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PROTEIN CONFORMATION AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The structure of a series of proteins has been investigated by circular dichroism, fluorescence and visible spectroscopy as well as by differential scanning calorimetry under reversed-phase high-performance liquid chromatography elution conditions. These studies show that 1-propanol, a typical eluent, induces a reversible conformational change in proteins to an apparently ordered, helical form. This structural transition occurs in the range of propanol concentrations that produces elution of a particular protein. The possible relationship between this conformational change and protein elution is considered.

INTRODUCTION

The analysis of protein mixtures by reversed-phase high-performance liquid chromatography (RP-HPLC) is complicated by the nature of the solutions usually employed to effect high-resolution separations. These conditions include low pH and the use of high concentrations of organic solvents as eluents[1], both of which are known to alter protein structure substantially[2–4]. In fact, it seems to be generally believed that RP-HPLC of proteins results in denatured proteins[1]. A second problem concerns an inability to predict the relative retention times of individual proteins. Unlike small peptides whose chromatographic behavior is directly related to their polarity[5], proteins are eluted in an unpredictable manner. This has led to an essentially empirical use of protein RP-HPLC. Both of these difficulties presumably reflect the existence of secondary and tertiary structure in native proteins, states whose chromatographic behavior is expected to be a function of macromolecular surface properties and other complex variables. To understand the behavior of proteins under RP-HPLC conditions better, we have conducted a study of the effect of several mobile phase variables on the structure of proteins under typical chromatographic conditions. We present evidence for reversible structural changes that appear in the range of organic modifier concentrations where elution occurs.

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MATERIALS AND METHODS

The proteins employed in this study were obtained from Sigma and were of the highest available commercial grade. The HPLC system used has been described elsewhere[6]. Post-column fluorescamine detection[7] and a Baker-Bond Wide-Pore Octyl (C_8) column (5 μm, 300 Å, 250 × 4.6 mm I.D.) were employed throughout. Proteins were eluted at room temperature with a 0.1 *M* phosphate buffer system (pH 4) and a 0–40% 1-propanol (double-distilled) gradient at a flow-rate of 0.75 ml/min. Proteins at a concentration of 0.1 mg/ml in pH 4 phosphate buffer were chromatographed. Elution times were found to be independent of protein concentration up to the 1.0 mg/ml concentrations examined.

Fluorescence, circular dichroism (CD) and absorbance spectroscopy were performed under precisely the same solution conditions as employed in chromatography [0.1 mg/ml protein, phosphate buffer (pH 4) and 1-propanol, as indicated]. Fluorescence spectra were obtained with a SLM 4000 spectrofluorometer interfaced with an HP85 microcomputer. All proteins were excited at 295 nm (>95% tryptophan emission) with the exception of ribonuclease where an excitation wavelength of 275 nm (tyrosine emission) was used. Excitation and emission monochromator resolutions of 8 nm were used in all fluorescence experiments. Emission at right angle to the excitation beam was detected except when examining the intrinsic fluorescence of cytochrome *c*, hemoglobin and myoglobin, where the presence of intense heme quenching necessitated the use of front surface geometry[8]. Circular dichroism was measured with a JASCO J500A spectropolarimeter, interfaced with an OKI Model 30 microcomputer, employing a 1-mm cell and a resolution of 1 nm. Spectra were obtained from 250–200 nm at a scan-rate of 5 nm/min. Since identical weight concentrations of proteins were used in all cases, CD data are simply plotted as observed ellipticity (θ) in millidegrees (m°) to enhance the accuracy of spectral comparisons. Visible absorption spectra were measured at 1-nm resolution with a Varian-Cary 210 spectrometer, interfaced with an Apple II microcomputer. In all three types of spectral measurements, data were obtained in triplicate with at least three independent sample preparations and averaged with resulting precisions of 3–5%. Peak positions and intensities were calculated from computer analysis of raw data. Light scattering was monitored by either measuring light attenuation outside the spectral region of interest (absorbance measurements) or by measurement of the Raleigh scattered excitation light in case of fluorescence studies. Scattering was judged to produce minor distortion of spectra under the conditions employed.

