Comparison of Protein Surface Hydrophobicity Measured at Various pH Values Using Three Different Fluorescent Probes

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The influence of type of fluorescent probe on the surface hydrophobicity values determined for three native and heated proteins was assessed using uncharged [6-propionyl-2-(*N*,*N*-dimethylamino)-naphthalene or PRODAN] versus anionic aliphatic (*cis*-parinaric acid or CPA) and aromatic (1-anilinonaphthalene-8-sulfonic acid or ANS) probes. Surface hydrophobicities of whey protein isolate, β-lactoglobulin, and bovine serum albumin under heated (80 °C for 30 min) and unheated conditions and at varying pH values (3.0, 5.0, 7.0, and 9.0) were measured using ANS, CPA, and PRODAN. ANS and CPA yielded opposing results for the effects of pH and heating on protein hydrophobicity. Hydrophobicity was lower at pH 3.0 than at other pH values for all proteins measured by PRODAN, whereas the values measured by ANS and CPA at pH 3.0 were quite high compared to those at other pH values, suggesting the influence of electrostatic interactions on anionic probe−protein binding. These results suggest that the presence or absence of a permanent charge as well as the aromatic and aliphatic nature of fluorescent probes can affect protein hydrophobicity values measured under various pH conditions.

Keywords: *Protein surface hydrophobicity; fluorescent probe; PRODAN; pH; heat*

INTRODUCTION

Due to their three-dimensional structures, food proteins are involved in many functional processes (Stryer, 1968). Over the years, food chemists have been trying to elucidate the mechanism of protein functionality. However, the food industry is still looking for ways to predict the functional properties of proteins. Hydrophobic, steric, and electrical parameters are the most important variables that affect the structure of proteins. Among these factors, hydrophobicity is known to be significantly related to the functional properties of proteins (Nakai, 1983). The tendency of nonpolar solutes to adhere to one another in an aqueous environment is called hydrophobicity (Cardamone and Puri, 1992).

One approach to quantify protein hydrophobicity is through fluorescent probe methods. The quantum yields of fluorescence and wavelength of maximum fluorescence emission of these compounds depend on the polarity of their environment (Li-Chan, 1999). Several fluorescent probes such as 1-anilinonaphthalene-8-sulfonic acid (ANS) and *cis*-parinaric acid (CPA) have been widely used to measure protein hydrophobicity. These probes have low quantum yield of fluorescence in aqueous solution. Upon binding of the probes to accessible hydrophobic regions of proteins, an increase in fluorescence is observed, which is used as a measure of protein surface hydrophobicity. However, due to the possible contribution of both electrostatic and hydrophobic interactions to the binding of these anionic probes, the interpretation based on these probes has not been easy. Therefore, there has been a need for an uncharged probe to circumvent this problem.

Due to high sensitivity, noninvasiveness, and availability of imaging techniques, fluorescence spectroscopy has been considered to be one of the most promising and potentially widely used techniques in medicine, biology, biochemistry, and molecular biophysics for the 21st century (Slavic, 1994; Royer, 1995). Fluorescent methods depend on the response of some fluorescent component, either intrinsic or extrinsic, to its environment following optical excitation (Damodaran, 1989; Hudson et al., 1986). ANS and 6-propionyl-2-(dimethylamino)-naphthalene (PRODAN) are known for sensing the polarity of the environment in biological materials (Rettig, 1993). The fluorescence emissions of ANS and some close analogues depend on the environment (Penzer, 1972). ANS is composed of aromatic rings, whereas CPA possesses an aliphatic hydrocarbon chain. The binding sites for CPA on protein molecules may therefore differ from the sites for ANS (Nakai and Li-Chan, 1988).

Both ANS and CPA are considered to be anionic probes, containing sulfonic acid and carboxylic acid groups, respectively. Depending on the p*K* of the groups and the pH of the environment, sulfonate and carboxylate groups may be formed. Yet, there is a need to conduct comprehensive studies of the structural and molecular properties of proteins such as β-lactoglobulin (β-lg) in the pH range 1−10 to predict the behavior of proteins in model systems in the presence of other variables (Phillips et al., 1994). Under these conditions of acidic and alkaline pH, a contribution of charged interactions on the measurement of surface hydrophobicity using the anionic fluorescent probes may be expected.

