Acknowledgments. We are grateful to the U.S. Public Health Service for Grant No. NIH-AM 17495, the Rutgers University Research Council, the Biomedical Research Support for financial aid, Dr. Gus Lienhard of Dartmouth Medical School for the generous gift of $CHDT^+Cl^-$, and Anheuser-Busch Brewing Co., Newark, N.J., for supplying the live brewer's yeast.

References and Notes

(1) Taken in part from the dissertation of D. J. Kuo presented in partial fulfillment of the requirements for the Ph.D. to the Rutgers University Graduate Faculty, 1977. Presented in part at the 173rd National Meeting of the American Chemical Society, New Orleans, La., March, 1977.
(2) L. O. Krampitz, *Annu. Rev. Biochem.*, **38**, 213 (1969).
(3) (a) L. O. Krampitz, I. Suzuki, and G. Greull, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **20**, 971 (1961); (b) H. Holzer and K. Beaucamp, *Biochim. Biophys. Acta*, **46**, 225 (1961); (c) G. L. Carlson and G. M. Brown, *J. Biol. Chem.*, **235**, Pc 3 (1960).
(4) S. Seltzer, G. A. Hamilton, and F. H. Westheimer, *J. Am. Chem. Soc.*, **81**, 4018 (1959).
(5) M. H. O'Leary, *Biochim. Biophys. Acta*, **235**, 14 (1970). M. H. O'Leary and J. A. Limburg, *Biochemistry*, **16**, 1129 (1977).
(6) M. H. O'Leary and R. L. Baughn, *J. Am. Chem. Soc.*, **94**, 626 (1972).
(7) M. H. O'Leary, P. T. Richards, and D. W. Hendrickson, *J. Am. Chem. Soc.*, **92**, 4435 (1970).
(8) M. I. Schimerlik, J. E. Rife, and W. W. Cleland, *Biochemistry*, 14, 5347 (1975).
(9) (a) J. Crosby, R. Stone, and G. E. Lienhard, *J. Am. Chem. Soc.*, **92**, 2891 (1970); (b) J. Crosby and G. E. Lienhard, *Ibid.*, **92**, 5707 (1970).
(10) J. Ullrich, *Methods Enzymol.* **18A**, 109 (1970).
(11) A. Schellenberger, G. Hubner, and H. Lehmann, *Angew. Chem., Int. Ed. Engl.*, 7, 886 (1968).
(12) W. W. Umbreit, R. H. Burris, and J. F. Stauffer, "Manometer Techniques", 4th ed, Burgess, Minneapolis, Minn., 1964.
(13) Operation and Maintenance Manual for Consolidated–Nier Isotope Ratio Mass Spectrometer Model 20-201, Pasadena, Calif. 1948.
(14) A. O. Nier, *Rev. Sci. Instrum.*, **18**, 398 (1947).
(15) (a) J. Bigeleisen, *Science*, **110**, 14 (1949); (b) J. Bigeleisen and T. L. Allen, *J. Chem. Phys.*, **19**, 760 (1951).
(16) G. E. Dunn, in "Isotopes in Organic Chemistry", E. Buncel and C. C. Lee, Ed., Elsevier, Amsterdam, 1977.
(17) M. H. O'Leary, *Biochem. Biophys. Res. Commun.*, **73**, 614 (1976).
(18) J. Bigeleisen and M. Wolfsberg, *Adv. Chem. Phys.*, **1**, 15 (1958).
(19) We note that the estimates of the magnitudes of secondary kinetic ^{13}C isotope effects carried out by Bigeleisen and Wolfsberg[18] are identical with those of the preequilibrium secondary isotope effect involving the free and the thiamin-bound substrate; i.e., $0.983 \leq k_1k_{-1}{}^*/k_1{}^*k_{-1} \leq 1.012$.
(20) J. P. Guthrie and F. Jordan, *J. Am. Chem. Soc.*, **94**, 9136 (1972).
(21) A similar suggestion was also made by a referee.
(22) W. Spindel, M. J. Stern, and E. U. Monse, *J. Chem. Phys.*, **52**, 2022 (1970).
(23) E. B. Wilson, J. C. Declus, and P. C. Cross, "Molecular Vibrations", McGraw-Hill, New York, N.Y., 1955.
(24) J. H. Schachtschneider and R. C. Snyder, *Spectrochim. Acta, Part A*, **19**, 117 (1963).
(25) M. Wolfsberg and M. J. Stern, *Pure. Appl. Chem.*, **8**, 225 (1964).
(26) J. Bigeleisen and M. G. Mayer, *J. Chem. Phys.*, **15**, 261 (1947).
(27) J. W. Bayles, J. Bron, and S. O. Paul, *J. Chem. Soc., Faraday Trans. 1*, **72**, 1546 (1976).
(28) D. E. Green, D. Herbert, and V. Subrahmanyan, *J. Biol. Chem.*, **138**, 327 (1941).
(29) T. P. Singer and J. Pensky, *J. Biol. Chem.*, **196**, 375 (1952).
(30) F. Jordan, D. J. Kuo, and E. U. Monse, *Anal. Biochem.*, in press.

Dependence of Cycloamylose–Substrate Binding on Charge

Raymond J. Bergeron,* Michael Almy Channing, and Kathy Ann McGovern

Contribution from the Department of Chemistry, University of Maryland, College Park, Maryland 20742. *Received July 30, 1977*

Abstract: The direction of sodium benzoate and benzoic acid penetration of the cyclohexaamylose cavity in aqueous solution is determined by a ^1H NMR study of the respective cycloamylose complexes. Chemical-shift changes in the host and guest molecules, as well as a strong intermolecular nuclear Overhauser effect, suggest that benzoic acid has a strong orientational preference for binding in the cycloamylose cavity, while sodium benzoate binding is somewhat more random. Both benzoic acid and sodium benzoate are shown to form 1:1 AB complexes with cyclohexaamylose, and the benzoic acid is shown to generate conformational changes in the cyclic oligosaccharide's glucose rings. The dependence of cycloamylose–substrate binding on charge is discussed in terms of the energy required to move a charged species from a medium of high dielectric to a medium of low dielectric. This "insertion energy" is approximated from free energy of solution studies.

