between protein and pectin contents.

The spectral pattern of galacturonic acids revealed wavenumbers at 1756 cm^{-1} (C=O ester) and 1608 cm^{-1} (COO^-). PCA split the broad band of the original spectra at 1645 cm^{-1} into amide I bands and a peak at 1608 cm^{-1} that was assigned to the galacturonic acids.

CONCLUSION

The similarity maps and the spectral patterns obtained by PCA give complementary information on the collection of spectra being studied. The maps enable spectra to be easily compared with each other. When the contents of different biochemical data are correlated, the observation of the similarity maps provided a global interpretation of the spectra. The directions in the PCA space characteristic of a given phenomenon are obtained by PCR. A representative spectral pattern can be associated by the described procedure to each direction so created. The spectral patterns reveal the spectral information that is significant about a particular prediction.

Middle-infrared spectroscopy is applicable to the study of the microbial degradation of natural products rich in cell wall components. Qualitative information may be obtained with the procedures presented. Previous studies (Isaksson and Naes, 1987) showed that PCR allows quantitative applications.

Registry No. Galacturonic acid, 685-73-4.

LITERATURE CITED

Association of Official Analytical Chemists. *AOAC Official Methods of Analysis*, 12th ed.; AOAC: Washington, DC, 1975; 130 pp.
Ben-Ghedalia, D.; Rubinstein, A. *J. Sci. Food Agric.* **1984**, *35*, 1159.
Ben-Ghedalia, D.; Miron, J. *J. Nutr.* **1984**, *114*, 880.
Bertrand, D.; Lila, M.; Furtoss, V.; Robert, P.; Downey, G. *J. Sci. Food Agric.* **1987**, *41*, 299.
Chesson, A.; Monro, J. A. *J. Sci. Food Agric.* **1982**, *33*, 852.
Cowe, I. A.; McNicol, J. W. *Appl. Spectrosc.* **1985**, *39*, 257.
Filippov, M. P.; Kohn, R. *Chem. Zvesti* **1975**, *29*, 88.
Filippov, M. P.; Shkolenko, G. A.; Kohn, R. *Chem. Zvesti* **1978**, *32*, 218.
Foucart, T.; Lafaye, J. Y. *Régression linéaire sur micro-ordinateurs*; Masson: Paris, 1983.
Gaillard, B. D. E. *J. Agric. Sci.* **1962**, *59*, 369.
Isaksson, T.; Naes, T. Proceedings of Euro Food Chemistry, IV, Loen-Norway, 1987; pp 509–513.
Jarrige, R., Ed. *Alimentation des Ruminants*; INRA: Versailles, 1978; pp 546–547.
Quin, J. I.; Van Der Wath, J. C.; Mybrugh, S. J. *Onderstepoort J. Vet. Res.* **1938**, *11*, 341.
Robert, P.; Bertrand, D.; Devaux, M. F. *Sci. Aliment.* **1988**, *8*, 113.
Sauvant, D.; Bertrand, D.; Giger, S. *Anim. Feed Sci. Technol.* **1985**, *13*, 7.
Theander, O.; Aman, P. *J. Sci. Food Agric.* **1980**, *31*, 31.
Thibault, J. F. *Lebensm. Wiss. Technol.* **1979**, *12*, 247.
Zundel, G.; Böhner, U.; Fritsh, J.; Merz, H.; Vogt, B. *Food Analysis, Principles and Techniques*; Gruenwedel, D. W., Whitaker, J. R., Eds.; Marcel Dekker: New York, 1984; Vol. 2, pp 438–442.

Received for review June 13, 1988. Accepted November 22, 1988.

Influence of Alcohol-Containing Spreading Solvents on the Secondary Structure of Proteins: A Circular Dichroism Investigation

David C. Clark* and Linda J. Smith

The conformational properties of a variety of proteins have been investigated in solution in the presence and absence of a propan-2-ol-based spreading solvent by circular dichroism spectroscopy. β-Casein, ovalbumin, α-lactalbumin, and β-lactoglobulin all exhibited large conformational changes consistent with the induction of α-helix into their structure. The influence of type and concentration of alcohol and temperature on the conformational properties of the "classical random protein", β-casein, has been investigated in detail. The effect of alcohol has been shown to be reversible upon dilution with biphasic kinetics. The slow rate of the second process indicates that in spreading experiments the protein is initially present at the interface in an altered conformation compared to that of adsorbed protein. This may have important consequences for the intermediate and final surface denatured state of the protein.

The physical measurement of interfacial properties of a wide variety of proteins has received considerable attention over the past few years. The principle aim of this effort has been to gain insight into the mechanism of protein stabilization of interfaces in foams and emulsions.

The most widely used approach adopted in this field has involved the study of macroscopic interfaces on Langmuir troughs. With use of this technique the formation of a protein layer at the air/water interface is possible by either of two methods, specifically by adsorption to the surface from the subphase or by spreading a protein monolayer directly onto the surface. It is the consequences of the latter technique that will be addressed in this study.

Chemical Physics Division, Institute of Food Research, Norwich Laboratory, Colney Lane, Norwich NR4 7UA, U.K.

Quantitative spreading of proteins has been most readily achieved by introducing the protein dissolved in an organic/aqueous solvent mixture as spreading solvent, where the protein is introduced to the interface with a microsyringe. Spreading from an aqueous solution is more difficult. In this case the protein solution is introduced to the surface by passing it down a perfectly wetted glass rod (Trurnit, 1960) at a moderate pH near the isoelectric point of the protein. These conditions reduce the energy barriers to adsorption.

