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.

References

Bunting, J. R., and Cathou, R. E. (1973), *J. Mol. Biol. 77,* 223.

Chan, L. M., and Cathou, R. E. (1977), *J. Mol. Biol.* (in press).

Chen, R. F., and Bowman, R. L. (1965), *Science 147,* 1.

Dale, R. E., and Eisinger, J. (1975), in Concepts in Biochemical Fluorescence, Vol. I, Chen, R. F., and Edelhoch, H., Ed., New York, N.Y., Marcel Dekker, p 115.

Eisen, H., and Siskind, G. W. (1964), *Biochemistry 3,* 996.

Gafni, A., Modlin, R. L., and Brand, L. (1975), *Biophys. J. 15,* 263.

Gollogly, J., and Cathou, R. E. (1974), *J. Immunol. 113,* 14.

Gratzel, M., Kalyanushundaram, K., and Thomas, J. K. (1974), *J. Am. Chem. Soc. 96,* 7869.

Holowka, D. A., and Cathou, R. E. (1976), *Biochemistry 15,* 3379.

Hudson, E. N. (1970), Ph.D. Thesis, University of Illinois, Urbana-Champaign, Ill.

Isenberg, I., and Dyson, R. (1969), *Biophys. J. 9,* 263.

Knopp, J. A., and Weber, G. (1969), *J. Biol. Chem. 244,* 6309.

Kremen, J., and Landa, I. (1974), *J. Anal. Chem. 46,* 1694.

Marquardt, D. W. (1963), *J. Soc. Indust. Appl. Math. 11,* 431.

Parker, C. W., Godt, S. M., and Johnson, M. C. (1967), *Biochemistry 6,* 3417.

Scatchard, G. (1949), *Ann. N.Y. Acad. Sci. 51,* 660.

Vaughan, W. M., and Weber, G. (1970), *Biochemistry 9,* 464–473.

Wahl, P. (1975), in Biochemical Fluorescence, Concepts, Vol. I, Chen, R. F., and Edelhoch, H., Ed., New York, N.Y., Marcel Dekker, p 1.

Weltman, J. K., Szaro, R. P., Frackelton, A. R., Jr., Dowben, R. M., Bunting, J. R., and Cathou, R. E. (1973), *J. Biol. Chem. 248,* 3173.

Yguerabide, J. (1972), *Methods Enzymol. 26C,* 498.

Yguerabide, J., Epstein, H. F., and Stryer, L. (1970), *J. Mol. Biol. 51,* 573.

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

Earle Stellwagen,* Lawrence M. Smith,‡ Robert Cass, Robin Ledger,§ and Harvey Wilgus

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

† From the Department of Biochemistry, The University of Iowa, Iowa City, Iowa 52242. *Received February 14, 1977.* This investigation was supported by Public Health Research Grant No. GM-13215 from the Institute of General Medical Sciences.

‡ Public Health Service Postdoctoral Fellow of the Institute of Arthritis, Metabolism and Digestive Diseases, Grant No. AM-05115, Division of Research Grants, National Institutes of Health, Bethesda, Md. Present address: Oregon Graduate Center, Beaverton, Ore. 97005.

§ On leave from the Department of Physiology and Medical Biochemistry, University of Cape Town Medical School, Observatory, 7900, South Africa.

of lysine residues in cytochrome *c* participate directly in electrostatic complexation with the anionic electron acceptors. Both arginine guanido groups, which provide the remaining two positive charges, participate in intraprotein interactions (Dickerson and Timkovich, 1975) and are unlikely to complex with the electron acceptors. Since the α-amino group is acetylated, it cannot provide a positive charge.

Specific guanidination of all 19 lysine residues of cytochrome *c* by reaction with *O*-methylisourea is known to preserve the structure and function of the protein (Hettinger and Harbury, 1964) since this modification, in contrast to other lysine modification reactions, retains a positively charged residue. In this report, we utilize guanidination with ^{12}C- and ^{13}C-enriched *O*-methylisourea to study the role of the lysine residues of cytochrome *c*, particularly lysine-79, in the complexation of cytochrome *c* with, and electron transfer to, electron acceptor molecules.

Experimental Procedures

Materials. Type III horse heart cytochrome *c* and 99.8% D_2O were purchased from the Sigma Chemical Co. *O*-Methylisourea was purchased from the Aldrich Chemical Co. and the reagent containing the guanido carbon 90% enriched in ^{13}C was obtained from Koch Isotopes, Inc. ^{14}C-Enriched sodium ferrohexacyanide having a specific activity of 21.8 mCi/mmol was obtained from Schwarz BioResearch, Inc. Cytochrome *c* peroxidase was purified from bakers' yeast using the procedure of Yonetani and Ray (1965). Cytochrome *c* hemepeptide 1–65 was purified from a cyanogen bromide digest by the procedure of Corradin and Harbury (1970). Guanidinate cytochrome *c* was routinely prepared by reacting the protein at a concentration between 50 and 100 mg/mL with 0.5 M *O*-methylisourea at pH 11.0 and 25 °C. Reaction for 10, 40, and 400 min under these conditions resulted in the guanidination of approximately 20, 50, and 98% of the lysine residues, respectively. The reaction was terminated by exclusion chromatography using Sephadex G-25 equilibrated with 50 mM phosphate buffer (pH 7.0). The eluent fractions containing protein were pooled, dialyzed against water, and lyophilized.

Methods. Samples for ^{13}C NMR[1] measurements were routinely dissolved in D_2O containing the desired buffer, filtered through a 0.20- or 0.45-μm Metricel filter, and placed in NMR tubes having a 10 mm o.d. The reference compound, dioxane, was placed in 1-mm o.d. capillaries inserted in the center of NMR tubes. All ^{13}C NMR spectra were obtained at 25 °C using a Bruker Model HX90E Fourier transform spectrometer operated at 22.63 MHz using a deuterium lock and broad band proton decoupling. Either 500- or 1000-Hz spectra were typically obtained having a digital resolution of about 1 data point every 0.25 Hz. The sample was pulsed for 7–15 μs every 2.1 s using a flip angle of 45°. The resolution of some spectra was enhanced by multiplying the free induction decay by an increasing exponential to remove two-thirds of the observed line width and then subjecting it to two successive fifth-order Wertheim smoothing functions based on a Lorentzian line function which is two-thirds of the observed line width (Pearson, 1977). Data for T_1 measurements were obtained by partially relaxed Fourier transformed spectroscopy using a 180° pulse for 3.3 μs, a 90° pulse for 11.6 μs, and τ values of 0.10 to 6 s.