PRODAN, on the other hand, is a solvent-sensitive probe and has no charge. This will eliminate possible electrostatic interaction contributions in the measure-

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ment of protein hydrophobicity (Hermetter et al., 1993). The first published literature on the synthesis and spectral properties of PRODAN dates back to 1979 (Weber and Farris, 1979). Studies on the binding and spectral properties of PRODAN with bacteriorhodopsin, membrane and protein interior, spectrin, tubulin, and horseradish peroxidase have been cited (Baasov and Sheves, 1987; Balter et al., 1988; Bruins and Epand, 1995; Catalan et al., 1991; Chakrabarti and Basak, 1996; Chakrabarti, 1996; Heisel et al., 1987; Krasnowska et al., 1998; Lasagna et al., 1996; MacGregor and Weber, 1986; Mazumdar et al., 1992). Wald et al. (1990) investigated the lipid domains in high-density lipoproteins, using PRODAN. Bunker et al. (1993) studied photophysical properties of PRODAN in solution. Royer (1995) stated that PRODAN is an excellent example of solvent relaxation phenomena, because it shows a very large excited-state dipole that renders the emission spectrum quite sensitive to the relaxation of the solvent. Prendergast et al. (1983) pointed out that the sensitivity of PRODAN to the polarity of solvents is due to the large dipole moment developed in the excited state as a consequence of facile charge delocalization between the 2-dimethylamino moiety and the carbonyl group in the 6-position of the naphthalene.

Recently the PRODAN probe was used by Haskard and Li-Chan (1998) for the quantitation of protein surface hydrophobicity. In that study, the influence of ionic interactions on the quantitation of protein surface hydrophobicity was assessed by comparing the binding of PRODAN versus ANS to bovine serum albumin (BSA) and ovalbumin at various ionic strengths. However, due to the low solubility (3.5 μM) of PRODAN in water [the PRODAN has to be stirred overnight to reach such solubility, as stated by Weber and Farris (1979)], the protocol established by Haskard and Li-Chan (1998) yielded low fluorescence readings and measurements were difficult to reproduce due to batch-to-batch variability in the solubility of the PRODAN aqueous stock solution (Alizadeh-Pasdar, unpublished data, 1998).

In response to a concern about the possible effect of anionic probes on the binding of proteins, the objectives of this study were to establish a fluorescent probe method using an uncharged probe (PRODAN), prepared as a methanol stock solution, to compare the values of protein surface hydrophobicity measured using this probe with those measured by aliphatic (CPA) and aromatic (ANS) anionic probes. Surface hydrophobicities of three protein samples [whey protein isolate (WPI), β-lg, and BSA] before and after heating (80 °C for 30 min) at various pH values (3.0, 5.0, 7.0 and 9.0) were measured using these three probes.

EXPERIMENTAL PROCEDURES

Materials. WPI, β-lg, and BSA were from Foremost Farms USA (Waukon, IA; Daritek NVB 389, lot 21-4080, containing 89.43% protein and 4.26% moisture; obtained as a gift from Canadian Inovatech Inc., Abbotsford, BC, Canada), Sigma (St. Louis, MO, lot L-2506), and Sigma (lot A-4503), respectively. Three fluorescent probes, ANS, CPA, and PRODAN were obtained from Sigma, Molecular Probes (Eugene, OR), and Molecular Probes, respectively.

Buffers were prepared according to the method of Dawson et al. (1969). For buffers at pH 3.0, 5.0, and 7.0, mixtures of 0.1 M citric acid (BDH, Toronto, ON) and 0.2 M Na_2HPO_4 (Fisher Scientific, Fair Lawn, NJ) in the following proportions (v/v), respectively, were used: 79.45:20.55, 48.5:51.5, and 17.65:82.35, yielding final buffer compositions of 0.079 M:0.041 M, 0.048 M:0.103 M, and 0.017 M:0.165 M citric acid/sodium phosphate, respectively. For pH 9.0 buffer, 50 mL of 0.025 M $Na_2B_4O_7$ (Fisher Scientific) was adjusted to pH 9.0 with 1 N NaOH (BDH) and was brought to 100 mL final volume (0.0125 M final concentration). In all buffers, 0.02% sodium azide (Sigma) was included to prevent the growth of microorganisms.