Differential scanning calorimetry (DSC) was performed with a Microcal MC-1 calorimeter at a protein concentration of 1 mg/ml and a sample volume of 0.7 ml at a scan-rate of 60°C/h. These experiments were performed at a protein concentration ten times greater than the spectroscopic measurements because of the small heats of the transitions involved, but the independence of protein RP-HPLC elution between 0.1 and 1 mg/ml would seem to justify this alteration from the standard HPLC conditions.

RESULTS

The chromatographic behavior of twenty proteins was initially examined to

permit selection of a number of well-characterized, homogeneous proteins with a range of elution times for further study. Three heme-containing proteins (cytochrome *c*, myoglobin and hemoglobin) and four relatively small globular proteins (ribonuclease, lysozyme, α-lactalbumin and β-lactoglobulin) were chosen on the basis of their chromatographic behavior. The percentage of propanol in the mobile phase necessary to elute each of these seven proteins from a C_8 RP-HPLC column as well as their calculated hydrophobicities[9] are summarized in Table I. Although an apparent correlation between the critical propanol concentration at which each protein is eluted and its calculated hydrophobicity can be ascertained, examination of all twenty proteins reveals no such clear correlation. This suggests that the relationship seen in Table I between polarity and elution is substantially coincidental. In the following structural studies, we chose to investigate two variables. It is known that low pH substantially enhances RP-HPLC resolution of protein mixtures[10]. Since 1-propanol was used to elute proteins in these experiments, the concentration of this organic solvent and pH were varied. All spectroscopic experiments were performed under solution conditions as identical to those used for RP-HPLC as possible to enhance the relevance of these structural studies to protein chromatographic behavior.

TABLE I

PERCENTAGES OF 1-PROPANOL NEEDED TO ELUTE PROTEINS FROM A C_8 COLUMN AND RELATED PROTEIN HYDROPHOBICITIES

Protein (abbr.) (source)	*Propanol (%)*	$H\Phi_{AVE}$*
Ribonuclease A (RNA) (bovine pancreas)	9.3	780
Lysozyme (LYS) (chicken egg white)	14.6	890
Cytochrome *c* (CYT) (equine heart)	18.6	1050
α-Lactalbumin (ALA) (bovine milk)	24.0	1050
Hemoglobin (HEM) (bovine)	25.3	1160
β-Lactoglobulin (BLA) (bovine milk)	26.7	1070
Myoglobin (MYO) (equine skeletal muscle)	26.7	1040

* Average hydrophobicity, calculated as described in ref. 9.

The CD spectra of the proteins hemoglobin and β-lactoglobulin at pH 4 are shown as a function of propanol concentration in Fig. 1. These macromolecules are chosen as examples of typical proteins containing α-helical (hemoglobin) and β-structures (β-lactoglobulin)[11] to illustrate eluent effects on secondary structure. Both proteins show increases in negative ellipticities as the propanol concentration is increased. In addition, the single negative peak at 217 nm seen with β-lactoglobulin in the absence of organic solvent is gradually converted to a typical double negative helical peak at 207 and 222 nm. Similar results were obtained with the five other

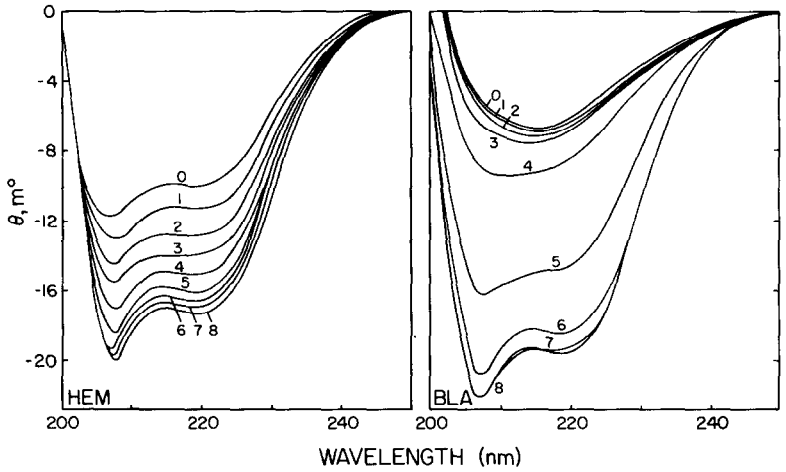

Fig. 1. Circular dichroism spectra of hemoglobin (HEM) and β-lactoglobulin (BLA) as a function of propanol concentration at pH 4: 0, 0% propanol; 1, 5%; 2, 10%; 3, 15%; 4, 20%; 5, 25%; 6, 30%; 7, 35%; 8, 40%.