The concentrations of homoarginine and lysine in acid hydrolysates of guanidinated cytochrome *c* were measured using the short column of a Spinco Model 120C amino acid analyzer. The measured concentration of homoarginine was corrected for a 1% conversion to ornithine during acid hydrolysis for 24 h at 110 °C.

The kinetics of the oxidation of guanidinated ferrocytochrome *c* by cytochrome *c* peroxidase were measured by generating both ferrocytochrome *c* and hydrogen peroxide in situ by reaction with superoxide anion produced by a xanthine oxidase generating system. The assay solution contained 50 mM phosphate buffer (pH 7.0), 50 μM xanthine, 100 μM EDTA, and 3 μM cytochrome *c* in a total volume of 1 mL. The reduction reaction was initiated by addition of an aliquot of xanthine oxidase. When the cytochrome was about 70% reduced, oxidation was initiated by addition of peroxidase sufficient to produce a rate of decrease in absorbance at 550 nm similar to the rate of increase. Subtraction of the observed oxidation rate from the observed reduction rate at comparable ferrocytochrome *c* concentrations provides the data for construction of double reciprocal kinetic plots.

Equilibrium dialysis measurements of the binding of ^{14}C-labeled ferrohexacyanide to various proteins were performed as described previously (Stellwagen and Cass, 1975).

Results

Reaction with O-Methylisourea. Conversion of protein lysine residues to homoarginine residues by reaction with *O*-methylisourea can be easily measured since lysine and homoarginine are stable to acid hydrolysis and resolved by standard amino acid procedures. Guanidination is a strongly pH-dependent reaction requiring the proton dissociated form of an amino group and the protonated form of *O*-methylisourea. At pH 11, the optimal pH for guanidination, 18 of the 19 lysine residues of horse heart ferricytochrome *c* should be equally reactive with the reagent since they extend outward from the surface of the protein into the solvent (Dickerson and Timkovich, 1975). The amino group of the remaining lysine residue is ligated with the heme iron at pH 11 in an axial coordination position. Recent hybridization results (Wilgus and Stellwagen, 1974) indicate that the lysine ligand is located at position 79 in the amino acid sequence. Accordingly, the rate of guanidination of lysine-79 should be measurably slower than the other 18 lysine residues since its guanidination requires deligation from the heme iron.

Spectral and EPR measurements (Morton, 1973) indicate that the lysine-79 ligand is replaced by a weak field ligand, most likely a hydroxide anion (Stellwagen et al., 1975), when lysine-79 is guanidinated. Thus, the guanidination of lysine-79 can be uniquely observed spectrally by the appearance of an absorbance band at 595 nm (Wilgus and Stellwagen, 1974) indicative of coordination of a weak field ligand in an axial position. The dependence of the extent of guanidination of all 19 lysine residues, measured chemically, on the guanidination of lysine-79, measured spectrally, is shown in Figure 1. This dependence was analyzed by a procedure described by Klee and Richards (1957) in which it was assumed that 18 lysine residues are guanidinated at the same rate, that the lysine-79 is guanidinated more slowly, and that the spectral change results only from guanidination of the lysine ligand. The curved line shown in Figure 1 was calculated using this model in which 18 lysines are guanidinated 3.3 times faster than the lysine ligand. While this model is certainly not unique in simulating the experimental values, it is the simplest model consistent with the properties of the protein and is consistent with the ^{13}C NMR measurements to be described below.

[1] Abbreviations used are: NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; Tris, tris(hydroxymethyl)aminomethane; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

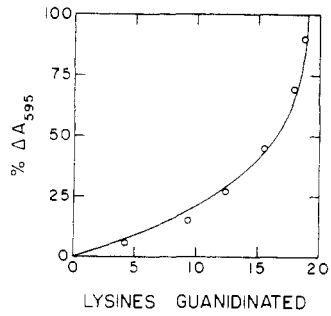

FIGURE 1: Effect of continued reaction of *O*-methylisourea on the increase in absorbance at 595 nm and on the guanidination of lysine residues. The guanidination reaction was done at 25 °C and at pH 11.0 for 8 h. The curved line was calculated using the equation $\log x = (k_f/k_s) \log y + \log 18$, where k_f/k_s was 3.3.

Additional support for this model is provided by a kinetic comparison of the rate of guanidination of groupings of the lysine residues. Several aliquots were removed from a guanidination reaction mixture during the first 10 min of reaction of ferricytochrome *c* with *O*-methylisourea at pH 11. The protein aliquots were cleaved with cyanogen bromide and peptides 1-65 and 81-104 purified from each aliquot. Analysis of the lysine and homoarginine content of each peptide indicates that the pseudo-first-order rates for initial guanidination of the 11 lysines in peptide 1-65 and the 5 lysines in peptide 81-104 are identical with the pseudo-first-order rate for initial guanidination of the 19 lysines in the intact protein.

^{13}C NMR Measurements of Guanidinated Cytochrome c. The ^{13}C NMR spectrum of the guanido carbon of a homoarginine residue not perturbed by tertiary structural effects was established using ferrihemepeptide 1-65 purified from a cyanogen bromide digest of horse heart ferricytochrome. This hemepeptide, which has little if any tertiary structure (Babul et al., 1972), was reacted with 90% ^{13}C-enriched *O*-methylisourea until 56% of the 11 lysine residues in the peptide had been converted to homoarginine residues. As shown in Figure 2, the ^{13}C NMR spectrum exhibits a single resonance in the guanido carbon region of the spectrum centered at 157.4 ppm, relative to tetramethylsilane, having a line width of about 2 Hz. Occurrence of the guanido carbon resonance of a homoarginine residue at this position compares favorably with the guanido carbon resonance positions of arginine and arginine residues in peptides and proteins which range from 157.4 to 158.1 ppm (Horsley et al., 1970; Glusko et al., 1972; Chien and Wise, 1973; Keim et al., 1974; Oldfield et al., 1975b). No evidence is seen for the natural abundance [^{13}C]guanido carbon resonance of the single arginine residue in the peptide. The intensity of the guanido carbon resonance of the 11 lysine residues guanidinated to the extent of 56% with 90% enriched *O*-[^{13}C]methylisourea should be about 500 times greater than the intensity of the ^{13}C resonance of the natural abundance guanido carbon of the intrinsic arginine residue. No evidence for the presence of a guanidinated α-amino group appears in the spectrum since the reactivity of the α-amino group of the peptide, as well as the intact protein, is negated by the presence of an *N*-acetyl group.