Preparation of Proteins. Stock protein solutions containing 1.5% (w/w) protein in double-distilled water, with 0.02% sodium azide, were prepared in duplicate. Protein concentrations of WPI, β-lg, and BSA were determined by absorbance at 280 nm using $E_{1cm}^{1\%}$ of 11.7 (Kitabatake et al., 1994), 9.6 (Fasman, 1992), and 6.61 (Fasman, 1992), respectively. The stock protein solutions were diluted, with appropriate buffers, to an intermediate concentration (0.03%) and then diluted to final concentrations either as is or after heat treatment for 30 min at 80 °C. All three proteins were soluble at the pH and ionic strength conditions studied; however, BSA showed slight turbidity after heating. Heat treatment of protein solutions in 50 mL flasks, which were covered with Parafilm to avoid evaporation, was done in a water bath (Blue M Magni Whirl). After heating, samples were cooled immediately, under running water. For the fluorometric probe assay, the stock proteins were diluted with required buffers at pH 3.0, 5.0, 7.0, and 9.0 to typical concentration ranges of 0.005−0.025% w/v (five concentrations) for measurements using ANS and CPA and 0.002−0.01% for measurements using PRODAN.

Hydrophobicity Determination. Protein surface hydrophobicity using ANS and CPA probes was determined according to a modification of the method of Kato and Nakai (1980). A similar approach was used to develop a new method using PRODAN. Stock solutions of 8×10^{-3} M ANS, 3.6×10^{-3} M CPA, and 1.41×10^{-3} M PRODAN were prepared in 0.1 M phosphate buffer (pH 7.4), ethanol, and methanol, respectively. For CPA, 10 μg of butylated hydroxyanisole (BHA) was added per milliliter of ethanol, as an antioxidant. CPA and PRODAN stock solutions were transferred to screw-capped vials, covered with aluminum foil, and the caps sealed with Parafilm to prevent evaporation of ethanol or methanol. The CPA and PRODAN stock solutions were stored in the freezer (≤ -10 °C) until the day of experiment, when they were held in ice throughout the experiment. ANS stock solution was stored in a screw-capped container at room temperature. All of the probes were wrapped in aluminum foil to avoid exposure to light. Under these conditions, the stock solutions were stable for at least 6 months as judged by the relative fluorescence intensity (RFI) values of the probes in the solvents used for standardization (data not shown). Concentrations of the ANS, CPA, and PRODAN stock solutions were determined spectrophotometrically at 350, 303, and 360 nm, respectively, using molar absorption coefficients of ϵ_{350} (ANS) $= 4.95 \times 10^3$ M^{-1} cm^{-1} (Weber and Young, 1964), ϵ_{303} (CPA) $= 7.6 \times 10^4$ (Haughland, 1996), and ϵ_{360} (PRODAN) $= 1.8 \times 10^4$ M^{-1} cm^{-1} (Chakabarti, 1996), respectively. All fluorescence measurements were made with a Shimadzu RF-540 (Shimadzu Corp., Kyoto, Japan) spectrofluorometer.

For hydrophobicity determination using PRODAN, the excitation/emission slits and wavelengths were set at 5 nm/5 nm and 365 nm/465 nm, respectively. To successive samples containing 4 mL of diluted proteins was added 10 μL of PRODAN stock solution, which was mixed well by vortexing. After 15 min in the dark, the RFI of each solution was measured, starting from buffer blank (buffer plus probe) and then the lowest to the highest protein concentration; the fluorometer quartz cell was rinsed between samples with a small volume of the solution to be measured. RFI values of buffer and protein dilution blanks (no PRODAN) were also measured. The RFI of each protein dilution blank was subtracted from that of corresponding protein solution with PRODAN to obtain net RFI. The initial slope (S_0) of the net RFI versus protein concentration (percent) plot was calculated by linear regression analysis with Microsoft Excel for Windows 95 (version 7.0) and used as an index of the protein surface hydrophobicity. To correct for day-to-day instrumental fluctuations in relative fluorescence intensity, standardization was

performed by measuring the RFI of 4 mL of methanol with 10 μL of PRODAN and correcting to a standard value of 50.

ANS and CPA probe methods were performed essentially according to the method of Kato and Nakai (1980). The procedure was the same as for PRODAN, with the following exceptions. Excitation and emission wavelengths were 390 and 470 nm, respectively, for ANS and 325 and 420 nm, respectively, for CPA. The excitation and emission slit widths were 5 and 5 nm, respectively, for ANS and 2 and 5 nm, respectively, for CPA. The amount of probe stock solution for measuring hydrophobicity using these two probes was 20 μL, to be added to 4 mL of diluted protein. For standardization of the ANS assay, the measured RFI for 10 mL of methanol with 10 μL of ANS was corrected to a value of 15. For the CPA assay, the measured RFI for 4 mL of *n*-decane with 10 μL of CPA was corrected to a value of 3.

