^{1}H, ^{113}Cd, and ^{31}P NMR of Osteocalcin (Bovine γ-Carboxyglutamic Acid Containing Protein)†

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Received December 27, 1984

ABSTRACT: The ^{1}H (500-MHz), ^{113}Cd (44-MHz), and ^{31}P (81-MHz) NMR spectra of the bovine γ-carboxyglutamate- (Gla-) containing protein osteocalcin and its Ca(II) and Cd(II) complexes in solution have been obtained. The ^{1}H NMR spectrum of the native protein shows narrow resonances and a highly resolved multiplet structure suggesting rotational freedom of the side chains. In comparison to the simulated ^{1}H NMR spectrum of a random polypeptide chain of the same amino acid composition, there is moderate chemical shift dispersion, indicating some conformational restraints to be present. Ca(II) binding broadens all ^{1}H resonances, so severely at four Ca(II) ions per molecule that few structural conclusions can be made. Cd(II) substituted for Ca(II) has the same effect, and ^{113}Cd NMR shows the Cd(II) to be in intermediate chemical exchange on the chemical shift time scale. Estimates of the chemical exchange rates required for ^{1}H and ^{113}Cd line broadening suggest a range of K_d values for the metal ion complexes from 10^{-6} M to as high as 10^{-3} M depending on the number of metal ions bound. Alternatively, ^{1}H line broadening could be explained by relatively slow conformational fluxes in the protein induced by labile metal ion binding to one or more sites. Cd(II) when used to form a cadmium–phosphate mineral analogous to hydroxylapatite results in a crystal lattice that removes osteocalcin from solution just as effectively as hydroxylapatite. $^{113}Cd(II)$ exchange at the binding sites of osteocalcin in solution is slowed dramatically by the addition of HPO_4^{2-}. ^{31}P NMR shows the interaction of phosphate with the protein to require the metal ion. Much of the original chemical shift dispersion in the ^{1}H NMR spectrum is removed by decarboxylation of the three Gla residues. The spectrum is no longer affected by Ca(II) and is more similar to that of a random coil, suggesting that the original conformational restraint may be dictated by the high negative charge density. These findings suggest that the extremely stable complex of this flexible protein with the hydroxylapatite lattice must be induced by conformational changes and chelate effects consequent to binding of the three Gla residues to the Ca(II) sites in the solid lattice.

Osteocalcin is a member of the recently discovered class of proteins that contain γ-carboxyglutamic acid residues (Gla). Gla residues are the product of a posttranslational vitamin K dependent carboxylation at the γ-position of glutamate residues. The dicarboxylate structure of the Gla residue allows for chelation of cations, and the Gla-containing proteins all exhibit a high affinity for Ca(II).

Osteocalcin is the most abundant noncollagenous protein found in bone, is the only bone protein known to contain Gla and is therefore also referred to as bone Gla protein or BGP.[1] There is a high degree of sequence homology between osteocalcins from different species (Hauschka & Carr, 1982). Bovine BGP contains three Gla residues and a single disulfide bridge and has a M_r of 5700 (Price et al., 1976a). The K_d for the BGP Ca(II) has been reported from 2×10^{-5} to 3×10^{-3} M depending on ionic strength and the number of Ca(II) ions present (Hauschka et al., 1977). The K_d for BGP bound to hydroxylapatite in the solid state has been estimated to be at least 10^{-7} M in 0.15 M NaCl (Poser & Price, 1979).

The high affinity of BGP for hydroxylapatite has been the focal point for investigations into the function of BGP. BGP is known to be synthesized within osteoblasts (Nishimoto & Price, 1979) and probably appears initially as a high molecular weight precursor (Hauschka et al., 1983). Reduction of total BGP to 2% of the normal level in warfarin-treated rat weanlings did not induce any detectable abnormality in bone mineralization or repair within 10 weeks (Price & Williamson, 1981). After 8 months of warfarin treatment, however, there was some abnormal calcification of the proximal tibial growth plate with consequent slowing of longitudinal growth (Price et al., 1982). It is known that BGP inhibits hydroxylapatite formation in vitro (Poser & Price, 1979). While BGP might slow hydroxylapatite formation in vivo, the precise effect of BGP on the in vivo hydroxylapatite equilibrium is unknown. Detailed discussions of the possible function of osteocalcin can be found in Price et al. (1976b, 1982), Price & Williamson (1981), and Lian et al. (1983).

The studies in this paper were directed toward elucidation of the principal structural elements of the bovine protein and its metal complexes in solution by NMR spectroscopy. ^{1}H NMR (500 MHz) was employed to observe structural differences between various forms of BGP, i.e., native, decarboxylated, and reduced. The dynamics of BGP interactions with Ca(II), Cd(II), and phosphate were explored with ^{1}H, ^{113}Cd, and ^{31}P NMR.

Materials and Methods

Bovine BGP was prepared according to published methods (Price et al., 1976b). Dialysis at pH 4 to remove calcium was

† This work was supported by NIH Grant AM32067. R.V.P. is the recipient of NIH Postdoctoral Fellowship AM07100. The 500-MHz NMR facility is supported by NSF Grant CHE79-16210.

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[1] Abbreviations: BGP, bone γ-carboxyglutamic acid containing protein; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/85/0424-6291$01.50/0

monitored by atomic absorption spectroscopy on an Instrumentation Laboratory 157 spectrophotometer. The calcium content was less than 5% of the BGP concentration. Homogeneity was checked by electrophoresis on a 20% polyacrylamide gel. The protein was dialyzed against 10 mM Tris-HCl, pH 8, lyophilized, dissolved in D_2O, and lyophilized again. When protein solutions were made for the NMR studies, protein concentration was determined with $E^{0.1\%1cm} = 1.06$ at 280 nm (Poser & Price, 1979). Decarboxylated and reduced BGP were prepared according to published procedures (Poser & Price, 1979). All other materials were analyzed reagent grade.

