Guinier, A., & Fournet, G. (1955) *Small-Angle Scattering of X-Rays*, p 126, John Wiley and Sons, New York.
Igarashi, T., Sato, M., Katsube, Y., Takio, K., Tanaka, T., Nakanishi, M., & Arata, Y. (1990) *Biochemistry 29*, 5727-5733.
Kashiwazaki, Y., & Ikai, A. (1985) *Arch. Biochem. Biophys. 237*, 160–169.
Katagiri, C. (1985) *Biochim. Biophys. Acta 834*, 139–143.
Katagiri, C., & Chino, H. (1973) *Insect Biochem. 3*, 429–437.
Katagiri, C., & de Kort, C. A. D. (1991) *Comp. Biochem. Physiol., B: Comp. Biochem.* (in press).
Katagiri, C., Kimura, J., & Murase, N. (1985) *J. Biol. Chem. 260*, 13490–13495.
Katagiri, C., Sato, M., & Tanaka, N. (1987) *J. Biol. Chem. 262*, 15857–15861.
Laggner, P., & Müller, K. W. (1978) *Q. Rev. Biophys. 11*, 371–425.
Pilz, I., Glatter, O., & Kratky, O. (1979) *Methods Enzymol. 61*, 175.
Robbs, S. L., Ryan, R. O., Schmidt, J. O., Keim, P. S., & Law, J. H. (1985) *J. Lipid Res. 26*, 241–247.
Shapiro, J. P., Keim, P. S., & Law, J. H. (1984) *J. Biol. Chem. 259*, 3680–3685.
Shapiro, J. P., Law, J. H., & Wells, M. A. (1988) *Annu. Rev. Entomol. 33*, 297–318.
Stuhrmann, H. B. (1974) *J. Appl. Crystallogr. 7*, 173–178.

Fourier Transform Infrared Spectroscopic Studies of Ca^{2+}-Binding Proteins†

Michael Jackson,*,‡ Parvez I. Haris,§ and Dennis Chapman§

Steacie Institute for Molecular Sciences, National Research Council of Canada, Ottawa, Ontario K1A 0R6, Canada, and Department of Protein and Molecular Biology, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, U.K.

Received January 28, 1991; Revised Manuscript Received June 14, 1991

ABSTRACT: The secondary structures of calmodulin and parvalbumin are well established from X-ray diffraction and nuclear magnetic resonance spectroscopic studies, which indicate that these proteins are predominantly α-helical in character. Recent infrared studies have nevertheless suggested that the helical structures present in these proteins in solution are not the standard α-helix but rather some kind of distorted helices [Trewhella, J., et al. (1989) *Biochemistry 28*, 1294]. The evidence for this was the unusually low amide I frequency for calmodulin and troponin C in ^2H_2O solution. The studies presented here, however, suggest that the helical structures in these proteins are not significantly distorted, for two reasons. First, distorted helical structures have weaker hydrogen bonds than the standard α-helix and would therefore be expected to absorb at a higher rather than a lower frequency. Second, distorted helical structures would absorb at an unusual frequency in H_2O solutions which is not the case for the proteins studied here. The band frequency of these proteins is observed to occur at a frequency observed with other proteins known to contain predominantly α-helical structures. Quantitative analysis of the FT-IR spectra of calmodulin (67% α-helix) and parvalbumin (68% α-helix) in H_2O in the presence of Ca^{2+} gives helical contents similar to those reported by X-ray studies. This raises the question as to why these proteins in H_2O show a normal frequency for the presence of α-helical structures and an abnormal frequency in ^2H_2O. Addition of deuterated glycerol to the proteins in ^2H_2O solutions results in a significant shift of absorbance to higher frequency. This is consistent with dehydration of the protein taking place. Circular dichroism spectra of the proteins in glycerol show no evidence of any structural rearrangements. The unusually low amide frequency for these proteins in ^2H_2O is interpreted on the basis of an unusual degree of solvent interaction with exposed helical structures, lowering the amide I maximum only in ^2H_2O due to the increased strength of deuterium bonds as compared to hydrogen bonds. Quantitative analyses of FT-IR spectra of calmodulin and parvalbumin show an increase in the helical content by approximately 6% for both proteins on addition of Ca^{2+}. A reduced rate of hydrogen–deuterium exchange in the Ca^{2+}-loaded state suggests the formation of a more compact structure. The most marked effect of Ca^{2+} is an enhanced thermal stability of these proteins. Elevation of the temperature to 70 °C in the absence of Ca^{2+} results in disruption of helical structures and formation of an intermolecular β-sheet. This rearrangement is prevented by Ca^{2+}.

