Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 4268–4272, June 1986
Biophysics

Role of conformational changes in the elution of proteins from reversed-phase HPLC columns

(surfaces/protein conformation/photoacoustic/infrared/fluorescence)

GIL E. KATZENSTEIN, SUSAN A. VRONA, REBECCA J. WECHSLER, BRYAN L. STEADMAN, RANDOLPH V. LEWIS, AND C. RUSSELL MIDDAUGH†

Department of Biochemistry, University of Wyoming, Box 3944, University Station, Laramie, WY 82071

Communicated by Henry Lardy, January 27, 1986

ABSTRACT **To test the hypothesis that conformational alterations might be involved in the elution of proteins from reversed-phase HPLC columns, the conformations of proteins bound onto a C-8 alkyl-bonded silica surface have been examined in the presence of increasing concentrations of the commonly employed eluent, 1-propanol. Using a combination of photoacoustic, diffuse reflectance deconvolution Fourier transform infrared and front face fluorescence spectroscopic techniques (to minimize interference from light scattering), the existence of surface-associated protein conformational changes induced by propanol is unequivocally demonstrated. The linear relationship found between the amount of propanol needed to elute proteins from C-8 columns and the midpoint of spectrally observed structural transitions is consistent with a role for conformational changes in the elution process.**

The now widespread use of reversed-phase high-performance liquid chromatography (RP-HPLC) to resolve mixtures of proteins makes it desirable to understand the physical basis of protein retention and elution. In the case of small polypeptides, binding and elution appear to be primarily controlled by bulk polarity (1). In contrast, the presence of secondary and tertiary structure in proteins is at least partially responsible for their much more complex and, consequently, less predictable elution behavior (2).

We have recently demonstrated, employing a variety of spectroscopic and calorimetric techniques, that the typical reversed-phase eluent 1-propanol induces significant conformational changes in many proteins in solution in the range of organic modifier concentrations where elution is induced (3). This suggests the possibility that such conformational changes may play a role in reducing the affinity of proteins for RP-HPLC bonded phases. There was not found, however, any apparent relationship between the amount of 1-propanol needed to produce these conformational effects and the order of elution of the proteins examined. This is not surprising since any conformational changes involved in the elution process presumably occur on the surface of the chromatographic matrix rather than in solution.

Therefore, to further test the hypothesis of a causal connection between eluent-induced protein conformational phenomena and chromatographic behavior, we have explored the possibility of similar conformational changes in proteins bound to a typical reversed-phase surface of octyl-silica. Spectral studies of this type are significantly complicated by the intense light scattering produced by the presence of the particulate C-8 silica beads. To overcome this problem we have employed a combination of photoacoustic (PAS) (4), diffuse reflectance deconvolution Fourier transform infrared (FTIR) (5, 6) and front face (FF) fluorescence (7) spectroscopic techniques that minimize potential light scattering artifacts.

MATERIALS AND METHODS

Proteins (Sigma), end-capped octyl-silica gel (Baker), 1-propanol (double-distilled), and buffer conditions (0.1 M potassium phosphate, pH 4.0) were identical to those employed in the previous solution spectral and chromatographic studies on which this present work is based (3).

Proteins were bound to octyl-silica by incubation with C-8 beads at 25°C at a weight ratio of 1:20 (protein:bead) in 0.1 M KH_2PO_4 buffer containing 10% 1-propanol at pH 4.0 for 1 hr. The protein-bead complex was then washed two or three times with the same buffer by centrifugation (40,000 × *g*) to remove unbound protein. Because the hydrophobic C-8 silica beads do not disperse well in aqueous buffer, 0% 1-propanol spectra were obtained by loading the beads in 10% 1-propanol (at which concentration protein elution is not yet evident) and subsequent removal of the propanol by centrifugation. Appropriate amounts of 1-propanol in buffer were then introduced and a second 1-hr incubation was performed at 25°C. Finally, the 1-propanol-equilibrated protein-bead complexes were washed in propanol-containing buffer three times by centrifugation and spectra were obtained as described below. After spectroscopic examination, samples were removed from detection cells and any free protein that appeared during spectral measurements was separated from bead-associated material by centrifugation. Spectra of free protein and the remaining bead-associated protein were then obtained. The spectra of bound protein obtained in the latter step were compared to spectra of bound protein derived subtractively by computer from spectra measured in the first step (1-propanol present) minus the free protein spectra. These two spectra of bound protein were found to be identical within experimental error.