^1H NMR spectroscopy was performed on a Bruker WM 500-MHz spectrometer. Decoupling experiments were performed with NOE suppression. pD was 8.4 and temperature 308 °K unless noted otherwise. The HDO resonance was set at 4.7 ppm. ^{111}Cd (42.4-MHz), ^{113}Cd (44.37-MHz), and ^{31}P (80.98-MHz) NMR spectroscopies were performed on a Bruker CXP-200 spectrometer with a broad-band tunable probe. The pulse angle was ~30°, and no decoupling was employed. Samples were 2 mL, 10% D_2O, pD 6.5, and run at 303 K. A vortex plug confined the sample and allowed for introduction of external standards in a capillary tube ($^{111}CdCl_2$, 95 ppm; methyl phosphonate, 29.5 ppm). The simulation of the BGP ^1H NMR spectrum was accomplished by using the Bruker PANIC program. Chemical shift and coupling constants for individual amino acids were taken from the following sources: for Gla, Märki et al. (1977); for Hyp, Abraham et al. (1962); for the rest of the residues, Bundi & Wüthrich (1979).

Results and Discussion

The ^1H NMR spectrum of calcium-free BGP is highly resolved (Figure 1A). Most of the proton resonances are similar to those expected for a random polypeptide of the same amino acid composition (Figure 1B). However, a number of the side-chain protons show chemical shift dispersion, suggesting that there is some structure present in the metal-free protein. Complexation of BGP by Ca(II), Cd(II) and HPO_4^{2-} is relatively weak, and the spectra of these complexes are modulated by chemical-exchange processes that cause significant line broadening. ^{113}Cd and ^{31}P NMR spectra of these complexes are consistent with such an exchange phenomenon.

The ^1H resonance assignments of the metal-free protein are presented first followed by the ^1H NMR spectra of the metal ion complexes. ^{113}Cd NMR is then presented as a probe of the formation of the complex with the metal ion, since $^{113}Cd(II)$ has an ionic radius similar to that of Ca(II) and readily substitutes for Ca(II). ^{31}P NMR was also used to follow the formation of metal-dependent phosphate complexes with the protein.

^1H NMR (500 MHz) of the Aromatic Residues of BGP. The ^1H spectrum of BGP in the region of the aromatic protons is given in Figure 1A and compared to a simulated aromatic ^1H spectrum of a random polypeptide of the same amino acid composition (Figure 1B). The proton assignments given for the BGP spectrum are based on decoupling and pH titration experiments discussed below. The most striking difference from the spectrum of a random polypeptide is shown by the tyrosyl protons. These appear to occur in roughly two groups of two residues each, one set with both 2,6 and 3,5 protons relatively unshifted (7.13 and 6.78 ppm, respectively) and a second set with both 2,6 and 3,5 protons shifted upfield (7.03 and 6.75 ppm, respectively). The two Tyr in the first set do not have precisely identical chemical environments (note

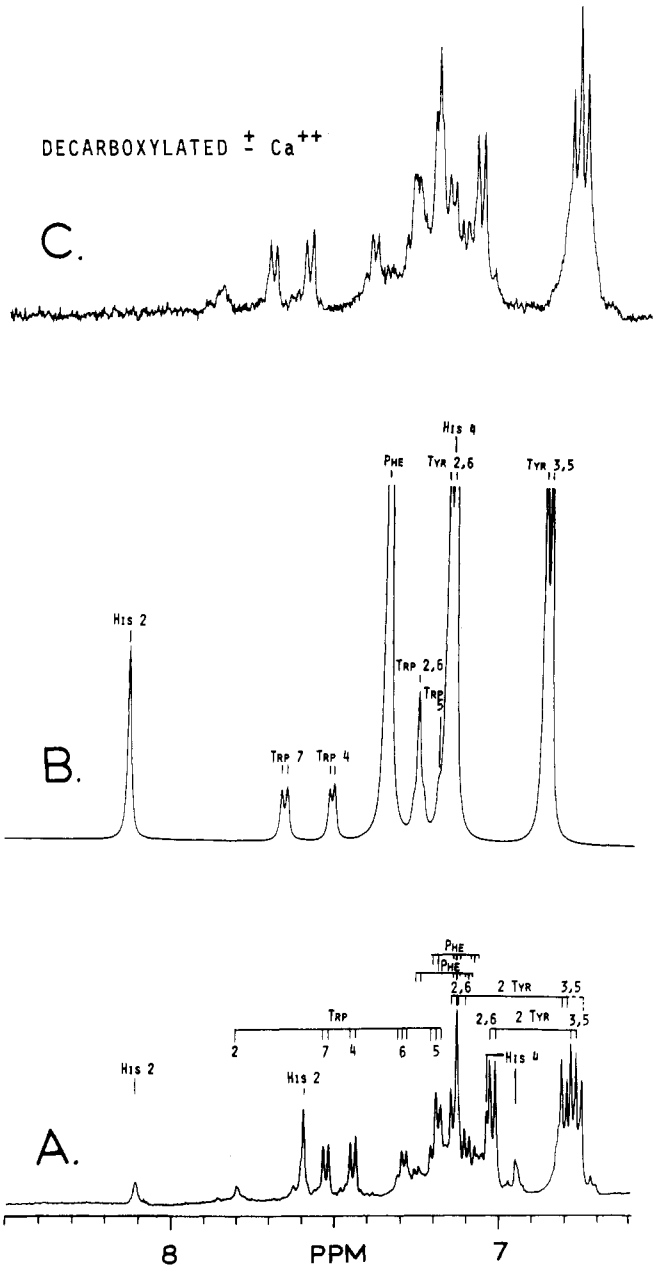

FIGURE 1: The 500-MHz ^1H spectrum of the aromatic residues of osteocalcin (BGP): (A) native BGP, (B) simulated spectrum (see Materials and Methods) and (C) decarboxylated BGP. In this and the following figures, the numbers refer to the ring position of the proton to which the resonance is assigned.

different chemical shifts for the 2,6 protons). The second set of Tyr residues with upfield-shifted protons appear to be identical giving a single doublet for both 2,6 and 3,5 protons (Figure 1A). The Tyr proton spectra, especially in the 3,5-proton region, however, are not always divisible into two subsets. A spectrum consisting of two sets of doublets (each four protons) for the 3,5 protons is nearly achieved at 308 °C (Figure 2), but at lower temperature overlapping doublets produce at least five peaks and two minor poorly resolved peaks slightly downfield. This suggests that at room temperature the Tyr residues exist in several slowly interconverting conformations that tend to disappear at higher temperature. Thus, BGP does not appear to exist in a single conformation in solution.