Many cytosolic proteins are targeted by Ca^{2+}. Of these, the so-called EF-hand proteins are by far the most widely studied. These are a homologous family of proteins containing the recurring helix–loop–helix or EF-hand structural motif described by Kretsinger and Nockolds (1973). Two helices are separated by a loop containing around 12 amino acids, many of which are acidic and are involved in Ca^{2+} coordination. The helices are oriented approximately perpendicular to each other.

The first EF-hand protein to be crystallized was parvalbumin (Kretsinger & Nockolds, 1973). Parvalbumin was shown by X-ray diffraction (Moews & Kretsinger, 1975) to consist of six α-helical regions, labeled A–F, helices A and B, C and

† This work was supported by the Wellcome Trust.
* To whom correspondence should be addressed.
‡ National Research Council of Canada.
§ Royal Free Hospital School of Medicine.

0006-2960/91/0430-9681$02.50/0

D, and E and F being separated by loops containing acidic amino acids. The C–D and E–F loops (but not the A–B loop) each bind one Ca^{2+} ion. The CD and EF loops are held in the correct orientation by two β-sheet hydrogen bonds between isoleucine-97 and isoleucine-58 and leucine-77 and lysine-64.

Subsequent to the elucidation of the crystal structure of parvalbumin, a range of proteins was suggested to contain the EF-hand domain, primarily on the basis of amino acid sequence homologies. Of these proposed EF-hand proteins, three have so far been crystallized: troponin C, calmodulin, and intestinal calcium-binding protein. The crystal structures of two of these proteins (calmodulin and troponin C) show a remarkable degree of similarity (Babu et al., 1985, 1988; Hertzberg & James, 1985).

The crystal structure of troponin C indicates an elongated, dumbbell-shaped molecule, some 70 Å in length (Hertzberg & James, 1985). The two globular regions contain the AB, CD (NH_3^+ terminus), EF, and GH (COO^- terminus) hands. These globular domains are separated by a nine-turn α-helix. X-ray diffraction data for calmodulin indicate a structure remarkably similar to that obtained for troponin C. Seven helical structures are seen, labeled I–VII. As in troponin C, four Ca^{2+}-binding loops are present, in two globular domains which are separated by an eight-turn helical region. The length of the calmodulin molecule is somewhat less than the troponin C molecule, at 65 Å. The Ca^{2+}-binding loops 1 and 4 are similar to those in parvalbumin, but loops 2 and 3 differ in that they share helix IV.

