Proton Magnetic Resonance Studies of *Desulfovibrio* Cytochromes c_3†

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ABSTRACT: Proton magnetic resonance spectra (pmr) (220 MHz) of cytochromes c_3 from *Desulfovibrio vulgaris* and *Desulfovibrio gigas* in D_2O were examined while varying temperature, pD, and oxidation state. Magnetic susceptibility measurements by a pmr method showed that ferricytochrome c_3 (*D. vulgaris*) is paramagnetic. The molar paramagnetic susceptibilities of ferricytochromes c_3 (*D. vulgaris*) and c_3 (*D. gigas*) are 6.85×10^{-3} and 7.82×10^{-3} cm^3 mol^{-1} at 22° indicating that all three or four heme iron atoms in each molecule are in the Fe^{3+} ($S = 1/2$) state. Spectra of ferrocytochrome c_3 (*D. vulgaris*) indicate four hemes per molecule with histidine coordinated to each heme iron at the 5th and 6th ligand positions, valine and leucine residues (10) mainly around the edge of heme groups, and lysine residues (20) mainly on the surface of the protein. Spectra of ferricytochromes c_3 contain many contact-shifted resonances and support the above conclusions. Resonances of heme methyl groups and meso protons are identified. Many common spectral features indicate the general structures of the two cytochromes are very similar. During reoxidation of ferrocytochrome c_3 (*D. vulgaris*) to ferricytochrome c_3, spectra of two partially oxidized intermediates were observed.

In 1956 Postgate isolated a protein from the bacterium, *Desulfovibrio vulgaris*, similar in molecular weight and absorption spectrum to mammalian cytochrome *c* but with a lower redox potential. It was designated cytochrome c_3. The amino acid sequence (Ambler, 1968) of c_3 shows no homology with mammalian cytochrome *c* except for four sequences: two Cys-(residue)$_2$-Cys-His, and two Cys-(residue)$_4$-Cys-His that resemble the Cys-(residue)$_2$-Cys-His sequence of cytochrome *C* that provides thioether attachments of the heme to the polypeptide chain and a histidine ligand to the heme iron atom. LeGall *et al.* (1965) isolated a similar C_3 protein (originally designated C_3') from *Desulfovibrio gigas*. The *D. gigas* C_3 protein is homologous with *D. vulgaris* C_3 at 49 residue positions out of 111 including homology at the four Cys–Cys-His sequence positions (Ambler *et al.*, 1969). The two proteins are similar in molecular weight ($\approx$14,000) and redox potential but have quite different isoelectric points. Similar C_3 proteins have been obtained from other *Desulfovibrio* species (Drucker *et al.*, 1970a) and certain heme proteins from photosynthetic bacteria exhibit related characteristics (Meyer *et al.*, 1971). The cytochromes c_3 exhibit ferri and ferro states; the latter state is readily autoxidizable. While the exact physiological role of these proteins is not established, they are implicated in the complex sulfate reduction metabolism of *Desulfovibrio* (Hatchikian *et al.*, 1972).

Initial studies of cytochrome c_3 indicated two hemes per molecule (Postgate, 1956; Horio and Kamen, 1961). Later work (Drucker *et al.*, 1970b) suggested three hemes per molecule but recent studies by direct analysis (Meyer *et al.*, 1971, Yagi and Maruzama, 1971), electron spin resonance (esr), spectroscopy (Der Vartanian, 1973), and chemical reactivity (Der Vartanian and LeGall, 1971) have led to the conclusion that there are four heme moieties per molecule in agreement with the number of presumptive attachment sequences (*i.e.*, Cys–Cys-His). The nature of the ligands at the 5th and 6th heme iron coordination sites are not known. The presence of histidines following the presumptive Cys–Cys attachment sequences as in mammalian-type cytochromes *c* implies that histidine binds at the 5th ligand position. The ligand at the 6th position cannot be methionine, however, since this residue does not occur in all cytochromes c_3 (Ambler *et al.*, 1969). The ferri form of cytochrome c_3 appears to be a very stable conformation as judged by resistance to denaturation or chemical reactivity (Der Vartanian and LeGall, 1971; LeGall *et al.*, 1971). The ferro form is less stable.

In the course of a general study of structures and interactions of cytochromes *c* by pmr spectroscopy (McDonald and Phillips, 1973), we have made a preliminary examination of characteristics of pmr spectra of *D. vulgaris* and *D. gigas* cytochromes c_3 which we present here. A brief summary of these results was reported earlier (McDonald *et al.*, 1970).[1]

Experimental Section

Methods for extraction and purification of cytochrome c_3 used for pmr studies have been described (LeGall *et al.*, 1971).

Pmr spectra were acquired with a Varian Associates high-resolution spectrometer which operates at a frequency of 220 MHz. Temperature of the samples was regulated to ±1°. Signal-to-noise characteristics of most of the spectra were enhanced by employing a Varian Associates C-1024 computer of average transients. Resonance positions were measured with respect to the methyl resonance of the sodium salt of 2,2-dimethyl-2-silapentanesulfonic acid used as an internal standard. Positive shifts are to low field from the reference resonance position.

Results and Discussion

Magnetic Susceptibilities. The molar paramagnetic susceptibility of *D. vulgaris* cytochrome c_3 (3.12×10^{-3} M in

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[1] Since completion of this manuscript, we have been informed by Professor R. J. P. Williams of Oxford University that he and his coworkers have recently studied the *Desulfovibrio vulgaris* C_3 protein and that a report of their work has been submitted to *Nature*.