The primary structure of bovine BGP (Price et al., 1976a) is as follows and shows that the aromatic residues are confined to the amino- and carboxy-terminal arms:

^{1}Tyr-Leu-Asp-His-Trp-Leu-Gly-Ala-Hyp-Ala-Pro-Tyr-Pro-Asp-Pro-
^{16}Leu-*Gla*-Pro-Lys-Arg-*Gla*-Val-Cys-*Gla*-Leu-Asn-Pro-Asp-Cys-Asp-
^{31}Glu-Leu-Ala-Asp-His-Ile-Gly-Phe-Gln-Glu-Ala-Tyr-Arg-Arg-Phe-
^{46}Tyr-Gly-Pro-Val

Therefore, temperature-induced mobility of the aromatic side chains is perhaps not surprising and indicates that there is some structure in the terminal regions of the peptide chain to impede side-chain mobility.

The chemical shift dispersion of the Tyr resonances also largely disappears on decarboxylation of the Gla residues (Figure 1C), suggesting that the structure present in metal-free BGP may reflect electrostatic repulsions or salt bridges in this highly negatively charged protein. The His protons show moderately different chemical shifts and titrate with pK_a's (6.8 and 7.8) different from those expected for a random polypeptide chain. The His sequences are Asp-His-Trp and Asp-His-Ileu, so sequence alone would not suggest such different pK_a values (Figure 3).

Typical examples of decoupling experiments used to assign the aromatic protons are shown in Figure 4. The minor most upfield Tyr 3,5 resonance does not decouple when either group of 2,6 proton resonances are saturated, suggesting that part of the molecules have at least one Tyr in a different environment at 278 °C. The resonance identified as a His-4 proton is not coupled to any other as expected. Studies of the solution conformation of metal-free osteocalcin from both chicken bone and bovine bone by circular dichroism of the peptide chromophores indicate a conformation that is not significantly different from that expected for a "random" polypeptide chain (Hauschka & Carr, 1982; Delmas et al., 1984; Gundläch & Voegeli, 1983). Circular dichroism of the preparations used in the present study carried out in this laboratory confirm this conclusion.

^{1}H NMR Spectra of the Aliphatic Protons of BGP. The aliphatic proton NMR spectrum of the protein shows little deviation from the ^{1}H NMR spectrum expected for a random polypeptide chain of the amino acid composition of BGP (Parts A and B of Figure 5, respectively). All the aliphatic proton resonances are relatively narrow, and many assignments can be made with reasonable confidence from the simulated spectrum. Proceeding from high field to low field in Figure 5, the Val, Leu, and Ile CH_3 protons (0.7–1 ppm) are only slightly shifted from those of the simulated spectrum (Figure 5B) and, like the Ala β-protons (1.2–1.3 ppm), show some chemical shift dispersion. There is also some chemical shift dispersion among the Leu β,γ-protons. However, the envelope of aliphatic proton resonances between 0 and 2.3 ppm (Figure 5A) exhibits strong homology to the simulated spectrum (Figure 5B). The resonances between 2.3 and 3.3 ppm (Figure 5A), comprised mostly of β-protons, are also similar to the simulation. The Pro and Hyp δ-protons appear well dispersed between 3.5 and 3.8 ppm. The Hyp and four of the six Pro amino acids occur between residue 9 and residue 18 in the BGP primary sequence (see above). These residues preclude the existence of any "classical" secondary structure within that region of the polypeptide chain; however, they may also prevent the peptide backbone from assuming all the possible conformations of a random structure. Likewise, the modest chemical shift dispersion in the α-proton resonances (4–5 ppm, Figure 5A) is indicative of some restriction to BGP taking on a completely random-coil solution structure.

The 500-MHz ^{1}H NMR Spectra of Decarboxylated and Reduced BGP. In order to assess the effect of the three dicarboxyl groups on the solution structure, the γ-carboxylglutamyl residues of metal-free BGP were decarboxylated by the previously described dry-heating method (Poser & Price, 1979). The ^{1}H NMR spectra of the decarboxylated protein are shown in Figures 1C and 5C. The most striking change is the loss of the chemical shift dispersion of the methyl protons of two of the four Ala residues at 1.15–1.3 ppm. None of the four Ala methyl groups are near the Gla residues in the sequence, so the original chemical shift dispersion of the Ala methyl groups most probably relates to a restricted secondary or tertiary structural element. A similar change is obvious in the region of the α-protons of the two Val and the one Ile, 4–4.1 ppm.

The above changes in the spectrum are the most dramatic, but the fact is that sharpening of the signals and loss of some of the chemical shift dispersion occur throughout the aliphatic spectrum upon decarboxylation. For example the β'-protons

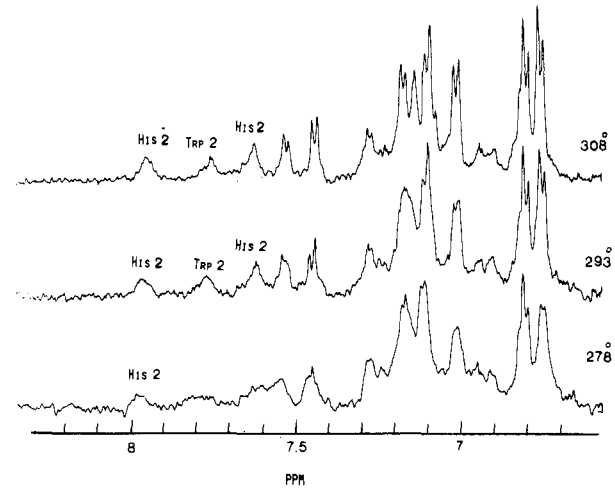

FIGURE 2: Effect of temperature on the resolution of the 500-MHz ^{1}H NMR spectrum of the aromatic residues of osteocalcin (BGP).