Separate samples of horse heart ferricytochrome *c* were reacted with 0.5 M 90% ^{13}C-enriched *O*-methylisourea at pH 11 until 19, 52, or 98% of the 19 lysine residues were converted to homoarginine residues as measured by amino acid analysis. The ^{13}C NMR spectra of these guanidinated proteins are shown in Figure 2. All significant additions to the natural abundance ^{13}C NMR spectrum of ferricytochrome following guanidination occur in this guanido carbon spectral region. In

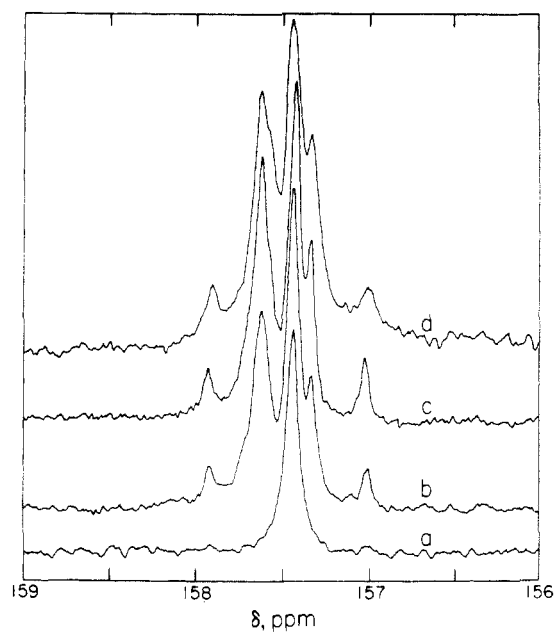

FIGURE 2: ^{13}C NMR spectra in the guanido carbon region. Spectrum a is guanidinated cytochrome *c*-ferriheme peptide 1-65 measured in 100 mM acetate buffer, pD 4.0, 6404 scans. Spectrum b is guanidinated ferricytochrome *c* having 19% of its lysine residues converted to homoarginine residues. This spectrum was obtained using a 4 mM solution of the guanidinated protein in 5 mM phosphate buffer, pD 7.0, after 40 279 scans. Spectrum c is guanidinated ferricytochrome *c* having 52% of its lysine residues converted to homoarginine residues. This spectrum was obtained using a 5 mM solution of the guanidinated protein in 50 mM phosphate buffer, pD 6.9, after 7219 scans. Spectrum d represents 98% guanidinated ferricytochrome *c* and was obtained using a 4 mM protein solution in 200 mM phosphate buffer, pD 7.0, after 5000 scans.

contrast to the spectrum of guanidinated ferriheme peptide 1-65, the spectrum of the guanidinated protein exhibits at least five resolved or partially resolved resonances extending over a range of 0.9 ppm in the guanido carbon region. It is unlikely that the natural abundance ^{13}C-labeled guanido carbon resonances of the two arginine residues located at 157.8 ppm (Oldfield et al., 1975b) contribute significantly to the spectra of the guanidinated protein since the intensity of a *single* ^{13}C-enriched homoarginine guanido carbon resonance should be 9 to 40 times greater (depending on the extent of guanidination) than the guanido carbon resonance of *both* arginine residues. Assuming then that the resonances in the guanido carbon region of the spectra of the guanidinated proteins originate from homoarginine guanido carbons and that each homoarginine guanido carbon has the same nuclear Overhauser enhancement the total resonance areas can be considered to represent the sum of 19 equivalent nuclei. The average numbers of homoarginine residues associated with the five resolved or partially resolved resonances are listed in Table 1. It should be noted that the guanido carbon resonances observed at 157.0 and 157.9 ppm each represent single homoarginine residues using this analytical procedure.

The spin-lattice relaxation times, T_1, for the five resolved or partially resolved guanido carbon resonances of guanidinated ferricytochrome were measured by partially relaxed Fourier transform spectroscopy. In order to interpret the magnitude of the experimentally measured T_1 values for homoarginine guanido carbons, T_1 values for a homoarginine guanido carbon experiencing unrestricted segmented motion and one having the rotatory correlation time of cytochrome *c* were calculated. Oldfield et al. (1975a) have found that the T_1 of arginine guanido carbons is dominated by ^{13}C-^{14}N di-

TABLE I: Guanido Carbons of Guanidinated Ferricytochrome *c*.

Chem shift (ppm)	No. of C	T_1 (s)
157.0	1.2 ± 0.7	1.8 ± 0.2
157.3	3.0 ± 1.3	2.2 ● 0.2
157.4	6.7 ± 1.2	2.3 ± 0.1
157.6	7.2 ± 0.4	1.9 ± 0.1
157.9	0.8 ± 0.3	1.3 ± 0.3

polar relaxation when D_2O is the solvent. The dependence of the T_1 of guanido carbons in D_2O relaxed by ^{13}C-^{14}N dipolar interaction on the rotational correlation time, τ_R, was calculated for the magnetic field employed, 21.1 kG, using eq 1:

$$\frac{1}{T_1} = \frac{2}{15}\hbar\gamma_C{}^2 3S_N(S_N + 1)(\gamma_N{}^2 r_{C\text{-}N}{}^{-6} X_N) \qquad (1)$$

where $\hbar$ is Plancks constant divided by 2π, γ_C and γ_N are the gyromagnetic ratios of ^{13}C and ^{14}N, respectively, S_N is the spin quantum number of ^{14}N, $r_{C\text{-}N}$ is the distance between the ^{13}C and ^{14}N nuclei in a guanido group, 1.31 Å, and X_N is given by eq 2:

$$X_N = \frac{\tau_R}{1 + (W_N - W_C)^2\tau_R{}^2} + \frac{3\tau_R}{1 + W_C{}^2\tau_R{}^2} + \frac{6\tau_R}{1 + (W_N + W_C)^2\tau_R{}^2} \qquad (2)$$

where W_N and W_C are the resonance frequencies of ^{14}N and ^{13}C at 21.1 kG, respectively. The calculated dependency indicates that the T_1 for a guanido carbon experiencing unrestricted segmented motion, such as homoarginine itself, having a τ_R value of about 0.7 μs, is about 4 s while a guanido carbon whose motion is that of cytochrome *c* with a τ_R value of 17 ns (Oldfield et al., 1975a) would have a T_1 of about 0.2 s. As shown in Table I, the T_1 values measured for the 19 homoarginine guanido carbon resonances of guanidinated ferricytochrome in D_2O range from 1.3 to 2.3 s indicating a range of segmental motion. Most significantly, the T_1 values decrease as the protein guanido carbon resonances vary from 157.4 ppm, the resonance position of all the homoarginine guanido carbons on the relatively structureless peptide 1–65. Thus, variance from the reference chemical shift correlates with decreased segmental motion.