D_2O, pD 6.0) was measured at 22° employing eq 1 based on an nmr method. The use of this procedure with the magnetic field geometry of the Varian Associates 220-MHz spectrometer has been described (Phillips and Poe, 1972). In eq 1, c is concen-

$$\chi_M^P = (3/4\pi c)(\Delta f/f) \quad (1)$$

tration in mol cm^{-3}, f is the spectrometer frequency (2.2×10^8 Hz), and Δf is the downfield *paramagnetic* shift (Hz) of an internal reference resonance (measured with respect to an external reference) that is caused by addition of the paramagnetic protein to the solution. The concentration was determined by optical absorption using $E(\mu M) = 411$ at 4090 Å (Drucker *et al.*, 1970a). An independent measurement of concentration based on comparison of the pmr intensity of the C_3 protein with the corresponding pmr intensity of a solution of hen egg-white lysozyme of known concentration (correcting for their different hydrogen atom components) gave a value of 3.26×10^{-3} M. The observed shift of the internal reference resonance on addition of the paramagnetic protein to the solution is the net of a downfield shift from the paramagnetism and an upfield shift from the diamagnetism of the protein. Thus, to determine Δf from the observed shift we must know the shift caused by the protein in a diamagnetic state at the same concentration. Ferrocytochrome c_3 is thought to be diamagnetic (all heme iron atoms in Fe^{2+} (S=O) states) because the similarity of its optical absorption spectrum to that of diamagnetic ferrocytochrome c and because it exhibits no esr (LeGall *et al.*, 1971). On anaerobic reduction of the ferricytochrome c_3 solution to ferrocytochrome c_3 with dithionite, a large upfield shift of the reference resonance was observed (-13.25 ± 0.25 Hz from the position with no protein in solution) confirming the diamagnetic state of the ferro protein and providing a diamagnetic shift correction for calculation of the paramagnetic shift Δf of the ferri protein. The magnitude of the diamagnetic shift is about twice that expected for a polypeptide of the same molecular weight and concentration; the additional diamagnetism must arise from the component porphyrin moieties. A value of 6.85 (± 0.20) $\times 10^{-3}$ cm^3 mol^{-1} was obtained for χ_M^P of ferricytochrome c_3.

Chemical and spectroscopic evidence from the various studies cited above indicates that the electron spin states of all the heme iron atoms in ferricytochrome c_3 are equivalent or nearly so. If this is the case, the effective magnetic moment per iron atom (μ) can be determined from eq 2, where n is the

$$\mu = [3kT\chi_M^P/nN\beta^2]^{1/2} \quad (2)$$

number of heme groups and k, T, N, and β have their usual significance (Phillips and Poe, 1972). If $n = 4$, $\mu = 2.01$ Bohr Magnetons, a value within the range of μ from 2.0 to 2.5 normally exhibited by Fe^{3+} ($S = 1/2$). This result supports the conclusion from earlier light absorption and esr studies (LeGall *et al.*, 1971) that the heme iron atoms of ferricytochrome c_3 are all in the low-spin ferric state as they are in ferricytochrome c. Further support, shown below, is provided by the fact that contact-shifted resonances of porphyrin hydrogen atoms in the pmr spectrum of ferricytochromes c_3 exhibit shifts and resonance widths typical of heme proteins in which the iron atoms are in the Fe^{3+} ($S = 1/2$) state. The satisfactory value of μ obtained by choosing $n = 4$ is not, however, unequivocal evidence for four hemes per protein molecule since $n = 3$ gives $\mu = 2.32$, a value still within the expectation range. Finally, the precision associated with the determination of χ_M^P reflects our estimate of the precision of the measurement of the reference resonance shifts and does not include possible errors from measurement of protein concentration, incomplete reduction of the protein, or the presence of paramagnetic impurities.

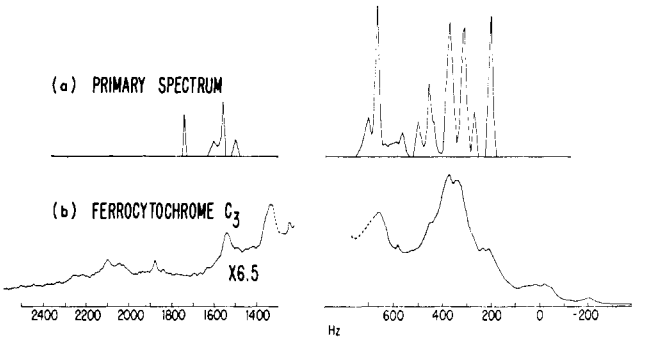

FIGURE 1: Pmr spectra of *D. vulgaris* ferrocytochrome c_3: (a) computed primary spectrum; (b) native protein.

The paramagnetic susceptibility of *D. gigas* ferricytochrome c_3 was examined in the same manner. We were not able to obtain a good value for the diamagnetic shift produced by the ferroform of the protein because of its rapid autoxidation. Consequently, the diamagnetic correction obtained from *D. vulgaris* ferrocytochrome c_3 was used. The value of χ_M^P determined for *D. gigas* ferricytochrome c_3 at 22° was 7.82×10^{-3} cm^3 mol^{-1}. The effective magnetic moment per iron atom is 2.48 or 2.14 Bohr Magnetons depending on choice of n as 3 or 4, respectively.

Pmr of Ferrocytochrome c_3. A computed "primary" pmr spectrum of the residue side chain CH protons of *D. vulgaris* ferrocytochrome c_3 is shown in Figure 1a. This is a representation of the spectrum that would be expected if the protein was extended in neutral D_2O so that resonance positions were not influenced by protein structure. The procedure for computing such a spectrum was described previously (McDonald and Phillips, 1969). Prominent features of the computed spectrum are: resonance at 205 Hz arising from all the methyl group resonances of eight valine residues and two leucine residues (60 protons), resonance at 270 Hz from five threonine methyl group resonances (three protons each), resonance at 310 Hz from methyl resonances of ten alanine residues, and γ-CH_2 protons of 20 lysine residues, resonance at 370 Hz primarily from lysine δ-CH_2 and β-CH_2 protons, resonance at 665 Hz from lysine ϵ-CH_2, and cysteine β-CH_2 protons, sharp resonances at 1740 Hz and 1555 from C-2 and C-4 resonances, respectively, of nine histidine residues (the remaining aromatic resonances arise from two phenylalanine residues and three tyrosine residues). Primary resonance positions of the porphyrin moieties are not included. Porphyrin substituents would contribute additional resonance absorption between 440 and 1000 Hz and the meso CH protons would provide a strong resonance at about 2310 Hz (McDonald and Phillips, 1973).