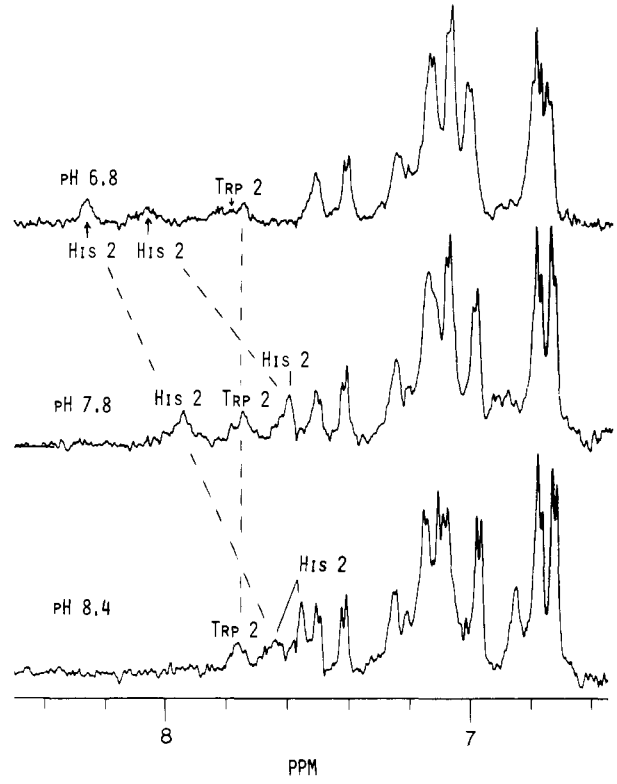

FIGURE 3: pH titration of the 500-MHz ^{1}H spectrum of the two His residues of osteocalcin (BGP). The His 2-protons shift upfield with increasing pH but exhibit different acidities consistent with pK_a's of 6.8 and 7.8.

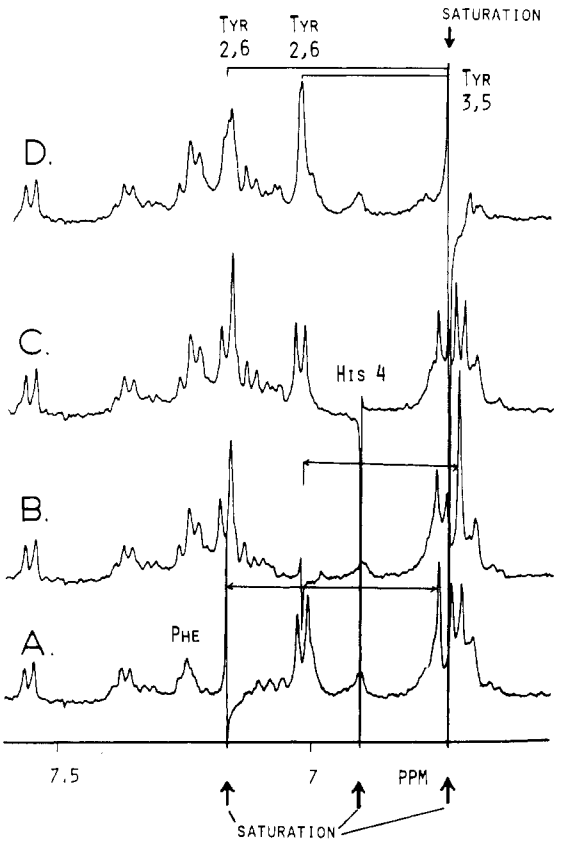

FIGURE 4: Decoupling experiments showing some spin–spin-coupled resonances in the 500-MHz ^{1}H NMR spectrum of the aromatic residues of osteocalcin (BGP). The vertical arrows indicate which multiplets are saturated. The saturated resonances are connected to their respective J-coupled multiplets by the horizontal arrows.

of the four Tyr and two Phe residues are now obvious at ~2.9 ppm and correspond much more to the simulated position. In addition, the Tyr aromatic proton resonances are now much more homogeneous (Figure 1C). There are two changes in the aliphatic spectrum that can reasonably be attributed to the loss of Gla, i.e., the loss of resonance at 2.15 (Gla β'-protons) and 3.45 ppm (Gla γ-protons) as indicated by the arrows in Figure 5A and the appearance of increased signal intensity at 2.2 (Glu γ- and γ'-protons) and 1.95 ppm (Glu β- and β'-protons) as indicated by the connected arrows in Figure 5C.

These results indicate that the negative charges provided by the dicarboxylic acid Gla residues, leading to a net 9– charge at pH 8 in the region spanning residues 14–34, restrict BGP conformation in solution. This would account for the moderate chemical shift dispersion in the apoprotein, which is greatly reduced in the decarboxylated form. The electrostatically induced structural tension appears to be relaxed upon decarboxylation, and BGP is then capable of assuming more of the conformations expected of a random polypeptide chain.

Reduction of the disulfide bridge between Cys residue 23 and Cys residue 29 with dithiothreitol results in few changes in the ^{1}H NMR spectrum (spectra not shown). The changes that do occur are largely confined to coalescence of the methyl protons from 0.9 to 1.35 ppm and a similar coalescence of the Gla β'-proton and Glu β-proton at 2.1 ppm. The aromatic protons also show little in the way of chemical shift change upon reduction. This is an interesting observation due to the fact that the disulfide bridge lies in the midst of a large number of acidic residues (see sequence). If a specific conformation were needed to bind cations, such as Ca(II) in hydroxylapatite, a disulfide bridge that forms a seven-residue loop in the region of a high density of negative charge should be a significant structural feature. However, in solution, the disulfide bridge appears to play little role in determining BGP conformation.

Solution Structure of Ca(II), Cd(II), and Phosphate Complexes of BGP as Assayed by ^{1}H, ^{113}Cd, and ^{31}P NMR. Addition of two Ca(II) ions/mol of BGP at millimolar concentrations severely broadens the aromatic and aliphatic proton NMR spectra (Figure 6A). The broadening increases when four Ca(II)/mol BGP are added. The best explanation appears to be that the Ca(II) complexes are quite labile, and therefore, exchanges between liganded and unliganded forms are fast enough for the intermediate chemical exchange condition to pertain.[2] Some chemical shift dispersion is probably also present, since the change between two and four Ca(II) ions (Figure 6A,B) suggests that it may be difficult to saturate all Ca(II) sites. Ca(II) could also cross-link one or more molecules of BGP, leading to further chemical shift dispersion and an increased rotational correlation time of the protein.

