethyl (Et), vinyl (V), hydrogen (H), and acetyl (Ac) modifying groups. The δ ^{13}CO data for myoglobins (Δ), the α ($\square$) and β ($\circ$) subunits of human adult hemoglobin as well as the β subunit ($\bullet$) of rabbit hemoglobin are shown. The σ_p values used were taken from McDaniel and Brown (1958), except for the vinyl group for which σ_p was estimated from the values of Taft et al. (1963a,b) in which $\sigma_p = \sigma_I + \sigma_R = 0.01 - 0.03$.

and the relative basicity of the trans ligand have been shown (Caughey, 1967) to significantly influence the interaction of carbon monoxide with iron(II) porphyrins. Results of various studies on modified porphyrins and hemes (Phillips, 1960; Alben and Caughey, 1962; Falk et al., 1966; Alben and Caughey, 1968) all suggest that electron withdrawal at the 2,4 position of the porphyrin ring reduces the strength of the iron-ligand bond for ligands such as carbon monoxide and oxygen. The possibility that such substituent effects could also be important in hemoglobin was explored by Sugita and Yoneyama (1971) who reported that reconstituted hemoglobins containing modified hemes were relatively stable and exhibited a reasonable degree of allosteric cooperativity. The oxygen affinities of these hemoglobins were influenced by the 2,4 substituents on the porphyrins with the affinities being ethyl > hydrogen > α-hydroxyethyl > vinyl in the ratio of 5:2:1.3:1. These data agreed with earlier kinetic studies of the binding of oxygen to hemoglobins containing modified hemes (Antonini and Gibson, 1960) and suggested that the oxygen af-

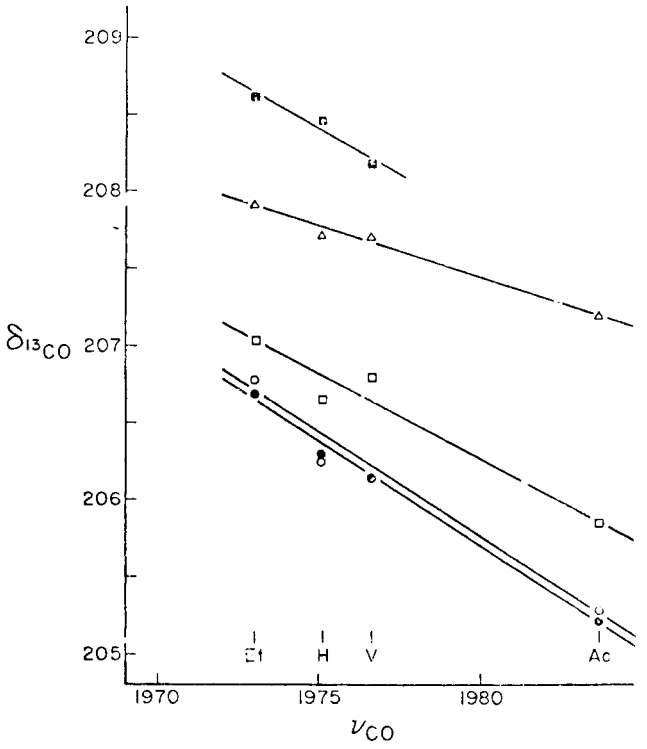

FIGURE 3: A plot of the relation between the $\sigma_{^{13}CO}$ data and the infrared carbonyl stretching frequencies (ν_{CO}) of 2,4-disubstituted pyridine carbonyl hemochromes. The $\sigma_{^{13}CO}$ data for myoglobin (Δ), α (□), and β (○) adult hemoglobin, and α (▲) and β (●) rabbit hemoglobin are shown. Infrared data were obtained from Alben and Caughey (1968).

finity increased with increasing inductive electron-donating character of the substituent.

More recent studies have, however, been interpreted as indicating that steric and not electronic effects dominate (Yonetani et al., 1974), particularly that the size of the group plays a crucial role (Yamamoto et al., 1974). Steric effects have also been raised as the mechanism by which variations in substituents influence hemoglobin cooperativity (Yamamoto and Yonetani, 1974), specifically that stereochemical effects induced in the heme pocket may be transmitted to the regions of intersubunit contacts and thereby affect cooperativity.