Surface hydrophobicity values were determined using at least duplicate analyses. In all cases, R^2 values of 0.99 were noted for the linear regression analyses used to calculate surface hydrophobicity (S_0) values. Quadruplicates of several samples were performed, and the coefficient of variation (CV) of the replicates were found to be <3%.

Statistical Analysis. Data were analyzed using an analysis of variance (ANOVA) procedure using the General Linear Model, with further analysis using Tukey's pairwise comparison test to determine differences ($p \leq 0.05$) between treatment means (Minitab for Windows, version 12, Minitab Inc., State College, PA). Due to the range in magnitude of data obtained, logarithmic transformation of the S_0 values was performed prior to Tukey's test.

RESULTS AND DISCUSSION

Comparison of Hydrophobicity Measured Using Anionic Probes ANS and CPA. The protein surface hydrophobicity (S_0) values measured using the two anionic fluorescent probes, ANS and CPA, are depicted in the top and middle panels, respectively, in Figures 1–3.

Surface hydrophobicity values based on the ANS probe method ranged from 78 for heated β-lg at pH 5.0 to 3020 for heated BSA at pH 3.0. Using the ANS method, significant ($p \leq 0.05$) increase in the S_0 value after heating was observed at pH 7.0 and 9.0 for WPI and β-lg. No significant effects of heating were observed for WPI or β-lg at pH 3.0 or 5.0. On the other hand, for BSA, heating significantly decreased the S_0 value at all pH values, except at pH 3.0.

At pH 3.0, 5.0, and 7.0, the hydrophobicity determined by ANS was in the order BSA > WPI > β-lg for unheated proteins, whereas the reverse was seen using CPA. For WPI and β-lg at pH 7.0 and 9.0, heating significantly affected the S_0 value, but ANS showed significant ($p \leq 0.05$) increase while CPA showed significant ($p \leq 0.05$) decrease.

CPA hydrophobicity values ranged from 12 for both heated and unheated BSA at pH 3.0 to 380 for unheated β-lg at pH 5.0. Using the CPA method, heating generally decreased the value of S_0 of the proteins at pH 7.0 and 9.0 and also at pH 5.0 for BSA.

Our results concerning the effect of heat on hydrophobicity of WPI at pH 7.0 and 9.0, measured using ANS probe, are in agreement with those of Mleko and Li-Chan (1997) and Monahan et al. (1995). However, the results of heating at pH 3.0 and 5.0 are inconsistent. Mleko and Li-Chan (1997) reported that heating WPI at pH 3.0, 5.0, 7.0, and 9.0 increased the S_0 values at each pH studied, whereas Monahan et al. (1995) reported decreases in surface hydrophobicity of WPI samples after heating at 80 °C at pH 3.0 and 5.0 and increases in surface hydrophobicity after heating at pH 7.0 and 9.0. We detected no significant changes by heating WPI at either pH 3.0 or 5.0. These inconsistencies could be due to differences in the WPI products studied. As reported in a review on the processing and functional properties of whey protein concentrates and isolates (Morr and Ha, 1993), widely different processing conditions of whey from different sources can result in products with varying compositions, degrees of protein denaturation, aggregation, and physicochemical and functional properties.