A 60:40 propan-2-ol–water solution was first used for spreading serum albumin by Stallberg and Teorell (1939). Other workers have used the same spreading solvent with the addition of small quantities of isoamyl alcohol or sodium acetate (MacRitchie and Alexander, 1963; Harrap, 1955; Dervichian, 1939) to spread proteins such as BSA, insulin, and ovalbumin, respectively. More recently milk proteins have been spread with a 66% propan-2-ol solution

0021-8561/89/1437-0627$01.50/0

alone (Murray, 1987). Differences in the surface behavior of spread and absorbed samples of proteins can be found in the literature. For example, using β-casein spread from 66% propan-2-ol–water mixtures, Murray (1987) reported a surface pressure of 23.5 $mN \cdot m^{-1}$ from an apparent surface concentration of 2 mg/m^2 (calculated from the amount spread and the surface area). In contrast, by adsorption techniques, Graham and Phillips (1979) reported surface pressures of 16.5 $mN \cdot m^{-1}$ at the same surface concentration, measured by radiocounting. Although these differences may be considered minor (30% lower surface pressure by adsorption), they were obtained with a highly flexible protein with an easily perturbed structure. These results suggest that spread monolayers contain more completely unfolded molecules than adsorbed layers. A similar conclusion was reached in an independent study comparing spread and adsorbed lysozyme (Adams et al., 1971).

One possible explanation of these effects arises from the influence of the organic spreading solvent on protein secondary structure. Herskovits et al. (1970) have examined the denaturation of globular proteins by optical rotatory dispersion (ORD) measurements and observed an increase in α-helix content at high alcohol concentrations. Alcohol-induced changes of protein structure have been examined by other techniques. Electron microscopy has shown that addition of small amounts of alcohol to casein micelles causes the "hairy" macropeptide layer to collapse (Horne, 1984). Further reduction of the static dielectric constant by addition of more alcohol eventually leads to precipitation (Horne and Parker, 1984). The effects of longer chain alcohols on protein structure have also been examined (Sadler et al., 1984; Katzenstein et al., 1986). Propan-1-ol induces helix formation at pH 4 and 7 in α-lactalbumin and β-lactoglobulin; however, quantitative measurements were not undertaken. The presence of the alcohol produced little effect in lysozyme and ribonuclease. Reversibility at pH 7 was found to be >80% for the affected proteins although the kinetics of this process were not reported. 2-Chloroethanol induces helix formation in α_s-casein and the whey proteins from milk. This conformational change has been interpreted in terms of the weakening and destruction of hydrophobic regions in the native protein (Herskovits and Mescanti, 1965).

In this paper we describe a series of circular dichroism experiments showing the effect of different alcohols, alcohol concentration, and temperature on the secondary structure of a variety of proteins. We have particularly concentrated on β-casein as this is the "classic" unstructured, random protein (Graham and Phillips, 1979) and, therefore, may be highly susceptible to alcohol-induced conformational change.

Finally, when spreading solvents are used to form protein layers at the air/water interface, it is assumed that any alcohol-induced changes in the secondary structure of the protein are reversible. We have examined this question in detail and include results obtained from rapid dilution experiments demonstrating the time dependence of the observed changes.

MATERIALS AND METHODS

All the protein samples and the 2,2,2-trifluoroethanol were purchased from Sigma Chemical Co. SDS–polyacrylamide gel electrophoresis was used to determine that the samples were free from protein contaminants. The ethanol and propan-2-ol were BDH Spectrosol grade. All other chemicals were Analar grade from British Drug Houses.

Circular dichroism (CD) spectra were measured on a Jasco J600 spectropolarimeter and recorded on-line with an IBM PC. Samples were made up in 10 mM sodium phosphate buffer, pH 7.5, and contained appropriate levels of various alcohols in the concentration range from 0 to 66% by volume. The final protein concentration was maintained at approximately 1 mg/mL. The spectra were recorded with silica quartz demountable cells of 0.1-mm path length at either 20 or 6 °C, and temperature regulation was achieved by a thermostatically controlled Grant heater/cooler system circulating water through the cell holder. Spectra were recorded in the far-UV region 260–190 nm) at a sensitivity of ±20 mdeg full scale and a time constant of 4 s. The data are presented as molar circular dichroism ($\Delta\epsilon$) based on a mean amino acid residue molecular weight of 113. Molar ellipticity may be calculated from

$$[\theta]_\lambda = 3300\Delta\epsilon \quad (1)$$

Analysis of the secondary-structure content of the spectra was performed on a VAX computer, using the Contin program of Provencher and Glockner (1981). This method is most accurate for determination of α-helix with a correlation coefficient between X-ray and circular dichroism methods of 0.96 for proteins used in the data bank. β-Sheet and random coil are determined with less certainty: the correlation coefficient for random was 0.49.

Stopped-flow CD measurements were made on a Hi-tech SFA-II rapid kinetics accessory. The experiment involved the rapid dilution of a sample of β-casein (0.1 mg/mL) in 10 mM sodium phosphate buffer, pH 7.5, containing 25% (v/v) trifluoroethanol (TFE) into phosphate buffer. The final concentrations of β-casein and trifluoroethanol after mixing were 0.05 mg/mL and 12.5% (v/v), respectively. The path length of the stopped-flow cell was 2 mm. Circular dichroism was measured as a function of time at a wavelength of 222 nm with a time constant of 0.25 s and a sensitivity of ±5 mdeg full scale. Data points were collected every 0.5 s. Instrument calibration was regularly checked during the course of the study with ammonium camphor-d_{10}-sulfonate (Takakuwa et al., 1985) and d-(−)-pantolactone (Konno et al., 1975).

The data were analyzed off-line on a VAX computer by a nonlinear least-squares fit to a two exponential expression (eq 2) with algorithms described previously. SDS–polyacrylamide

$$A(t) = A_0 - A_1 \exp(-k_{obs1}, t) - A_2 \exp(-k_{obs2}, t) \quad (2)$$

gel electrophoresis was performed according to the method of Laemmli (1970), except that the gel buffers contained 6 M urea rather than 0.2% SDS and the electrophoresis buffer was adjusted to pH 9.1 with NaOH.