Fluorescence spectra were obtained with an SLM4000 fluorometer interfaced to an HP85 microcomputer for subtractive analysis. All proteins were excited at 290 nm (primarily tryptophan emission) with the exception of ribonuclease, which lacks tryptophan, where excitation at 275 nm was employed to produce tyrosine emission. FF geometry employing an angle of 60° was used to permit sufficient light to reach the photomultiplier for measurement purposes (7). Corrections for Raman and Raleigh scattering were made subtractively as described above. Twenty percent (wt/vol) sucrose was included in all solutions to increase solvent density to keep the protein-bead complexes suspended for the time course of the spectral measurements. The presence of sucrose did not detectably perturb the protein-bead binding equilibrium under the indicated experimental conditions.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: RP-HPLC, reversed-phase HPLC; PAS, photoacoustic; FTIR, Fourier transform infrared; FF, front face.

†To whom all correspondence should be addressed.

Visible photoacoustic spectra of protein-bead slurries were obtained with an EDT Research OAS-4000 PAS spectrometer as described (8). To minimize drying during analysis, spectra were obtained at the rapid scanning rate of 50 nm/min in small intervals of 50 nm with periodic removal of the sample from the exciting beam.

FTIR spectroscopy was performed with an Analect 6260 instrument in the diffuse reflectance mode. A resolution of 2 cm^{-1} was employed with a Hg/Cd/Te detector. To permit the amide I band (1600–1700 cm^{-1}) to be directly observed, spectra were obtained in ^2H_2O throughout. Proteins were exchanged into ^2H_2O for 48 hr at room temperature ($p^2H \approx 5$). As in the PAS studies, the surface of a gelatinous pellet was examined and drying was again minimized, in this case by obtaining only a 128-scan interferogram. A number of such spectra were obtained and averaged until signal-to-noise ratios were sufficient for partial deconvolution (5, 6). All spectra were obtained relative to a C-8 silica background under solution conditions (temperature, p^2H, propanol, buffer) identical to those employed in obtaining sample spectra. The resultant spectra were normalized by the Kubelka–Munk method and partial deconvolution was performed by the method of Kauppinen *et al.* (9) employing values of $K = 2.5$ and $\sigma = 6.5$.

RESULTS

Seven representative proteins were examined in this study based on the previously described criteria of known crystal structure, RP-HPLC behavior, and spectroscopic properties (3). Each of the proteins shows evidence of a relatively abrupt red shift in front surface intrinsic fluorescence emission maxima at specific 1-propanol concentrations on the C-8 surface (Fig. 1), although the magnitude and shape of these spectral transitions are somewhat variable. Exchange of bead-bound protein into buffer solution without 1-propanol causes a >90% return of fluorescence maxima to their original positions. In general, the fluorescence transitions of these proteins bound to C-8 silica occur at somewhat higher (3–5%) 1-propanol concentrations than solution protein, although the significance of this observation is unclear since the actual surface concentration of eluent is unknown. Furthermore, in the absence and presence of the organic eluent, the fluorescence spectra of the bound protein are generally blue-shifted 5–20 nm when compared to the same proteins in solution (solution data in ref. 3).

To confirm the presence of these 1-propanol-induced conformational changes on the RP-HPLC matrix surface, concentrated slurries of certain bead-bound proteins were also examined by using UV/visible PAS spectra (4). Only the three proteins containing the heme chromophore could be examined by this method, but clear evidence for transitions similar to those observed in the fluorescence experiments are illustrated in Fig. 2. In this case, intensities of the porphyrin Soret band (400 nm) rather than peak positions are plotted because of the relatively small shifts (1–2 nm) in this absorption peak. The small shifts in the Soret peak are to higher wavelength in contrast to the much more substantial blue shifts seen in dilute solution (3). All PAS intensities are shown relative to the invariant 280-nm aromatic protein peak to correct for the decreasing amount of bound protein at the higher 1-propanol concentrations.

