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Nanosecond Fluorescence Spectroscopy of Pyrenebutyrate–Anti-Pyrene Antibody Complexes†

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ABSTRACT: The utility of the long-lived fluorophore, pyrene, as a probe in nanosecond fluorescence depolarization measurements was investigated using pyrenebutyrate bound in the combining sites of rabbit antipyrenebutyrate immunoglobulin G. The time dependent anisotropy decay data points showed very little scatter in the time interval 0–350 ns, which is more than three times the comparable time interval observed with ϵ-1-dimethylamino-5-naphthalenesulfonyllysine (DNS-lysine) bound in the combining sites of anti-DNS antibodies [Holowka, D. A., and Cathou, R. E. (1976), *Biochemistry 15,* 3379]. Thus, the use of pyrene can significantly extend the range of macromolecular rotational correlation times that can be measured by the single photon technique. In the present investigation, we confirmed the presence of Fab segmental flexibility in immunoglobulin G molecules specific for a hapten different from DNS-lysine. We obtained a value of about 135 ns for the longer rotational correlation time which probably represents global rotation of the entire molecule. In the course of these experiments, we have also found that the combining sites of antipyrenebutyrate antibodies are, as expected, relatively nonpolar.

Nanosecond fluorescence spectroscopy is one of the most sensitive techniques currently available for the analysis of the conformation and dynamic properties of macromolecules. It has been successfully utilized to yield information on size, shape, and segmental flexibility (Yguerabide, 1972). Such studies of rotational motion require that the lifetime of the first excited singlet state of the fluorescent moiety be comparable to the rotational correlation times to be measured (Yguerabide, 1972). Substituted naphthalenesulfonates, such as DNS[1], which exhibit lifetimes of up to about 24 ns, depending on the

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[1] Abbreviations used: PBA, pyrenebutyric acid; PB-KLH, pyrenebutyryl-keyhold limpet hemocyanin; PB-HSA, pyrenebutyryl-human serum albumin; DNS, ϵ-1-dimethylamino-5-naphthalenesulfonyl; PBS, 0.02 M sodium phosphate–0.15 M NaCl buffer, pH 7.4.

environment, have been employed for the measurement of correlation times in the range of 10 to 150 ns (Yguerabide et al., 1970; Wahl, 1975, Holowka and Cathou, 1976). However, for accurate measurement of rotational correlation times of large macromolecules, such as immunoglobulin M, with molecular weights in the range of 1×10^6, fluorescent probes with longer lifetimes must be employed.

Pyrenebutyric acid (PBA) exhibits a lifetime of about 100 ns in air-saturated solutions (Hudson, 1970) and, hence, should be eminently suitable for this purpose. PBA has been used for steady-state fluorescence polarization measurements on bovine serum albumin, IgG, and IgM (Knopp and Weber, 1969), and synthesis of several reactive forms, suitable for conjugation to proteins, has been reported (Hudson, 1970; Weltman et al., 1973).

In this paper, we report time-dependent anisotropy measurements which utilized PBA as a fluorophore and IgG anti-PBA antibodies as a model system. IgG antibodies were specifically chosen for this purpose because, although they have been previously studied by the nanosecond depolarization technique (Yguerabide et al., 1970; Chan and Cathou, 1977), a much shorter lived fluorophore, DNS-lysine, was utilized so that there was some ambiguity about the value of the longer rotational correlation time. Furthermore, we felt it important to establish whether or not the Fab segmental flexibility suggested by the anti-DNS antibody studies could be demonstrated in antibodies of entirely different specificity.

In this paper, we report time-dependent anisotropy measurements which utilized PBA as a fluorophore and IgG anti-PBA antibodies as a model system. Our results show that the useful time range of nanosecond depolarization measurements can be extended to at least 350 ns, which is much larger than that observed with DNS. With respect to antibody structure, we have found that Fab segmental flexibility is probably generally present and that anti-DNS antibodies do not represent a special case.

Materials and Methods

Pyrenebutyryl-human serum albumin (PB-HSA) and pyrenebutyryl-keyhole limpet hemocyanin (PB-KLH) were prepared from pyrenebutyrylsulfuric anhydride and HSA or KLH by the method of Hudson (1970). The average molar substitution ratio of pyrene to protein was 9. Rabbits were immunized either subcutaneously or in the footpads with 0.5–1.0 mg of PB-KLH in complete Freund's adjuvant; anti-PB activity was measured in precipitin tests with PB-HSA as antigen and was evident 2 months after initial immunization. The rabbits were then boosted with PB-KLH at intervals of 1 to 3 months.