The ^{13}C NMR spectrum of guanidinated cytochrome *c* was measured under a variety of conditions in an attempt to deduce the sources for the observed range of homoarginine guanido carbon resonances. The characteristic spectrum for guanidinated ferricytochrome *c* at pH 7.0 illustrated in Figure 2 is not altered by placing the protein in solvents containing either phosphate or Tris-HCl buffers having concentrations ranging from 5 to 200 mM. Thus, the range of chemical shifts is not produced either by bulk solvent effects or by specific binding of phosphate or chloride ions. The same ^{13}C NMR spectrum is observed using guanidinated protein concentrations ranging from 0.5 to 5 mM indicating that the range of chemical shifts is not likely due to protein intermolecular interactions. Reduction of the heme ion to form guanidinated ferrocytochrome alters the ^{13}C NMR guanido carbon spectrum as shown in Figure 3A. Specifically, the single carbon resonance at 157.9 ppm is lost, multiple changes occur in the central envelope, and a new single carbon resonance appears at 157.2 ppm. These changes could be the result of the loss of the paramagnetic contribution to the spectrum of guanidinated ferricytochrome *c*, to changes in the relative positions of surface residues accompanying formation of ferrocytochrome *c* (Mandel et al., 1977), or both. The guanido carbon ^{13}C NMR spectrum of guanidinated ferricytochrome *c* measured at 5 °C is essentially equivalent to that shown in Figure 2 indicating that none of the resonances are preferentially shifted by contact or pseudocontact interactions with the paramagnetic heme iron. Formation of the alkaline isomer of guanidinated ferricytochrome *c* alters the appearance of the two satellite single carbon resonances as shown in Figure 3B. These changes could result from the known conformational differences between ferricytochrome *c* and its alkaline isomer (Margalit and Schejter, 1973), from the change in spin state accompanying formation of the alkaline isomer of guanidinated ferricytochrome *c* (Morton, 1973), or both. Taken together, these spectra support the view that the range of guanido carbon resonances of guanidinated ferricytochrome has a conformational origin.

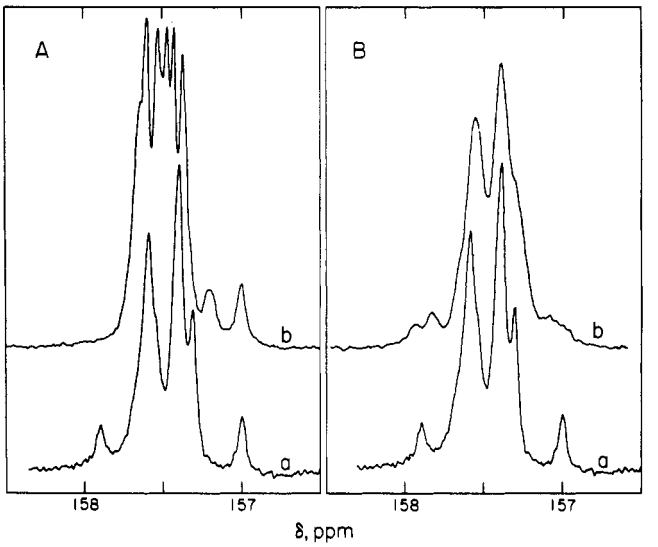

FIGURE 3: The ^{13}C NMR spectra of different forms of guanidinated cytochrome *c*. (A) Comparison of guanidinated ferri- and ferrocytochrome *c*. Spectrum a was obtained using a 4 mM solution of 52% guanidinated ferricytochrome *c* in 50 mM phosphate buffer, pD 7.0, after 7219 scans. Spectrum b was obtained using a 10 mM solution of 52% guanidinated ferrocytochrome *c* in 50 mM phosphate buffer, pD 7.1, after 28 990 scans. (B) Comparison of the neutral and alkaline isomers of ferricytochrome *c*. Spectrum a is the same as shown in the A panel. Spectrum b was obtained using a 5 mM solution of 50% guanidinated ferricytochrome in 70 mM phosphate buffer, pD 10.2, after 27 138 scans.

The virtual identity of the guanido carbon resonance region after guanidination of 19, 52, or 98% of the 19 lysine residues shown in Figure 2 supports the model discussed above which assumed that the reactivity of 18 lysine residues is equivalent at pH 11.0. Thus, it is not possible to preferentially guanidinate any of these 18 lysine residues on the protein. However, the ^{13}C content of the remaining lysine, lysine-79, can be preferentially increased by guanidinating the protein with *O*-[^{12}C]methylisourea prior to use of the ^{13}C-enriched reagent. Accordingly ferricytochrome *c* was guanidinated with the ^{12}C reagent at pH 11.0 until 50% of the increase in absorbance at 595 nm had occurred. The guanidination reaction was then stopped and the ^{12}C reagent removed. The guanidination reaction was then completed using 90% ^{13}C-enriched *O*-methylisourea. According to the analysis of Figure 1, this protein should contain 18 homoarginine residues whose guanido carbons are each enriched 16% with 90% ^{13}C and a single homoarginine at position 79 whose guanido carbon is enriched 50% with 90% ^{13}C. As shown in Figure 4, the spectrum of this guanidinated protein appears to have a preferentially intense 157.6-ppm component. The comparison of the calculated and observed areas of the five spectral components listed in Table II is remarkably similar