In recent years, the cycloamyloses have received a great deal of attention as enzyme active-site models.[1-3] However, little of this attention has been focused on the forces responsible for cycloamylose–substrate binding. Although there have been a number of suggestions as to the nature of the complexation driving forces (release of high-energy cavity water,[3] release of ring strain,[4] and London dispersion forces[3]) there is relatively little experimental evidence available in support of any one of these concepts. Furthermore, any explanation of the binding forces must now take into consideration Breslow's remarkable discovery that substrates bind in the cycloamylose cavity in nonaqueous solvents.[5] Although we have been unable to find any support from our solution studies for either the strain energy or high-energy water concepts, we have accumulated some evidence in favor of the London dispersion forces arguments.

One fact is undeniable: both the charge on the substrate and its direction of penetration are of great importance in regulating the stability of the cycloamylose–substrate complexes formed.[6,7] The relationship between these factors was not a clear one; e.g., sodium *p*-nitrophenolate binds 13 times more tightly in the cycloamylose cavity than the neutral phenol, while just the opposite is true of benzoic acid and its anion with the carboxylate anion binding 82 times more loosely.[6,8] It seemed likely that an understanding of this apparent anomaly would help clarify the relationship between the direction of substrate penetration and charge on substrate binding.

In this paper, we report on the direction in which benzoic acid and sodium benzoate penetrate the cyclohexaamylose cavity and compare these results with our earlier findings on the sodium *p*-nitrophenolate and *p*-nitrophenol complexes. This comparison suggests the importance of "insertion energy" in substrate binding, i.e., the energy required to move the carboxylate anion from water, a medium of high dielectric, to the cycloamylose cavity, a medium of low dielectric. This is further verified by free energy of solution studies of the guest molecules in solvents whose properties approximate those of the cyclohexaamylose cavity.

Experimental Section

Materials. The cyclohexaamylose, benzoic acid, sodium benzoate, and deuterium oxide, 99.8%, were obtained from Aldrich Chemical

0002-7863/78/1500-2878$01.00/0

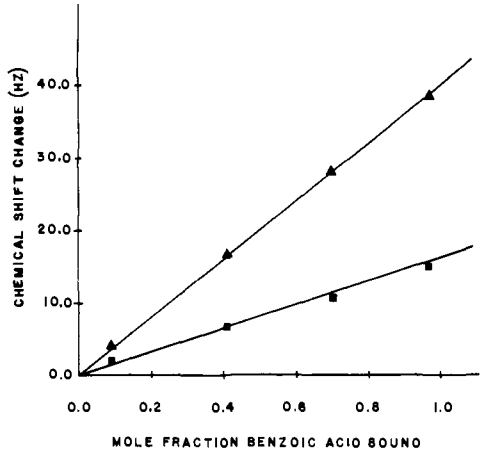

Figure 1. A plot of the change in chemical shifts of the aromatic protons of benzoic acid vs. the percent bound benzoic acid: (▲) A protons; (■) Bc protons.

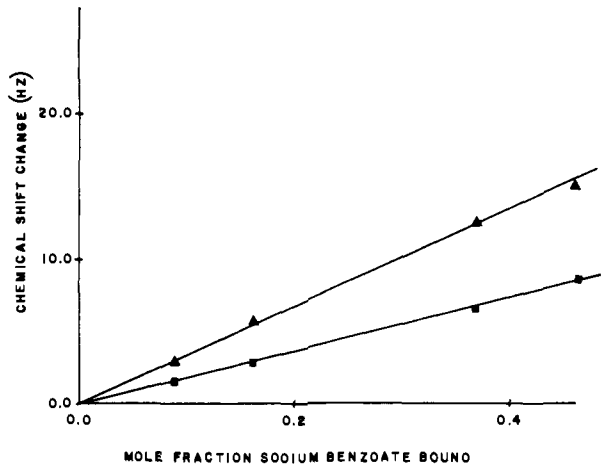

Figure 2. A plot of the change in chemical shifts of the aromatic protons of sodium benzoate vs. the percent bound sodium benzoate: (▲) A protons; (■) Bc protons.

Co. The 1,4-dioxane was obtained from J. T. Baker Chemical Co. and purified by refluxing over sodium for 6 h followed by distillation.

Sample Preparation for Nuclear Magnetic Resonance. The cyclohexaamylose hydroxyl protons were exchanged for deuterium by lyophilizing 600 mg of the carbohydrate from 40 mL of D_2O three times. This helps to minimize the HOD in the final sample. The buffer solutions were made up with anhydrous Na_3PO_4 and NaD_2PO_4 in D_2O. The pD values were 11.00 ± 0.02 and 3.00 ± 0.02, with I = 0.5 in both buffers. These pD values were obtained by adding 0.4 to the pH meter reading,[9] using a combination electrode which had been standardized with pH 10.0 ± 0.003 buffer in H_2O and then rinsed with D_2O.