proteins of this study and are summarized in terms of changes in the observed 207 nm ellipticity minimum in Fig. 2. With the exception of ribonuclease, all of the proteins show evidence of a propanol-induced transition at both acidic and neutral pH. Although this transition shows both qualitative and quantitative variations, it is of significant magnitude with the exception of lysozyme. In general, the midpoint of this transition occurs between 15 and 25% propanol in the proteins examined and

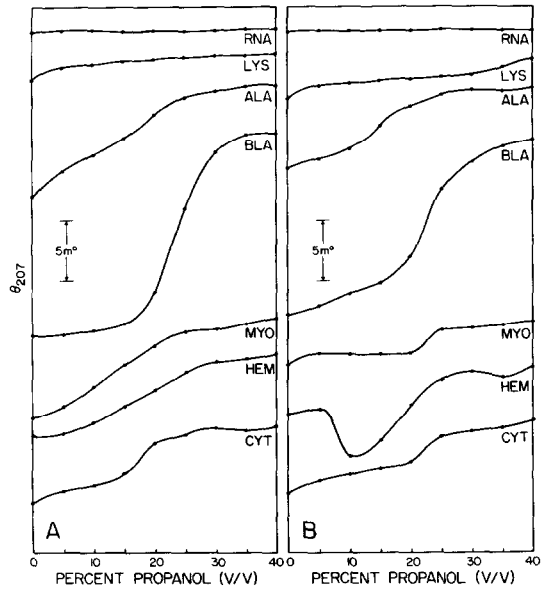

Fig. 2. Observed CD ellipticity at 207 nm as a function of propanol concentration at pH 4 (A) and pH 7 (B). Abbreviations are defined in Table I.

appears to initiate at slightly lower propanol concentrations at the lower pH. These spectral changes were found to be >95% reversible at pH 4 and >80% reversible at pH 7, as examined either by dilution or dialysis procedures to remove propanol.

To explore this propanol-induced conformational effect further, the intrinsic fluorescence emission spectra of the proteins were also examined. This procedure provides additional information about tertiary structure in terms of changes in the environment of atomatic amino acid sidechains[12]. The effects of propanol concentration at pH 4 and 7 on peak intensity and position are presented in Figs. 3 and 4, respectively. As found in the CD experiments, both ribonuclease and lysozyme demonstrate evidence for only weak transitions, while the other five proteins manifest major transitions over a range of propanol concentrations. Because several of the proteins possess a heme chromophore, the visible absorbance spectra at pH 4 of these proteins as a function of propanol concentration were also obtained (Fig. 5). Evidence for propanol-induced transitions is again apparent. Removal of propanol in all cases again produced at least a 90% return to the unperturbed spectra in both fluorescence and absorbance measurements.

To examine the effect of propanol upon the seven proteins further, the stability of several of the proteins was examined by the non-spectroscopic technique of dif-

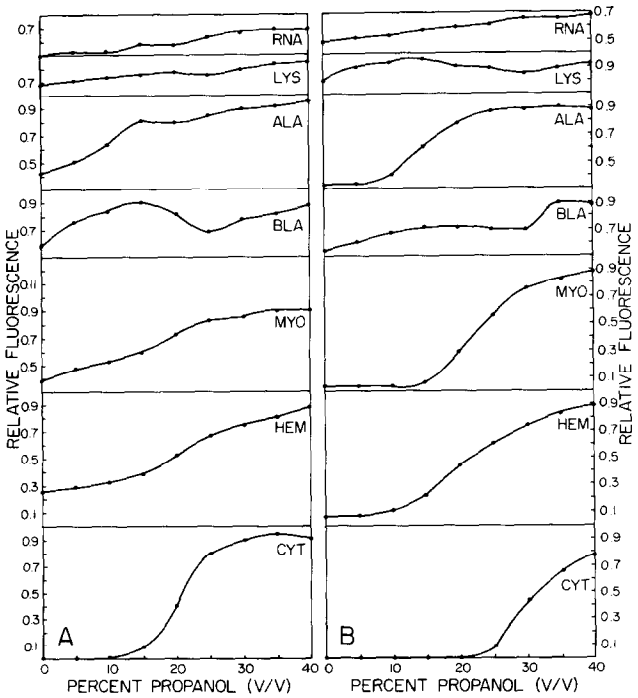

Fig. 3. Relative intrinsic fluorescence intensities at the emission peak as a function of propanol concentration for the seven proteins of Fig. 2. Excitation conditions are described in the text. pH 4 (A), pH 7 (B).