PAS data are also shown for the same three proteins in solution at the high protein concentration of 100 mg/ml (Fig. 2). This permits a more direct comparison between the high surface protein concentrations and proteins in a concentrated solution state. Again, similar propanol-induced conformational changes are seen, although they are noticeably shifted relative to the bound protein. Interestingly, the propanol-induced spectral shifts are now blue-shifted (not illustrated)

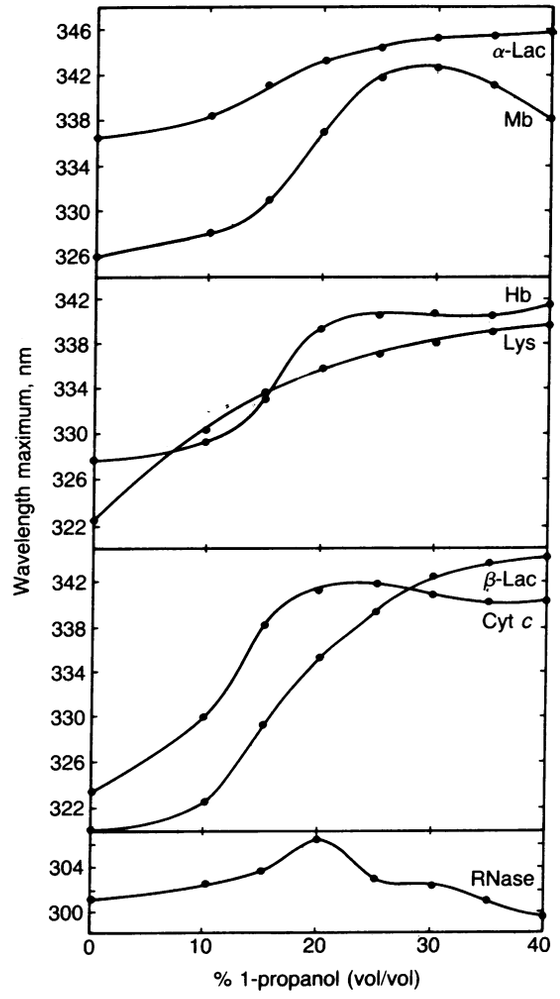

FIG. 1. Effect of 1-propanol on the intrinsic fluorescence maximum of proteins adsorbed on the surface of octyl-silica. Mb, equine skeletal muscle myoglobin; Hb, bovine hemoglobin; Cyt *c*, equine heart cytochrome *c*; Lys, chicken egg white lysozyme; RNase, bovine pancreas ribonuclease A; α-Lac, bovine milk α-lactalbumin; β-Lac, bovine milk β-lactoglobulin.

as observed for these proteins in solution, although the magnitude of these shifts (3–5 nm) is less than that observed in a more dilute state (3). The spectral changes of the bound and solubilized heme proteins are again reversed by removal of 1-propanol (>90%).

To further characterize the nature of the protein 1-propanol-induced surface transitions, bound proteins were examined in the amide I region of the mid-infrared by the method of diffuse reflectance FTIR and then partially deconvoluted to enhance resolution (5, 6, 9). As illustrated for four proteins in Fig. 3, substantial changes in the deconvoluted spectra are evident with each protein as the 1-propanol concentration is increased. With the exception of myoglobin, the proteins show a broadening and general loss of defined peaks at higher cosolvent concentrations. Spectra of surface-bound protein at 0% and 10% propanol were very similar and therefore only the 10% results are shown. For purposes of comparison, solution spectra of the proteins in concentrated solution (100 mg/ml) are shown as the 0% spectra. The binding of the proteins to the C-8 silica surface have clearly produced a loss of spectral resolution, although with at least three of the proteins (ribonuclease, hemoglobin, and myoglobin) the same deconvoluted peaks are evident in the solution and 10% 1-propanol spectra. It seems highly probable that much of the loss of resolution is due to the