A solution of *D. vulgaris* ferricytochrome c_3 (15% in D_2O, pD 8.0) was reduced under nitrogen with sodium dithionite. The pmr spectrum was obtained at 35°; regions of the spectrum are shown in Figure 1b. Resonance widths (width at half-height) of resolved resonances at the fringes of the spectrum are broader than expected for a diamagnetic protein with a molecular weight of 14,000. For example, a methyl resonance at -203 Hz has a resonance width of about 40 Hz and a single proton resonance (probably a histidine C-2 resonance) at 1877 Hz has a resonance width of about 20 Hz. Single resolved resonances of hen lysozyme or ferrocytochrome c, diamagnetic proteins of similar size, have resonance widths of 7–10 Hz. The lack of resolved detail in the pmr spectrum of ferricyto-

chrome c_3 indicates that most of the resonances are broadened by some means. Several sources of such broadening can be proposed: aggregation, multiple forms of the protein, residual paramagnetism, and paramagnetic impurities. Resolution was not improved by lowering protein concentration, increasing the amount of reductant, or adding EDTA to chelate paramagnetic impurities. Although the reduced protein was stable for the relatively short intervals required for magnetic susceptibility measurements, it does autoxidize appreciably to partially oxidized paramagnetic intermediate (discussed below) forms over the period of hours required to acquire several time-averaged pmr spectra. This oxidation is slowed but still persists at 5°. We conclude that the resonance broadening evident in Figure 1b arises from fast exchange of the diamagnetic ferrocytochrome c_3 with paramagnetic oxidized states. As autoxidation proceeds, many resonances broaden and shift, contact-shifted resonances appear, and finally most of the features of the spectrum of Figure 1b become altered. Protein in this intermediate state of oxidation can be partially but not completely rereduced. The spectra of Figure 1b are representative of the best reduction we achieved. Quantities of protein available were insufficient to fully explore conditions (*e.g.*, pH) that might provide more complete or stable reduction.

Because of the relatively poor resolution of the pmr spectra of ferrocytochrome c_3, these spectra must be interpreted with some caution. Nevertheless, some tentative conclusions can be drawn. The spectrum in Figure 1b is quite different from the primary spectrum. Most of these differences are probably caused by the large ring-current magnetic fields of the porphyrin moieties and by the smaller ring-current fields of the many aromatic residues. Resonances of protons close to the faces of the aromatic rings experience high-field shifts whereas resonances of protons around the periphery of the aromatic structures are shifted to low field. Studies (McDonald and Phillips, 1973) of ferrocytochrome *c* have shown that protons of ligands at the 5th and 6th ligand positions of the heme iron that are close to the porphyrin face and sixfold symmetry axis experience high-field shifts of up to 7 ppm. First, examining the high-field spectrum of Figure 1 we note that the methyl resonances of valine and leucine residues that provide a strong peak at 205 Hz in the primary spectrum are dispersed in the spectrum of native ferrocytochrome c_3 so that only a low broad resonance band is observed around 200 Hz. The interesting point is that few of these methyl resonances have been shifted very far to high field by ring currents in spite of the large heme content of the protein. The highest field resonance that was detected is a methyl resonance at -203 Hz. The resonance absorption from -100 to 100 Hz accounts for about another 20 protons. Thus, most of the valine and leucine residues are in locations that do not bring their methyl groups close to the faces of the porphyrin rings or aromatic residues (primarily histidines). The strong resonances at 310 and 370 Hz in the primary spectrum arise mainly from lysine residues and these appear to persist largely unchanged in the spectrum of the native protein. This indicates (as would be expected for a highly water soluble protein) that most of the lysine residues are on the surface of the protein extending into the solvent. Resonances of the γ and methyl protons of methionine bound at the 6th ligand position of a diamagnetic heme protein exhibit characteristic resonances in the field region from about -400 to -800 Hz. Since methionine does not occur in *D. gigas* cytochrome c_3 it is not likely to be a heme ligand in *D. vulgaris* cytochrome c_3. This assumption is confirmed by the absence of resonances above -200 Hz in Figure 1b. It also appears unlikely that lysine or cysteine residues are heme iron ligands on the following basis. From pmr studies of ferrocytochrome *c* (McDonald and Phillips, 1973) we found that protons bonded to carbon atom next to the liganding atom are shifted 4.4–6.9 ppm to high field by the ring-current field of the porphyrin ring. Thus, if lysine or cysteine is a ligand in cytochrome c_3, we would expect resonances of the lysine ϵ protons or the cysteine β protons to appear from -300 to -850 Hz. Since no resonances were detected above -200 Hz, either these residue types are not ligands or their resonances are too broad to be detected in the pmr spectra that we examined.

Next, we consider the aromatic region of the pmr spectrum of ferrocytochrome c_3 shown in Figure 1b. The amplification of this low-field region is 6.5 times greater than that of the high-field spectrum. The fairly well-resolved resonance at 1877 Hz appears to have intensity of about 1 proton and is probably a C-2 proton resonance of a histidine residue. The corresponding C-4 resonance is part of the resonance absorption at 1540 Hz (see Figure 3). The resonance absorption from 2000 to 2300 Hz is too broad and weak to integrate accurately but it appears to correspond to about ten protons. These resonances are probably porphyrin meso CH protons that are shifted somewhat to high field from their primary position at about 2300 Hz. Other meso CH resonances may be too broad to detect or are shifted much farther to high field by ring-current fields. The resonances at about 1340 and 1740 Hz are assumed to arise primarily from the aromatic resonances of the two phenylalanine and three tyrosine residues. If this assumption is correct most of the aromatic residue protons experience modest to large high-field ring-current shifts such as would be expected if the aromatic residues are packed more or less between faces of porphyrin rings. The C-2 resonances of eight histidine residues are missing from the resonance region around 1700 Hz and appear to have been shifted to high field, perhaps beyond the aromatic region entirely. This situation would be expected if all eight histidines are ligands of the iron atoms of four heme groups. In fact, if this is the case, the histidine C-4 resonances would occur at about 0 Hz (McDonald and Phillips, 1973) and may account for some of the resonance absorption that is observed from -100 to 100 Hz.