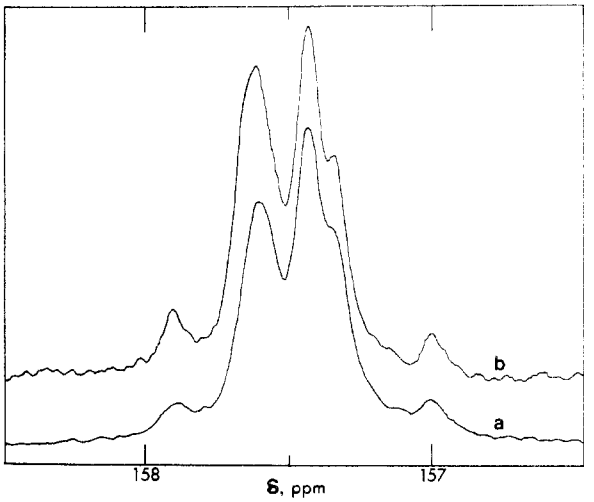

FIGURE 4: Comparison of the ^{13}C NMR spectrum of totally guanidinated ferricytochrome *c*. Spectrum a represents ferricytochrome after guanidination of 98% of its lysine residues with ^{13}C-enriched *O*-methylisourea. This spectrum was obtained using a 4 mM protein solution in 200 mM phosphate, pD 7.0, after 5000 scans. Spectrum b represents ferricytochrome *c* in which the last 16% of the reactive lysine residues were guanidinated with ^{13}C-enriched *O*-methylisourea. This spectrum was obtained using a 3.6 mM protein solution in 5 mM phosphate buffer, pD 7.0, after 26 070 scans.

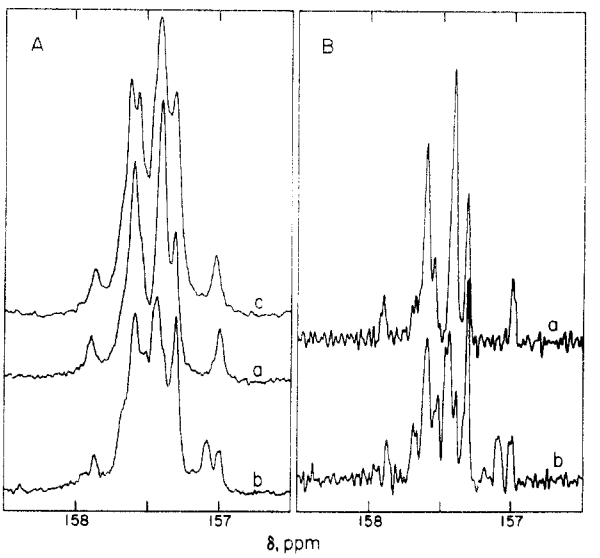

FIGURE 5: Effect of iron hexacyanides on the ^{13}C NMR spectra of guanidinated ferricytochrome *c*. (A) Raw spectra. Spectrum a was obtained using a 4 mM solution of 52% guanidinated ferricytochrome *c* in 50 mM phosphate buffer, pD 7.0, after 7219 scans. Spectrum b represents the same protein solution containing 50 mM ferrihexacyanide after 7219 scans. Spectrum c represents an 8 mM solution of the same protein in 50 mM phosphate buffer, pD 6.5, containing 50 mM ferrohexacyanide after 3673 scans. (B) Resolution enhancement of spectra a and b in panel A.

TABLE II: Analysis of the Spectrum of Guanidinated Ferricytochrome *c* Preferentially Enriched in ^{13}C at Position 79.

Chem shift (ppm)	Calcd ^{13}C content[a]	% total area	
		Calcd[b]	Obsd
157.0	0.19	4	3
157.3	0.48	14	14
157.4	1.08	31	34
157.6	1.15 + 0.45	46	45
157.9	0.13	4	4

[a] The ^{13}C content of each of the five components in the guanido carbon ^{13}C NMR spectrum was calculated in the following manner. The average number of carbons in each component listed in Table I was multiplied by 0.16, the expected fraction of each homoarginine residue containing ^{13}C-labeled guanido carbon, and by 0.90, the enrichment of the guanidination reagent. One residue was subtracted from the resonance at 157.6 ppm prior to this calculation to treat lysine-79 separately. According to the analysis of Figure 1, homoarginine should have 50% of its guanido carbon enriched with 90% enriched guanidination reagent thus producing a residue having 45% of its guanido carbon containing a ^{13}C-labeled guanido carbon. [b] The calculated area was obtained by computing the percentage of total ^{13}C content which is expected in each of the five components.

suggesting rather strongly that homoarginine-79 has a guanido carbon resonance at 157.6 ppm.

Interaction with Iron Hexacyanides. Previous measurements (Stellwagen and Shulman, 1973; Miller and Cusanovich, 1975) have indicated that ferrohexacyanide forms a complex with ferricytochrome *c* which is productive in electron transfer between the protein and hexacyanide and that ferrihexacyanide competes with ferrohexacyanide for this protein binding site. As shown in Figure 5A, the presence of 50 mM diamagnetic ferrohexacyanide produces small changes in some of the guanido carbon resonances located in the central region of the ^{13}C NMR spectrum of guanidinated ferricytochrome *c*. These changes must reflect complexation of ferrohexacyanide since addition of either phosphate or Tris-HCl to the same ionic strength does not alter the guanido carbon ^{13}C NMR spectrum of the guanidinated protein. Addition of paramagnetic ferrihexacyanide to a concentration of 50 mM produces more marked changes in the central guanido carbon region and, in addition, shifts a single guanido carbon resonance upfield to 157.1 ppm as shown in Figure 5A. The more dramatic changes produced with ferrihexacyanide most likely result from paramagnetic effects since electrostatic complexation of anionic ferrihexacyanide with one or more cationic guanido groups would place the paramagnetic ferrihexacyanide iron within about 7 Å of the guanido carbon(s). The changes in the central region of the guanido carbon resonance resulting from complexation of ferrihexacyanide were examined in detail by comparing the areas of resolution-enhanced spectra of the guanidinated protein in the presence and absence of ferrihexacyanide shown in Figure 5B. Such comparison suggests that one of the six guanido carbon resonances at 157.6 ppm shifts downfield by about 0.1 ppm and a second shifts upfield by about 0.1 ppm and that one of the seven guanido carbon resonances at 157.4 ppm shifts upfield about 0.05 ppm, a second shifts upfield about 0.1 ppm, and a third shifts upfield about 0.3 ppm. Addition of increasing concentrations of ferrihexacyanide ranging from 20 to 200 mM results in a systematic upfield shift in the single guanido carbon resonance located at 157.1 ppm in the spectrum of the guanidinated protein containing 50 mM ferrihexacyanide shown in Figure 5. Analysis of the dependence of the chemical shift of this carbon resonance on the concentration of ferrihexacyanide indicates that this resonance originates at 157.4 ppm in the protein and will attain a resonance position of 157.0 ppm in the saturated protein-ferrihexacyanide complex using an association constant of 50 M^{-1} measured for the complex in the solvent ionic strength used for these NMR measurements (Stellwagen and Cass, 1975). These results indicate that three to five homoarginine guanido groups resonance are shifted by complexation of iron hexacyanides. However, use of the guanidinated ferricytochrome preferentially enriched in ^{13}C at position 79 described above indicates that the shifted resonance at 157.6 ppm is not that of homoarginine-79.