However, the nonequivalence of the α and β subunits of hemoglobin (Ogata and McConnell, 1972; Moon and Richards, 1974; Johnson and Ho, 1974) coupled with the effects of hemoglobin allostery can complicate discriminations based on observations of overall thermodynamic or kinetic behavior. In contrast the ^{13}CO chemical shifts of the ligand bound to the α and β subunits can be easily distinguished and are relatively independent of hemoglobin quaternary structure (Moon and Richards, 1974; 1976; Vergamini et al., 1973). Hence a comparison of the chemical shifts for ^{13}CO bound to different subunits with various hemes and the expected electron-donating or -withdrawing properties of these substituents might more reliably discriminate between electronic and steric effects.

The conclusions from the chemical shift data obtained in this work can be summarized: (i) the electronic nature of the substituents is reflected in the observed chemical shift; (ii) different globin units continue to exert influences on the modified hemes similar to those experienced by protoheme itself; (iii) the sensitivity of the chemical shift of bound ligand to changes in porphyrin substituents varies as a function of protein; and (iv) electronic effects which might be induced in the porphyrin ring by steric interactions between the porphyrin ring and peripheral residues of the heme pocket are not responsible for the differences (as experienced by bound ^{13}CO) between native myoglobins and the α and β subunits of modified hemoglobins.

Heme Reconstitution. Does introduction of modified hemes so grossly distort the protein that ligand binding characteristics are likewise grossly perturbed? Indeed Hill coefficients are invariably lower for reconstituted than for native hemoglobins (Sugita and Yoneyama, 1971; Yonetani et al., 1974; Yamamoto and Yonetani, 1974). Thus a simple explanation for the apparent absence of electronic substituent effects in reconstituted myoglobins and hemoglobins containing both iron and cobalt hemes (Yonetani et al., 1974; Yamamoto et al., 1974) could be that reconstitution led to large steric effects which overwhelmed any smaller electronic effects. However, EPR studies of reconstituted proto-, meso-, and deuteromyoglobins (Hori, 1972) show no change in heme plane orientation.

Our results offer further evidence that the protein is not grossly distorted when reconstituted with a modified heme, for the inherent differences between native myoglobin and the subunits of human and rabbit hemoglobins are fully retained in the reconstituted proteins. Thus the heme–protein interactions responsible for the observed differences between the native proteins are apparently largely unchanged in the reconstituted proteins. At the same time, these reconstituted proteins show a linear relationship with the electronic nature of the substituent groups. Further, the steric size of the substituent does not appear to be the dominant influence as ethyl and acetyl substituents, though of similar size, shift the resonances of bound ^{13}CO in opposite directions from that of deuteroheme-containing proteins.

The nature of the chemical shifts for ^{13}CO bound to reconstituted proteins provides further evidence that electronic effects dominate. In general, ^{13}C chemical shifts are most influenced by paramagnetic contributions to nuclear shielding (Karplus and Pople, 1963), where distortion of the carbon p orbitals generates a downfield shift. In the present case, the 2,4 substituents of the heme have been shown to vary the basicity of the pyrrole nitrogens and should, therefore, also influence the electron density at the iron. An increase in the electron density at the iron should cause a resultant increase in bonding between the heme and the ^{13}CO ligand due to increased d–π overlap between the orbitals of the iron and the carbon (Alben and Caughey, 1968). Increasing this overlap should increase the distortion of the p orbitals of carbon, thereby causing a downfield shift. Thus electron donation by groups on the periphery of the porphyrin ring should lead to downfield shifts for bound ^{13}CO. Mesoheme-containing proteins, where the ethyl group substituents are electron donating, show higher ^{13}CO binding and downfield shifts, whereas proteins reconstituted with 2,4-diacetyldeuteroheme (electron withdrawing) bind ^{13}CO less strongly and have resonances shifted upfield. Other metal carbonyl compounds also show upfield chemical shifts with increasing electropositive character of the metal (Levy and Nelson, 1972).

In a preliminary report, Caughey et al. (1973) have also shown that, as the substituents on the heme become more electron donating, the ν_{CO} of the carbon monoxide bound to the reconstituted heme decreases. This result indicates that increased electron density on the iron atom results in increased d overlap.