IgG, containing the antibody, was purified from serum by QAE-Sephadex A-50 (Pharmacia, Piscataway, N.J.) ion-exchange chromatography (see Figure 1), followed by ammonium sulfate salting out (33% w/v) and gel filtration. The resulting material had the same elution profile from Sephadex G-200 as a DNS conjugate of IgG and did not contain aggregates. The preparations contained active antibody of the IgG class as determined by immunodiffusion, immunoprecipitin assay with PB-HSA, sodium dodecyl sulfate gel electrophoresis, and fluorescence binding titrations (see Results and Discussion), and will be referred to below as anti-PBA antibody.

PBA-anti-PBA complexes were typically prepared by adding 0.5 mL of PBA (9.4×10^{-6} M) to 1.5 mL of antibody (4.8×10^{-6} M), and then dialyzing the solution against 8 L of PBS overnight. The solution was then filtered through 0.22-μm Millipore filter. The final concentrations of PBA and protein were 1.3×10^{-6} M and 3×10^{-6} M, respectively. Addition of PBS to nonspecific IgG at comparable concentrations resulted in no measurable bound PBA so that all of the PBA added to the antibody solution was most likely specifically bound in the combining sites.

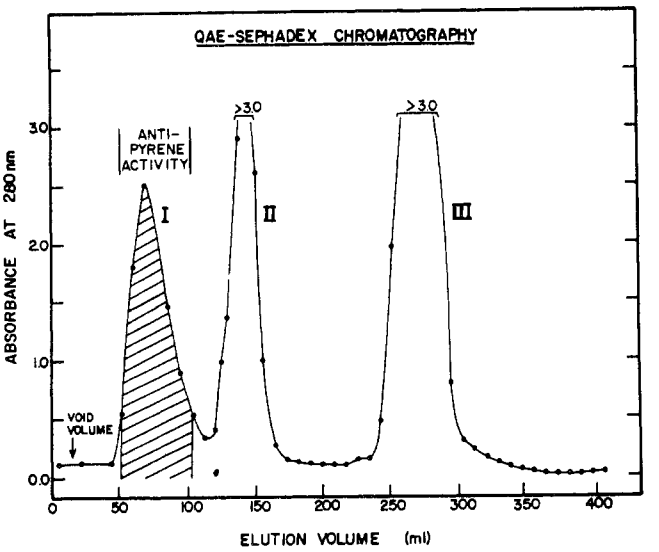

FIGURE 1: Elution profile of anti-PBA antibody on QAE-Sephadex. The column dimensions were 50 cm by 2.5 cm. Serum, diluted with ethylenediamineacetic acid buffer ($I = 0.1$, pH 7.0), was loaded onto the column and eluted with the same buffer. The fractions in the shaded area under peak I were pooled for further purification.

All steady-state fluorescence spectra and polarization measurements were made on an AMINCO SPF-1000 corrected excitation and emission spectrofluorometer (Kremen and Landa, 1974). Absorption measurements were made on either a Cary 14 or a Zeiss PMQ II spectrophotometer. Concentrations were determined spectrophotometrically; the following molar extinction coefficients were used: PBA, $\epsilon_{342} = 4.0 \times 10^4$ (Knopp and Weber, 1969); IgG, $\epsilon_{280} = 2.30 \times 10^5$.

Time-dependent fluorescence depolarization measurements were performed on a modified Ortec 9200 nanosecond fluorescence spectrometer as previously reported (Bunting and Cathou, 1973; Holowka and Cathou, 1976). The spark-gap excitation lamp (Ortec Model 9352) was pulsed at about 25 kHz. The excitation wavelength was selected with a 355-nm glass band-pass filter with a 54-nm spectral bandwidth (Oriel G-774-3550, Oriel Corp., Stamford, Conn.). The emission light was selected with a 400-nm narrow band-pass interference filter (Oriel Corp. G-522-4000).

The total number of counts collected for a lifetime decay experiment typically was about 2×10^7 with 5×10^4 to 1×10^5 counts in the peak channel. For depolarization experiments, the total counts for $F_\parallel$ and $F_\perp$ were about 8×10^7 each, with 1.5×10^5 to 3.0×10^5 counts in the peak channel and up to 1×10^4 counts in the channels at the end of the decay curve. Deconvolution and analysis of the lifetime data were performed by the method of moments (Isenberg and Dyson, 1969); in some cases, the Laplace transform (Gafni et al., 1975) was also used. The lifetime decay data were fit to the equation:

$$F(t) = \sum_{i=1}^{N} \alpha_i e^{-1/\tau_i} \qquad (1)$$

where $F(t)$ is the fluorescence, α_i is the preexponential weighting factor, and τ_i is the fluorescence lifetime.