Interaction with Cytochrome c Peroxidase. Addition of a

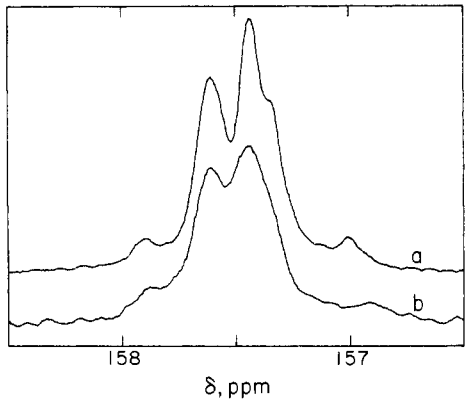

FIGURE 6: Effect of cytochrome *c* peroxidase on the ^{13}C NMR spectrum of guanidinated ferricytochrome *c*. Spectrum a was obtained using a 2 mM solution of 98% guanidinated ferricytochrome *c* in 5 mM phosphate buffer, pD 7.0, after 3000 scans. Spectrum b was obtained using a 2 mM solution of 98% guanidinated ferricytochrome containing 2.2 mM yeast cytochrome *c* peroxidase in 70% D_2O adjusted to pD 7.0, after 3800 scans.

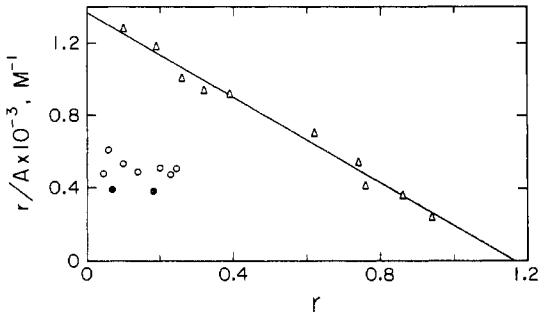

FIGURE 7: Scatchard plots of the binding of ferrohexacyanide. The abscissa, *r*, represents the moles of ferrohexacyanide bound per total moles of protein while the ordinate represents *r* divided by *A*, where *A* is the moles of unbound ferrohexacyanide. The protein compartments contained: (△) 0.2 μmol of ferricytochrome *c*; (●) 0.092 μmol of cytochrome *c* peroxidase; or (○) 0.082 μmol of ferricytochrome *c* and 0.078 μmol of cytochrome *c* peroxidase. The ligand compartments initially contained between 0.1 and 5.0 μM ferrohexacyanide containing ^{14}C. The solvent for all measurement was 50 mM Pipes buffer, pH 7.0, and measurements were done at room temperature.

10% molar excess of the electron acceptor protein yeast cytochrome *c* peroxidase to guanidinated ferrihexacyanide produces some discernible changes in the guanido carbon resonances as shown in Figure 6. The guanido carbon resonances of the protein–protein complex are broadened due in part to the presence of 30% H_2O which allows ^{13}C–^1H dipolar relaxation, increasing T_1 and T_2, and due in part to the larger τ_R of the complex which has four times the mass of ferricytochrome *c* itself. Resolution-enhanced spectra of the protein–protein complex indicate that the single guanido carbon resonance of guanidinated ferricytochrome at 157.0 ppm is shifted 0.1 ppm upfield, that a new approximately single carbon resonance occurs at about 157.2 ppm, and that small shifts occur in the central region of the spectrum.

These results suggest but hardly demonstrate that ferrohexacyanide and ferriperoxidase produce similar perturbation in the guanido carbon resonances of ferricytochrome *c* upon complexation. Competition between ferrohexacyanide and peroxidase for a common site on ferricytochrome *c* was examined by equilibrium dialysis measurements using ^{14}C-labeled ferrohexacyanide. As shown in Figure 7, ferricytochrome *c* binds a single ferrohexacyanide under the conditions employed with an association constant of about 1.2×10^4 M^{-1}, while peroxidase binds ferrohexacyanide much more weakly.

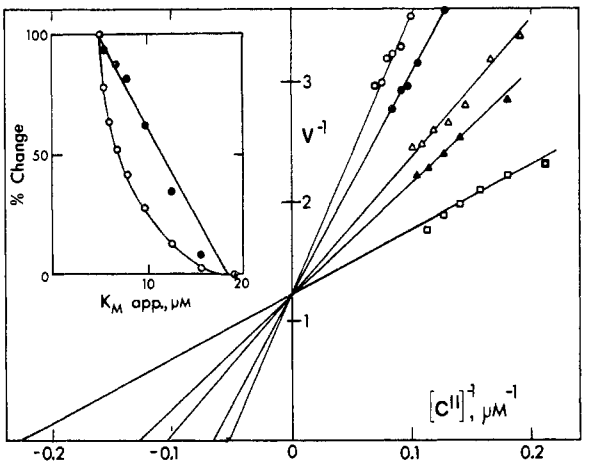

FIGURE 8: Kinetic measurements of the peroxidase-catalyzed electron transfer from ferrocytochrome *c* (C^{II}) to hydrogen peroxide. A solution of ferricytochrome *c* was reacted with 0.5 M *O*-methylisourea at pH 11.0 and 25 °C. Aliquots were removed during the course of the reaction, immediately adjusted to pH 7.0, and dialyzed against water to remove the *O*-methylisourea. Both the change in absorbance of each dialyzed sample at 595 nm and its ability to serve as a substrate for cytochrome *c* peroxidase were measured. The illustrated samples were reacted with *O*-methylisourea for: (○) 0; (●) 15; (△) 55; (▲) 80; and (□) 350 min. The insert describes the correlation of the measured changes in the apparent K_M of guanidinated ferrocytochrome *c* with percentage change in the absorbance at 595 nm (○), indicative of guanidination of lysine-79, and with the percentage of guanidination of all 19 lysine residues (●).