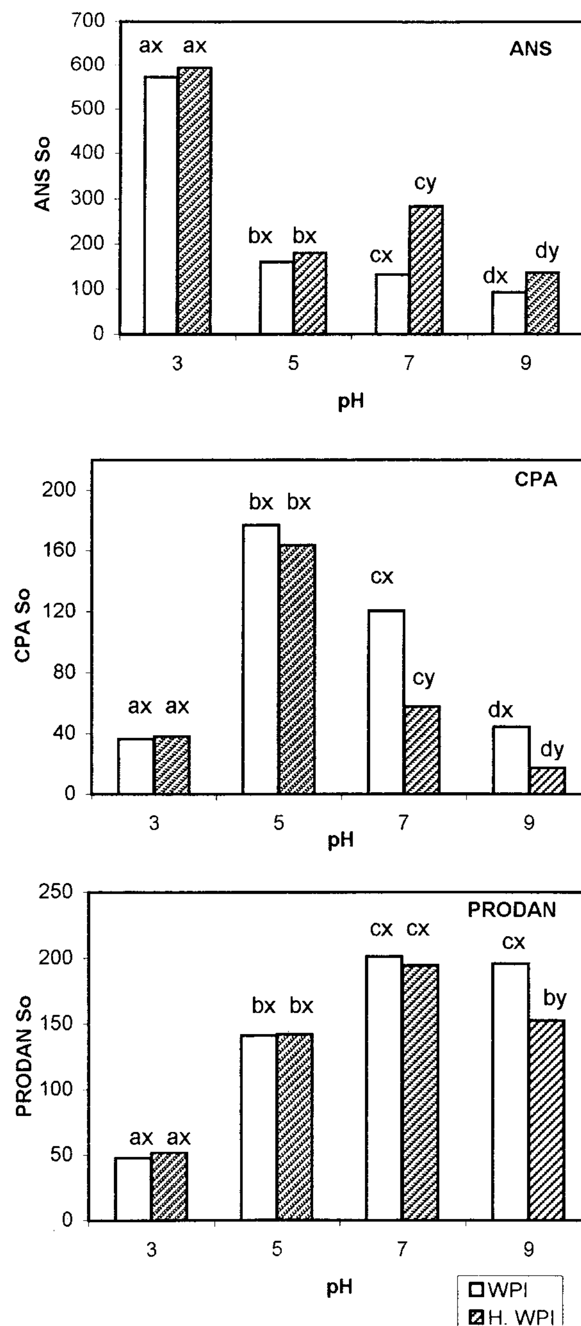

Figure 1. Surface hydrophobicity (S_0) of WPI measured at pH 3.0–9.0 with ANS, CPA, and PRODAN (top, middle, and bottom graphs, respectively). Open and shaded bars show mean values of duplicate determinations for unheated and heated samples, respectively. Bars with different letters (a–d) represent significant ($p \leq 0.05$) differences in S_0 values within heated or unheated samples as a function of pH. Bars with different letters (x, y) represent significant ($p \leq 0.05$) differences in S_0 values between heated and unheated samples at a given pH.

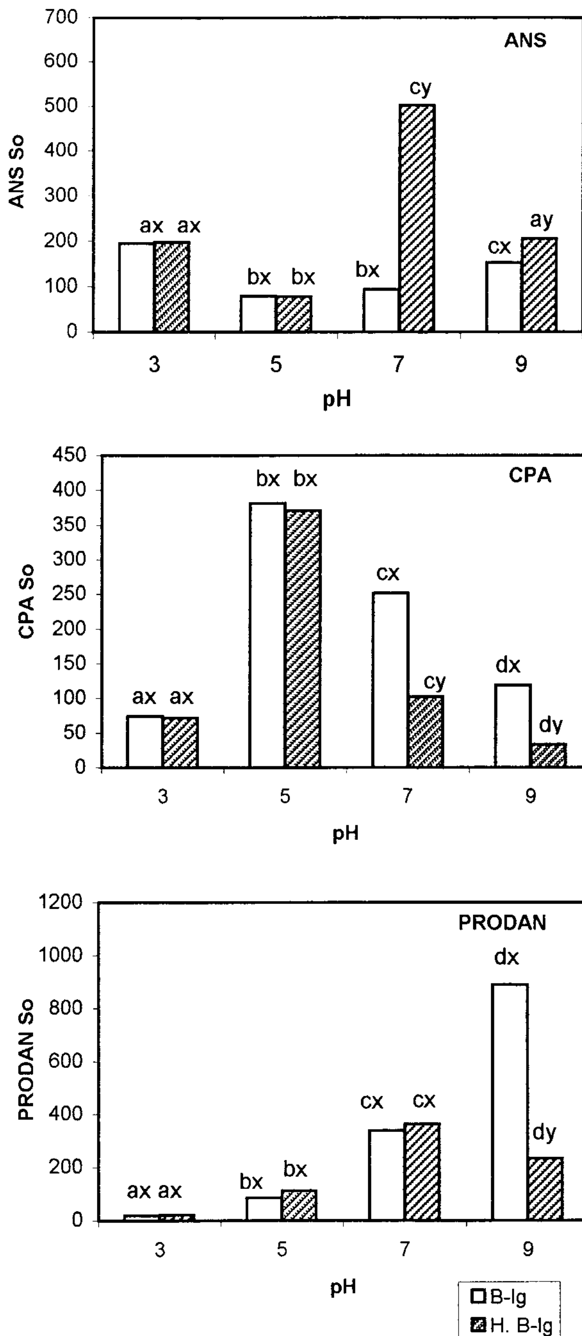

Figure 2. Surface hydrophobicity (S_0) of β-lg measured at pH 3.0–9.0 with ANS, CPA, and PRODAN (top, middle, and bottom graphs, respectively). Open and shaded bars show mean values of duplicate determinations for unheated and heated samples, respectively. Bars with different letters (a–d) represent significant ($p \leq 0.05$) differences in S_0 values within heated or unheated samples as a function of pH. Bars with different letters (x, y) represent significant ($p \leq 0.05$) differences in S_0 values between heated and unheated samples at a given pH.