In depolarization experiments, equal total counts for $F_\parallel$ and $F_\perp$ were collected in order to give similar statistical accuracy

TABLE I: Fluorescence Lifetimes of PBA, Free in Solution and Bound to Anti-PBA Antibody.

Environment	Month[a]	Deoxygenated[b]	τ_1 (ns)	α_1[c]	τ_2 (ns)	α_2[c]
Antibody site (rabbit 1285)	5.5	−	59	0.37	138	0.63
		+	78	0.54	155	0.46
Antibody site (rabbit 1285)	11	−	118	1.0		
		+	157	1.0		
Antibody site (rabbit C-72)	8	−	35	0.44	137	0.56
0.02 M PBS, pH 7.4		−	101	1.0		
		+	121	1.0		

[a] Month after initial immunization that antibody was obtained. [b] Solutions were deoxygenated by slowly bubbling N_2 through them for 90 min. [c] $\alpha_1 + \alpha_2 = 1.0$.

for both decays. Emission anisotropy (A) was calculated as a function of time from the raw experimental data by the standard equation:

$$A(t) = \frac{f_\parallel(t) - f_\perp(t)}{f_\parallel(t) + 2f_\perp(t)} \quad (2)$$

where $f_\parallel(t)$ and $f_\perp(t)$ are the fluorescence intensities of the vertically and horizontally polarized emission components, respectively, as a function of time (Yguerabide, 1972). Because the lamp intensity and rate vary slightly during the course of an experiment, $f_\parallel(t)$ and $f_\perp(t)$ curves must be normalized after data collection. Although this can be done by comparing the total counts in the $f_\parallel(t)$ and $f_\perp(t)$ decay curves over short time intervals on the nanosecond apparatus (Yguerabide et al., 1970), the steady-state fluorescence determination of this normalization ratio $(\bar{F}_\parallel/\bar{F}_\perp)$[2] is more reproducible and is the method which we employed (Dale and Eisinger, 1975). Excitation and emission light were polarized by Polacoat ultraviolet polarizing filters with both steady-state and nanosecond instruments, and steady-state monochromator polarization effects were corrected for (Chen and Bowman, 1965). Wavelengths and spectral bandwidths were nominally the same as those employed in the nanosecond apparatus, and good agreement was obtained between the ratio measured by the two instruments. Normalization was accomplished by scaling $f_\parallel(t)$ and $f_\perp(t)$ to make the ratio of the areas under their curves equal to $\bar{F}_\parallel/\bar{F}_\perp$.

As the simplest interpretation for a curved log ($A(t)$) decay, two major modes of Brownian rotation were assumed to account for the decay, and the data were fit to the sum of two exponential terms of a nonlinear, least-squares analysis (Marquardt, 1963) weighted with $1/A_i(t)$ (Massachusetts Institute of Technology Subroutine LSMARQ IBM Applications Program 84). The form of the question employed was:

$$A(t) = A_0(f_S e^{-t/\phi_S} + f_L e^{-t/\phi_L}) \quad (3)$$

where ϕ_S and ϕ_L represent the short and long rotational correlation times which together describe the time-dependent depolarization, and f_S and f_L are their respective preexponential terms (Yguerabide et al., 1970; Holowka and Cathou, 1976). The subroutine LSMARQ is a nonlinear least-squares fitting routine which determines the values of A_0, f_i, and ϕ_i by minimizing the value X^2 in the equation:

$$X^2 = \sum_{i=1}^{n} W_i(A_i - A(t))^2 \quad (4)$$

[2] $\bar{F} = \int_0^\infty F(t)dt$, where $F(t)$ is the deconvoluted fluorescence.