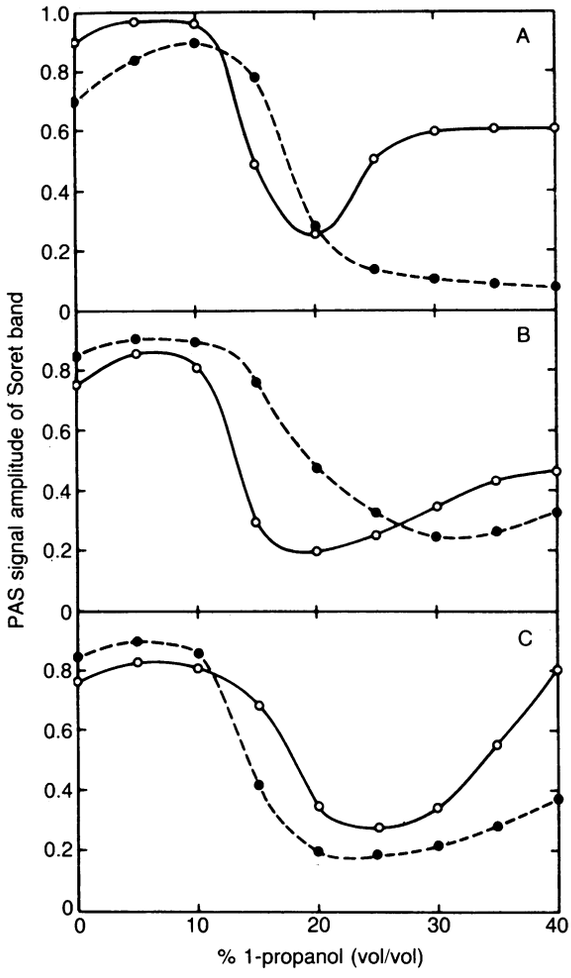

FIG. 2. Effect of 1-propanol on the PAS intensity of the Soret band (400–410 nm) of three heme proteins adsorbed onto a C-8 silica surface (●----●) and in solution at a concentration of 100 mg/ml (○—○). (*A*) Myoglobin. (*B*) Hemoglobin. (*C*) Cytochrome *c*.