Determination of Cycloamylose–Substrate Dissociation Constants by Nuclear Magnetic Resonance. ^{1}H-pulsed Fourier transform NMR spectra (100.1 MHz) were obtained on a Varian XL-100 spectrometer at 25 ± 0.5 °C. The change in chemical shift of the substrate aromatic protons was measured as a function of changing cycloamylose concentration. The spectra were referenced to an internal capillary of 0.050 M sodium acetate. The sodium benzoate and benzoic acid were made up in phosphate buffer at pD 11.00 ± 0.02 and pD 3.00 ± 0.02, I = 0.5, respectively. The concentrations of sodium benzoate and benzoic acid were held constant at 0.002 and 0.010 M, respectively, and the cyclohexaamylose concentrations were varied between 0.010–0.090 and 0.001–0.050 M, respectively. The data were treated according to a modified Hildebrand–Benesi program.[6,10]

Benzoic Acid and Sodium Benzoate Induced Chemical-Shift Changes in the ^{1}H NMR of Cyclohexaamylose. The measurements were made on a 220.02-MHz Varian NMR at 25 ± 1 °C. The cyclohexaamylose concentration was held constant at 0.005 M. The benzoic acid concentrations were varied between 0.001 and 0.011 M at pH 3.00 ± 0.02, I = 0.5, and the sodium benzoate concentrations were varied between 0.002 and 0.019 M at pH 11.00 ± 0.02, I = 0.5.

^{1}H Homonuclear Overhauser Enhancements (NOE's). ^{1}H homonuclear Overhauser enhancements (NOE's) are reported as the percentage difference in integrated intensity of the resonance being observed when the second radiofrequency (rf) was first applied at the resonance frequency to be irradiated, and then set in a vacant region of the spectrum. Peak intensities were determined by planimetry.

Benzoic Acid Induced Changes in the Cyclohexaamylose $J_{1,2}$ Coupling Constant. ^{1}H-pulsed Fourier transform NMR spectra (220.02 MHz) were obtained on a Varian super con spectrometer. The 8K spectra were obtained with a spectral width of 2564 Hz. The change in the anomeric coupling constant was measured as a function of changing benzoic acid concentration. The benzoic acid and cyclohexaamylose samples were made up in sodium phosphate buffer at pD 3.00 ± 0.02, I = 0.5. The concentration of cyclohexaamylose was held constant at 0.005 M and the benzoic acid concentrations ranged from 0.001 to 0.011 M, i.e., 0 to 84.4% bound. The coupling constant changes were plotted against the benzoic acid–cyclohexaamylose mole ratio and treated according to a linear least-squares program.

^{1}H-pulsed Fourier transform NMR spectra (100.1 MHz) were also obtained on a Varian XL-100 spectrometer for both free and 82.5% benzoic acid bound cyclohexaamylose. The two free induction decays (FID) were treated with a resolution enhancement program giving 8K spectra having spectral widths of 1024 Hz.[11]

Beer's Law Plots. All measurements were made at 24.5 °C on a Perkin-Elmer Coleman 139 spectrometer at 260 nm. For each Beer's law plot, two stock solutions were prepared and a series of dilutions was taken from each. The benzoic acid and sodium benzoate samples were made up in pH 3.00 and pH 11.00 phosphate buffers, I = 0.5, respectively. All samples in dioxane were kept out of light and used within 48 h of the dioxane's distillation.

Concentration of Solutes in Saturated Solutions. Saturated solutions were prepared by adding excess solute to the appropriate solvent and allowing the solutions to stir for 24 h. Aliquots of each sample were then filtered through Teflon millipore filters, allowed to settle for 4 h, and filtered as before. The resulting solutions were appropriately diluted and their concentrations determined from their absorbances at 260 nm.

Because of the minimal solubility of sodium benzoate in dioxane, Beer's law plots were obtained for sodium benzoate in dioxane/water solvent systems of varying mole percentages (mol % H_2O: 12.8, 19.9, 34.5, 45.5, 100). The saturated sodium benzoate concentration in each mixed solvent system was then determined as described above. To determine the solubility of the sodium benzoate in pure dioxane, a graph of mol % H_2O vs. sodium benzoate solubility was plotted and the line extended to 0 mol % H_2O.

Results

Cyclohexaamylose Induced Chemical-Shift Changes in Benzoic Acid. The dissociation constant, k_D, for the benzoic acid–cyclohexaamylose complex was measured in pD 3.00 ± 0.02 phosphate buffer, I = 0.5, at 25 ± 0.5 °C by observing changes in the chemical shift of the benzoic acid protons as a function of increasing cycloamylose–substrate ratios. The data were analyzed according to a modified Hildebrand–Benesi equation for an A + B ⇌ AB equilibrium. The result, 1.25 ± 0.13 × 10^{-3} M, was in good agreement with the literature value, 9.6 × 10^{-4} M.[3]

The magnitude of the chemical-shift changes induced in the benzoic acid substrate was largest for the ortho protons; see Figure 1.

Cyclohexaamylose Induced Chemical-Shift Changes in ^{1}H NMR of Sodium Benzoate. The sodium benzoate–cyclohexaamylose dissociation constant determined at 25 ± 0.5 °C in phosphate buffer at pD 11.00 ± 0.02, I = 0.5, 1.02 ± 0.30 × 10^{-1} M, was in good agreement with the literature value of 8.0 ± 1 × 10^{-2} M.[8] Again, the ortho protons experienced the largest chemical shifts. The ratio of ortho to meta chemical shifts is greater for the benzoic acid complex than for the sodium benzoate complex, 2.47 vs. 1.87, respectively; see Figure 2.