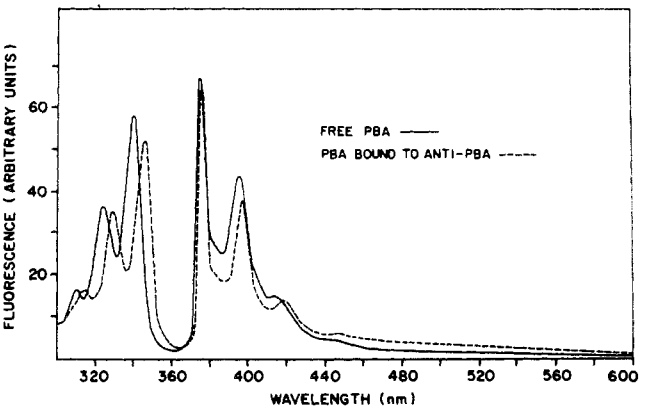

FIGURE 2: Corrected excitation and emission spectra of free and antibody bound PBA in PBS. The excitation spectra (left half) were obtained by measuring the emission at 374 nm for free PBA and at 376 nm for bound PBA; spectral band widths were 2 and 10 nm for excitation and emission, respectively. The emission spectra (right half) were obtained by exciting at 341.5 nm for free PBA and at 347 nm for bound PBA; spectral band widths were 10 and 2 nm for excitation and emission, respectively. The concentration of PBA was 3.3×10^{-8} M.

where the weighting factor W_i is $1/A_i$, and $A(t)$ and A_i are the calculated and experimental time dependent emission anisotropies, respectively. The accuracy of the fit to the data is reported in terms of the standard deviation.

Results and Discussion

Fluorescence Properties of PBA–Anti-PBA Complexes. PBA is a long-lived fluorophore with a single exponential lifetime of 101 ns in an air-saturated aqueous solution of 0.02 M PBS, pH 7.4 (see Table I). When the PBA solution was deoxygenated by slow bubbling with N_2, the lifetime increased to 121 ns. Both values agree very well with those reported by Hudson (1970).

When bound in the combining site of anti-PBA, the lifetime of PBA was increased for either part or all of the population, indicating in general either a more hydrophobic or oxygen protected environment for the ligand (see Table I). That bound PBA was still accessible to oxygen in solution, however, was shown by the observation that deoxygenation increased the lifetime still further. Interestingly, antibody produced at 11 months appeared to have homogenous combining sites, at least by the criterion of PBA fluorescence lifetime, whereas earlier antibody was clearly heterogeneous. In the latter case, at least two populations of antibody were present: one population had active sites which stabilized the excited state of PBA, whereas the other had sites which clearly destabilized it.

Additional spectroscopic changes occurred on binding of PBA. The major fluorescence excitation maxima were red shifted from 326.5 to 330.5 nm and from 341.5 to 347 nm while the major emission maxima were shifted from 375 to 376 nm and 395 to 396 nm (see Figure 2). Similar, although smaller, red shifts have been reported for PBA conjugated to BSA (Vaughan and Weber, 1970) and have been correlated with a change from an aqueous to a more nonpolar environment (Vaughan and Weber, 1970; Gratzel et al., 1974). Visual comparison of the major peaks in the emission spectra of free and bound PBA suggested that the fluorescence of bound hapten was somewhat quenched (see Figure 2). However, measurement of the relative quantum yield of bound PBA, by integration of the emission spectrum from 365 to 500 nm, gave a value of 0.98 compared with that of free PBA. The decrease in emission of bound PBA in the bands at 376 and 396 nm thus appears to be compensated for by an increase in the small but

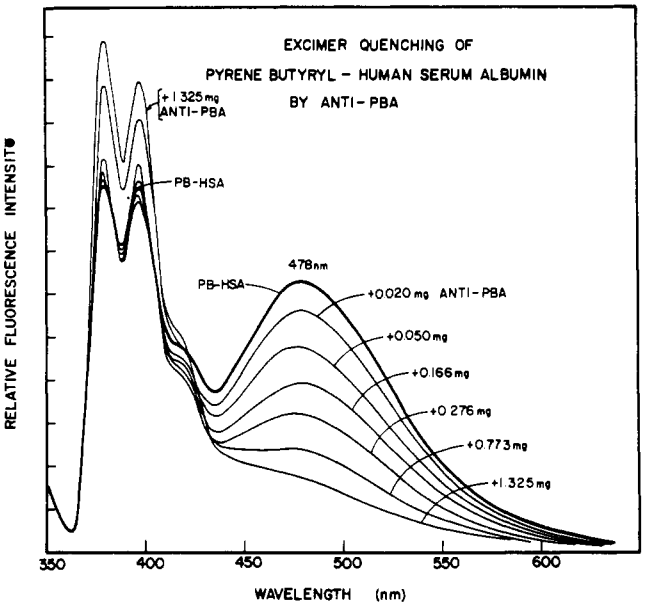

FIGURE 3: Excimer quenching of PB-HSA by anti-PBA. Excitation was at 346 nm. The heavy line shows the emission spectrum of PB-HSA (0.016 mg). Upon addition of antibody (final quantity in each case), the excimer emission band at 478 nm decreased in intensity while the monomer emission bands at 378 nm and 396 nm first decreased in intensity (first two additions of anti-PBA) and then increased.