The ^{1}H NMR of the decarboxylated protein shows no changes upon Ca(II) addition and confirms that the three dicarboxylated side chains make the major contribution to the formation of the Ca(II) binding sites (Figures 1C and 5C). Without these extra carboxyl groups, both the initial conformation and the Ca(II) binding sites are lost. The His residues may contribute to the Ca(II) sites, since these resonances shift in the complex (see Figure 6C), but once the Gla residues are gone, no shift in these resonances is induced by Ca(II).

^{111}Cd, ^{113}Cd, and ^{31}P NMR of the Cd(II) Complexes of BGP. Cd(II) has been shown to bind to a large number of Ca(II) binding proteins and provides a useful NMR probe for Ca(II) binding sites in the form of ^{113}Cd ($S = {}^1/_2$) or ^{111}Cd ($S = {}^1/_2$) isotopes (Forsén et al., 1979, 1980). That Cd(II) interaction with osteocalcin is not an unreasonable model for Ca(II) binding is supported by the observation that if Cd(II) is substituted for Ca(II) in the formation of a cadmium–phosphate compound formed by following the standard steps leading to hydroxylapatite formation (Main et al., 1959), a solid is obtained with the same solution properties and the same (Cd(II):phosphate ratio as the Ca(II):phosphate ratio in true hydroxylapatite. While we have not physically characterized the lattice, the Cd(II) analogue removes BGP from solution

[2] The K_d and therefore $k_{exchange}$ for Ca(II) that would satisfy the intermediate chemical exchange condition for ligand ^{1}H resonances are difficult to estimate precisely. The ^{113}Cd(II) NMR signals from the Cd(II) complex of BGP (to be shown) show chemical exchange broadening, reasonably explained by direct exchange of the central metal ion. The minimum $k_{exchange}$ estimated for this process is 10^3 s^{-1}. The maximum $\Delta\omega$ between exchanging species for the protons, however, cannot be over 0.2 ppm (100 Hz), and the exchange rate required to broaden these resonances is nearer 10^2 s^{-1}. On the other hand at 10^{-3} M protein, some metal binding sites are not saturated until at least 4 mM metal ion is added (Figure 6). Thus, K_d for at least one class of sites must be near 1 mM, which implies a $k_{exchange}$ of 10^5 s^{-1}, fast exchange for ^{1}H resonances but still sufficient to broaden ^{113}Cd resonances since $\Delta\omega$ is much larger. It is possible that a range of binding constants applies for the Ca(II) and Cd(II) complexes of BGP in solution. The first metal ion may be bound with a dissociation constant approaching 10^{-6} M, while a K_d of 10^{-3} M may apply to the third metal ion bound. For example, one might invoke all three Gla residues as being involved in binding the first metal ion followed by rearrangement on further addition of metal ions. Such a model may correlate with our observation that it is extremely difficult to remove the last mole of Ca(II) from BGP with EDTA. It is also possible that general exchange broadening of all ^{1}H resonances results not from direct metal ion exchange but from conformational flux of the flexible BGP on a relatively slow time scale induced by more rapid exchange of Ca(II) ions in several possible ligand configurations of the protein.

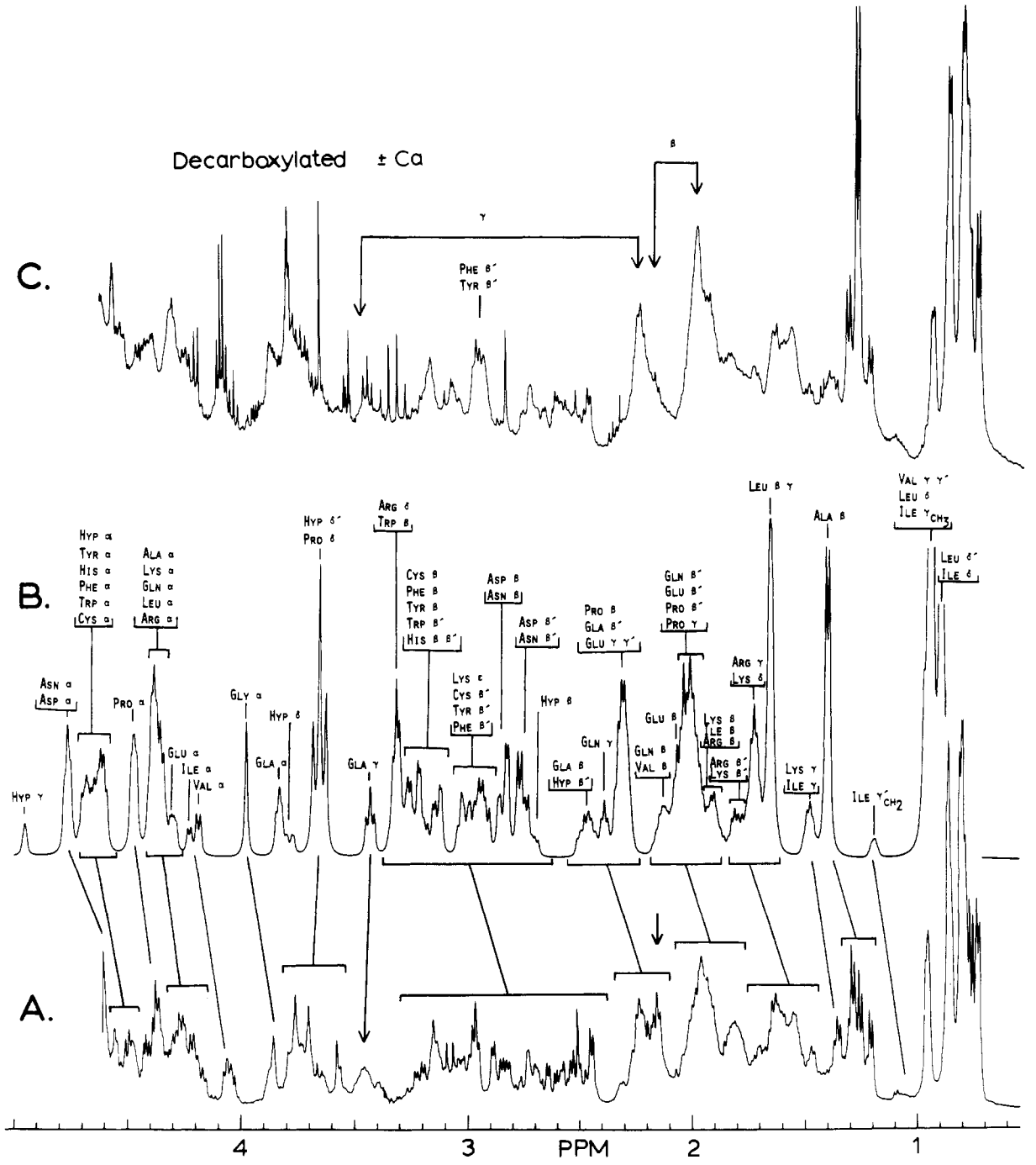

FIGURE 5: The 500-MHz ^{1}H spectra of the aliphatic residues of osteocalcin (BGP): (A) native BGP, (B) simulated spectrum (see Materials and Methods), and (C) decarboxylated BGP.