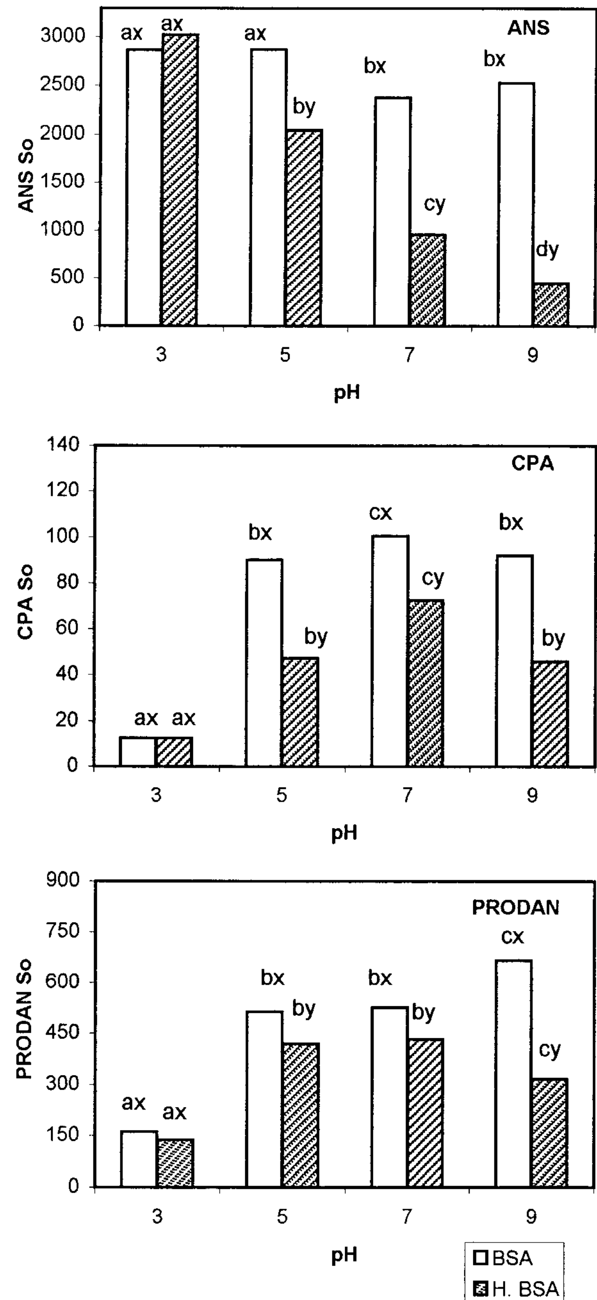

Figure 3. Surface hydrophobicity (S_0) of BSA measured at pH 3.0–9.0 with ANS, CPA, and PRODAN (top, middle, and bottom graphs, respectively). Open and shaded bars show mean values of duplicate determinations for unheated and heated samples, respectively. Bars with different letters (a–d) represent significant ($p \leq 0.05$) differences in S_0 values within heated or unheated samples as a function of pH. Bars with different letters (x, y) represent significant ($p \leq 0.05$) differences in S_0 values between heated and unheated samples at a given pH.

Das and Kinsella (1989) measured the fluorescence intensity of protein-bound ANS at different pH values (2.8, 4.3, 5.0, 7.6, and 9.7). Their results indicated the highest hydrophobicity at pH 2.8 and showed that with increase in pH, the hydrophobicity decreased drastically. However, they did not find any correlation between hydrophobicity and surface area of the emulsions stabilized by β-lg. They speculated that this may be due to the fact that hydrophobicity is usually determined at very low concentrations and the hydrophobicity values may change at the higher concentrations used for studying functionality.