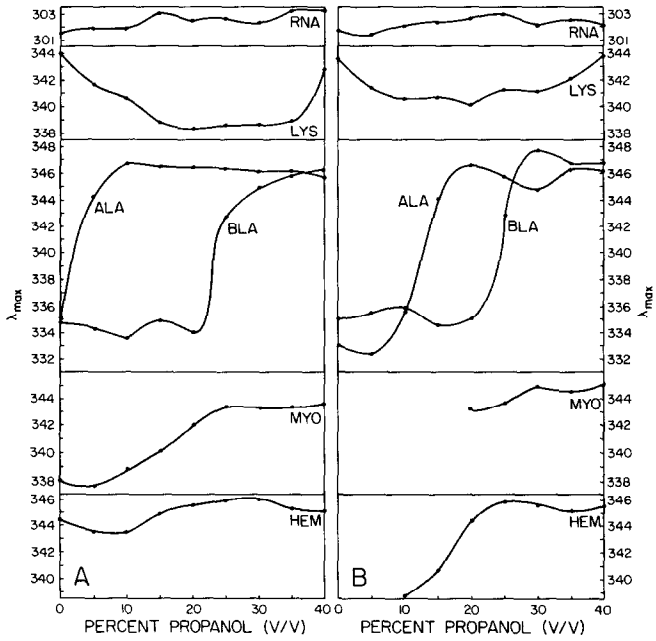

Fig. 4. Fluorescence peak positions at pH 4 (A) and pH 7 (B) at various propanol concentrations for the seven proteins described. Myoglobin data below 20% propanol are not shown because of the weak intensities of the fluorescence. Data for cytochrome *c* are not illustrated because of the poor reproducability of measurements of the peak position.

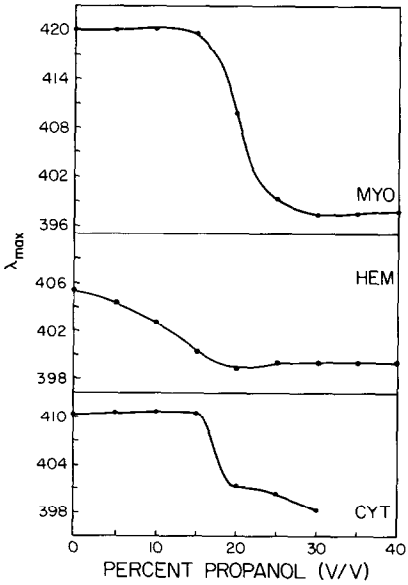

Fig. 5. Position of the absorbance maximum as a function of propanol concentrations at pH 4 for three heme containing proteins.

ferential scanning calorimetry (Fig. 6A). In all cases studied, propanol caused a linear decrease in the stability of the proteins, as measured by a reduction in the midpoint of the denaturational transitions temperature midpoint. Evidence for a pH dependent maximum was also observed in the proteins studied (Fig. 6B). Results were limited to those shown in Fig. 6 because of protein precipitation at the higher protein concentrations necessary to perform these experiments.

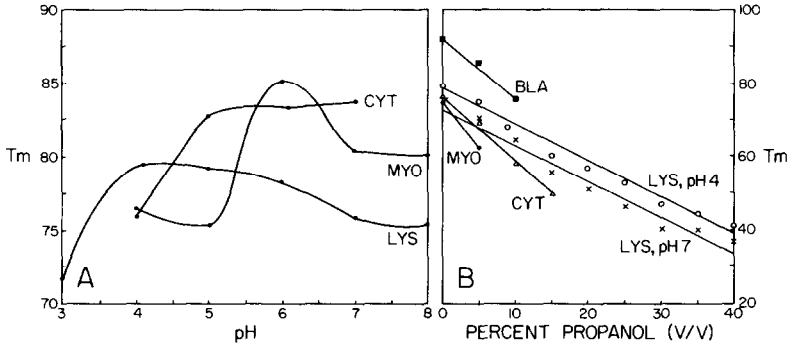

Fig. 6. The midpoint of the denaturation transition as a function of (A) pH for three proteins and (B) propanol concentration for four proteins, as determined by differential scanning calorimetry. Conditions are described in the text. In B, all data were obtained at pH 4, except as indicated.