Protein concentrations were measured spectrophotometrically on a Perkin-Elmer Lambda 9 UV/vis/near-IR spectrophotometer. Absorbance coefficients of 0.66 mL mg^{-1} cm^{-1} at 280 nm for BSA (Daniel and Weber, 1966), 0.46 mL mg^{-1} cm^{-1} at 278 nm for β-casein (Graham et al., 1984), 0.96 mL mg^{-1} cm^{-1} at 278 nm for β-lactoglobulin (Bell et al., 1970a), 2.02 mL mg^{-1} cm^{-1} at 281.5 nm for α-lactalbumin (Bell et al., 1970b), 0.668 mL mg^{-1} cm^{-1} at 280 nm for ovalbumin (Ansari et al., 1975), and 2.58 mL mg^{-1} cm^{-1} at 280 nm for lysozyme (Halford, 1975) were used.

RESULTS

The far-UV circular dichroism properties of a variety of proteins have been investigated, and the spectra are shown in Figure 1. In each panel, the spectrum of the native protein in 10 mM sodium phosphate, pH 7.5, is compared with that obtained in the presence of propan-2-ol. In most cases, we examined the effect of exposure of the protein to 66% propan-2-ol; however, for lysozyme and bovine serum albumin this concentration resulted in protein precipitation, and thus the alcohol concentration was reduced to tolerable levels of 40% and 50%, respectively. Examination of the spectra reveals that, with the exception of lysozyme and BSA, all the proteins behave in a qualitatively similar manner upon exposure to the alcohol with the development of negative minima at 222 and 208 nm consistent with an increase in α-helical structure.

The spectra were analyzed for secondary-structure content by the Contin method (Provencher and Glockner, 1981), and the results are shown in Table I. In the absence

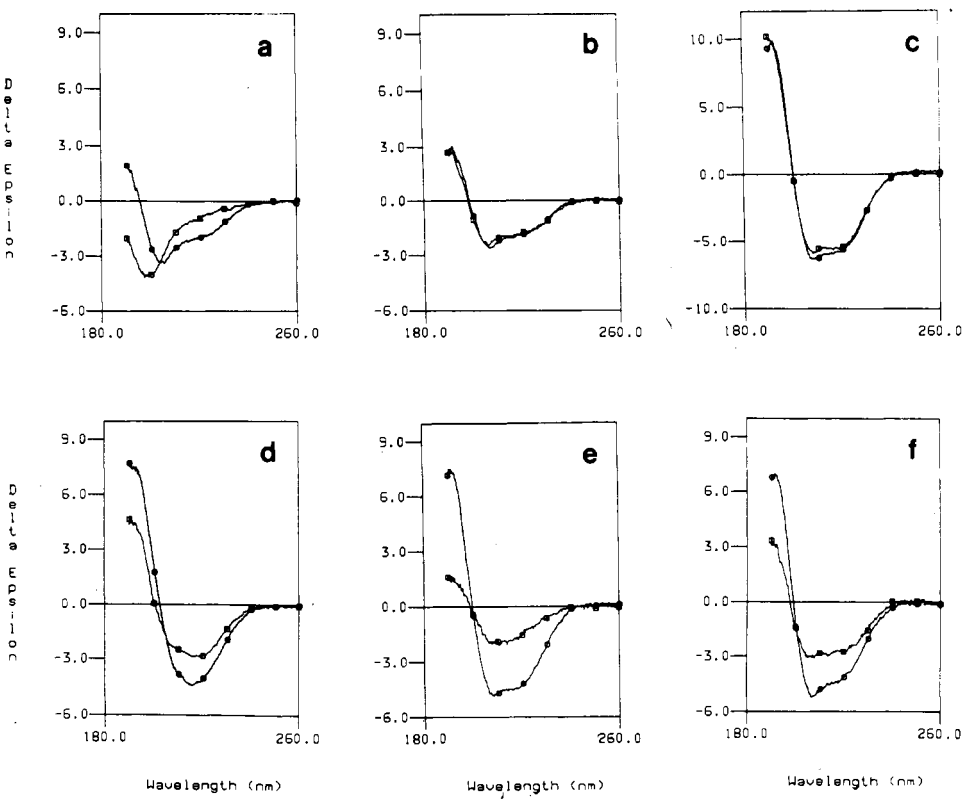

Figure 1. Far-UV circular dichroism spectra of a range of proteins in the presence and absence of propan-2-ol: (a) β-casein (□) 0% and (○) 66% propan-2-ol; (b) lysozyme (□) 0% and (○) 40% propan-2-ol; (c) bovine serum albumin (□) 0% and (○) 50% propan-2-ol; (d) ovalbumin (□) 0% and (○) 66% propan-2-ol; (e) lactoglobulin (□) 0% and (○) 66% propan-2-ol; (f) lactalbumin (□) 0% and (○) 66% propan-2-ol. All protein solutions were made up in 10 mM sodium phosphate buffer, pH 7.5. Spectra were recorded at 20 °C.

Table I. Contin Analysis of Far-UV Circular Dichroism Spectra of Proteins in the Presence and Absence of Propan-2-ol at 20 °C[a]

protein	% P-2-ol[b]	helix, %	sheet, %	random, %
β-casein	0	5 (0.5)	42 (0.8)	53 (0.9)
	66	18 (0.7)	39 (1.0)	42 (1.1)
lysozyme	0	24 (1.6)	59 (1.8)	17 (3.0)
	40	23 (1.6)	58 (1.8)	19 (3.0)
BSA	0	50 (0.9)	34 (1.4)	16 (1.6)
	50	54 (1.1)	46 (1.2)	0 (0.0)
ovalbumin	0	23 (1.5)	63 (1.7)	14 (2.9)
	66	29 (1.6)	49 (1.8)	21 (3.0)
lactoglobulin	0	16 (2.1)	58 (2.4)	25 (3.9)
	66	41 (2.0)	53 (2.2)	7 (3.7)
lactalbumin	0	24 (1.1)	41 (1.6)	35 (1.9)
	66	47 (1.0)	53 (1.0)	0 (0.0)

[a] Standard errors are shown in parentheses. [b] P-2-ol is propan-2-ol.