Although detailed X-ray diffraction data are available for a number of EF-hand proteins, these data relate to a static, Ca^{2+}-loaded structure. Crystallographic data cannot provide information concerning the native, non-Ca^{2+}-loaded state, the effects of Ca^{2+} upon the secondary structure of the protein, or information relating to the effects of Ca^{2+} on protein stability. Such studies rely on spectroscopic techniques, which can probe the conformation of proteins in an aqueous (native) environment. A number of these spectroscopic techniques have recently been applied to the study of EF-hand proteins, including NMR (Levine et al., 1983), fluorescence, circular dichroism (CD)[1] (Gerday & Closet, 1975), and FT-IR spectroscopy (Haris et al., 1987; Trewhella et al., 1989). In general, agreement between the spectroscopic techniques has been good. A predominantly helical structure is proposed in the Ca^{2+}-loaded state, in agreement with X-ray diffraction data. Removal of Ca^{2+} leads to a decrease in the proportion of helical structures within the molecule, although α-helices remain the main structural motif. However, discrepancies between X-ray diffraction results and other techniques have been reported. For example, it has been recently suggested that the calmodulin central helix is not straight, but is bent in solution, bringing the Ca^{2+}-binding domains closer together (Heidorn & Trewhella, 1989). Furthermore, FT-IR spectroscopic studies have demonstrated an unusual feature of the infrared spectrum of calmodulin and troponin C, namely, a markedly lower amide I maximum in ^2H_2O solutions than has previously been observed for predominantly α-helical proteins (Haris et al., 1987; Trewhella et al., 1989). This has been attributed to the presence of an unusual type of helical structure (Trewhella et al., 1989) together with an unusual degree of hydration. The proportions of distorted helix were suggested to increased upon Ca^{2+} binding.

[1] Abbreviations: FT-IR, Fourier transform infrared; CD, circular dichroism; NMR, nuclear magnetic resonance; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CIRCOM, computerized infrared characterization of materials.

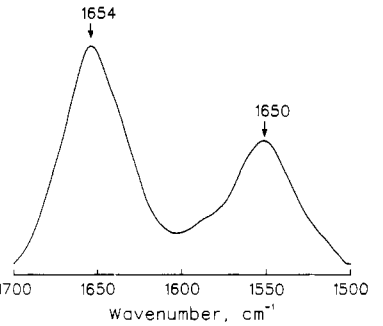

FIGURE 1: Absorbance spectrum in the amide I and II regions of parvalbumin in 5 mM EGTA, pH 5.5.

There exists, therefore, an apparent paradox between the structure of calmodulin and troponin C as determined by FT-IR spectroscopy and all other techniques. In this paper, we show that this paradox exists for parvalbumin also. We present experimental evidence which suggests that the low frequency of the IR absorption of these proteins in ^2H_2O is indeed related to the degree of solvent interaction. However, we find no evidence for significant distortions of the helical structure. Furthermore, the addition of Ca^{2+} to each protein did not result in the formation of distorted structures as suggested, but rather exerted a stabilizing effect on the proteins.

Materials and Methods

Parvalbumin, EGTA, and ^2H_2O were purchased from Sigma. Deuterated glycerol was obtained from Aldrich. Calmodulin was the generous gift of the late Prof. Gabriela Sarzala (Nencki Institute of Experimental Biology, Warsaw, Poland).

Samples were prepared for infrared spectroscopy as follows. Protein solutions (0.25–2.0% w/v) were prepared in ^2H_2O or H_2O buffers containing 5 mM EGTA/20 mM phosphate, and the pD (or pH) was adjusted to 5.5, 7.5, or 9.5. Approximately 40 μL of each sample was placed between a pair of CaF_2 windows separated by a 50-μm tin spacer. The windows were mounted in a Beckman FH-01 cell holder, and the temperature was maintained at 20 or 70 °C by a circulating water jacket.

FT-IR spectra were recorded on a Perkin-Elmer 1750 FT-IR spectrometer equipped with a fast-recovery TGS detector and continuously purged with dry air. For each sample, 100 interferograms were recorded and Fourier-transformed to produce a spectrum with a nominal resolution of 4 cm^{-1}. Protein absorbance spectra were generated by interactive digital subtraction of the appropriate buffer spectrum. Quantitative analyses of the spectra of parvalbumin and calmodulin recorded in H_2O were carried out by factor analysis using the CIRCOM program supplied by Perkin-Elmer [see Lee et al. (1990) for further details regarding this method].

For samples analyzed in the presence of deuterated glycerol, protein solutions were left at 4 °C for several days to allow complete hydrogen–deuterium exchange. Complete exchange was verified by the absence of an amide II absorption in FT-IR spectra. Deuterated glycerol was then added to a final concentration of 60% (w/v), and spectra were recorded at 20 °C. Circular dichroism measurements were carried out on the same samples after appropriate dilution.