Shimizu et al. (1985), who measured structural properties of β-lg at different pH values, also reported that the highest value of surface hydrophobicity measured by ANS was found at pH 3.0. On the other hand, Phillips et al. (1994) cited a number of studies indicating a compact structure of β-lg and increased thermostability under low pH conditions, in contrast to enhanced susceptibility to surface denaturation at pH 9.0, suggesting a more open flexible molecular structure at

alkaline pH values compared to that at pH 3.0. Our results using the ANS probe (Figure 2) also indicate that unheated β-lg has higher S_0 values at pH 3.0 and 9.0 compared to those at pH 5.0 and 7.0. However, the S_0 values measured with the CPA probe showed the opposite trend, with hydrophobicity in the following order: pH 5.0 > pH 7.0 > pH 9.0 > pH 3.0.

According to Laligant et al. (1991), at pH 7.0, β-lg contains a high proportion of hydrophobic amino acid side chains, which are turned, preferably, toward the inside of the molecule. He also stated that ANS does not constitute a sensitive or practical probe to study β-lg hydrophobicity, due to the low affinity of β-lg for ANS. Molecular rigidity rather than solvent polarity is the dominant factor influencing the energy and quantum yield of ANS (Penzer, 1972). The fluorescence properties of ANS are determined by both intramolecular configuration of substituents that can undergo torsional motions and the dipolar properties of the probe environment, so it cannot yield information regarding binding site polarity (Prendergast et al., 1983).

Ibrahim et al. (1993), using the CPA method, showed a decrease in S_0 value when β-lg samples (pH 7.4) were heated at 80 °C in a dry state. The decrease in S_0 could be due to burial of effective hydrophobic regions due to the interaction of partially denatured molecules by dry-heat denaturation (Ibrahim et al., 1993). Kato et al. (1983) also showed a decrease of S_0 values of β-lg and BSA solutions (pH 7.4) when heated from 20 to 80 °C at the rate of 1 °C/min, using the CPA method. These results are similar to those observed in the present study after WPI and β-lg were heated at pH 7.0 and 9.0 (Figures 1 and 2).

Similarly, decreases in S_0 measured by both ANS and CPA probes were observed after heating of BSA solutions at pH 5.0, 7.0, and 9.0 (Figure 3). However, no change in S_0 measured by either probe was observed in BSA solutions at pH 3.0 after heating. According to Takeda et al. (1989), the BSA molecule expands in the acidic pH range, so the helical content decreases. The tertiary structure of BSA is in the expanded or loosened state at acidic pH, whereas it is in a compact state at pH 7.0.

In summary, different observations were noted using ANS and CPA probes for the effect of pH and heating on surface hydrophobicity of these three protein samples. Similar discrepancies between results obtained by these two fluorescent probes have been noted previously (Hayakawa and Nakai, 1985). These discrepancies may be partly attributed to differing probe chemistry arising from the aromatic versus aliphatic nature of the probes, but also may have been due to interference of electrostatic interactions in the hydrophobicity measurement (Li-Chan et al., 1985).

Comparison of Hydrophobicity Measured Using an Uncharged Probe, PRODAN, to Those Measured Using the Anionic Probes, ANS and CPA. The surface hydrophobicity values of the three proteins measured using PRODAN are shown in the bottom panels in Figures 1−3. The pH significantly affected S_0 values in all three proteins ($p = 0.0000$), with the lowest S_0 values being found at pH 3.0. Heating had no effect on S_0 value of WPI or β-lg at pH 3.0, 5.0, or 7.0, nor for BSA at pH 3.0. Heating WPI and β-lg at pH 9.0 significantly decreased S_0 ($p \leq 0.05$). Heating of BSA at pH 5.0, 7.0, and 9.0 caused significant decreases in S_0 ($p \leq 0.05$).

PRODAN is an aromatic hydrophobic probe, similar to ANS but without an ionizable group. The general trends of protein hydrophobicity measured using PRODAN were more similar to those of ANS than to those of CPA. For example, at pH 5.0 and 7.0, both ANS and PRODAN showed higher S_0 for BSA than either WPI or β-lg. However, major differences between the PRODAN and ANS results were observed, especially at acidic and alkaline pH values. For example, using either the CPA or PRODAN method, BSA had higher hydrophobicity at pH 5.0, 7.0, and 9.0 than at pH 3.0. However, using ANS, the hydrophobicity of BSA was higher at pH 3.0 and 5.0 than at pH 7.0 or 9.0. Furthermore, heating decreased the hydrophobicity measured by PRODAN for all three proteins at pH 9.0, similar to the results observed using CPA. In contrast, hydrophobicity measured by ANS increased after heating of β-lg and WPI but decreased after heating of BSA.