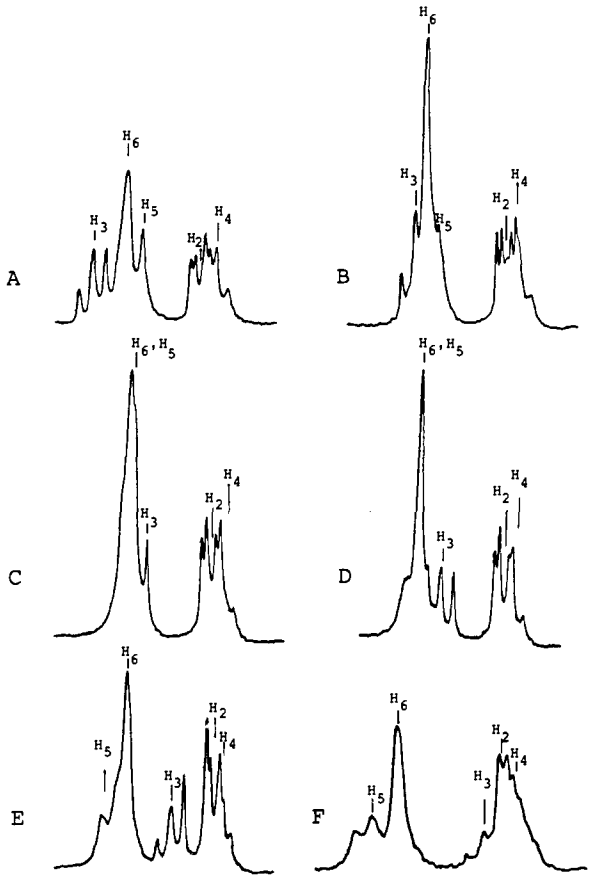

Figure 3. Spectral changes induced in cyclohexaamylose on benzoic acid complexation. The percent bound cyclohexaamylose is (A) 0.0, (B) 15.4, (C) 29.5, (D) 41.9, (E) 61.0, and (F) 82.5. Anomeric protons are not shown.

Benzoic Acid Induced Chemical-Shift Changes in the ^1H NMR of Cyclohexaamylose. The changes in the chemical shifts of the cyclohexaamylose protons, measured at 220.02 MHz, on addition of benzoic acid are substantial. The cyclohexaamylose concentration was held constant at 0.005 M and the benzoic acid concentration was varied between 0.001 and 0.011 M. The C-3 protons sustained the largest chemical-shift changes, moving about 87 Hz upfield, while C-5 protons move downfield about 23.9 Hz. The C-6 protons, however, move very little, ±1 Hz; see Figure 3.

Sodium Benzoate Induced Chemical-Shift Changes in the ^1H NMR of Cyclohexaamylose. Because of the weak binding of sodium benzoate in the cyclohexaamylose cavity, it is difficult to measure the changes in chemical shift of the cycloamylose protons under highly bound conditions. However, at 62.5% bound, the cyclohexaamylose C-3 and C-5 methine protons shift 41.8 and 1.0 Hz upfield, respectively. The shifts in the C-3 and C-5 methine protons for the corresponding benzoic acid–cyclohexaamylose complex, with the cyclohexaamylose 61.0% bound, are 57.1 Hz upfield and 18.6 Hz downfield, respectively.

Intermolecular Nuclear Overhauser Effects in the Cyclohexaamylose–Benzoic Acid Complexes. A ^1H homonuclear Overhauser experiment was performed on the cyclohexaamylose–benzoic acid complex, 0.010 M benzoic acid and 0.050 M cyclohexaamylose, i.e., 19.4% cyclohexaamylose and 97% benzoic acid bound, respectively. The ortho protons experienced a 31% enhancement and the meta protons a 2% enhancement.

Intermolecular Nuclear Overhauser Effects in the Cyclohexaamylose Sodium Benzoate Complexes. Because the substrate must be highly bound to observe an intermolecular NOE, owing to the weak binding of sodium benzoate in the cyclohexaamylose cavity, we have been unable to measure an NOE with any precision.

Free Energies of Solution of Benzoic Acid in *p*-Dioxane and in Phosphate Buffer. The concentration of benzoic acid in a saturated *p*-dioxane solution at 24.5 °C is 3.07 M and in saturated pH 3.00, $I = 0.5$ phosphate buffer solution at 24.5 °C it is 1.80×10^{-2} M. Because the free energy of the solid benzoic acid is equal to zero, the free energies of solution can be determined from the approximation $\Delta G = RT \ln C$. They are −0.663 and +2.37 kcal/mol, respectively.

Free Energies of Solution of Sodium Benzoate in *p*-Dioxane and in Phosphate Buffer. The concentration of sodium benzoate in *p*-dioxane was approximated graphically by plotting the concentration of sodium benzoate in solvent systems consisting of varying molar ratios of dioxane/water and extending the line to 0 mol % water. Each of the different solutions was, of course, saturated with sodium benzoate. The solubility of sodium benzoate in *p*-dioxane at 24.5 °C is 2.13×10^{-4} M. In pH 11.00, $I = 0.5$ phosphate buffer at 24.5 °C, the sodium benzoate is soluble to the extent of 3.41 M. The respective free energies of solution are +5.00 and −0.725 kcal/mol.

Discussion

Benzoic Acid–Cyclohexaamylose Complex Formation. It is clear from the changes in the chemical shifts of both the benzoic acid guest and the cyclohexaamylose host on varying the host to guest ratio and from the rates of formation and dissociation of similar cyclohexaamylose complexes[12] that the cyclohexaamylose–benzoic acid system is in the NMR chemical-shift fast exchange limit.[13] This means that the benzoic acid and cyclohexaamylose resonances appear at the average of their free and bound forms, weighted by the fractional population of the molecules in each environment.

The protons inside of the cyclohexaamylose cavity, the C-3 and C-5 methine protons, experience the largest changes in chemical shift on benzoic acid complexation (Figure 1). Furthermore, the dissociation constant, K_D, for the benzoic acid–cyclohexaamylose complex, determined by observing the changes in the chemical shift of the benzoic acid protons as a function of changing the cycloamylose to benzoic acid ratios ($1.25 \pm 0.13 \times 10^{-3}$ M), is in good agreement with the K_D determined by observing the changes in the chemical shift of the cycloamylose C-3 and C-5 methine protons (4.3×10^{-3} M).