In the pmr spectrum of horse cytochrome *c* in D_2O, certain slowly exchanging NH proton resonances are observed from 1750 to 2000 Hz (McDonald and Phillips, 1973). They have been assigned to peptide NH protons of residues in the interior of the protein that are shielded from the solvent and remain unexchanged. In the low-field pmr spectrum of ferrocytochrome c_3 no resonances occur that because of location or slow exchange are inferred to be NH protons. Either all NH protons have ready access to the solvent because of their location at the protein surface or because the molecule is subject to transient partial unfolding or nonexchanging NH protons are located in porphyrin ring-current fields such that their resonances are shifted substantially to high field out of the characteristic NH region.

No attempt was made to study the pmr spectrum of *D. gigas* ferrocytochrome c_3 because of the rapid autoxidation of this protein observed in magnetic susceptibility experiments.

Pmr of Ferricytochrome c_3. Regions of the pmr spectrum of *D. vulgaris* ferricytochrome c_3 (15% in D_2O, pD 8.0) at 35° are shown in Figure 2. Spectra of these same regions acquired at pD values from 4 to 9 and at temperatures from 5 to 45° exhibited the same general pattern but substantial shifts of many resonances. Vertical bars beneath the spectra in Figure 2 represent resonances that were clearly resolved at some temperature or pD value. The taller bars represent methyl group

resonances, the shorter bars represent individual protons. Dashed bars are less certain in location or intensity than the solid bars. Subscript numbers are for identification in discussion.

The pmr spectrum of ferricytochrome c_3 is greatly different from that of native ferrocytochrome c_3 or the primary spectrum of Figure 1. The major sources of these differences are hyperfine contact shifts and pseudocontact shifts arising from the paramagnetism of the three or four heme iron atoms of the protein. It is not known whether there is a conformational change when the protein is oxidized from the ferro form that also contributes in a lesser fashion to differences between pmr spectra of the oxidized and reduced proteins.

Several methyl resonances are observed in the field region from 2500 to 6500 Hz, that resemble in location and resonance width ($\approx$70 Hz) contact-shifted resonances of porphyrin methyl substituents of mammalian cytochrome *c*. From this we infer that the nature and magnitudes of the contact shifts of cytochrome c_3 and cytochrome *c* are rather similar and interpret the spectra in Figure 2 on that basis.

Considering Figure 2b first, we note that a strong band of resonance absorption remains in the 300–425-Hz region that is similar to the region of lysine resonances in the primary spectrum. Again this supports the postulate that the lysine residues are mostly extended into solvent at the surface of the protein and their resonances remain close to their primary positions. Comparing the methyl pmr regions of the ferri and ferro proteins it is clear that in the case of the oxidized protein there is a much greater shift of resonance absorption to high field from the primary positions. The high-field region above 100 Hz comprises resonances of about 75 protons for ferricytochrome c_3 as contrasted with about 23 for ferrocytochrome c_3. The resolution of the resonance region from 100 to -600 Hz is insufficient to permit determination of the components as to methyl resonances and single proton resonances. However, the series of partially resolved peaks indicates that most of this absorption arises from methyl groups. If the situation is parallel to that in cytochrome *c* (McDonald and Phillips, 1973) protons around the edges of the porphyrin rings are subject to high-field pseudocontact shifts and protons near the faces of the porphyrins are subject to low-field shifts. That is, these shifts for cytochrome *c* are larger and opposite in direction to the ring-current shifts. Consequently, we expect the absorption above 100 Hz to comprise resonances of porphyrin meso CH protons and thioether bridge methyls that are subject to hyperfine and/or pseudocontact shifts and resonances of aliphatic and methyl protons that lie close to the edges of the porphyrin rings in ferricytochrome c_3 and are subject to pseudocontact shifts. The 23 protons in this field region in the spectrum of ferrocytochrome *c* have been associated with ligands and residues close to the porphyrin faces. In all probability they are mostly shifted to low field out of this spectral region in the ferricytochrome c_3 spectrum. Thus, if there are four heme groups, we may expect 16 heme meso CH resonances in the high-field region of Figure 2. The three of four broad resonances from -2000 to -3500 Hz are thus assigned. Their very large widths indicate that the protons are close to the heme iron. The remaining meso CH resonances probably fall to lower field in the overlapped region or are too broad to detect. Thioether bridge methyls may contribute up to 24 proton resonances to the high-field region. Thus, about 35 proton resonances remain to be assigned to protons and particularly methyl groups of residues close to the edges of the heme groups. This conclusion supports the analysis of the pmr spectrum of ferrocytochrome c_3 in which it was proposed that most