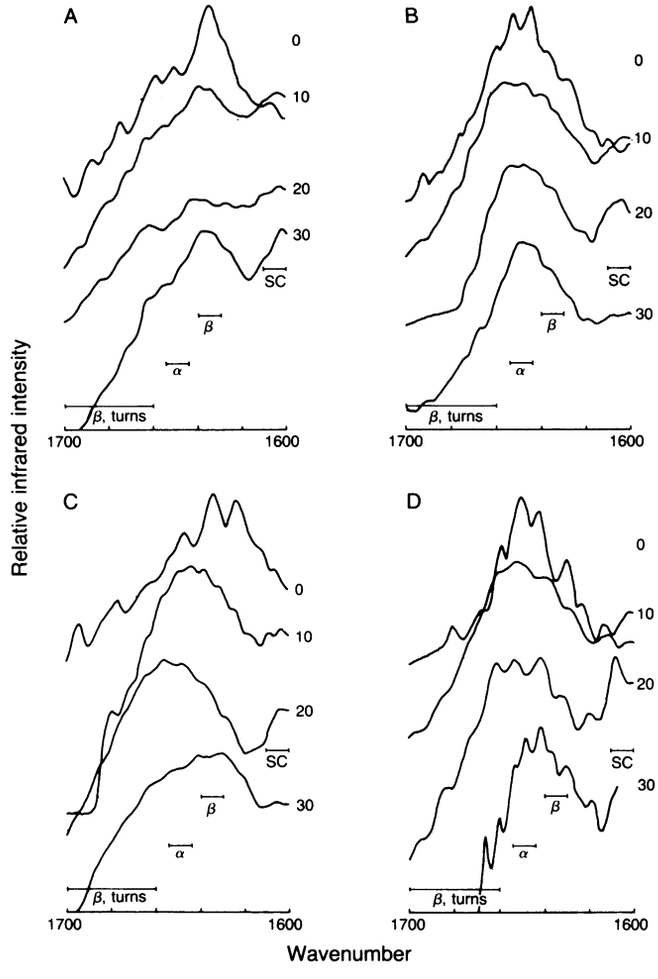

FIG. 3. Partially deconvoluted FTIR spectra of the amide I band of RNase A (*A*), hemoglobin (*B*), β-lactoglobulin (*C*), and myoglobin (*D*) adsorbed onto an alkyl-silica surface at 10%, 20%, and 30% 1-propanol concentrations. All spectra were obtained in ^2H_2O and the 0% spectra are of protein at 100 mg/ml in ^2H_2O solution. Assignments (turn, α, β, side chain) are from ref. 5.

Kubelka–Munk linearization of the spectra. A careful examination of Fig. 3 and the indicated assignments from ref. 5 show no obvious correlation between the major spectral changes that occur between 10% and 30% 1-propanol and the type of secondary structure involved.

The possibility of a correlation between the propanol-induced spectral transitions and the elution of proteins from the RP-HPLC matrix was examined by plotting the amount of 1-propanol necessary to elute each protein from the bonded phase (under the solvent conditions employed in the spectral studies) and the midpoints of the fluorescence and PAS transitions (Fig. 4). Quantitative propanol titration data could not be obtained from the FTIR studies, although the major portion of these spectral changes occurs in the same propanol range seen with the other approaches. A strikingly linear relationship is observed between the concentration of propanol necessary to produce protein elution and the spectral transition midpoints when these midpoints are operationally defined as the propanol concentrations that induce one-half of the total spectral change.

DISCUSSION

In a typical protein RP-HPLC separation, the processes taking place potentially involve a complex series of equilibria between protein, surface, solvent, and eluent (10–15). Based on the experiments described in this study and our previous work (3), the binding and elution scheme outlined in Fig. 5 describes one possible simple model that would account for the observed conformational behavior of chromatographed proteins.

The precise nature of the binding of proteins to the alkyl-bonded phase (equilibria 1) is only indirectly addressed by these experiments. At least three different, but not mutually exclusive, modes of binding can be envisioned in this regard. In the first, ligate binds to already existent apolar binding sites on the native protein's surface (16). In the second, ligate actually penetrates into the hydrophobic interior of the protein (16). In the third, the ligate's hydrocarbon chain induces a conformational change in the protein (P*) that leads to significant apolar interactions (10–15, 17, 18). The surface fluorescence results (Fig. 1) and solution fluorescence studies reported previously (3) generally indicate that the intrinsic fluorescence of proteins bound to this alkyl-silica surface are significantly blue-shifted relative to solution spectra. This implies that the indole side chains of these proteins are transferred to a more apolar environment upon surface binding. This is consistent with either the second or third mechanism and suggests that the first mechanism is less likely since indole side chains are found primarily in protein interiors (19).

The deconvoluted FTIR spectra of the amide I band of adsorbed proteins are also consistent with either penetration of the alkyl-bonded phase into protein interiors or some type