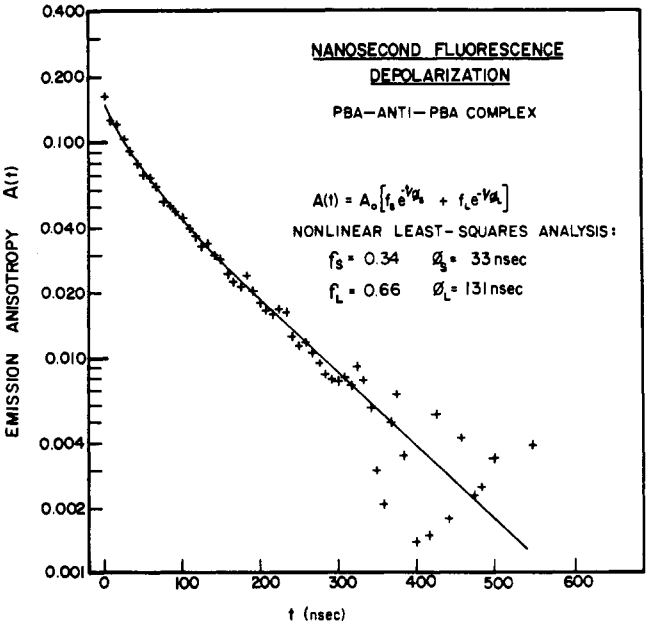

FIGURE 4: Time dependent emission anisotropy of PBA in the combining site of anti-PBA antibody obtained from rabbit 1285. The concentration of PBA was 1.3×10^{-6} M. (+) Representative data points; the solid line is the least-squared best fit of eq 3 to the observed data. See Table II for values of rotational correlation times.

very broad band(s) above 430 nm. This region corresponds to the emission of pyrene excimer; although the majority of anti-PBA combining sites specifically *quench* excimer fluorescence (see below), a small percent of the population may still enhance excimer formation and fluorescence.

The spectroscopic changes discussed above occurred only in binding of PBA to specific antibody. Solutions of PBA and nonspecific rabbit IgG at similar concentrations exhibited spectra identical with those of free PBA.

We took advantage of the spectral red shift which occurs on binding of PBA to antibody to estimate an average binding constant. A fluorescence titration was performed in which small increments of PBA were added to a solution of antibody and the excitation spectra were recorded. At very low hapten to antibody ratios, the hapten was essentially all bound, the excitation maximum of the lowest energy band was at 347 nm, and the ratio of $F_{347}/F_{341.5} = 2.0$, where F = fluorescence intensity. For free PBA, this ratio was 0.313. If one assumes that *all* of the bound PBA exhibits an excitation maximum at 347 nm, then the concentration of bound hapten, h_b, can be obtained from:

$$h_b = h_T \frac{F_{347}/F_{341.5} - 0.313}{2.00 - 0.313} \qquad (5)$$

where h_T is the total hapten concentration present. The concentration of free hapten, h_f, is then equal to $h_T - h_b$. Analysis of the data obtained with antibodies from rabbit C-72 (see Table I) by the Scatchard relationship (Scatchard, 1949) gave a value of 5×10^8 M^{-1} for the average binding constant. This value is similar to those observed for fluorescein binding to anti-fluorescein (Gollogly and Cathou, 1974), ϵ-DNS-lysine to anti-DNS (Parker et al., 1967), and ϵ-DNP-lysine to anti-DNP (Eisen and Siskind, 1964). PBA, however, is a unique ligand because the binding forces must be almost entirely hydrophobic in nature. The PBA-anti-PBA complex would thus be an excellent system for measurement of thermodynamic parameters associated with these forces.

PB-HSA exhibits a broad excimer band of emission with a maximum at 478 nm (Hudson, 1970) which is thought to be due to rapid multiple reaction of PB-SO_3 with adjacent sites of HSA on initial contact (Hudson, 1970). As can be seen in Figure 3, on binding of anti-PBA to PB-HSA, a striking decrease in the excimer emission, and a corresponding increase in the monomer emission, took place. These results suggest that antibody binds primarily to the monomer form of pyrene on the HSA surface and probably dissociates the excimers which are present. Thus, free and bound pyrene can be easily distinguished, and such measurements could provide a rapid determination of the binding of anti-PB to an antigen such as PB-HSA.