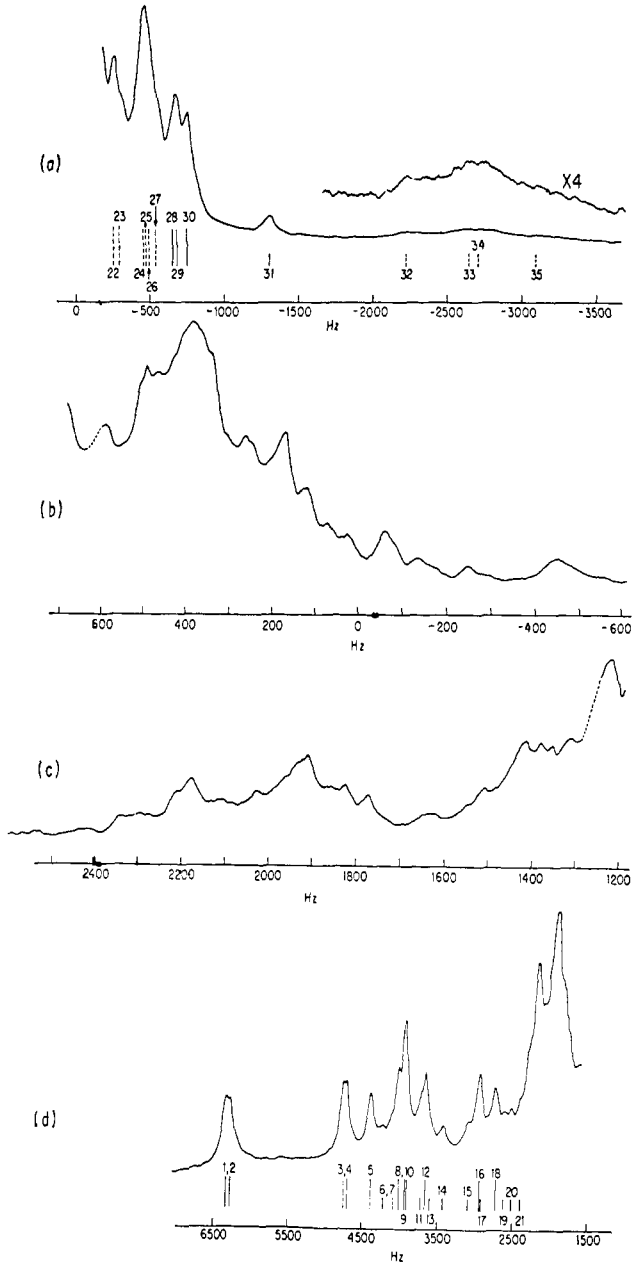

FIGURE 2: Regions of the pmr spectrum of *D. vulgaris* ferricytochrome c_3.

of the methyl-bearing residues are wrapped around the porphyrin edges rather than intertwining between faces of porphyrin moieties.

Turning next to the low-field spectra in Figure 2, we note that there are 11 methyl resonances from 2500 to 6500 Hz. Comparison with previous studies of low-spin ferric heme proteins (Wüthrich, 1970) suggests that these resonances that are greatly shifted from their primary positions can only be assigned to methyl groups attached to the heme rings. These protons are thought to be subject to large hyperfine contact interactions (Wüthrich, 1970). At four methyl groups per heme, the 11 methyl resonances require a minimum of three hemes per molecule but do not exclude four hemes since the missing five methyl resonances may occur in the overlapped resonance region from 1700 to 2300 Hz. This latter region comprises resonances of about 30 protons but is not sufficiently resolved to permit identification of methyl resonances. It should be mentioned that resonances in this region in the spectrum of the reduced protein appear to broaden and shift out as the

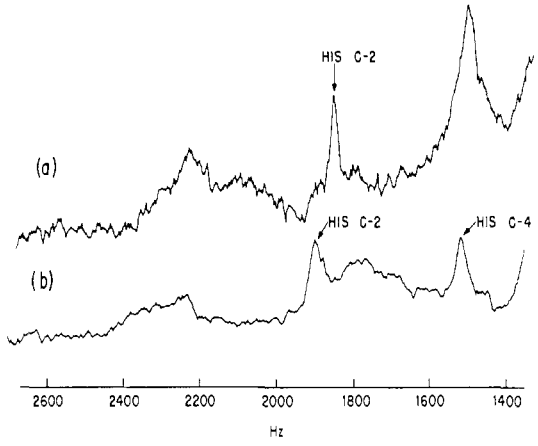

FIGURE 3: Aromatic resonance region of *D. vulgaris* cytochrome c_3 (15% in D_2O, pD ≈8, 40°): (a) reduced; (b) early stage of autoxidation of reduced from.

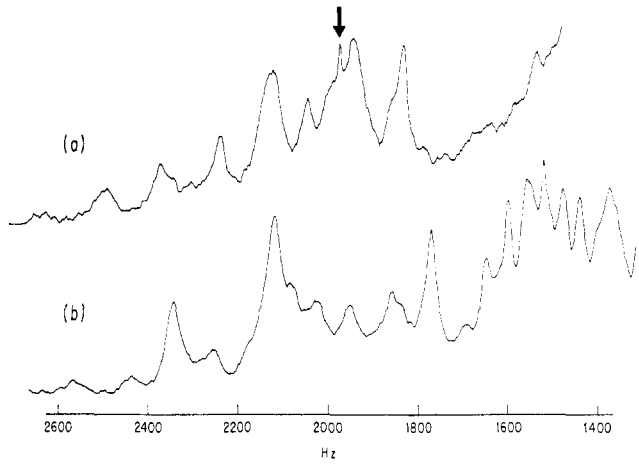

FIGURE 5: Normal histidine C-2 resonance regions for ferricytochromes c_3 (15% in D_2O, pD 6.0, 32°): (a) *D. vulgaris* c_3; (b) *D. gigas* c_3.