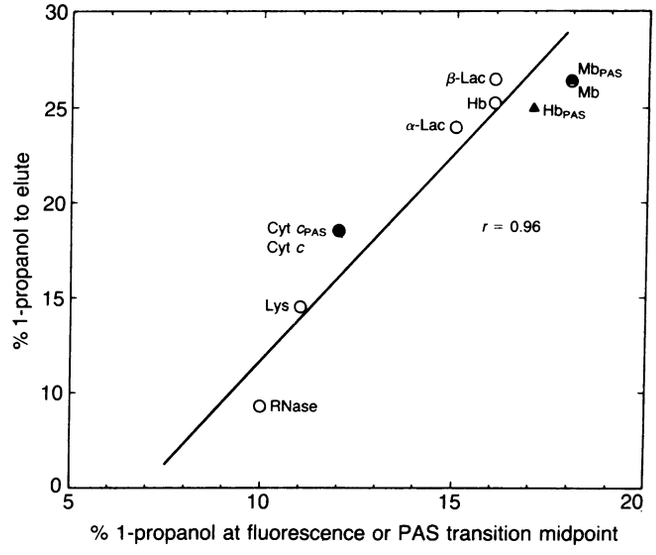

FIG. 4. Relationship between the amount of 1-propanol necessary to elute the proteins of this study from a C-8 column (3) and the propanol concentrations that produce one-half of the spectral changes illustrated in Figs. 1 and 2. RNase, ribonuclease A; Lys, lysozyme; Cyt *c*, cytochrome *c*; α-Lac, α-lactalbumin; Hb, hemoglobin; β-Lac, β-lactoglobulin; Mb, myoglobin; *r*, correlation coefficient of the line; PAS, PAS spectra.

of conformational change. Binding itself, however, may alter carbonyl stretching vibrations through direct surface interactions (20). Despite this reservation, very marked, repeatable differences are evident in the partially deconvoluted amide I peaks of all proteins examined. This is consistent with changes in protein secondary structure upon surface binding, although side chain contributions in this region (21) make more detailed conformational analysis problematic. The PAS spectra of heme proteins adsorbed on C-8 silica when compared to concentrated solution state proteins also show small differences in visible peak positions consistent with conformational alteration upon protein binding. In general, however, all of the above evidence supports the view that conformational changes induced upon binding are small (i.e., nondenaturational), consistent with the fact that some

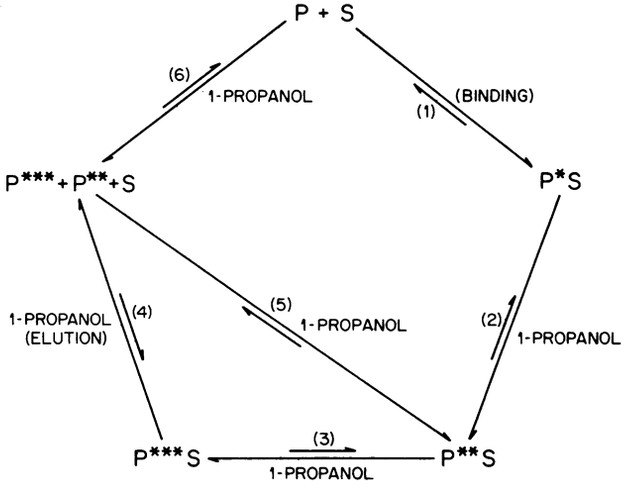

FIG. 5. Proposed model for conformational equilibria involved in the binding and elution of proteins from alkyl-silica RP-HPLC columns. S, C-8 silica surface; P, native protein; P*, initially bound form of protein; P**, intermediate conformational form(s); P***, helical eluted form. The length of the arrows reflects the postulated dominant equilibria. The numbers are referred to in the text.

enzymes maintain activity upon surface adsorption. It should be noted that the binding of most proteins to pure (unmodified) silica surfaces does not generally show such changes (unpublished results) directly implicating the alkyl-ligate in the conformational effects.