Circular dichroism measurements were performed on a Jasco 600 CD spectrometer using silica cuvettes with path lengths of 0.01–0.1 cm, at concentrations ranging from 0.02 to 2.0 mg/mL, in H_2O and ^2H_2O buffers in the range 190–260 nm.

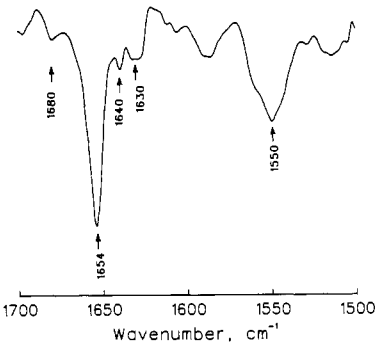

FIGURE 2: Second-derivative spectrum of the amide I and II regions of parvalbumin in 5 mM EGTA, pH 5.5.

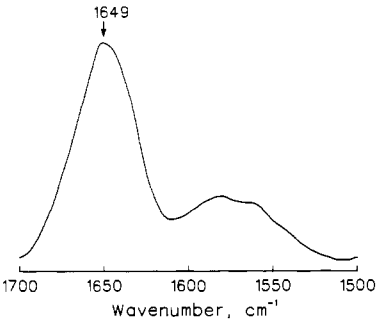

FIGURE 3: Absorbance spectrum in the amide I and II regions of parvalbumin in 5 mM EGTA, pD 5.5, and ^2H_2O.

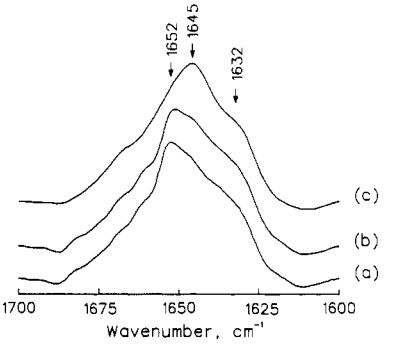

FIGURE 4: Deconvolved spectra of the amide I region of parvalbumin in ^2H_2O/5 mM EGTA, pD 5.5 (a), 7.5 (b), and 9.5 (c).

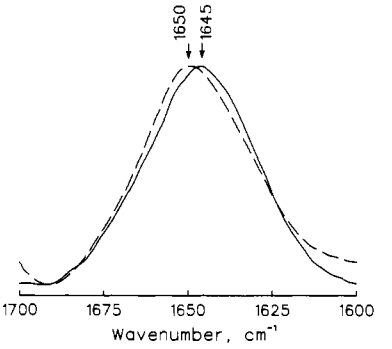

FIGURE 5: Absorbance spectra in the amide I region of calmodulin in the presence (dashed line) and absence (solid line) of 60% deuterated glycerol.

RESULTS

FT-IR Spectroscopy. (*A*) *Proteins in H_2O in the Absence of Ca^{2+}.* The absorbance spectrum of parvalbumin in 5 mM EGTA, pH 5.5, is shown in Figure 1. The amide I and II maxima are observed at 1654 and 1550 cm^{-1}, respectively. Deconvolution (not shown) and second-derivative (Figure 2) analysis of the amide I band reveals the presence of further absorptions at 1630–1640 and 1680 cm^{-1}. Similar spectra were obtained at pH 7.5 and 9.5 (not shown). Qualitatively similar spectra were obtained for calmodulin. Quantitative analysis of the spectra of calmodulin and parvalbumin was carried out by using factor analysis of their infrared spectra using the CIRCOM program. Calmodulin was found to have 61% α-helix and 15% β-sheet. Values obtained for parvalbumin were 62% α-helix and 17% β-sheet.