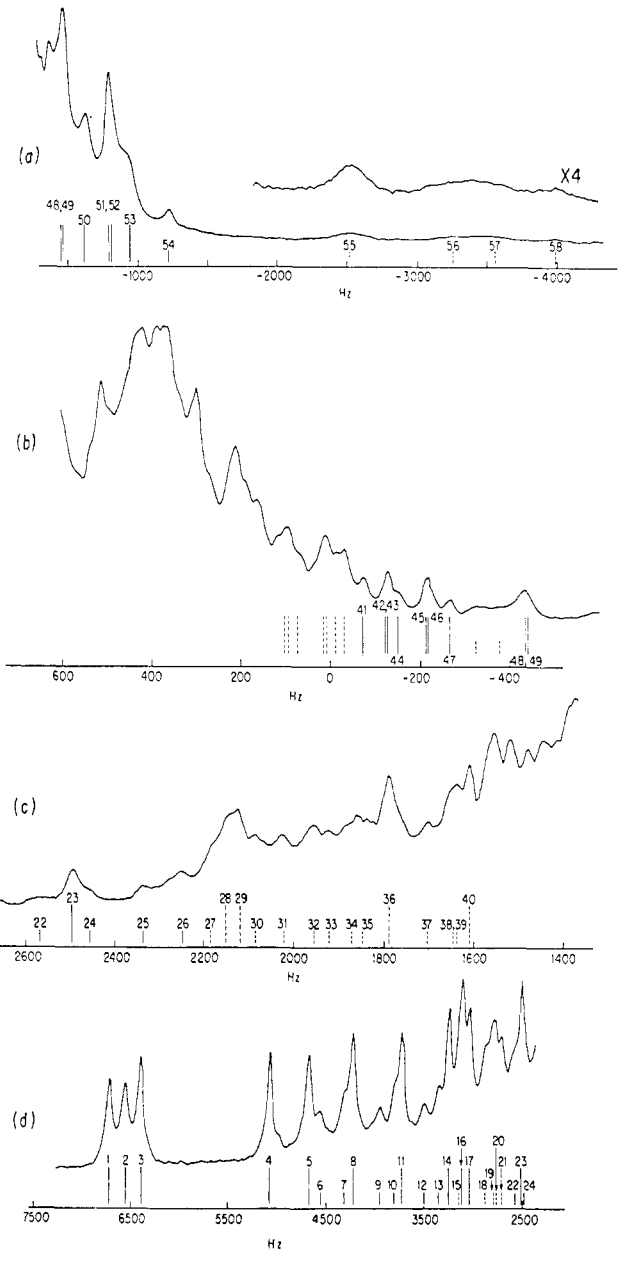

FIGURE 4: Regions of the Pmr spectrum of *D. gigas* ferricytochrome c_3.

protein starts to autoxidize (Figure 3) so we can reasonably conclude that all resonances below 1700 Hz arise from protons subject to contact shifts.

In the pmr spectrum of ferricytochrome *c* the resonances of the heme methyl resonances are well separated into two groups of two resonances each, one pair from 6500 to 7500 Hz, the other from 1500 to 2500 Hz (Figure 4). No such distribution is apparent in the spectrum of the heme methyl resonances of ferricytochrome c_3 and on the average they are shifted considerably to high field from the positions in ferricytochrome *c*. This situation may arise because there are differences in spin density distributions on the various heme rings. An alternative and more likely reason is that some (or all) of the heme groups are stacked in the molecule with their porphyrin rings approximately parallel so that some of the heme methyls are subject to high-field shifts induced by pseudocontact interactions with the neighboring heme or hemes. The hemes cannot be closely stacked along a common axis because of the space required for ligands at the 5th and 6th ligand positions.

The low-field region below 2400 Hz contains ten single proton resonances. In the same spectral region of ferricytochrome *c* five nonexchangeable single proton resonances are observed, three assigned to porphyrin CH substituents, and two unassigned. The origin of the single proton resonances in Figure 2d is probably the same, and the missing additional resonances may be located in the unresolved region to high field. The positions of the histidine C-2 and C-4 resonances of the histidine ligands have not been located in the spectra of ferricytochrome *c* or ferricytochrome c_3. Since these protons are quite close to the heme iron, they may be too broad to detect readily.

A single sharp resonance can be observed in the spectral region from 1900 to 2000 Hz as the pH is lowered from 7 (designated by arrow in Figure 5a). This resonance is assigned to a histidine C-2 proton and probably arises from the same histidine residue that was observed in the spectrum of the reduced protein.

The resolved contact-shifted resonances in the high-field and low-field regions of the spectrum of ferricytochrome c_3 exhibited a linear increase in shift with the inverse of the absolute temperature from 5 to 35° as expected for uncomplicated contact-shifted resonances (Figure 6).

Positions of many of the contact-shifted resonances are also somewhat dependent on pD as this parameter is changed from 8 to 5. Representative data for the low-field resonances are

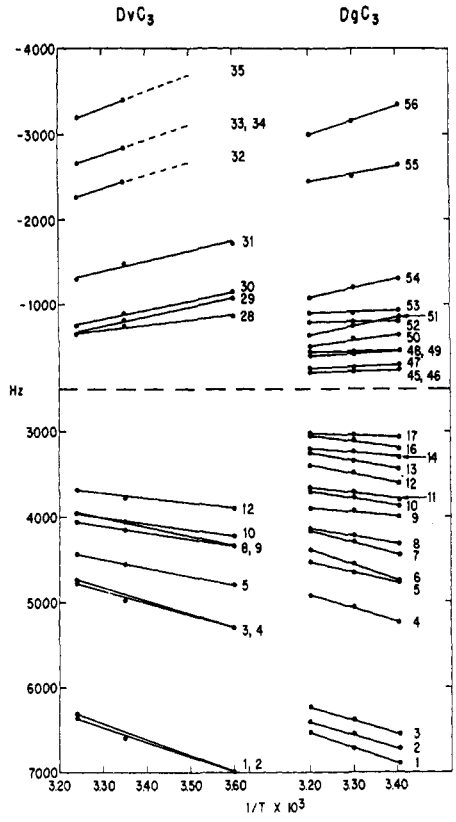

FIGURE 6: Dependence of resonance positions on T^{-1} (left) *D. vulgaris* ferricytochrome c_3, pD 8.0; (right) *D. gigas* ferricytochrome c_3, pD 7.1.

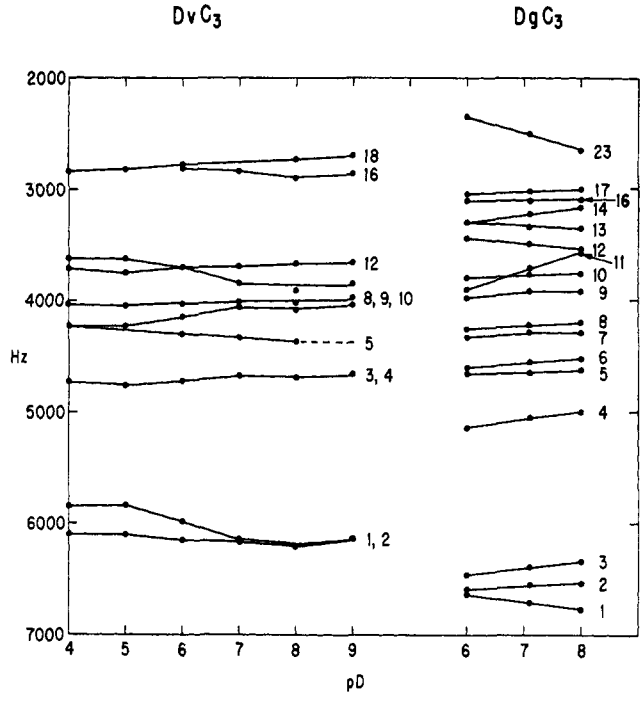

FIGURE 7: Dependence of resonance positions on pD: (left) *D. vulgaris* ferricytochrome c_3, 40°; (right) *D. gigas* ferricytochrome c_3, 32°.

shown in Figure 7. This result implies either that some of the heme ligands are changing their relationship to the heme iron atoms as pD is changed or, more likely, that the molecule undergoes a small conformational change that modifies the relationship of the hemes to each other and the effects of interheme pseudocontact interactions.