of alcohol, β-casein (Figure 1a) is composed of an approximately 50:50 mix of β-sheet and random coil with only a minor contribution from α-helix. We consistently see considerably more β-structure in β-casein than predicted by the Chou and Fasman algorithm using the sequence (Swaisgood, 1982) or by previous CD work analyzed by the method of Brahms and Brahms (1980). However, our results are in good agreement with previous CD data analyzed by the method of Provencher and Glockner (1981) as reported by Graham et al. (1984). Upon addition of 66% propan-2-ol the protein increases its helical content from 5 to 18% mainly at the expense of the random-coil component. Lysozyme (Figure 1b) is a highly structured, compact, globular protein and does not exhibit any detectable change in secondary structure upon exposure to 40% propan-2-ol. There is also very little qualitative change observed in the spectrum of BSA upon exposure to 50% alcohol (Figure 1c); however, the Contin analysis shows that both α-helix and β-sheet components increase by approximately 5 and 10% of the total structure, respectively, at the expense of the random coil present in the structure in aqueous solution. This is very close to the expected experimental error and therefore may not be significant. Ovalbumin shows an increase in helical fraction in propan-2-ol apparently at the expense of the β-sheet fraction of the protein structure (Figure 1d). The largest structural changes are observed with the two whey proteins from milk, namely β-lactoglobulin and α-lactalbumin (Figure 1e, f). This is evident from examination of the spectra and from the Contin analysis (Table I). Both proteins show dramatic increases in helical content of approximately 25% of the total structure, and this principally arises from apparent induction of structure into the random elements of the protein present in aqueous solution.

These results indicate that the secondary structure of the majority of proteins is altered in the presence of a commonly used, alcohol-containing, spreading solvent. If we were to examine the interfacial properties of one of the proteins susceptible to a structural change in alcohol, the initial conformation of the protein at the start of the experiment would be dependent on whether the protein was spread on the interface from a solvent or adsorbed at the interface from an aqueous subphase (i.e., the conformation of β-casein, α-lactalbumin, β-lactoglobulin, and ovalbumin in the spreading solvent contains more α-helix than that in aqueous solution). This difference in initial conformation may influence the course and extent of surface denaturation observed and alter surface pressure and concentration profiles (Murray, 1987; Graham and Phillips, 1979).

To investigate this possibility further, we examined the influence of alcohols in more detail. To simplify matters, we have concentrated on one of the proteins investigated

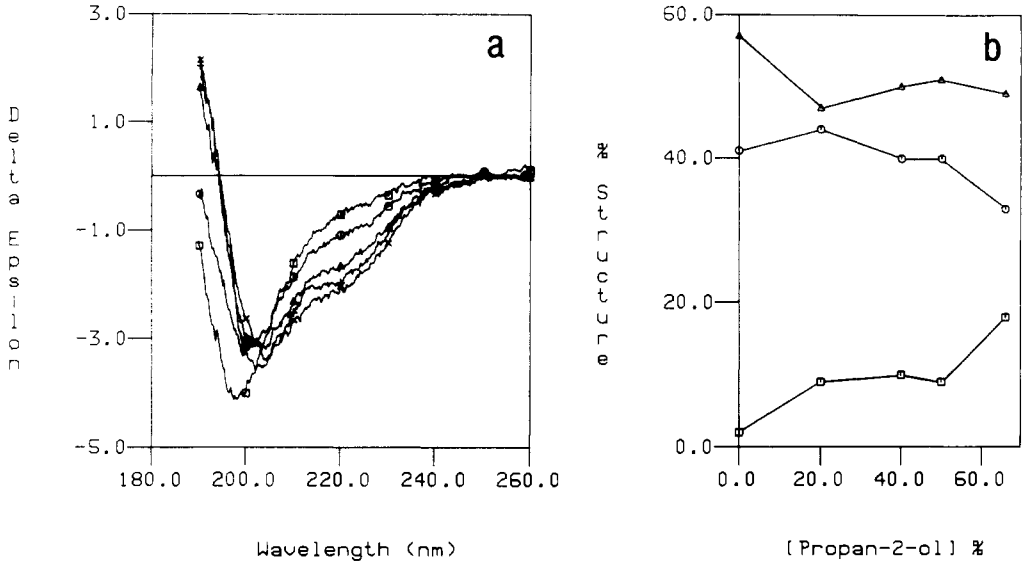

Figure 2. Effect of propan-2-ol on the secondary structure of β-casein at 20 °C. (a) Far-UV circular dichroism spectra as a function of propan-2-ol concentration: □, 0%; ○, 20%; △, 40% +, 50%; ×, 66% propan-2-ol. (b) Contin analysis for β-casein spectra shown in (a): □, α-helix; ○, β-sheet; △, aperiodic. Protein concentration was 1.011 mg/mL.

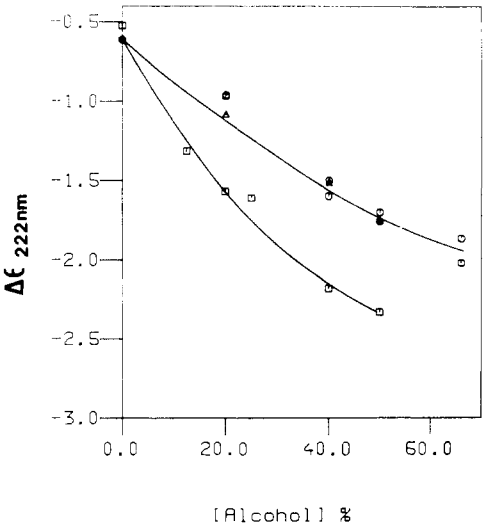

Figure 3. Effect of different alcohols on the secondary structure of β-casein: □, 2,2,2-trifluoroethanol; ○, propan-2-ol; △, ethanol.