at the same low protein concentrations as does authentic hydroxylapatite.

From pH 6 to pH 9 broad or undetectable ^{113}Cd or ^{111}Cd resonances are observed for Cd(II) ions in the presence of BGP, $\nu_{1/2}$ = 500–1500 Hz, δ = 0–40 ppm (Figure 7A). The best resolved ^{111}Cd resonance ($\nu_{1/2}$ = 192Hz) that can be observed is one near 0 ppm when one ^{111}Cd(II) ion and one inorganic phosphate ion are added to metal-free BGP as shown in Figure 7B. The narrow line at 95 ppm is a $^{111}CdCl_2$ standard (100 mM) in an external capillary. The absence of a ^{111}Cd (^{113}Cd) line or at best a broad line is most likely due to the presence of intermediate chemical exchange between two or more environments. If the chemical shift difference between free and protein-bound ^{111}Cd(II) is 10–100 ppm and the K_d is 10^{-5}–10^{-3} M, then exchange rates of 10^3–10^5 s^{-1} (τ = 10^{-3}–10^{-5} s), assuming a diffusion controlled on rate (10^8 s^{-1}), would result in severe exchange broadening of the ^{111}Cd (^{113}Cd) resonance, depending on the precise chemical shift difference between free and bound ^{113}Cd(II). A more detailed analysis of ^{113}Cd (^{111}Cd) exchange broadening in protein sites is given in Coleman et al. (1979). The relatively labile Cd(II) complex of BGP in solution is in marked contrast to other Ca(II) binding proteins like troponin C (Forsén et al., 1979) or calmodulin (Forsén et al., 1980), which give sharp ^{113}Cd resonances in solution when Cd(II) is substituted for Ca(II). In general, the ^{1}H spectra of the Ca(II) or Cd(II) complexes of a protein like calmodulin show narrow resonances, although a few specific resonances are moderately broadened due to metal ion exchange (Klevit et al., 1984). While the Ca(II) and Cd(II) complexes of calmodulin show almost identical ^{1}H NMR spectra, greater broadening of specific resonances suggests that Cd(II) may exchange faster than Ca(II). Such