When the cyclohexaamylose is 83% bound by benzoic acid, the C-3 methine protons move upfield approximately 87 Hz, while the C-5 methine protons move downfield 24 Hz. However, the chemical shift of the C-1 anomeric protons and the C-6 methylenes hardly change at all. The shielding of the cycloamylose's C-3 methine protons is likely a result of their being within the magnetic field of the benzoic acid's aromatic π cloud; however, the mechanism for the C-5 methine deshielding is not nearly as obvious. This C-5 methine deshielding has also been observed for the *p*-iodoaniline and sodium 2,6-dimethyl-4-nitrophenolate complexes of cyclohexaamylose.[6,14] There are a number of possible mechanisms which could explain this: diamagnetic anisotropy of particular bonds or regions of the host,[15] van der Waals shifts,[16] or steric perturbation.[17] However, because of the diverse electronic nature of the guest molecules causing the deshielding, it seems likely that either the van der Waals shifts or steric perturbation, not diamagnetic anisotropic shielding, is or are responsible for the shifting.

The fact that the C-6 methylenes are not being shifted at all confirms two structural features about the complex: first, that the C-6 methylenes do not lie within the deshielding magnetic field of the benzoic acid's aromatic π cloud, and, secondly, that they are too far from the carboxyl group to experience van der

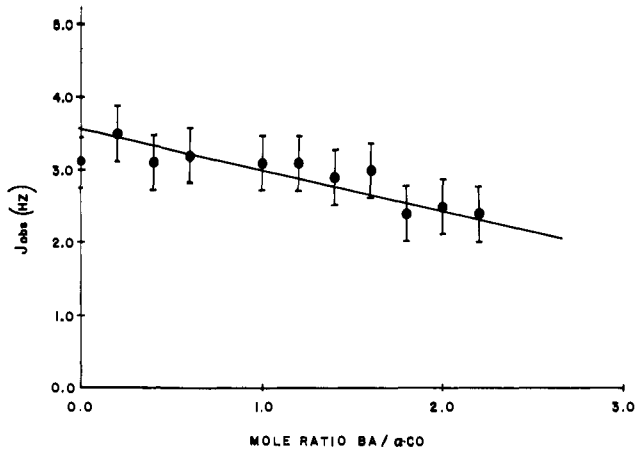

Figure 4. A plot of the changes in the cyclohexaamylose's coupling constants vs. the molar ratio of benzoic acid to cyclodextrin.

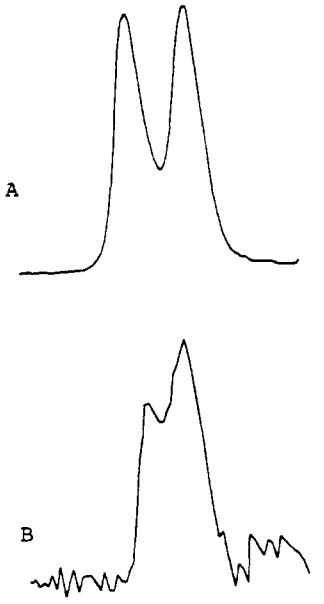

Figure 5. Resolution-enhanced 100.1-MHz ^{1}H Fourier transform NMR spectra of the anomeric doublet of (A) free and (B) 82% bound cyclohexaamylose with benzoic acid.

Waals or steric perturbation induced shifts. In a recent study Lehn has shown that there is considerable freedom of movement about the cyclohexaamylose C-5–C-6 bond.[18] Therefore, the absence of any change in the chemical shift of the C-6 methylenes cannot be attributed to the protons being held "pointing away" from the substrate.

The most notable feature of the changes in the ^{1}H NMR spectra of benzoic acid on cycloamylose complexation is in the relative magnitudes of the changes in the ortho and meta protons. The ortho protons which penetrate the cavity sustain about 2.5 times the change in chemical shift as the meta protons (Figure 1). We observed a similar phenomenon for the changes in the chemical shifts of the meta and ortho protons of sodium *p*-nitrophenolate on cyclohexaamylose complexation; i.e., the penetrating meta protons experience about 2.8 times the change in chemical shift as the ortho protons. The downfield ^{1}H magnetic resonance shifts of the substrate could, as with the cycloamylose's C-5 methine proton downfield shifts, be explained by several physical mechanisms: diamagnetic anisotropy of particular bonds or regions of the host,[15] van der Waals shifts,[16] or steric perturbation.[17] However, owing to the limited amount of experimental data available, assignment of a particular mechanism is not yet possible.

Based on changes in ^{1}H chemical shifts of the host and guest molecules and on an intermolecular nuclear Overhauser effect, we were able to establish conclusively that the sodium *p*-nitrophenolate is sitting in the cyclohexaamylose cavity nitro end first at the 2,3-hydroxyl side. In light of these findings, our observation that the benzoic acid ortho protons sustained the largest change in chemical shift on cyclohexaamylose complexation suggested it might be penetrating the cavity carboxyl group first. Furthermore, this idea was borne out by the intermolecular nuclear Overhauser experiment, in which the ortho protons' area was enhanced by 31% while the meta-para proton multiplet remained effectively unchanged.

Cycloamylose Conformational Changes on Benzoic Acid Complexation. In addition to the changes in chemical shifts of the cycloamylose protons on benzoic acid complexation, there are also changes in the cycloamylose's coupling constants indicating that a conformational change in the cyclohexaamylose is occurring on benzoic acid complexation. Because of the complexity of the cyclohexaamylose's ^{1}H NMR spectra, we were able to accurately measure only the change in the coupling of the anomeric C-1 proton to the C-2 proton, about 0.7 Hz. That the coupling constant change was in fact real was verified in two ways. The change was observed as a function of varying substrate-cycloamylose ratios employing normal FT NMR data analysis (Figure 4). In addition, a special resolution enhancement FT NMR analysis was employed (Figure 5).