Regions of the pmr spectrum of *D. gigas* ferricytochrome c_3 (15% in D_2O, pD 7.1) at 32° are shown in Figure 4. Similar spectra were acquired at pD values from 6 to 8 and at temperatures from 5 to 47°. Positions of many of the contact-shifted resonances exhibited dependences on temperature and pD similar to those observed for *D vulgaris* ferricytochrome c_3 (see Figures 6 and 7).

The spectra of *D. vulgaris* C_3 and *D. gigas* C_3 differ considerably in detail as expected because of the many differences in their amino acid sequences. However, the general features of the spectra of the two proteins have so many common features that there can be little doubt that their structures are very similar. The analysis applied to the spectra in Figure 2 appears to apply equally well to the spectra of Figure 4. Broad resonances from meso CH protons are observed from −2000 to −4000 Hz. Resonances of approximately 20 methyl groups are tentatively identified from 100 to −1000 Hz and there are undoubtedly also a considerable number of unresolved single proton resonances in this field region. In the low-field spectra, 11 methyl resonances assigned to heme methyl substituents are clearly resolved between 2500 and 6700 Hz and four additional methyl resonances are tentatively identified in the overlapped resonance region from 1600 to 2200 Hz. In this spectrum, the low-field contact-shifted resonances merge into the lower resonances of the high-field aliphatic resonances. The distribution of the heme methyl resonances is rather different for the two proteins perhaps indicating some differences in the geometrical relationship of the hemes to each other and consequent differences in effects of pseudocontact interactions between heme groups.

The single histidine C-2 resonance observed as a sharp peak in the normal histidine resonance region for *D. vulgaris* ferricytochrome c_3 is not observable in the spectrum of *D. gigas* ferricytochrome c_3 (Figure 5b). Accordingly we suggest that this resonance arises from His-66 of cytochrome c_3 which is substituted by lysine in *D. gigas* cytochrome c_3 and that the other eight histidine residues common to both proteins are ligands of four heme iron atoms. There are, of course, other explanations for absence of the histidine C-2 resonances from their normal resonance region.

Resonances of slowly exchanging NH resonances have not been detected in the pmr spectra of either cytochrome c_3. Either all the NH protons exchange immediately on addition of the protein to D_2O or slowly exchanging NH protons do not exchange appreciably during the experimental examination of the protein solutions. Inasmuch as the protein solutions were held for many hours at temperatures as high as 45°, the former explanation seems most probable but some NH resonances of ferrocytochrome *c* exchange very slowly at even higher temperatures (McDonald and Phillips, 1973). It is of course quite possible that the disappearance of resonances in poorly resolved regions of the spectrum went undetected.

Pmr of Intermediate Redox States of Cytochrome c_3. The low-field pmr region from 2000 to 8000 Hz was monitored as an anaerobic solution of *D. vulgaris* ferrocytochrome c_3 (15% in D_2O, pD 8) at 5° slowly oxidized over a period of 3 days. Representative spectra acquired during this period are shown in Figure 8. Spectra a and b were amplified more than the others by factors of 5 and 2, respectively. Spectrum a was obtained from the fully reduced protein and no contact shifted resonances were observed. Spectra b–g acquired in sequence at intervals of several hours show a developing and changing pattern of contact-shifted resonances. Spectrum h was obtained at the conclusion of the experiment on addition of air to the sample and represents the fully oxidized protein.

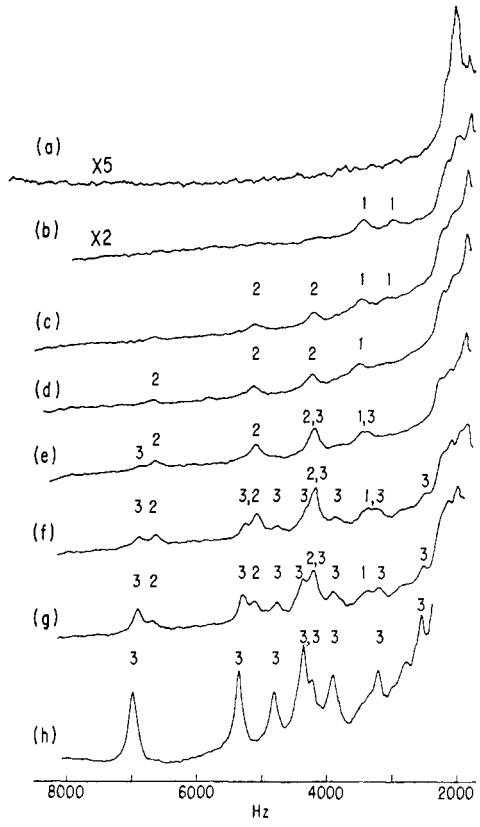

FIGURE 8: Low-field contact-shifted resonances of *D. vulgaris* cytochrome c_3 during oxidation (5°, pD 8).