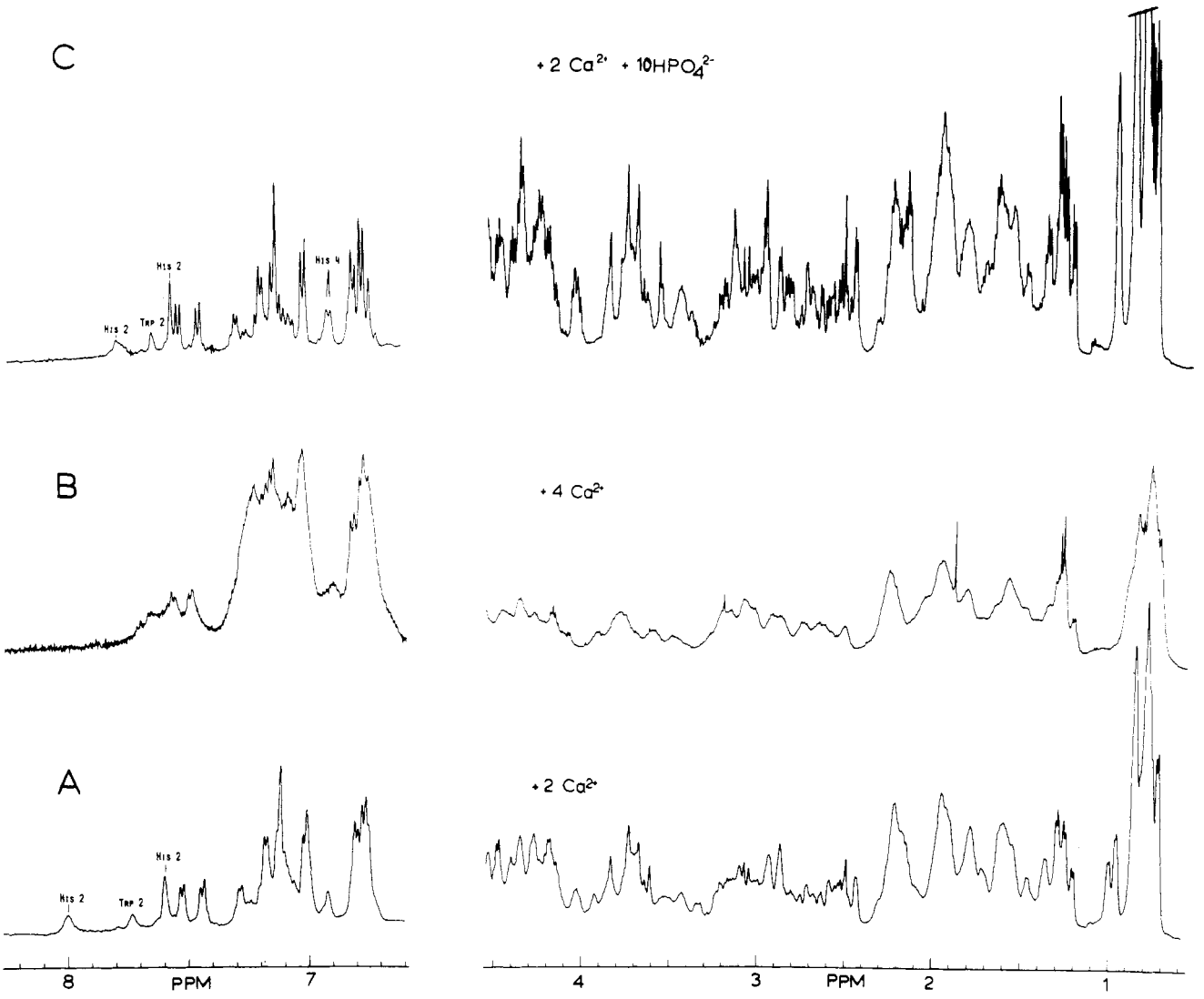

FIGURE 6: The 500-MHz ^1H NMR spectrum of BGP complexes with Ca(II) and/or HPO_4^{2-}: (A) BGP–Ca(II) in a 1:2 complex, (B) BGP–Ca(II) in a 1:4 complex, and (C) BGP–Ca(II)–HPO_4^{2-} in a 1:2:10 complex.

a differential exchange rate, were it to apply to the BGP complexes, might contribute to a different time scale of chemical exchange on the basis of the ^{113}Cd resonances vs. the ^1H resonances of the Ca(II) complex. The exchange rates accounting for the broadening in Figures 6 and 7 do seem to be slightly different.[2]

Phosphate must add to the Cd(II) complex and stabilize the bound metal ion, since phosphate addition removes a large part of the exchange broadening of the ^{111}Cd resonance (Figure 7B). Confirming this picture is the finding that the ^{31}P NMR signal from inorganic phosphate shows no change in chemical shift or broadening when phosphate is added to metal-free BGP. When Cd(II)–BGP is added, the ^{31}P resonance shifts upfield from 3 to ~1 ppm and broadens (Figure 7D). Addition of a second phosphate ion (Figure 7E) broadens the line even further and moves it back downfield slightly, suggesting that bound phosphate is in rapid exchange with free P_i. As additional phosphate is added, the line continues to move downfield, reaches a maximum width of 130 Hz at 20 mM P_i, and then narrows rapidly from 20 to 100 mM P_i (spectra not shown.)

Formation of the ternary phosphate complex is also reflected in the ^1H spectrum of the protein (Figure 6C). Much of the broadening of the proton resonances is removed by the addition of phosphate to the Ca(II) protein and reveals that significant changes have occurred in the chemical shifts of the His C2, His C4, and Trp C2 protons. We first concluded that this simply reflected deprotonation of the His residues, since Ca(II) binding could have induced deprotonation, hidden by the broadening. This may be a partial explanation, since the shifts are in the correct direction, but not expected of course at pH 6.8 (see Figure 3). The upfield shift in Trp C2 suggests an additional change in conformation. We considered that the loss of broadening on phosphate addition could have the trivial explanation that phosphate removes Ca(II) from BGP in solution. This seems unlikely because of the changes in the aromatic protons and the ^{113}Cd and ^{31}P NMR signals for the Cd(II) analogue are not explained by Cd(II) complexation with free phosphate. It would appear that tighter less exchange-labile metal binding induced by phosphate (Figure 6) removes exchange broadening and probably chemical shift dispersion as well because of more homogeneous metal site occupancy. If so, the more highly resolved ^1H NMR spectrum of the ternary complex suggests that Ca(II) binding to BGP in solution has little effect on the overall structure of the protein (Figure 6C).

The ^1H, ^{113}Cd, and ^{31}P NMR indicate that the conformation of BGP in solution is flexible and the metal ion complexes are labile. In contrast, it seems likely that the conformation of BGP, bound to hydroxylapatite, will be dictated by the geometry of the charged lattice to which it binds. What the solution NMR study suggests is that neither Ca(II) nor

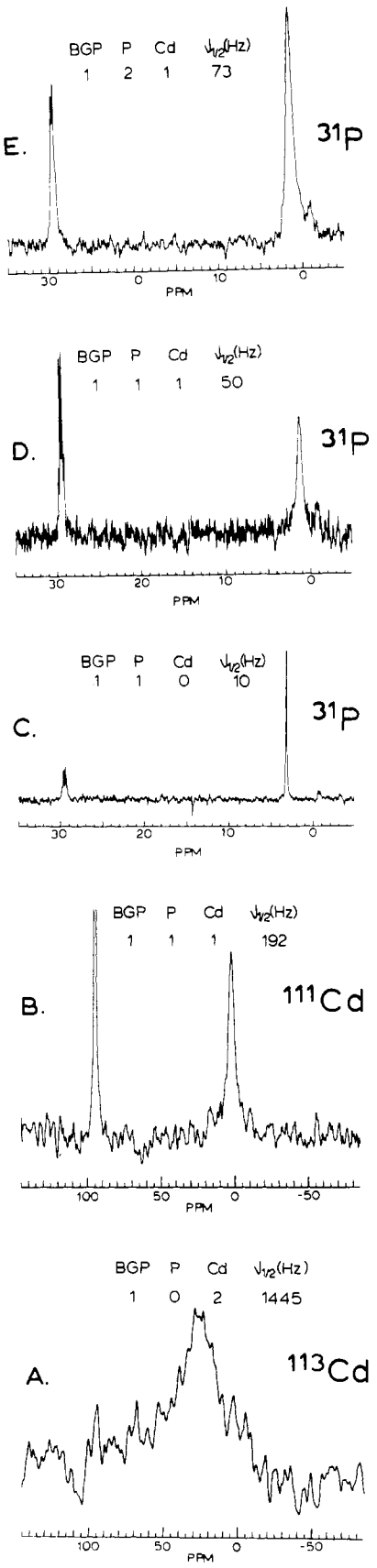

FIGURE 7: ^{111}Cd, ^{113}Cd, and ^{31}P NMR spectra of various complexes of osteocalcin (BGP). $\nu_{1/2}$ is the line width at peak half-height. See Materials and Methods for details. (A) ^{113}Cd NMR spectrum of a 1:2 complex of BGP–Cd(II), (B) ^{111}Cd NMR spectrum of a 1:1:1 complex of BGP–Cd(II)–P_i, (C) ^{31}P NMR spectrum of a 1:1 ratio of BGP to P_i, (D) ^{31}P NMR spectrum of a 1:1:1 complex of BGP–Cd(II)–P_i, and (E) ^{31}P NMR spectrum of a 1:1:2 complex of BGP–Cd(II)–P_i.