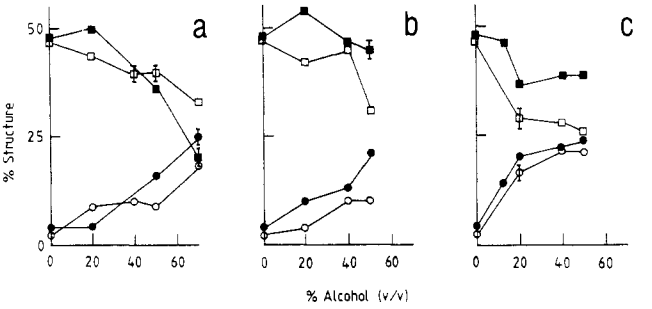

Figure 4. Effect of temperature on the secondary structure of β-casein as a function of alcohol concentration: ●, ■, helix and sheet present 6 °C; ○, □, helix and sheet at 20 °C. Solvents: (a) propan-2-ol; (b) ethanol; (c) TFE. Errors bars are included where the standard deviation exceeded the size of the symbol.

in Figure 1, namely β-casein. This protein was chosen because its interfacial properties have been studied by several groups, and historically, it has always been referred to as the "classical random protein" (Graham and Phillips, 1979).

The influence of the concentration of propan-2-ol on the far-UV CD spectrum of β-casein is shown in Figure 2a. Inspection of the spectra in the 222-nm region shows an increase in negative intensity with increasing alcohol concentration. This region of the spectrum is sensitive to contributions from α-helix in the protein. The results of secondary-structure analysis of the spectra by the Contin method are plotted in Figure 2b. The α-helix component of the structure increases with propan-2-ol concentration whereas both β-sheet and random-coil contributions decrease with increasing alcohol.

These effects are not limited to the influence of propan-2-ol on the secondary structure of β-casein. Similar experiments have been performed with other alcohols including ethanol and trifluoroethanol, and the results are presented in Figure 3. In this figure the CD intensity at 222 nm ($\Delta\epsilon$(222 nm)) is plotted as a function of alcohol concentration. Examination of the plots shows that propan-2-ol, a secondary alcohol, behaves in a manner similar to that of ethanol. In contrast, trifluoroethanol induces a much larger negative increase in intensity than equivalent levels of propan-2-ol and ethanol and is therefore considerably more effective in promoting the formation of helical structure.

β-Casein forms micellar aggregates at temperatures exceeding 6 °C (Swaisgood, 1982). At temperatures below this threshold the protein exists primarily as a monomer. Thus, most interfacial studies have been performed on the protein in its micellar form as examined in Figures 1–3; however, we thought it would be of interest to examine the effect of temperature and hence protein aggregation on the effectiveness of alcohols at causing conformational changes. The Contin analysis of the spectra obtained is presented in Figure 4.

All the alcohols behave in a similar manner and cause an increase in the negative signal at 222 and 208 nm signifying an increase in helix content with increasing concentration of the alcohol. Ethanol and propan-2-ol show a similar concentration dependence for this effect, the extent of which appears greater at 6 than 20 °C. TFE induces more helix at lower concentrations than the other alcohols, and this correlates with the more negative intensities at 222 nm observed with this alcohol as shown in Figure 3. Only a very minor and possibly insignificant temperature dependence of α-helix formation was detected with TFE.

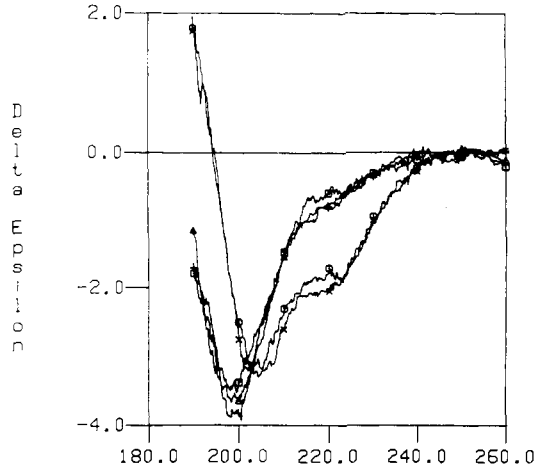

Figure 5. Reversibility of secondary structure induced by treatment of β-casein with 66% propan-2-ol: □, β-casein stock in buffer; ○, β-casein in 66% propan-2-ol; △, as ○ but after 5-h evaporation to remove propan-2-ol; +, control-1, β-casein after 5-h incubation at room temperature; X, control-2, β-casein incubated at room temperature in 66% propan-2-ol without evaporation.

Identification of the origin of the temperature dependence of the secondary-structure content as a function of alcohol concentration is complicated since the alcohol may induce association or dissociation of the casein micelles independently. For example, in experiments with α_s-casein C, Swaisgood and Timasheff (1968) observed a monomer to trimer transition upon increasing the methanol concentration from 26 to 56%. Thus, more detailed interpretation of these results must await data concerning the state of aggregation of β-casein micelles as a function of alcohol concentration.

Finally, we have addressed the question of the reversibility of the alcohol-induced conformational change observed with β-casein. The experiment was performed with a series of controls in order to ensure that any irreversible changes that were observed could be attributed to the influence of the alcohol and not time-dependent changes in the protein. The experiment was conducted in the following manner: A sample of β-casein was prepared in 10 mM sodium phosphate buffer, pH 7.5, and the far-UV CD spectrum was recorded. The sample was then divided into two fractions. One fraction (control-1) was retained and stored at room temperature; the other fraction was made 66% in propan-2-ol, and its far-UV CD spectrum was recorded. This sample was then divided into two fractions. One fraction (control-2) was stored in a sealed vessel such that the propan-2-ol could not evaporate away, while the second fraction was placed on a watch glass and the propan-2-ol was allowed to evaporate over a period of 5 h. After this period the weight of the sample had reduced to a plateau level consistent with the evaporation of >98% of the propan-2-ol. At this stage, the far-UV CD spectra of this sample and the two controls were recorded and the data analyzed after adjustments had been made to compensate for changes in the protein concentration where necessary.