Sodium Benzoate–Cyclohexaamylose Complexation. Noggle and Schirmer[19] have presented the theory describing intermolecular NOE's in rapidly exchanging systems. Furthermore, the specific case of observing the resonances of a small molecule exchanging between an environment in which it is bound to the macromolecule and an environment in which it is free in solution, while saturating the macromolecule's resonances, has been considered by Balaram et al.[20] Although quantitative interpretation of intermolecular NOE's can be complicated, things are simplified since the chemical-shift fast-exchange approximation holds for the present system.[19] The magnitude of the observed NOE is dependent on the extent to which the nucleus whose resonance is being observed is relaxed by the nucleus being saturated. This then means that the lifetime of the species being observed, i.e., benzoic acid or sodium benzoate in its bound environment, must be long enough for significant intermolecular relaxation to occur. More importantly, the mole fraction of this observed species must be large enough so that the NOE intensity changes will be seen in the averaged, free plus bound spectrum. Consequently, we have been unable to observe intermolecular nuclear Overhauser effects with any precision simply because it is difficult to get either species in a highly enough bound state. However, the changes in the chemical shifts of both the cycloamylose and sodium benzoate protons do provide substantial evidence regarding the nature of substrate penetration.

The sodium benzoate–cyclohexaamylose complex is substantially less stable than the corresponding benzoic acid complex. This is reflected in the magnitude of the chemical-shift changes generated in the host and guest molecules on complexation. However, the dissociation constant determined by measuring the changes in the sodium benzoate's ^{1}H chemical shifts ($1.02 \pm 0.3 \times 10^{-1}$ M) is in good agreement with the literature value ($8.1 \pm 1.0 \times 10^{-2}$ M).[8] Although the direction of the changes in chemical shift of the guest molecules is the same for the cycloamylose–sodium benzoate and cycloamylose–benzoic acid complexes, there are some differences in the magnitude of the changes in chemical shifts. As with benzoic acid, the sodium benzoate's ortho protons are shifting more than the meta protons. However, the ratio of ortho to meta proton shifts (calculated from the Q value)[6] for the fully

bound substrates is greater for the benzoic acid complex, 2.56 vs. 1.41. This can be interpreted as a more random penetration of the cyclohexaamylose cavity by the sodium benzoate. Penetration of the cavity by the carboxylate anion is likely to be somewhat less favorable than penetration by the carboxyl group because of the ion's solvation requirements. There are also differences in the changes induced in the cycloamylose ^1H NMR spectrum by benzoic acid and by sodium benzoate for effectively the same percent bound cycloamylose. When the cycloamylose is 63% bound by sodium benzoate or 61% bound by benzoic acid, the C-3 methine protons move upfield, 41.8 and 57.1 Hz, respectively. However, in the sodium benzoate–cycloamylose complex, the C-5 protons move only 1 Hz while in the corresponding benzoic acid complex, they move downfield 23.9 Hz. Although the carboxylate anion is likely to generate a different shift in the C-5 methine protons than the corresponding neutral carboxyl group, it is difficult to explain why the C-5 methines only move 1 Hz. However, we observed a similar phenomenon in the sodium *p*-nitrophenolate–cyclohexaamylose complexes.[6] Finally, we did not observe any change in the C-6 methylene protons.

Again, for technical reasons attributable to the weak binding of sodium benzoate in the cyclohexaamylose cavity, we were unable to observe the cyclohexaamylose in a highly bound state, i.e., no more than 70% bound. Consequently, we were unable to accurately measure a change in the cycloamylose's $J_{1,2}$ coupling constant.

Benzoic Acid– vs. Sodium Benzoate–Cyclohexaamylose Binding. The NMR evidence clearly supports the idea that both benzoic acid and sodium benzoate penetrate the cavity at the 2,3-hydroxyl side, carboxyl group first, although the sodium benzoate penetration is more random. In light of this finding, the difference in dissociation constants between the cyclohexaamylose–benzoic acid and the cyclohexaamylose–sodium benzoate complexes is understandable. The energy required to take a charged species from a medium of high dielectric and insert it into a medium of lower dielectric might well make up the difference in binding energy between the neutral and charged substrates. In a study on the effect of various solvent systems on the visible spectra of cycloamylose–substrates, Bender showed that the environment experienced by the substrate bound in the cycloamylose cavity could be approximated by *p*-dioxane.[8] In keeping with this observation, we felt that the difference in the free energies of solution of sodium benzoate in pH 11.00 phosphate buffer, ΔG_1, and in dioxane, ΔG_2, as compared with the difference in the free energies of solution of benzoic acid in pH 3.00 phosphate buffer, ΔG_3, and in dioxane, ΔG_4, Table I, should reflect the difference in sodium benzoate- and benzoic acid–cyclohexaamylose binding constants. These numbers would then provide some idea of the magnitude of the insertion energy described above. The results suggest that movement of benzoic acid out of pH 3.00 phosphate buffer into *p*-dioxane, a cyclodextrin-like environment, is favored by $\Delta G_4 - \Delta G_3 = -3.03$ kcal/mol (ΔG_5), a free energy very close to the free energy of formation for the cyclohexaamylose–benzoic acid complex, −3.5 kcal/mol. However, movement of sodium benzoate out of the phosphate buffer solution into dioxane is an unfavorable process by $\Delta G_2 - \Delta G_1 = +5.73$ kcal/mol (ΔG_6). This, of course, means that the insertion energy is greater for the charged species, sodium benzoate, by 8.73 kcal. This difference, although much larger than the difference in the free energies of formation for the cyclohexaamylose–benzoic acid and cyclohexaamylose–sodium benzoate complexes, 2.60 kcal/mol, can be attributed to the fact that the sodium benzoate would not be completely stripped of its water of solvation when in the cyclohexaamylose cavity, no matter how it penetrates the cavity. If the sodium benzoate is sitting in the cavity at the 2,3-hydroxyl side carboxylate first, water molecules could still partially solvate the carboxylate anion and the sodium cation at the 6-hydroxyl side of the cavity, thus lowering the "insertion energy". Alternatively, if the sodium benzoate is oriented such that only the aromatic ring is penetrating the cycloamylose cavity, solvation of the anion and cation would not be substantially different than solvation of the ions in bulk solvent.