HPO_4^{2-} interaction separately in solution accounts for a rigid protein–metal ion–ligand complex. When both calcium and phosphate are present at specific and separate lattice points as in hydroxylapatite, it is possible that energetically favorable "chelate" effects are possible involving Gla binding to the Ca(II) lattice. The chelate could involve the extended rigid rings formed by the three Gla–Ca(II) sites, the protein backbone, and the hydroxylapatite lattice. While additional interactions between the three Arg and one Lys positive charges and the phosphate lattice could be postulated, we have modified the three Arg residues with cyclobutanedione and phenylglyoxal (Lundblad & Noyes, 1984), and there is relatively little effect of either modification on the removal of the protein from solution by hydroxylapatite crystals. This is consistent with the lack of phosphate binding to metal-free osteocalcin (Figure 7C). Thus, the Gla–Ca(II) interaction alone appears to account for most of the binding interaction.

Added in Proof

Since submission of this paper, we learned of an independent ^{1}H (500-MHz) NMR study of osteocalcin carried out by R. J. P. Williams and his colleagues of Oxford University. We appreciate helpful discussions with these investigators.

Registry No. Cd-113, 14336-66-4.

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Influence of Detergent Polar and Apolar Structure upon the Temperature Dependence of Beef Heart Cytochrome *c* Oxidase Activity†

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Received January 14, 1985; Revised Manuscript Received May 21, 1985

ABSTRACT: The temperature dependence of lipid-depleted beef heart cytochrome *c* oxidase activity was studied in a series of chemically homogeneous detergents. The detergents that were tested included C_{10} to C_{18} maltosides, C_8 to C_{12} glucosides, C_8 to C_{16} Zwittergents, and C_{12} poly(oxyethylene) ethers. The observed rates of electron transport were dependent upon the structure of the polar head group and the length of the hydrocarbon tail. Of the detergents tested, the alkyl maltosides were the best in terms of both high rates of electron transport and superior enzyme stability. With the maltosides, changing the length of the alkyl tail affected the activity of cytochrome *c* oxidase in a manner quite similar to that reported with synthetic phosphatidylcholines and phosphatidylethanolamines [Vik, S. B., & Capaldi, R. A. (1977) *Biochemistry 16*, 5755–5759], suggesting that the alkyl maltosides can mimic some of the features of the membrane environment. In each of the detergents, the activation enthalpy (determined from the slope of an Arrhenius plot) was nearly identical, suggesting that the same electron-transfer step within cytochrome *c* oxidase is rate limiting. This result has been interpreted as evidence for the existence of two or more conformers of cytochrome *c* oxidase, one of which is significantly more active than the other(s). The enzyme turnover number, which changes by 2 orders of magnitude depending upon the structure of the bound detergent, may reflect the ability of each detergent to alter the equilibrium between the active and nearly inactive conformers.

The electron-transport activity of cytochrome *c* oxidase is known to be influenced by either the phospholipid or detergent environment that surrounds its two hydrophobic, intramembrane domains. Reconstituting the purified enzyme into a variety of phosphatidylcholines and phosphatidylethanolamines has shown that the fatty acid composition of the boundary layer phospholipids affects the enzymatic turnover number more so than does the nature of the polar head group (Vik & Capaldi, 1977). Limited activity measurements using the detergent-solubilized enzyme, i.e., enzyme solubilized in either lysophosphatidylcholines or in Tween detergents, have shown a similar but less pronounced dependence of the enzymatic activity upon the structure of the detergent's hydrocarbon tail and polar head group (Vik & Capaldi, 1977; Robinson & Capaldi, 1977). Unfortunately, extension of this type of study to other classes of detergents in order to more fully understand the structural requirements at the protein–amphiphile interface has been difficult since most commercially available detergents, including the Tween detergents used in the studies mentioned above, are heterogeneous mixtures and contain significant amounts of inherent impurities, especially peroxides and free radicals. Therefore, it is almost impossible to determine whether the enzymatic rate changes that are observed in each detergent are caused by important structural differences in the detergents or are the result of inactivation of cytochrome *c* oxidase by the different amounts or types of impurities that are present in the detergents.

In the past several years, a variety of homogeneous, structurally defined detergents have become available that potentially could be used to assess the structure–function relationships at the hydrophobic surface of cytochrome *c* oxidase. Some of the most promising classes of highly purified detergents are the alkyl glycosides, Zwittergents,[1] and homogeneous

† This investigation was supported by research grants from the National Institutes of Health (GM 24795). A preliminary report of some of this work was presented at the Canadian Biochemical Society Symposium held in Banff, Alberta, in May 1984.
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[1] Abbreviations: Tris-HCl, tris(hydroxymethyl)aminomethane base titrated to the appropriate pH with HCl; EDTA, ethylenediaminetetraacetic acid; cmc, critical micelle concentration; $C_{12}E_8$, octylethylene glycol dodecyl ether; $C_{12}E_9$, nonaethylene glycol dodecyl ether; octyl glucoside, octyl β-D-glucopyranoside; decyl glucoside, decyl β-D-glucopyranoside; lauryl glucoside, dodecyl β-D-glucopyranoside; decyl maltoside, decyl β-D-maltopyranoside; lauryl maltoside, dodecyl β-D-maltopyranoside; myristyl maltoside, tetradecyl β-D-maltopyranoside; cetyl maltoside, hexadecyl β-D-maltopyranoside; stearyl maltoside, octadecyl β-D-maltopyranoside; Zwittergent 3-10, 3-(*N*-decyl-*N*,*N*-dimethylammonio)-1-propanesulfonate; Zwittergent 3-12, 3-(*N*-dodecyl-*N*,*N*-dimethylammonio)-1-propanesulfonate; Zwittergent 3-14, 3-(*N*-tetradecyl-*N*,*N*-dimethylammonio)-1-propanesulfonate; Zwittergent 3-16, 3-(*N*-hexadecyl-*N*,*N*-dimethylammonio)-1-propanesulfonate; CL, cardiolipin.

0006-2960/85/0424-6298$01.50/0