The spectra obtained are presented in Figure 5 and fall into two distinct categories: First, three samples have spectra similar to that observed previously for β-casein in aqueous solution (Figure 2, □), and Contin analysis reveals that they contain β-sheet (40–50%) and random coil. This category includes the spectrum of the stock protein solution at time zero, control-1, and the β-casein after evaporation of the alcohol. Only some minor variations in the intensities of these spectra are observed in the 222-nm spectral region. Second, the spectra include that of the protein in the presence of propan-2-ol and control-2 (also in propan-2-ol). These spectra clearly exhibit enhanced contributions from α-helix estimated at 18% by Contin analysis. It would appear from these data that the effect of exposure to 66% propan-2-ol is reversible although some changes in the β-sheet and random-coil contributions are present. Also minor time-dependent changes are observed in the control samples. Sadler et al. (1984) reported that propan-1-ol-induced conformational changes observed in a number of globular proteins also showed a high degree of reversibility.

Table II. Kinetic Data for the Conformational Change on Dilution of β-Casein–TFE Mixtures at 20 °C[a]

time base, s	K_{obs1}, s^{-1}	A_1, Δε	K_{obs2}, s^{-1}	A_2, Δε	A_0, Δε
500	0.068	−1.91	0.0040	1.62	−0.87
	(0.019)	(0.25)	(0.0008)	(0.07)	(0.10)
150	0.067	−1.81	0.0040[b]	1.62[b]	−1.18
	(0.012)	(0.19)			(0.10)

[a] Standard errors are shown in parentheses. [b] Parameter was held constant at the indicated value. The results shown were the averages of eight determinations.

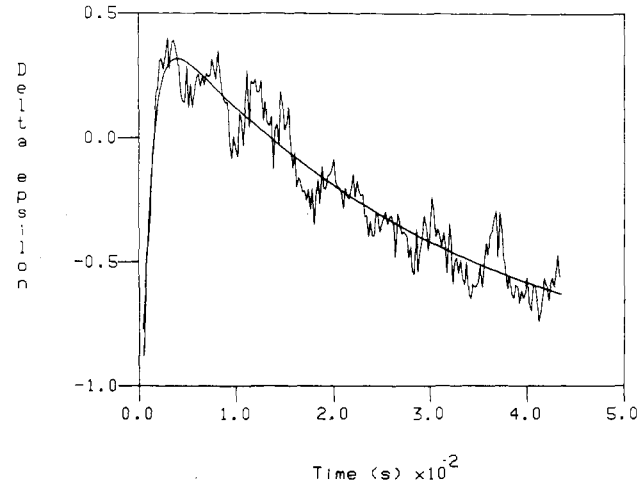

Figure 6. Stopped-flow circular dichroism trace for rapid dilution of β-casein in 25% 2,2,2-trifluoroethanol to 12.5% 2,2,2-trifluoroethanol at 20 °C. The experimental curve and computed biexponential fit are both shown on the plot. Returned values for the fit were as follows: k_{obs1} = 0.097 s^{-1}; A_1 = −2.08; k_{obs2} = 0.032 s^{-1}; A_2 = 1.56; plateau = −1.01. Total time 500 s.

Given that the effect of alcohol appeared to be reversible, we examined the kinetics of this process. Propan-2-ol has significant absorbance in the far-UV. Since the optical cell used for stopped flow had a path length of 2 mm, the experiments were conducted with trifluoroethanol, which has negligible absorption in this region. Stopped-flow experiments were performed to examine the kinetics of the conformational change observed upon dilution of the alcohol from 25% to 12.5%. The results of the experiments are shown in Table II, and a typical stopped-flow trace is presented in Figure 6.

The observed conformational change upon dilution was biphasic with a fast positive process followed by a slow negative process. The experiments were performed over two different time domains to obtain accurate data for both phases. Initially, data were collected over a period of 500 s (Figure 6) and were fitted with a two-exponential expression with five variables (Table II). This gave poor fits to the fast process but good fits to the slow process. Having determined the rate and amplitude of the slow process as 0.0040 ± 0.0008 s^{-1} and (1.62 ± 0.07)Δε, the time

base was reduced and data were collected for a period of 150 s. Again these data were fitted with a two-exponential expression, but in this case the parameters describing the slow process were held constant at the values quoted above and the fast process was fitted using three variables (K_{obs1}, A_1, A_0).

Several control experiments were performed to ensure that the observed CD signal was not an artifact. No transient was observed when 25% TFE was mixed with phosphate buffer nor when β-casein was diluted into buffer in the absence of TFE.

Examination of the transient suggests that initially there is a large and rapid reduction in the levels of helix present in the protein upon dilution of the alcohol, followed by a slow re-formation of helix. The estimation of the plateau value of the process (A_0) of −0.87 to −1.18 is consistent with β-casein in 12.5% TFE (Figure 3). The positive CD signal of approximately 0.75 (i.e. −0.87 + 1.62) observed at the end of the fast phase is consistent with the formation of significant amounts of random coil during this process. Indeed a positive value for $\Delta\epsilon$ at 222 nm is indicative of the complete absence of α-helix in the sample. Thus, the reduction in α-helix in β-casein following dilution of the alcohol appears to follow an unusual mechanism whereupon rather than the helix content decreasing in proportion to the reduction in alcohol concentration in a single step, the mechanism is biphasic with an initial complete loss of helix followed by helix re-formation to a level in proportion to the new alcohol concentration. It is notable however that the amplitude of the fast phase is probably subject to significant error as it was impossible to accurately define time zero in these experiments. Thus, the conformational change observed in the presence of 25% TFE is reversible upon dilution to 12.5% TFE although the kinetics of the transition are rather slow.

DISCUSSION

Data are presented showing significant conformational changes in β-casein, ovalbumin, α-lactalbumin, and β-lactoglobulin upon exposure to a commonly used spreading solvent. These results may at least in part serve to explain some inconsistencies observed when the interfacial properties of these proteins have been compared by adsorption and spreading techniques (Murray, 1987; Graham and Phillips, 1979; Adams et al., 1971; Mitchell et al., 1970).