Table I. Free Energies of Solution for Benzoic Acid and Sodium Benzoate in Various Solvents at 24.5 °C

ΔG_n	ΔG, kal/mol	Solute	Solvent	pH
ΔG_1	−0.725	$C_6H_5CO_2Na$	H_2O, $I = 0.5$	11.00
ΔG_2	+5.00	$C_6H_5CO_2Na$	$C_4H_8O_2$	
ΔG_3	+2.37	$C_6H_5CO_2H$	H_2O, $I = 0.5$	3.00
ΔG_4	−0.663	$C_6H_5CO_2H$	$C_4H_8O_2$	

Finally, we would like to point out that an x-ray study published at the submission of this article on benzoic acid– and *p*-nitrophenol–cyclohexaamylose complexes supports our suggestion for cycloamylose–substrate disposition.[21]

Conclusion

Prior to this work, the fact that sodium *p*-nitrophenolate binds in the cyclohexaamylose cavity 13 times more effectively than *p*-nitrophenol,[22] while sodium benzoate binds in the cavity 82 times less effectively than benzoic acid, seemed somewhat curious.

Our earlier studies clearly showed that both sodium *p*-nitrophenolate and *p*-nitrophenol related substrates penetrated the cyclohexaamylose cavity nitro end first, at the 2,3-hydroxyl side with the hydroxyl or the hydroxyl oxanion pointing out into solution. With the substrate in this orientation, i.e., with the oxanion pointing out into solution and thus hydrated, it is understandable that, if cycloamylose–substrate binding is controlled by an induced dipole–dipole mechanism, the phenolate should bind more tightly in the cavity than the neutral phenol. However, if as in the sodium benzoate–cyclohexaamylose complex, the carboxylate anion is in the cycloamylose cavity, out of solution, and partially desolvated, or forced to adopt an orientation which reduces host–guest contact, i.e., with the carboxylate anion pointing out of the cavity, other factors must be considered regarding the stability of the neutral vs. charged cycloamylose–substrate complexes. The energy required in transferring a charged species such as the sodium benzoate from a medium of high dielectric to a medium of low dielectric seems to be a major factor controlling the stability of the complexes.

Acknowledgments. Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, and to the Alfred P. Sloan Foundation for support of this research.

References and Notes

(1) F. Cramer and H. Hettler, *Naturwissenschaften*, **54**, 625 (1967).
(2) D. W. Griffiths and M. L. Bender, *J. Am. Chem. Soc.*, **95**, 1679 (1973).
(3) D. W. Griffiths and M. L. Bender, *Adv. Catal.*, **23**, 209 (1973).
(4) P. C. Manor and W. Saenger, *J. Am. Chem. Soc.*, **96**, 3690 (1974).
(5) B. Siegel and R. Breslow, *J. Am. Chem. Soc.*, **97**, 6869 (1975).
(6) R. J. Bergeron, M. A. Channing, G. J. Gibelly, and D. M. Pillor, *J. Am. Chem. Soc.*, **99**, 5146 (1977).
(7) R. J. Bergeron and R. Rowan, *Bioorg. Chem.*, **4**, 290 (1976).
(8) R. L. Van Etten, J. F. Sebastian, G. A. Clowes, and M. L. Bender, *J. Am. Chem. Soc.*, **89**, 3242 (1967).
(9) P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, **64**, 188 (1960).
(10) H. A. Benesi and J. H. Hildebrand, *J. Am. Chem. Soc.*, **71**, 2703 (1949).
(11) I. D. Campbell, C. H. Dobson, R. J. P. Williams, and A. V. Xavier, *J. Magn. Reson.*, **11**, 172 (1973).
(12) F. Cramer, W. Saenger, and H. Spatz, *J. Am. Chem. Soc.*, **89**, 14 (1967).
(13) B. D. Sykes and M. D. Scott, *Annu. Rev. Biophys. Bioeng.*, **1**, 27 (1972).
(14) D. J. Wood, F. E. Hruska, and W. Saenger, *J. Am. Chem. Soc.*, **99**, 1735 (1977).

(15) J. W. Apsim, W. G. Craig, P. V. DeMarco, D. W. Mathleson, L. Saunders, and W. B. Whalley, *Tetrahedron*, **23**, 2339 (1967).
(16) B. B. Howard, B. Linder, and M. T. Emerson, *J. Chem. Phys.*, **36**, 485 (1962).
(17) B. V. Cheney and D. M. Grant, *J. Am. Chem. Soc.*, **89**, 5319 (1967).
(18) J. P. Behr and J. M. Lehn, *J. Am. Chem. Soc.*, **98**, 1743 (1976).
(19) J. H. Noggle and R. E. Schirmer, "The Nuclear Overhauser Effect", Academic Press, New York, N.Y., 1971.
(20) P. Balaram, A. A. Bothner-By, and E. Breslow, *Biochemistry*, **12**, 4695 (1973).
(21) K. Harata, *Bull. Chem. Soc. Jpn.*, **50**, 1416–1424 (1977).
(22) We wish to point out that we previously and unintentionally published the K_D for the *p*-nitrophenol–cyclohexaamylose complex as 5.3×10^{-2} M. This error escaped our attention. The number should read 5.23×10^{-3} M and therefore sodium *p*-nitrophenolate binds 13.0 and not 130 times more tightly in the cyclohexaamylose cavity than *p*-nitrophenol does.