TABLE II: Anisotropy of PBA-Anti-PBA Complexes.

Antibody from rabbit No.	No. of exponents in fit	A_0	f_s	ϕ_s (ns)	f_L	ϕ_L (ns)	SD ($\times 10^3$)
1285	1	0.101	1.00	103			2.01
	2	0.111	0.34	33	0.66	131	0.75
C-72	1	0.110	1.00	124			2.71
	2	0.139	0.30	24	0.70	140	1.37

Time Dependent Anisotropy. The time dependent anisotropy of the PBA-anti-PBA complex is shown in Figure 4. It can be seen that there is remarkably little scatter in the anisotropy data points up to a time of about 350 ns. In contrast, with DNS-lysine as a probe, comparable data can be obtained only to about 100-125 ns (Holowka and Cathou, 1976; Chan and Cathou, 1977). When the data obtained with several antibody preparations were analyzed by eq 3, the values of the rotational correlation times shown in Table II were obtained. These values are in general agreement with those obtained with DNS-Lys-anti-DNS IgG antibodies (Yguerabide et al., 1970; Chan and Cathou, 1977) which have been interpreted in terms of two *major* modes of rotation in the IgG molecule: Fab segmental flexibility and global rotation of the entire molecule. Since the time decay of fluorescence depolarization with the PBA-anti-PBA system could be examined over a time scale

in which the longest decay component could be unambiguously determined, these measurements represent an important check on the validity of the conclusions drawn from the anti-DNS studies. Furthermore, the measurements were carried out on an antibody population with a different specificity. Thus, Fab segmental flexibility is probably generally present in IgG molecules.

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Characterization of Guanidinated Cytochrome *c* by ^{13}C Nuclear Magnetic Resonance Spectroscopy†

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ABSTRACT: All 19 lysine residues in horse heart ferricytochrome *c* were guanidinated using 90% ^{13}C-enriched *O*-methylisourea. The guanido carbon resonances of the resultant 19 homoarginine residues are distributed over a range of about 1 ppm and include two discrete single guanido carbon resonances. Distinct changes in the ^{13}C NMR spectrum are observed upon complexation of guanidinated ferricytochrome *c* with ferrihexacyanide, upon conversion of the guanidinated ferricytochrome *c* to its alkaline isomer, and upon reduction to guanidinated ferrocytochrome *c*. Analysis of the changes in the visible absorbance spectrum accompanying guanidination of ferricytochrome *c* with *O*-methylisourea indicates that 18 lysine residues are guanidinated about 3 times more rapidly than the single remaining lysine residue, most likely lysine-79. The guanido carbon of this residue was preferentially enriched with ^{13}C-enriched *O*-methylisourea. Analysis of the ^{13}C-enriched guanido carbon spectrum of this derivative provided assignment of the guanido carbon resonance of homoarginine-79. This resonance is not shifted by complexation with ferrihexacyanide, indicating that lysine-79 does not participate in the binding of iron hexacyanides as previously proposed. Guanidination of all 19 lysine residues of cytochrome *c* decreases the K_M but does not change the V_{max} for transfer of an electron from ferrocytochrome *c* to peroxide catalyzed by cytochrome *c* peroxidase. The decrease in K_M does not correlate with the extent of guanidination of lysine-79 indicating that this residue is also not involved in functional complexation with cytochrome *c* peroxidase. These results taken together suggest that the lower portion of the exposed heme edge of cytochrome *c* is not directly involved in electron exchange reactions.

Ferricytochrome *c* is known to form discrete complexes which facilitate electron exchange with electron acceptors such as ferrohexacyanide (Stellwagen and Shulman, 1973; Miller and Cusanovich, 1975) and cytochrome *c* peroxidase (Orii et al., 1962; Yonetani, 1962; Nicholls, 1964; Van Gelder and Muijsers, 1966; Nicholls and Mochan, 1971; Mochan and Nicholls, 1972; Gupta and Yonetani, 1973). The dependence of the formation constant for complexation on ionic strength and pH indicates that the interactions stabilizing complex formation are principally electrostatic in which cytochrome *c* is the cation and the electron acceptor is the anion. Since 19 of the 21 cationic groups on cytochrome *c* at neutral pH, where complexation occurs, are supplied by the ϵ-amino groups of the lysine residues, it is very likely that one or probably a cluster

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