Generally, heating affected S_0 values of WPI and β-lg in a similar way, except in the case of β-lg heated at pH 9.0, which showed a large decrease in S_0, using PRODAN and CPA methods, whereas a smaller decrease in S_0 was observed for WPI under similar conditions. At pH 8.0 and above, β-lg can be regarded as unstable, forming aggregates of denatured protein (Bottomley et al., 1990). β-lg is the major (~50%) protein constituent of whey (Marshall, 1982); thus, similar changes in S_0 due to effect of environmental conditions are expected. It has been shown that the thermal behavior of whey proteins is mainly governed by the properties of β-lg (de Wit, 1981). However, differences between WPI and β-lg can be attributed to different processing conditions, such as method of concentration and isolation, which may cause denaturation of proteins (Kinsella, 1976).

It has been shown that the least heat-sensitive pH range for whey proteins lies between pH 2.5 and 3.5, where proteins retain their good solubility (de Wit, 1981). This is in agreement with our results using PRODAN, ANS, and CPA, which show little or no significant change in surface hydrophobicity after heating at pH 3.0 of any of the three proteins.

It is often expected that surface hydrophobicity should increase when the molecule unfolds during heating. However, unfolding may be followed by protein aggregation, through hydrophobic interactions or through SH/SS interchange reactions (Laligant et al., 1991). These intermolecular interactions could lead to decreases in surface hydrophobicity. In other words, heating may have two different effects on the protein hydrophobicity, including unfolding of molecules, thus exposing hydrophobic sites, and heat-induced aggregation with decrease in the exposure of hydrophobic sites and then loss of solubility (Nakai and Li-Chan, 1989). Of course, food proteins also may differ in their response to heat treatment, as is the case for the proteins in this study.

At pH 3.0 and 5.0, the hydrophobicity determined by PRODAN was in the following order: BSA > WPI > β-lg. At pH 7.0, the order was BSA > β-lg > WPI. At pH 9.0, the hydrophobicity of β-lg was greatly increased, and the order was β-lg > BSA > WPI. For all three proteins, the lowest hydrophobicity values using the PRODAN method were found at pH 3.0. The low hydrophobicity of β-lg at pH 3.0 and marked increase in hydrophobicity at pH 9.0 observed using PRODAN are consistent with observations reported for this pro-

tein, indicating the higher thermostability at acidic conditions and greater susceptibility to surface denaturation at alkaline conditions (Phillips et al., 1994). It has been speculated that the β-lg molecule undergoes specific structural transitions characterized by a tighter conformation at acidic pH, compared to a more hydrophobic and flexible molecule at pH values >7.5 (Phillips et al., 1994).

In contrast, using the anionic probes ANS and CPA, hydrophobicity values were generally higher at acidic pH compared to neutral or alkaline pH. The anionic probes may interact with positively charged sites on the proteins at low pH, thus overestimating the hydrophobicity. This supports the advantage of using an uncharged probe (PRODAN) for measurement of protein surface hydrophobicity, especially under conditions of varying pH.

The nondissociable nature of the PRODAN probe is an advantage in enabling investigation of the effects of changes in protein surface hydrophobicity over a broad range of pH. Nevertheless, the electron absorption and emission transitions of PRODAN are highly sensitive to the solvent acidity (Catalan et al., 1991). Use of an appropriate blank consisting of buffer and probe (without protein) is necessary to correct for the effect of the buffer on the probe and the subsequent estimation of the protein hydrophobicity values in different buffers.

Conclusions. The uncharged aromatic fluorescent probe PRODAN may be used to determine the surface hydrophobicity of proteins over a wide range of pH values. Differences obtained in this study for the relative surface hydrophobicity values for three proteins when measured by this probe compared to two anionic probes, ANS and CPA, confirm the importance of considering not only the aromatic or aliphatic nature but also the presence or absence of a permanent charge when using fluorescent probes for measurement of protein hydrophobicity.

ABBREVIATIONS USED

ANS, 1-(anilino)naphthalene-8-sulfonic acid; CPA, *cis*-parinaric acid; PRODAN, 6-propionyl-2-(*N*,*N*-dimethylamino)naphthalene; WPI, whey protein isolate; BSA, bovine serum albumin; β-lg, β-lactoglobulin; BHA, butylated hydroxyanisole; RFI, relative fluorescence intensity; CV, coefficient of variation.

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