Exposure of C-8 silica-bound protein to increasing concentrations of 1-propanol causes a spectroscopically detectable conformation change in the surface-associated protein in all cases examined (equilibria 2 and 3). These transitions are sufficiently narrow to suggest the highly cooperative structural alterations commonly manifested by proteins as an immediate consequence of a variety of environmental perturbations (22–25). Solution studies of the same proteins provide direct evidence for related propanol-induced transitions, with red-shifted intrinsic fluorescence emission spectra encountered on and off the surface (3). Thus, partial exposure of aromatic side chains is apparently induced by 1-propanol with proteins free in solution and bound to the RP-HPLC matrix, although the extent of exposure cannot be immediately evaluated since the presence of the propanol itself would tend to blue-shift the spectra. Circular dichroism demonstrates that the altered solution conformations are primarily of a helical nature, an observation well established with a variety of organic agents and detergents (26). FTIR spectra of bound protein in the amide I region that primarily reflect secondary structure contributions do not show any evidence of increased helicity (1650 cm^{-1}) of bound protein. This strongly suggests the existence of at least two clearly distinguishable propanol-induced conformations of bound proteins. This first conformational state (P**) is of unknown nature, whereas the second is similar to the helical form (P***) found in solution but not present on the surface in detectable amounts. This scheme is, in fact, in general agreement with previous solution studies that hypothesize a three-state organic cosolvent-induced transition of the form $N \rightleftharpoons I \rightleftharpoons H$, where N, I, and H are native, intermediate, and helical protein conformations (22, 27), as well as with investigations of the kinetics of possible conformational changes of proteins on interaction with RP-HPLC surfaces (14). The broadness and shapes of the fluorescence and PAS transitions of the surface-associated proteins (Figs. 1 and 2) are entirely consistent with the presence of one or more distinct intermediate forms (27) as observed in solution (i.e., $PS^* \rightleftharpoons PS^{**} \rightleftharpoons PS^{***}$).

A direct role for 1-propanol-induced conformational changes in the HPLC elution process (equilibria 4 and 5) is suggested by the linear relationship between the amount of 1-propanol necessary to elute the protein and the amount needed to produce one-half of the total spectral change. This strikingly linear correlation can most simply be explained by a lowered affinity of one or more of the propanol-induced conformational states for the alkyl-bonded phase with the propanol contributing an essentially constant weakening of protein/surface interactions. The major alternative to this hypothesis is that the propanol causes differential weakening of protein/surface interaction forces and that this primarily controls the relative elution of proteins (15). This is not supported by Fig. 4, but our data cannot yet be considered to refute retention-type models of elution (15). The presence of the helical form of the protein in solution suggests that this conformational state has the lowest affinity for the matrix and constitutes the eluted form. The inability to detect significant quantities of helical protein on the surface especially at lower propanol concentrations supports this view and implies that equilibria 5 is primarily shifted to the bound form (P**S) while equilibria 4 is shifted to the unbound, eluted (primarily P***) state. There is currently no evidence to support a significant equilibrium between P*S and any unbound form. This argues that solution spectra in the presence of 40% propanol should be similar to the spectra of the small amount

of proteins remaining on the matrix at similar propanol concentrations. In fact, the fluorescence emission maxima of solution state and bound protein at these high cosolvent concentrations are similar for all proteins examined.

What do these experiments tell us about the use of RP-HPLC as a tool in protein separation? First, it is clear that the structure of eluted proteins is significantly altered by this chromatographic procedure (10–14), as also suggested by the appearance of multiple peaks upon the chromatography of homogeneous proteins (12, 14). In the very interesting work of Karger and his colleagues, it has been shown that certain proteins at very low pH migrate as two peaks, apparently corresponding to denatured and native forms (12–14). These are essentially kinetic experiments conducted under stronger denaturing conditions than those employed in this study and are therefore difficult to compare to our equilibrium surface studies. Our results at higher pH, however, do not support the view of complete surface unfolding of bound protein but are consistent with the well-known laboratory observation of complete or partial loss of biological activity upon RP-HPLC of proteins (2) and should further encourage caution in the characterization of previously chromatographed proteins. It is especially important to note, however, that the structural changes detected in this and our previous study appear to be reversible (equilibria 2, 3, and 6), at least when 1-propanol is used as an eluent at pH 4. These results are also consistent with the known difficulty of prediction of protein elution times based on any single physical property (28). Despite this unpredictability, it seems possible that the surface (and elution) behavior of proteins may itself eventually provide us with unique information about protein structure as the interaction of proteins with surfaces becomes better understood (10–15). In this regard, extension of these conformation studies of bound protein needs to include variation of ligate, cosolvent, pH, and ionic strength. In preliminary studies we have found that another common cosolvent, acetonitrile, induces conformational changes similar to those produced by 1-propanol. These changes occur at higher acetonitrile than propanol concentrations, in agreement with the higher concentrations of acetonitrile necessary to elute proteins from alkyl-silica columns (14), and further support the proposed role of conformational changes in the elution of proteins from RP-HPLC matrices.