Steric factors induced by substituents about the porphyrin ring may play a role in kinetic and thermodynamic properties of ligand binding by reconstituted protein (Yonetani et al.,

1974; Yamamoto et al., 1974) without affecting the bound ligand, for example, because of interactions between the substituents at the heme periphery and the protein. In this regard, though the σ_p values for hydrogen and vinyl substituents are quite similar, differences are observed between the ligand affinities of deuteroheme and protoheme proteins; these differences may well reflect steric effects operating independently of those factors dominating the chemical shift of bound ^{13}CO.

Magnitude of Substituent Effects. There has been considerable ambiguity as to the origin of the degree to which the magnitude of the effects brought about by changing substituent groups of the heme can be influenced by the nature of the protein within which the heme is bound (hemoglobin vs. myoglobin, for example). In fact studies of the oxygen affinities for heme-modified hemoglobins containing iron (Sugita and Yoneyama, 1971) and cobalt (Yonetani et al., 1974) showed differences in the relative order of oxygen affinities compared with the order observed for the corresponding reconstituted iron and cobalt myoglobins. This led Yonetani et al. (1974) to emphasize the importance of the apoprotein in modulating the effect of substituent groups and, further, to consider that stereochemical as distinct from electronic effects where dominant. Our data (Figures 2 and 3) also show that the apoprotein can influence the degree to which the electronic effects of substituent group variations can be transmitted to the iron–ligand bond. Substituent effects are more pronounced in hemoglobin subunits than in myoglobin, but the order of these effects is not inverted. Thus, as probed by the environment experienced by ^{13}CO as a ligand, the apoprotein does modulate the degree to which bound ligand experiences electronic changes in the porphyrin ring.

One possible mechanism for this modulation of substituent effect by protein concentrates on the interaction between the heme iron and the proximal histidine (F8). The stronger the interaction between histidine (F8) and the iron, the more strongly will the substituent effects be transmitted to the ligand by the iron–ligand bond. This argument suggests, therefore, that histidine F8 interacts more strongly with the heme iron in hemoglobin subunits than in myoglobin.

Direct steric interaction between side-chain groups of the protein and atoms of the ligand may also influence the coupling between the ligand and the substituent group on the porphyrin by way of the iron. For example, the more the iron–ligand bond is bent, the more the iron–ligand bond order may be reduced, and the less strongly will the ligand be coupled to the substituent groups of the heme. In this regard, one should note that x-ray diffraction studies of the monomeric oxygen-carrying heme proteins from *Chironomus* (Huber et al., 1971) and *Glycera* (Padlan and Love, 1974) indicate that the Fe–C–O bond is bent and not perpendicular to the heme plane as might have been expected.

Subunit Nonequivalence. The factors responsible for the different ^{13}CO chemical shifts observed for native myoglobins and the α and β subunits of native hemoglobins must arise from some combination of steric and electronic effects acting directly on the heme and bound ligands. These effects are observable by our studies of the ^{13}CO chemical shifts even though not manifested by change in the electronic spectra of the α and β subunits. In the present studies, substituent changes which do not produce appreciable changes in the electronic spectra (Soret bands) of the hemes do, nevertheless, produce changes in the ^{13}CO chemical shifts which are comparable to, but not greater than, those observed between the α and β subunits of various hemoglobins. We conclude, therefore, that the electronic substituent effects which influence the ^{13}CO chemical shifts may not be the same as those which influence the electronic spectra of the porphyrin rings.

The infrared frequencies are also instructive in regard to subunit nonequivalence. Rabbit carboxyhemoglobin exhibits two carbon–oxygen stretching bands at 1951 (β subunit) and 1928 cm^{-1} (α subunit) (Matwiyoff et al., 1973). These differences, as well as those in the ^{13}CO chemical shifts, could arise, as discussed previously, by interaction between the proximal histidine and the heme, an interaction which may influence the iron–ligand bond without strongly influencing the Soret spectrum of the porphyrin ring. Also, this difference could reflect changes in the interaction between the ligand and the distal histidine (E7) in the ligand binding pocket (Moon and Richards, 1976). In this regard, heme proteins which lack a distal histidine such as *Glycera* hemoglobin (Padlan and Love, 1974) and the α subunits of opossum hemoglobin (Waterman and Stengel, 1974) show anomalous shifts of their ^{13}CO resonances relative to those observed in hemoglobins which do possess this distal histidine.