(*B*) *Proteins in H_2O in the Presence of Ca^{2+}.* In the presence of 5 mM Ca^{2+} spectra of calmodulin and parvalbumin in H_2O are identical with those recorded in 5 mM EGTA. Quantitative analysis of the FT-IR spectrum of calmodulin in the presence of Ca^{2+} was also carried out. The results obtained gave 67% α-helix and 11% β-sheet for calmodulin. For parvalbumin, values of 69% α-helix and 12% β-sheet were obtained.

(*C*) *Proteins in ^2H_2O in the Absence of Ca^{2+}.* The absorbance spectrum of parvalbumin obtained 15 min after dissolution in ^2H_2O, pD 5.5, in the presence of 5 mM EGTA is shown in Figure 3. The amide I maximum is seen to be shifted to 1649 cm^{-1}, with the amide II band much reduced in intensity. Deconvolved spectra of parvalbumin at pD 5.5, 7.5, and 9.5 are shown in Figure 4. At pD 5.5 and 7.5, the amide I maximum is seen at 1652 cm^{-1} with additional absorptions at 1632 and 1670–1680 cm^{-1}. Similar deconvolved spectra were obtained for calmodulin (not shown) at these pD values. Upon elevation of the pD to 9.5, spectra of each protein recorded 15 min after dissolution exhibit amide I maxima in absorbance spectra at 1644 cm^{-1}. Derivative and deconvolution analysis reveals the presence of a major component at 1644 cm^{-1}. Minor bands are unaffected by deuteration. No residual amide II band was apparent.

The second-derivative spectra of parvalbumin and calmodulin recorded at pD 5.5, 7.5, and 9.5 in 5 mM EGTA 24 h after dissolution were identical with spectra of parvalbumin recorded 15 min after dissolution at pD 9.5 (not shown).

(*D*) *Proteins in ^2H_2O in the Presence of Ca^{2+}.* In the presence of 5 mM Ca^{2+} at 20 °C, parvalbumin and calmodulin spectra recorded 24 h after dissolution in ^2H_2O exhibit amide I maxima in absorbance, derivative, and deconvolved spectra at 1646–1647 cm^{-1} (not shown). Unlike samples in the absence of Ca^{2+}, these spectra still show significant absorbance in the amide II region, indicative of reduced hydrogen–deuterium exchange. The minor bands are unaffected by the presence of ligand. Addition of 5 mM Ca^{2+} to parvalbumin and calmodulin after complete deuteration of the proteins produced spectra identical with those recorded after complete deuteration in the presence of EGTA.

(*E*) *Effect of Protein Concentration and Glycerol on Spectra of Proteins in ^2H_2O.* FT-IR spectra of parvalbumin and calmodulin were obtained at a range of concentrations (0.25–2.5% w/v). Both absorbance and derivative spectra were found to be unaffected by variations in the concentration of the protein (not shown).

FT-IR spectra of parvalbumin and calmodulin were recorded in the presence of 60% glycerol. Figure 5 compares the absorbance spectrum of calmodulin in ^2H_2O in the presence and absence of 60% deuterated glycerol. An increase in the amide I maximum of calmodulin from 1645 cm^{-1} in ^2H_2O to 1650 cm^{-1} in 60% glycerol in ^2H_2O is seen.

(*F*) *Effect of Heating on the Spectra of Proteins in ^2H_2O.* Spectra of parvalbumin and calmodulin in 5 mM EGTA, pD 5.5, recorded at 70 °C are characterized by an amide I maximum at 1650 cm^{-1}. Figure 6 shows the deconvolved spectrum of parvalbumin recorded at 70 °C. Two additional

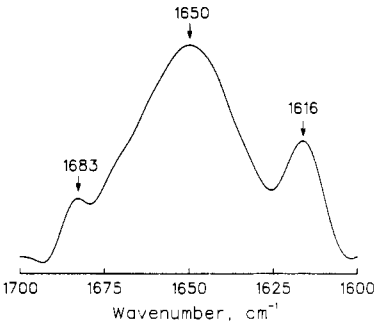

FIGURE 6: Deconvolved spectrum in the amide I region of parvalbumin at 70 °C in ^2H_2O/5 mM EGTA. Half-width = 18 cm^{-1}, resolution enhancement factor = 1.8.

bands are apparent at 1616 and 1683 cm^{-1} in addition to the main band at 1650 cm^{-1}. Similar results were obtained for calmodulin.