Bifunctional Substrates of Erythrocyte Carbonic Anhydrase. Enzyme-Catalyzed Hydration and Hydrolysis of Pyruvate Esters

Y. Pocker,* J. E. Meany,[1a] Brian C. Davis,[1b] James Arrigoni,[1c] and John E. Stein

Contribution from the Department of Chemistry, University of Washington, Seattle, Washington 98195. *Received September 17, 1976*

Abstract: The present investigation shows that carbonic anhydrase from bovine erythrocytes catalyzes both the *hydration* and the *hydrolysis* of pyruvate esters. Both enzymatic processes exhibit sigmoidal pH–rate profiles with a point of inflection around neutrality. Both obey Michaelis–Menten kinetics. For a given ester, under similar experimental conditions, turnover numbers for the enzymatic hydration are much larger than those for the corresponding hydrolysis. However, it was observed that the values of K_m for hydrolysis were significantly smaller than those for hydration. Both reactions of the bifunctional substrates appear to be strongly inhibited by acetazolamide. The experimental inhibition constants, however, differ widely: K_i(hydrolysis) $< 3 \times 10^{-7}$ M vs. K_i(hydration) $= 2 \times 10^{-5}$ M. It is suggested that the dual function of bovine carbonic anhydrase on one and the same substrate may involve somewhat different modes of binding and hence different courses of enzyme action despite a number of kinetic similarities between enzymatic hydration and hydrolysis.

Erythrocyte carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) possesses wide catalytic versatility both in terms of its binding capacity and turnover efficiency. The enzyme acts both as a hydrase[2a-d] and an esterase.[3] However, never before has a single substrate served to demonstrate both types of activity.

Alkyl pyruvate esters possess a carbonyl which undergoes hydration (reaction 1) and an adjacent alkyl carboxylate group which undergoes hydrolysis (reaction 2).[4]

$$CH_3COCO_2R + H_2O \rightleftharpoons CH_3C(OH)_2CO_2R \quad (1)$$

$$\begin{bmatrix} CH_3COCO_2R \\ H_2O \uparrow\downarrow -H_2O \\ CH_3C(OH)_2CO_2R \end{bmatrix} + H_2O \longrightarrow \begin{bmatrix} CH_3COCO_2^- \\ H_2O \uparrow\downarrow -H_2O \\ CH_3C(OH)_2CO_2^- \end{bmatrix} + H^+ + ROH \quad (2)$$

In our earlier work, we have compared the catalytic properties of bovine carbonic anhydrase (BCA) as a hydrase[2b] with its catalytic properties as an esterase.[3] The present work shows that BCA catalyzes not only the hydration (reaction 1), but also the hydrolysis (reaction 2) of methyl and ethyl pyruvate. These bifunctional substrates offer a unique opportunity to investigate the enzymatically catalyzed consecutive reactions. Thus, a common substrate allows the direct comparison of kinetic parameters associated with the known hydrase and esterase activities of the enzyme in the presence and absence of certain inhibitors. A detailed knowledge of the kinetic behavior of pyruvate systems is made even more important by the use of some related compounds as enzyme modification agents, e.g., bromopyruvate, and by the very special properties of the N^{τ}-carboxyketoethylated histidine residue in the enzyme.[5,6]

For reactions 1 and 2, the experimental techniques necessary to obtain accurate rate data are far simpler than those involved in similar studies pertaining to the reversible hydration of carbon dioxide. Furthermore, the solubilities of the low molecular weight alkyl pyruvates in water are such that conveniently measured amounts of substrate can be added to buffered enzyme solutions to obtain accurate Michaelis parameters.

Experimental Section

Lyophilized BCA was obtained from Mann Research Laboratories. The methods of purification[6] and standardization[7] of the enzyme solutions were described in earlier publications. The substrates (Aldrich Chemical Co.) were distilled through a Vigreux column: bp (methyl pyruvate) 43 °C (19 Torr); bp (ethyl pyruvate) 54 °C (19 Torr). Buffer solutions were prepared in deionized water from reagent grade buffer components. The ionic strength of all solutions was adjusted by adding the appropriate quantities of sodium sulfate. Acetazolamide was obtained from American Cyanamid (Lederle Laboratory Division). The instruments employed for spectrophotometric and pH measurements and for temperature control were described in an earlier publication.[4]

The reactions were initiated by adding the appropriate volumes of the pyruvate esters to 3 mL of the reaction solution by means of a calibrated Hamilton syringe. The initial equilibration between the pyruvate esters and their respective hydrates (eq 1) results in a relatively rapid diminution of absorbancy ($\tau_{1/2} < 2.0$ s at 25.0 °C) at 340 nm. The pyruvate ion formed in the much slower subsequent hydrolysis step has a considerably lower fraction of hydration, $\chi^{25\,°C}_{\text{pyruv. ion}} = 0.06$,[8] than that of the pyruvate esters, $\chi^{25\,°C}_{MP} = 0.74$,[4] $\chi^{25\,°C}_{EP} = 0.70$.[4] Thus, the hydrolyses of the pyruvate esters were monitored spectrophotometrically at 340 nm by the subsequent increase in absorbancy which occurs with the formation of the less hydrated pyruvate ion.[4,8] The observed velocities (in M min^{-1}), $v_{obsd} = v_{buffer} + v_{enz}$, were calculated by dividing the initial increasing slopes associated with the hydrolysis by the difference in extinction coefficients between the pyruvate ion and the ester under consideration: $\Delta\epsilon^{MP}_{340nm} = 16.1$ M^{-1} cm^{-1}, $\Delta\epsilon^{EP}_{340nm} = 15.5$ M^{-1} cm^{-1}. The initial slopes of increasing

0002-7863/78/1500-2883$01.00/0