DISCUSSION

The results of these studies provide direct evidence that 1-propanol, used to elute protein from a C_8 RP-HPLC column, produces conformational changes in proteins under normal, chromatographic elution conditions. Furthermore, the results are consistent with the interesting possibility that these conformational changes may play a role in the elution process itself. This is suggested by the early elution times of the two proteins which show little evidence of significant conformational alteration, and the fact that the elution of the remaining proteins occurs at propanol concentrations where such conformational changes are strongly manifested. These simple considerations ignore, of course, the possibly crucial role of the direct surface interactions between protein and matrix (although see below). The lack of major propanol-induced conformation effects in ribonuclease and lysozyme probably reflect their highly disulfide-crosslinked structures. No clear correlation between a conformational effect and pH-enhanced chromatographic resolution[10] is immediately obvious from these studies.

The nature of these solvent-induced conformational transitions is somewhat unclear. The propanol-induced changes in the CD spectra are very similar to those observed by others in the presence of a variety of apolar compounds, such as detergents and organic solvents, like trifluoroethanol[13]. The production of a new helical structure, as evidenced by a double negative ellipticity minimum having a 207 nm peak of greater strength than the 222 nm peak has been consistently demonstrated to be characteristic of proteins upon reduction of solvent polarity[14]. While no general agreement exists concerning the precise structure of these reordered conformations,

most studies suggest an extended, intermittent coil-like structure as a direct consequence of the presence of apolar agents[13,14]. Although our CD results strongly suggest the induction of increased helical structure, fluorescence peak positions consistently manifest red shifts during the corresponding transitions. This indicates extensive exposure of previously buried tryptophan side chains[12] induced by the presence of propanol. Since protein helices have their side chains exposed on their outer surface, this result supports an extended helical structure for these conformations. This conclusion is also consistent with the calorimetric results, which imply significant tertiary structure destabilization by propanol. In the few cases where actual transition enthalpies can be measured (results not illustrated), this enthalpy appears to pass through a maximum within 5% of the propanol concentration where elution occurs in further support of propanol-induced destabilization. Overall, these results are not consistent with the idea that true protein denaturation occurs, but rather with the induction of a significantly altered, but highly ordered conformation at the secondary structural level[13] by the eluting solvent. It is interesting to note that, unlike denaturation to a disordered form, this structure can frequently be returned to a conformational state similar to that of the native protein by simple removal of the organic agent. This suggests the possibility that removal of eluent after chromatography may in some cases permit RP-HPLC to be used in the isolation of biologically active proteins.

If the propanol-induced altered form of a protein displays lower affinity than the native form for the bonded phase, then elution as an immediate consequence of such a conformational change would appear to offer a plausible albeit overly simplified hypothesis. This idea may at first seem surprising, since the fluorescence results clearly indicate that the propanol-induced reordered helical form possesses apolar aromatic side chains that are more exposed than in the native protein. Such residues might be expected to interact more favorably with the hydrophobic ligate. This apparent contradiction can most simply be explained by the existence of either substantial apolar pockets in the native protein or significant penetration of the C_8 chain into the hydrophobic interior of the native macromolecule, which leads to a tight interaction between the unaltered proteins and the packing material. Disruption of these stereochemically created macromolecular apolar environments then produces a periodic but dispersed polar–apolar helical exterior[15]. This form would then display a decreased affinity for ligate because of the loss of cooperative (extended) interaction between protein and bonded phase.

While the data presented above are consistent with the single hypothesis that elution may be conformationally induced, it must be strongly emphasized that the relevant conformational transitions probably occur while proteins are actually physically bound to the column matrix. This follows if there is no appreciable equilibrium between bound and unbound protein in the absence of eluent[16]. We are therefore currently examining the structure of the proteins of this study while they are adsorbed onto a C_8 silica matrix. In preliminary studies by visible photo-acoustic spectroscopy for heme-containing proteins and front surface fluorescence intrinsic emission spectroscopy for the rest of the proteins of this study, evidence for a conformational transition similar to that described in this work has been obtained.

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