References

Alben, J. O., and Caughey, W. S. (1962), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 21,* 46.

Alben, J. O., and Caughey, W. S. (1968), *Biochemistry 7,* 175.

Antonini, E., Brunori, M., Caputo, A., Chiancone, E., Rossi Fanelli, A., and Wyman, J. (1964), *Biochim. Biophys. Acta 79,* 284.

Antonini, E., and Gibson, Q. H. (1960), *Biochem. J. 76,* 534.

Atassi, M. Z., and Caruso, D. R. (1968), *Biochemistry 7,* 699.

Brockman, H., Bliesner, K. T., and Inhoffen, H. H. (1968), *Justus Liebigs Ann. Chem. 718,* 148.

Caughey, W. S. (1967), *Annu. Rev. Biochem. 36,* 611.

Caughey, W. S., Alben, J. O., Fujimoto, W. Y., and York, J. L. (1966), *J. Org. Chem. 31,* 2631.

Caughey, W. S., Barlow, C. H., O'Keefe, D. H., and O'Toole, M. C. (1973), *Ann. N.Y. Acad. Sci. 205,* 296.

Falk, J. E. (1964), Porphyrins and Metalloporphyrins, New York, N.Y., Elsevier Publishing Co., p 135.

Falk, J. E., Phillips, J. N., and Magnusson, E. A. (1966), *Nature (London) 212,* 1531.

Fischer, H., and Orth, H. (1937a,b), Die Chemie des Pyrrols, Vol. 2, Leipzig, Akad. Verlagsgesellschaft, pp 304, 416.

Hapner, K. D., Bradshaw, R. A., Hartzell, C. R., and Gurd, F. R. N. (1968), *J. Biol. Chem. 243,* 683.

Hori, H. (1972), *Biochim. Biophys. Acta 278,* 399.

Huber, R., Epp, O., Steigemann, W., and Formanek, H. (1971), *Eur. J. Biochem. 19,* 42.

Huh, T. E., and Gurd, F. R. N. (1970), *J. Biol. Chem. 245,* 1931.

Johnson, M. E., and Ho, C. (1974), *Biochemistry 13,* 3653.

Karplus, M., and Pople, J. A. (1963), *J. Chem. Phys. 38,* 2803.

Levy, G. C., and Nelson, G. L. (1972), Carbon-13 Nuclear Magnetic Resonance for Organic Chemists, New York, N.Y., Wiley, p 143.

Matwiyoff, N. A., Vergamini, P. J., Needham, T. E., Gregg, C. T., Volpe, J. A., and Caughey, W. S. (1973), *J. Am. Chem. Soc. 95,* 4429.

McDaniel, D. H., and Brown, H. C. (1958), *J. Org. Chem. 23,* 420.

Moon, R. B., and Richards, J. H. (1974), *Biochemistry 13,*

3437.
Moon, R. B., and Richards, J. H. (1976), in preparation.
Ogata, R. T., and McConnell, H. M. (1972), *Biochemistry 11,* 4792.
Padlan, E. A., and Love, W. E. (1974), *J. Biol. Chem. 249,* 4067.
Perutz, M. F. (1970), *Nature (London) 228,* 726.
Phillips, J. N. (1960), *Rev. Pure Appl. Chem. 10,* 35.
Rossi-Fanelli, A. E., and Antonini, A. (1957), *Arch. Biochem. Biophys. 72,* 243.
Rossi-Fanelli, A. E., Antonini, E., and Caputo, A. (1959), *Arch. Biochem. Biophys. 85,* 37.
Stun, D. F., and Hixana, R. H. (1956), *Org. Synth. 16,* 77.
Sugita, Y., and Yoneyama, Y. (1971), *J. Biol. Chem. 246,* 389.
Taft, R. W., Price, E., Fox, I. R., Lewis, J. C., Andersen, K. K., and Davis, G. T. (1963a), *J. Am. Chem. Soc. 85,* 709.
Taft, R. W., Price, E., Fox, I. R., Lewis, I. C., Andersen, K. K., and Davis, G. T. (1963b), *J. Am. Chem. Soc. 85,* 3146.
Vergamini, P. J., Matwiyoff, N. A., Wohl, R. C., and Bradley, T. (1973), *Biochem. Biophys. Res. Commun. 55,* 453.
Waterman, M. R., and Stengel, P. (1974), *Biochim. Biophys. Acta 359,* 401.
Winterhalter, K. H., and Huehns, E. R. (1964), *J. Biol. Chem. 239.*
Winterhalter, K. H., Iopollo, C., and Antonini, E. (1971), *Biochemistry 10,* 3790.
Yamamoto, H., Kayne, F. J., and Yonetani, T. (1974), *J. Biol. Chem. 249,* 691.
Yamamoto, H., and Yonetani, T. (1974), *J. Biol. Chem. 249,* 7964.
Yonetani, T., Yamamoto, H., and Woodrow, G. V., III (1974), *J. Biol. Chem. 249,* 682.