We thank J. T. Baker Research Products for their gift of octyl-silica gel. This work was supported by National Institutes of Health Grants GM32650 and AI00663.

1. Meek, J. L. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1632–1636.
2. Regnier, F. E. (1983) *LC* **1,** 350–352.
3. Sadler, A. J., Micanovic, R., Katzenstein, G. E., Lewis, R. V. & Middaugh, C. R. (1984) *J. Chromatogr.* **317,** 93–101.
4. Rosencwaig, A. (1980) *Annu. Rev. Biophys. Bioeng.* **9,** 31–54.
5. Susi, H. & Byler, D. M. (1983) *Biochem. Biophys. Res. Commun.* **115,** 391–397.
6. Purcell, J. M. & Susi, H. (1984) *J. Biochem. Biophys. Methods* **9,** 193–199.
7. Eisinger, J. & Flores, J. (1979) *Anal. Biochem.* **94,** 15–21.
8. Sadler, A. J., Horsch, J. G., Lawson, E. Q., Harmatz, D., Brandau, D. T. & Middaugh, C. R. (1984) *Anal. Biochem.* **138,** 44–51.
9. Kauppinen, J. K., Moffat, D. J., Mantsch, H. H. & Cameron, D. G. (1981) *Anal. Chem.* **53,** 1454–1457.
10. Lau, S. Y. M., Taneja, A. K. & Hodges, R. S. (1984) *J. Chromatogr.* **317,** 129–140.
11. Ingraham, R. H., Lau, S. Y. M., Taneja, K. & Hodges, R. S. (1985) *J. Chromatogr.* **327,** 77–92.
12. Cohen, S. A., Benedek, K. P., Dong, S., Tapuhi, Y. & Karger, B. L. (1984) *Anal. Chem.* **56,** 217–221.
13. Cohen, S. A., Benedek, K., Tapuhi, Y., Ford, J. C. & Karger, B. L. (1985) *Anal. Biochem.* **144,** 275–284.
14. Benedek, K., Dong, S. & Karger, B. L. (1984) *J. Chromatogr.* **317,** 227–243.
15. Geng, X. & Regnier, F. E. (1984) *J. Chromatogr.* **296,** 15–30.
16. Melander, W. R. & Horvath, Cs. (1982) *Chromatographia* **15,** 86–90.
17. Morrissey, B. W. (1977) *Ann. N.Y. Acad. Sci.* **283,** 50–64.
18. MacRitchie, F. (1978) *Adv. Protein Chem.* **32,** 283–326.
19. Burstein, E. A., Vedenkina, N. S. & Ivkova, M. N. (1973) *Photochem. Photobiol.* **18,** 263–279.
20. Morrissey, B. W. & Stromberg, R. R. (1974) *J. Colloid Interface Sci.* **46,** 152–164.
21. Chirgadze, Y. N., Fedorov, O. V. & Trushina, N. P. (1975) *Biopolymers* **14,** 679–694.
22. Herskovits, T. T., Gadegbeku, B. & Jaillet, H. (1970) *J. Biol. Chem.* **245,** 2588–2598.
23. Finney, J. L., Gellatly, B. J., Golton, I. C. & Goodfellow, J. (1980) *Biophys. J.* **32,** 17–30.
24. Privalov, P. L. (1979) *Adv. Protein Chem.* **33,** 167–241.
25. Privalov, P. L. (1982) *Adv. Protein Chem.* **35,** 1–104.
26. Wu, C. S. C. & Yang, J. T. (1981) *Mol. Cell. Biochem.* **40,** 109–122.
27. Arakawa, T. & Goddette, D. (1985) *Arch. Biochem. Biophys.* **240,** 21–32.
28. Hearn, M. T. W. (1984) *Methods Enzymol.* **104,** 190–212.