The major part of this study has focused on the influence of a variety of alcohols on β-casein. It has been demonstrated that a spreading solvent containing propan-2-ol alters the secondary structure of β-casein to the same extent as comparable levels of ethanol. It would therefore appear that propan-2-ol is less effective at perturbing protein structure than propan-1-ol (Griffin et al., 1986) and considerably less effective than TFE, which is well-known for its helix-promoting properties (Bayley et al., 1983). Nonetheless, its effect could not be considered insignificant. The influence of alcohol on β-casein secondary structure is somewhat sensitive to temperature but may also affect state of aggregation, especially at high alcohol concentrations.

The alcohol-induced structural change in β-casein appears to be completely reversible within the limits of experimental error; however, some minor time-dependent changes in the protein concentration were observed. The kinetics of the conformational change were biphasic. The slow rate of the second process means that the protein will not revert back to its native conformation immediately after the spreading solvent has evaporated. The initial conformation of a protein, prior to arrival or delivery at the interface, determines its effect on surface tension. Comparison of native and heat-denatured α-lactalbumin and β-lactoglobulin revealed significant differences in the surface pressure observed for coverages of 2 mg/m^2 in both spreading and adsorption experiments (Mitchell et al., 1970). Proteins undergo large conformational changes upon arrival at an interface. The time scale of these changes is slow. Graham and Phillips (1979) observed that the kinetics of surface pressure–time and surface concentration–time isotherms for several proteins did not follow the same time course. The slow increase in surface pressure over a period of several hours was attributed to conformational changes in the adsorbed protein. Spread proteins also show a time dependence or effect of aging in their surface pressure–surface concentration plots (Murray, 1987). Thus, the initial altered conformation of the protein spread from alcohol may influence the course and extent of surface denaturation observed and therefore alter surface pressure and surface concentration profiles. Further experiments are being designed to investigate the conformation of the protein in situ at the interface.

Registry No. Lysozyme, 9001-63-2; ethanol, 64-17-5; 2-propanol, 67-63-0; 2,2,2-trifluoroethanol, 75-89-8.

LITERATURE CITED

Adams, D. J.; Evans, M. T. A.; Mitchell, J. R.; Phillips, M. C.; Rees, P. M. Adsorption of lysozyme and some acetyl derivatives at the air-water interface. *J. Polym. Sci., Part C* **1971**, *34*, 167–179.

Ansari, A. A.; Kidwai, S. P.; Salahuddin, A. Acetylation of amino groups and its effect on the conformation and immunological activity of ovalbumin. *J. Biol. Chem.* **1975**, *250*, 1625–1632.

Bayley, P. M.; Clark, D. C.; Martin, S. R. Conformational properties of microtubule protein: Their relation to the self-assembly process *in vitro*. *Biopolymers* **1983**, *22*, 87–91.

Bell, K.; McKenzie, H. A.; Murphy, W. H.; Shaw, D. C. β-Lactoglobulin (Droughtmaster): A unique protein variant. *Biochim. Biophys. Acta* **1970**, *214*, 427–436.

Bell, K.; Hopper, K. E.; McKenzie, H. A.; Murphy, W. H.; Shaw, D. C. A comparison of bovine α-lactalbumin A and B of droughtmaster. *Biochim. Biophys. Acta* **1970b**, *214*, 437–444.

Brahms, S.; Brahms, J. Determination of protein secondary structure in solution by vacuum ultraviolet circular dichroism. *J. Mol. Biol.* **1980**, *138*, 149–178.

Daniel, E.; Weber, G. Co-operative effects in binding by bovine serum albumin I. The binding of 1-Anilino-8-naphthalene sulphonate. Fluorimetric titrations. *Biochemistry* **1966**, *6*, 1893–1900.

Dervichian, D. A new technique for the spreading of proteins and the 'Spreading number'. *Nature* **1939**, *144*, 629–630.

Graham, D. E.; Phillips, M. C. Proteins at liquid interfaces. I-III Molecular structures of adsorbed films. *J. Colloid Interface Sci.* **1979**, *70*, 403–439.

Graham, A. R. B.; Malcolm, G. N.; McKenzie, H. A. On the isolation and conformation of β-casein A. *Int. J. Biol. Macromol.* **1984**, *6*, 155–161.

Griffin, M. C. A.; Price, J. C.; Martin, S. R. The effect of alcohols on the structure of caseins. Circular dichroism studies of κ-casein. *Int. J. Biol. Macromol.* **1986**, *8*, 367–371.

Halford, S. E. Stopped flow fluorescence studies on saccharide binding to Lysozyme. *Biochem. J.* **1975**, *149*, 411–422.

Harrap, B. S. The molecular weight of insulin, determined by the surface balance technique. *J. Colloid Sci.* **1955**, *10*, 351–361.

Herskovits, T. T.; Mescanti, L. Conformation of proteins and polypeptides. II Optical rotary dispersion and conformation of the milk proteins and other proteins in organic solvents. *J. Biol. Chem.* **1965**, *240*, 639–644.

Herskovits, T. T.; Gadegbeku, B.; Jaillet, H. On the structural stability and solvent denaturation of proteins. *J. Biol. Chem.* **1970**, *245*, 2588–2598.

Horne, D. S. Steric affects in the congulation of casein micelles by ethanol. *Biopolymers* **1984**, *23*, 989–993.

Horne, D. S.; Parker, T. G. Factor effecting the ethanol stability of bovine casein micelles: 3. Substitution of ethanol by other organic solvents. *Int. J. Biol. Macromol.* **1984**, *3*, 399–402.