The binding of ferrohexacyanide to an equimolar mixture of cytochrome *c* and peroxidase is not additive but characteristic of the weak binding to peroxidase. These results are consistent with the view that complexation of cytochrome *c* with peroxidase is about three orders of magnitude stronger than complexation with hexacyanide, and that complexation with peroxidase masks the hexacyanide binding site on cytochrome *c*.

The effects of guanidination on the kinetic parameters for electron transfer from ferrocytochrome *c* to peroxide catalyzed by cytochrome *c* peroxidase were also measured. Previous measurements (Wada and Okunuki, 1969) have shown that guanidination of cytochrome *c* increases the rate of electron transfer to cytochrome oxidase. As shown in Figure 8, guanidination of cytochrome *c* does not change the V_{max} for the catalyzed reaction but does decrease the apparent K_M by greater than a factor of three. the decrease in the apparent K_M correlates with the extent of guanidination of the protein and does not correlate with the extent of guanidination of lysine-79 as indicated by the increase in absorbance at 595 nm.

Discussion

The distribution of the 19 homoarginine guanido carbon resonances of ferricytochrome *c* over a range of 0.9 ppm is somewhat unexpected since the parent lysine residues are all located on the protein surface. This observation is, however, not unprecedented in that the guanido carbon resonances of the 11 arginine residues of lysozyme are distributed over 0.3 ppm (Oldfield et al., 1975a,b) even though 10 of the 11 arginine residues extend into the protein solvent. In addition, the ^{19}F NMR spectrum of ferricytochrome *c* whose lysine residues are all trifluoroacetylated exhibits about a 1-ppm range of resonances distributed both upfield and downfield from the resonances for trifluoroacetate (Staudenmayer et al., 1976). The observed range of chemical shifts for guanidinated ferricytochrome *c* must be due to conformational effects such as the orientation and distance of individual guanido carbons

relative to the paramagnetic heme iron, to heme and aromatic ring currents, and/or to the proximity of polar or ionic groups on adjacent residues.

Precise evaluation of the contribution of each of these sources to the observed guanido carbon chemical shifts is not possible for at least two reasons. Firstly, it is not certain that native and guanidinated cytochrome *c* have the same conformations although the correspondence of their properties in solution suggests this to be the case. Secondly, the segmental motion of the majority of the external homoarginine side chains, evident in the T_1 values measured for their guanido carbons, makes calculation of their average positions difficult. Nonetheless, examination of the crystallographic models for tuna heart ferri- and ferrocytochrome *c* (Takano et al., 1977) is instructive regarding the potential of the paramagnetic heme iron, the ring currents, and hydrogen bonding to perturb the guanido carbon chemical shifts.

The ϵ-amino nitrogens of each of the 19 lysine residues range between 10.9 and 23.9 Å from the paramagnetic heme iron. Reference to changes in the chemical shifts of nonprotonated carbon resonances of cytochrome *c* accompanying reduction of paramagnetic ferricytochrome *c* to diamagnetic ferrocytochrome *c* (Oldfield et al., 1975b) indicates that carbons located more distant than 12 Å from the heme iron would be expected to be paramagnetically shifted by less than 0.1 ppm. Thus, the guanido carbons of at most two residues, homoarginines-27 and -79, would be expected to be paramagnetically shifted. Assignment of the guanido carbon resonance of homoarginine-79 at 157.6 ppm in guanidinated ferricytochrome *c* is consistent with a 0.2-ppm downfield shift from the reference peptide guanido carbon resonance. The anticipated 0.1-ppm shift of the guanido carbon of homoarginine-27 is inconsistent with either the larger shifts of the single carbon resonance of guanidinated ferricytochrome *c* located at 157.9 ppm or the constancy of the other single carbon resonance located at 157.0 ppm.

Inspection of the crystallographic model indicates that the ϵ-amino groups of at least seven lysine residues are within 7.0 Å from an oxygen of a neighboring carboxylate. Considering the added length of a homoarginine side chain compared with that of a lysine side chain and considering the better steric and electrostatic mating of a guanido compared with an amino to a carboxylate, we are of the opinion that many of the guanido groups are hydrogen bonded with carboxylates in the guanidinated protein and that such hydrogen bonding is responsible in part for the observed range of guanido carbon shifts, particularly those downfield from the reference resonance. Inspection of the crystallographic model also indicates that at least six ϵ-amino nitrogens are within 6 Å of adjacent atoms bearing a positive charge. Again given the added length of a homoarginine residue it is possible that such electrostatic environments could produce measurable upfield shifted guanido carbon resonances.

Detection of shifts in the guanido carbon resonances of guanidinated ferricytochrome *c* commensurate with complexation of ferrihexacyanide should provide a procedure for identification of the complexation site on the surface of the protein. Assignment of the shifted resonances, particularly that shifted to 157.0 ppm, will require preparation of a variety of guanidinated cytochromes *c* in which different homoarginine residues will be selectively enriched in ^{13}C. The relatively high pH required for the guanidination reaction precludes use of the electron acceptor molecules for masking the reactivity of those lysines participating in complexation, since the electron acceptor molecules do not complex with cytochrome *c* at these high pH values. The apparently equivalent reactivity of at least 18 lysine residues makes it unlikely that partially guanidinated cytochrome can be fractionated to yield cytochromes *c* with selectively guanidinated lysine residues beyond the manipulations that were used in this study. However, it is possible to generate a series of both covalent (Wilgus and Stellwagen, 1974) and noncovalent (Wilgus et al., 1977) peptide hybrids of cytochrome *c* in which the lysine residues in one of the peptides in the hybrid are all guanidinated while the lysine residues in the other peptides are not guanidinated. Since all cytochromes *c* are considered to have the same conformation and can be used interchangeably in electron-transfer reactions, it is likely that the positively charged residues which participate directly in electron-transfer interactions occupy invariant positions in the sequences of cytochromes *c*. Given the replacement of certain lysine residues by arginine or trimethyllysine residues, neither of which can be guanidinated, in the sequence of some cytochromes *c* (Dickerson and Timkovich, 1975) and the ability to hybridize guanidinated peptides 1–38, 1–65, or 66–104 with the nonguanidinated peptides, it should be possible to assign the ^{13}C-labeled guanido carbon resonances and catalytic effects to individual homoarginine residues in cytochrome *c*. Such an assignment should provide the necessary information for not only identifying and observing those lysine (homoarginine) residues involved in complexation of cytochrome *c* with iron hexacyanide and peroxidase but also in principal for any lysine residues involved in complexation with electron donor proteins as well. In addition, the involvement of individual lysine (homoarginine) residues in the conformational isomerizations of cytochrome, such as the alkaline isomerization, as well as the binding of small ligands can be studied. These potentialities lend encouragement to the considerable task of obtaining such hybrids for assignment.