Magnetic Resonance Studies of the Binding Site Interactions between Phosphorylcholine and Specific Mouse Myeloma Immunoglobulin†

A. M. Goetze and J. H. Richards*

ABSTRACT: The interaction of phosphorylcholine-binding mouse myeloma protein M603 and the isotopically substituted hapten phosphoryl[*methyl*-^{13}C]choline has been investigated using ^{13}C and ^{31}P nuclear magnetic resonance (NMR) spectroscopy. Upon binding to antibody, upfield shifts of 0.7 and 1.5 ppm are observed for the hapten ^{13}C and ^{31}P resonances, respectively, and both spectra are in the "slow" exchange limit. Linewidth analysis indicates some immobilization of the phosphate group but essentially unrestricted methyl group rotation for the bound hapten. Hapten–antibody dissociation rate constants of 10 and 38 s^{-1} are calculated from ^{13}C and ^{31}P NMR spectra, respectively, suggesting the possibility of differential dissociation rates for the two opposing ends of the phosphorylcholine molecule. The NMR data are entirely consistent with the known x-ray structure of the M603 Fab′–phosphorylcholine complex (Segal, D. M., Padlan, E. A., Cohen, G. H., Rudikoff, S., Potter, M., and Davies, D. R. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4298).

Antibodies, the primary molecules of the immune response, are responsible for the specific recognition of antigen and also for other, biologically important, effector functions which include initiation of the complement cascade, release of histamine from mast cells, and activation of the differentiation of B lymphocytes into antibody-producing plasma cells (Metzger, 1974). X-ray crystallographic techniques have recently revealed the three-dimensional structures of some antibodies and their Fab fragments (Davies et al., 1975; Poljak, 1975) and correlation of antibody structure with various biological functions has attracted interest (Yasmeen et al., 1976; Hurst et al., 1974). A precise knowledge of the molecular details (both structural and dynamic) of the interactions between antigen and antibody is central to a thorough understanding of antibody specificity and the relationship between the structure of an antibody and its function. Various physical techniques have been employed to elucidate such information including circular dichroism (Rockey et al., 1972), chemical modification (Grossberg et al., 1974), and magnetic resonance (Dwek et al., 1976). We have initiated a systematic study of the correlation between antibody structure and hapten binding properties of a group of myeloma proteins which have specificity for phosphorylcholine and related substances (Potter, 1972). The study of these antibodies offers several advantages: (i) the immunoglobulins can easily be obtained as homogeneous proteins in relatively large (gram) quantities; (ii) extensive amino acid sequence data are available for many of them (Barstad et al., 1974; Hood et al., 1975); (iii) the three-dimensional structure of the Fab′ fragment of a typical member of this group, M603, has recently been reported (Segal et al., 1974). This knowledge allows the results of binding experiments to be interpreted in terms of known and inferrable molecular structures.

Magnetic resonance affords a physical technique for studying hapten–antibody interactions which is particularly well suited to phosphorylcholine as one can observe events occurring at either end of the hapten using the signals from ^{31}P (natural abundance) in the phosphoryl group and ^{13}C (enriched) in the trimethylammonium group without significant interference from protein signals. We report here the results

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