Katzenstein, G. E.; Vrona, S. A.; Wechsler, R. J.; Steadman, B. L.; Lewis, R. V.; Middaugh, C. R. Role of conformation changes in the elution of proteins from reversed phase HPLC columns. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 4268–4272.
Konno, T.; Meguro, H.; Tuzimura, K. D-pantolactone as a circular dichroism (CD) calibration. *Anal. Biochem.* **1975**, *67*, 226–232.
Laemmli, U. K. *Nature* **1970**, *227*, 680–685.
MacRitchie, F.; Alexander, A. E. Kinetics of adsorption of proteins at interfaces. Part 1. The role of bulk diffusion in adsorption. *J. Colloid Sci.* **1963**, *18*, 453–457.
Mitchell, J. R.; Irons, L.; Palmer, G. J. A study of the spread and adsorbed films of milk proteins. *Biochim. Biophys. Acta* **1970**, *200*, 138–150.
Murray, E. K. Interfacial behaviour of protein mixtures at air-water interfaces. *Food Emulsions and Foams*; Dickinson, E., Ed.; The Royal Society of Chemistry: London, 1987; pp 170–187.
Provencher, S. W.; Glockner, J. Estimation of globular protein secondary structure from circular dichroism. *Biochemistry* **1981**, *20*, 33–37.
Sadler, A. J.; Micanovic, R.; Katzenstein, G. E.; Lewis, R. V.; Middaugh, C. R. Protein conformation and reversed phase high-performance liquid chromatography. *J. Chromatogr.* **1984**, *317*, 93–101.
Stallberg, S.; Teorell, T. A new method of spreading monolayers of albumin and lipoid-albumin mixtures. *Trans. Faraday Soc.* **1939**, *35*, 1413–1420.
Swaisgood, H. E. Chemistry of milk protein. In *Developments in dairy chemistry 1*; Fox, P., Ed.; Applied Science: London, 1982; pp 1–59.
Swaisgood, H. E.; Timasheff, S. N. Association of α_s-Casein C in the alkaline pH range. *Arch. Biochem. Biophys.* **1968**, *125*, 344–361.
Takakuwa, T.; Konno, T.; Meguro, H. A new standard substance for calibration of circular dichroism: Ammonium d-10-camphorsulphonate. *Anal. Sci.* **1965**, *1*, 215–218.
Trurnit, H. J. A theory and method for the spreading of protein monolayers. *J. Colloid Sci.* **1960**, *15*, 1–13.

Received for review August 31, 1987. Revised manuscript received June 13, 1988. Accepted October 14, 1988.

Effect of Storage Temperatures on the Formation of Disulfides and Denaturation of Tilapia Hybrid Actomyosin (*Tilapia nilotica* × *Tilapia aurea*)

Shann-Tzong Jiang,* Pau-Chen San, and Lenda Sudarty Japit

Effects of frozen storage temperatures on the formation of disulfides and denaturation of actomyosin (AM), extracted from tilapia hybrid (*Tilapia nilotica* × *Tilapia aurea*) dorsal muscle, were investigated. The activities of Ca-ATPase and Mg(Ca)-ATPase, Ca sensitivity, and AM solubility in 0.6 M KCl decreased at a higher rate at −20 °C than at −40 °C. The insoluble proteins increased at a much higher rate at −20 °C than at −40 °C. The total SHs of sample at −20 °C significantly decreased during freezing and subsequent storage. However, the total SHs at −40 °C slightly decreased during the early 2 weeks of storage, and no significant changes were observed during prolonged storage. This suggested that more disulfides formed in samples at −20 °C than at −40 °C.

Among the factors affecting the stability of fish muscle proteins, the nature of fish muscle and freezing and storage temperatures are considered to be the most important factors (Arai et al., 1973; Arai, 1977; Suzuki et al., 1964, 1965; Suzuki, 1967; Hatano, 1968; Tokiwa and Matsumiya, 1969; Seki and Hasegawa, 1978; Fukuda et al., 1981; Fukuda, 1986; Jiang, 1977; Matsumoto, 1980). The stability of muscle proteins of tilapia hybrid during frozen storage at various temperatures was greater than that of milkfish (Tsai et al., 1989). The inactivation rate constant (K_D) of actomyosin (AM) Ca-ATPase of frozen mackerel at −15 °C was 5-fold that at −40 °C (Fukuda, 1986). The stability of AM of cod, halibut, plaice, and rosefish was greater when stored at −23 °C than at −12 and −18 °C (Dyer and Morton, 1956; Dyer et al., 1956). Jiang (1977) and Jiang et al. (1985) reported that mullet and amberfish muscle proteins were much more stable when stored at −40 °C than at −20 °C. No significant changes in extractability, sedimentation constant, and intrinsic viscosity of AM were found in fish muscle frozen by liquid nitrogen (Dyer, 1951; Segran, 1956; Suzuki et al., 1964, 1965; Noguchi and Matsumoto, 1970). However, protein denaturation occurred during frozen storage when the storage temperature was not low enough (Suzuki et al., 1965). The freeze denaturation of AM was found to be dominantly caused by formation of disulfide, hydrogen, and hydrophobic bonds during freezing and storage (Jiang et al., 1988a). More disulfides formed and denaturation occurred in milkfish AM frozen at −20 °C than at −35 °C (Jiang et al., 1988b).

This study aimed to investigate the effects of storage temperature and added reductants ($NaBH_4$ and $NaNO_2$) on the formation of disulfides, solubility, and ATPase activity of freeze–thawed tilapia hybrid AM.

MATERIALS AND METHODS

Preparation of the Actomyosin. Actomyosin was extracted from tilapia hybrid (*Tilapia nilotica* × *Tilapia aurea*) dorsal muscle according to Noguchi and Matsumoto (1970). To investigate the effects of storage temperatures on AM, 30 mL of extracted AM (4.80 mg/mL) was placed in plastic tubes, stoppered, frozen, and stored at −20 and −40 °C for 12 weeks. At definite time intervals, samples were removed, thawed to 0 °C with running tap water (about 25 °C), and subjected to the following analyses.

Department of Marine Food Science, National Taiwan College of Marine Science & Technology, Keelung, Taiwan 20224, R.O.C.

0021-8561/89/1437-0633$01.50/0