The results obtained here together with the absorbance spectral measurements of Power et al. (1975) indicate that lysine-79 is not directly involved in the complexation of iron hexacyanides as previously proposed by ourselves (Stellwagen and Cass, 1975). We have confirmed the measurements of Power et al. (1975) and further found that loss of the 695-nm band accompanying the displacement of the methionine-80 ligand in the alkaline isomerization of ferricytochrome is not due to ligation of a hydroxide anion in the presence or ferrihexacyanide since no band at about 600 nm appears characteristic for ligation of a weak field ligand. It is likely that the presumed equivalence of the alkaline isomer and carboxymethyl derivative of ferricytochrome erroneously led to our proposing an involvement of lysine-79 in iron hexacyanide complexation.

The diminution in the association constant for ferrihexacyanide complexation with ferricytochrome *c* in the presence of cytochrome *c* peroxidase suggests but does not establish that ferrihexacyanide and peroxidase share a common binding site on the surface of cytochrome *c*. The decrease in the apparent K_M for ferrocytochrome *c* in the peroxidase-catalyzed electron transfer to peroxide resulting from guanidination of cytochrome *c* provides a sensitive procedure for assignment of the participating lysine/homoarginine residues. It is anticipated that the hybrids to be used in the assignment of the shifted guanido carbon resonances described above will be of use in determination of the lysines whose guanidination changes the apparent K_M for cytochrome *c* in the peroxidase-catalyzed reaction.

Acknowledgment

The authors are indebted to Professor Richard E. Dickerson for supplying manuscripts describing crystallographic analyses of tuna heart ferri- and ferrocytochrome *c* in advance of pub-

lication and to Dr. Gerald A. Pearson for assistance in obtaining and interpreting the NMR spectra.

References

Babul, J., McGowan, E. B., and Stellwagen, E. (1972), *Arch. Biochem. Biophys. 148,* 141–147.

Chien, J. C. W., and Wise, W. B. (1973), *Biochemistry 12,* 3418–3424.

Corradin, G., and Harrbury, H. A. (1970), *Biochim. Biophys. Acta 221,* 489–496.

Davis, L. A., Schejter, A., and Hess, G. P. (1974), *J. Biol. Chem. 249,* 2624–2632.

Dickerson, R. E., and Timkovich, R. (1975), *Enzymes, 3rd Ed., 11,* 397–547.

Glusko, V., Lawson, P. J., and Gurd, F. R. N. (1972), *J. Biol. Chem. 247,* 3176–3185.

Gupta, R. K., and Yonetani, T. (1973), *Biochim. Biophys. Acta 292,* 502–508.

Hettinger, T. P., and Harbury, H. A. (1964), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2892–2894.

Horsley, W., Sternlicht, H., and Cohen, J. S. (1970), *J. Am. Chem. Soc. 92,* 680–686.

Keim, P., Vigna, R. A., Nigen, A. M., Morrow, J. S., and Gurd, F. R. N. (1974), *J. Biol. Chem. 249,* 4149–4156.

Klee, W. A., and Richards, F. M. (1957), *J. Biol. Chem. 229,* 489–504.

Mandel, N., Mandel, G., Turs, B. L., Rosenberg, J., Carlson, G., and Dickerson, R. E. (1977), *J. Biol. Chem.,* submitted for publication.

Margalit, R., and Schejter, A. (1973), *Eur. J. Biochem. 32,* 492–499.

Miller, W. G., and Cusanovich, M. A. (1975), *Biophys. Struct. Mech. 1,* 97–111.

Mochan, E., and Nicholls, P. (1972), *Biochim. Biophys. Acta 267,* 309–319.

Morton, R. A. (1973), *Can. J. Biochem. 51,* 465–671.

Nicholls, P. (1964), *Arch. Biochem. Biophys. 106,* 25–48.

Nicholls,P, and Mochan, E. (1971), *Biochem. J. 121,* 55–67.

Oldfield, E., Norton, R. S., and Allerhand, A. (1975a), *J. Biol. Chem. 250,* 6368–6380.

Oldfield, E., Norton, R. S., and Allerhand, A. (1975b), *J. Biol. Chem. 250,* 6381–6402.

Orii, Y., Sekuzu, I., and Okunuki, K. (1962), *J. Biochem. (Tokyo) 51,* 204.

Pearson, G. A. (1977), *J. Magn. Resonance* (in press).

Power, S. D., Choucair, A., and Palmer, G. (1975), *Biochem. Biophys. Res. Commun. 66,* 103–107.

Staudenmayer, N., Smith, M. B., Smith, H. T., Spies, F. K., Jr., and Millett, F. (1976), *Biochemistry 15,* 3198–3205.

Stellwagen, E., Babul, J., and Wilgus, H. (1975), *Biochim. Biophys. Acta 405,* 115–121.

Stellwagen, E., and Cass, R. D. (1975), *J. Biol. Chem. 250,* 2095–2098.

Stellwagen, E., and Shulman, R. G. (1973), *J. Mol. Biol. 80,* 559–573.

Takano, T., Trus, B. L., Mandel, N., Mandel, G., Kallai, O. B., Swanson, R., and Dickerson, R. E. (1977), *J. Biol. Chem. 252,* 776–785.

Van Gelder, B. F., and Muijsers, A. O. (1966), *Biochim. Biophys. Acta 118,* 47–57.

Wada, K., and Okunuki, K. (1969), *J. Biochem. (Tokyo) 66,* 249–262.

Wilgus, H., Ranweiler, J. S., Wilson, G. S., and Stellwagen, E. (1977), *Biochemistry,* submitted for publication.

Wilgus, H., and Stellwagen, E. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2892–2894.

Yonetani, T. (1962), *J. Biol. Chem. 237,* 550–559.

Yonetani, T., and Ray, G. S. (1965), *J. Biol. Chem. 240,* 4502–4508.