In the spectra of Figure 8, resonances of three different oxidation states (labeled 1, 2, and 3) appear to develop in turn as oxidation proceeds and oxidation state 3 corresponds to the fully oxidized protein. At the stage of oxidation of spectrum g it appears from intensity considerations that about $^1/_3$ of the protein is oxidized above the fully reduced form and all three levels of oxidation coexist. Thus, electron exchange between the reduced state and the three oxidized states is too slow to cause "exchange" averaging of the respective pmr spectra (but, as discussed above, probably is fast enough to cause broadening of the resonances of ferrocytochrome c_3). It is attractive to postulate that the three states of oxidation represent oxidation of one, two, and three heme groups in the protein. One should not conclude, however, that the protein contains only three hemes since the spectra are weak and intensities of the resonances of the intermediate spectra are uncertain. We may have failed to detect the spectrum of one intermediate state or two heme groups may oxidize together. We favor the latter interpretation; our tentative conclusion is that oxidation state 1 represents oxidation of one heme, oxidation state 2 represents oxidation of two hemes, and oxidation state 3 which appears to add a much larger increment of contact-shifted resonances represents oxidation of all four hemes believed to be in the molecule. It seems clear that as additional heme groups become oxidized, the positions of contact-shifted resonances of already oxidized heme groups are shifted (*i.e.*, resonances corresponding to oxidation state 2 do not appear in the spectrum of the fully oxidized protein). This effect probably results from inter-heme pseudocontact interactions but could also arise from influences of one heme group on the spin density delocalization in a neighbor or from conformational changes that accompany oxidation.

Electron spin resonance studies on cytochrome c_3 have led to the conclusion that the protein contains four nonequivalent heme groups and that the four hemes reoxidize from the reduced state at different rates (Der Vartanian, 1973). A half-oxidized intermediate was detected (Der Vartanian and LeGall, 1971; LeGall *et al.*, 1971).

Hypothetical Structure of Cytochromes c_3. The conclusions drawn from this preliminary pmr study of cytochromes c_3 are insufficient to permit unequivocal statements about the structures of these proteins. However, the pmr data are compatible with the following hypothetical model which is based in part on results from earlier chemical, electron spin resonance, optical, and sequence studies referred to above.

The model comprises four heme groups each having histidines at the 5th and 6th ligand positions. The heme groups are in the core of the protein but may have some access to the surface as in cytochrome *c*. They may be stacked to some extent but space requirements for the histidine ligands and associated peptide chain do not permit close parallel packing. The heme groups may be contiguous in certain regions if the stacking is staggered or heme planes are not parallel. Aromatic residues are mainly packed between heme faces, valine and leucine residues are mainly around the heme edges, and most lysine residues are on the surface of the protein extending into the aqueous medium. In the ferrocytochrome the iron atoms are all in the Fe^{2+} ($S = 0$) state and in the ferricytochrome the iron atoms are all in the Fe^{3+} ($S = ^1/_2$) state. On oxidation from the ferroprotein at least two hemes are oxidized in separate, discrete steps.

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Energetics and Spectral Changes in Ligand Binding by Homogeneous Rabbit Anti-Lactose Antibody†

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ABSTRACT: Homogeneous isoelectric fractions of rabbit anti-lactose antibodies induced with a killed vaccine of *Streptococcus faecalis*, strain N, have been characterized with respect to their interaction with a lactose-containing ligand. This has been done by measurement of the association constants as a function of temperature, using equilibrium dialysis, to obtain the thermodynamic parameters of the binding reaction. In addition, structural differences among the combining sites have been recognized by optical analyses of the antibody–ligand complex. All of the isoelectric fractions were similar with respect to the energetics of the binding reaction, namely, the predominance of the ΔH term and the minor contribution of the ΔS_u term. It was inferred, therefore, that the stability of the complex arises primarily from multiple hydrogen bonding between the lactosyl group and donor and acceptor groups of the contact amino acids. In contrast, the difference spectra associated with the binding of the chromophoric ligand provided a unique, phenotypic characterization of each homogeneous antibody population although all fractions exhibited hyperchromicity. This common spectral feature may be the result of the enhanced stabilization of the planar structure of the resonance system. Induced circular dichroism of the bound ligand has also been found, exhibiting differences among antibody populations from different animals. The common feature has been a positive circular dichroism (CD) band reflecting, presumably, a generally similar asymmetric environment. The identity of the CD band for three fractions derived from a single animal and the close similarity of their thermodynamic properties have provided the basis for the suggestion of identical V_L and V_H germ-line genes in the emergence of the clones producing these fractions. Finally, it was found that the binding of lactose to the antibody site could lead either to enhancement or quenching of the tryptophan fluorescence. It is evident that optical probes can provide a highly characteristic identification of the monoclonal product and serve as tools for the recognition of the corresponding clone.

In a recent publication (Ghose and Karush, 1973) we have utilized a bacterial vaccine of *Streptococcus faecalis*, strain N (Pazur *et al.*, 1971, 1973), to induce in rabbits the formation of IgG anti-lactose antibody of restricted heterogeneity. The specifically purified antibody was further fractionated by preparative isoelectric focusing (Freedman and Painter, 1971) into functionally homogeneous populations, presumably monoclonal in origin. The specific binding properties of these fractions were evaluated and the temporal variation of their distribution over the period of 1 year was studied in relation to the maturation of the immune response.

The affinity of the isoelectric fractions spanned a range of 100-fold in association constant although in some animals the range was much more restricted. The availability of these functionally homogeneous populations of common specificity but different affinity has made feasible the examination of the microenvironment of the combining sites of the antibodies. This has already been done to a limited extent in our previous investigation (Ghose and Karush, 1973) by the measurement of the association constants for a lactose-containing ligand. A finer analysis of the combining sites appeared desirable because of the possibility that a more detailed phenotypic characterization would emerge than that provided by the affinity values. It was anticipated that this further characterization of the isoelectric fractions would provide clues to the origins of the variable region genes (V_L and V_H) selected for the expression of anti-lactose specificity.

The present study describes the examination of the combining sites of homogeneous antibody fractions by a thermodynamic analysis of their interaction with a lactose-containing ligand (Lac dye)[1] and by the use of optical probes. The latter involved the measurement of difference spectra arising from changes in the absorption spectrum of the Lac dye when complexed with antibody and the measurement of circular dichroism (CD) of the Lac dye induced by complex formation. In addition changes in the tryptophan fluorescence of the antibody arising from the occupancy of the combining site by lactose were also evaluated. It has become apparent from the results of these analyses that the optical probes are particularly

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[1] Abbreviations used are: Lac dye, *p*-(*p*-dimethylaminophenylazo)-phenyl β-lactoside; PBS, 0.15 M NaCl–0.02 M phosphate (pH 7.4).