In the presence of 5 mM Ca^{2+}, spectra of parvalbumin and calmodulin recorded at 70 °C were similar to those recorded at 20 °C; no new bands were evident in the spectra.

Circular Dichroism Spectroscopy. CD spectra were recorded for calmodulin and parvalbumin as a function of pH in the presence of EGTA. The typical spectrum was observed, consistent with a high proportion of α-helix (Chang et al., 1978; Martin & Bayley, 1986). No systematic changes were observed as function of pH (or pD) from 5.5 to 9.5. CD spectra in the concentration range 0.02–2.0 mg/mL at all three pD values showed no indication of concentration dependence. Similarly, the presence of 60–80% glycerol did not affect the far-UV CD of the proteins.

These results indicate that both proteins retain their highly α-helical conformation under these physical conditions.

Discussion

The secondary structures of parvalbumin and calmodulin are well established from X-ray diffraction and NMR spectroscopy. Both proteins are predominantly α-helical with small amounts of β-sheet.

The amide I maxima of parvalbumin and calmodulin are observed at 1654–1655 cm^{-1} in difference spectra recorded in aqueous buffer containing 5 mM EGTA at pH 5.5–9.5. Such an absorption is characteristic of a protein containing α-helical and/or unordered polypeptide segments (Lee et al., 1985; Surewicz & Mantsch, 1988; Jackson et al., 1989). Second-derivative spectra of these proteins also show bands characteristic of the presence of β-sheet (1630–1640 and 1680 cm^{-1}).

Quantitative analysis of the secondary structure of parvalbumin and calmodulin in the Ca^{2+}-loaded state by factor analysis of their IR spectra gives results which agree quite well with the X-ray diffraction results. This suggests that the predominantly α-helical structure of these proteins is retained in aqueous solution, in agreement with NMR spectroscopic results. Quantitative analysis in the absence of Ca^{2+} suggests a reduction in the α-helical content of 6% and an increase in the β-sheet content of 4–5% for each protein. However, these differences are small and taken together with the error involved in the method may not be significant.

Deuterium substitution experiments are routinely employed in the FT-IR spectroscopic analysis of protein secondary structure to separate the overlapping absorptions from helical and unordered polypeptide chains. Following deuteration, the absorption arising from the rapidly exchanging unordered segments is shifted to 1644–1646 cm^{-1}, while deuterated α-helices can be shown to absorb in the region 1648–1652 cm^{-1}. Upon immediate dissolution of each protein in ^2H_2O at pD 5.5 in the absence of Ca^{2+}, the amide I maximum is seen at 1649–1650 cm^{-1}, suggesting a predominantly helical structure for these proteins. This is in agreement with X-ray diffraction (Kretsinger & Noews, 1986; Kumar et al., 1990), NMR (Levine et al., 1983), and CD spectroscopy (Gerday & Closset, 1975). Minor bands attributable to β-sheet structures are seen in derivative spectra.

A broad series of bands at 1560–1580 cm^{-1} arises from ionized side chains of acidic amino acids. The strength of these bands is such that is is not possible to accurately assess the intensity of the residual amide II band in order to estimate the extent to which the protein has been deuterated. However, from the relatively weak nature of the shoulder on the band at 1560–1580 cm^{-1}, the protein appears to be highly deuterated. In view of the high degree of solvent exposure conferred by the very open structure of these proteins, this is not surprising. Similar results were obtained at pD 7.5.

Elevation of the pD to 9.5 produced spectra characteristic of highly disorganized proteins, with amide I maxima in difference, deconvolved, and derivative spectra at 1644 cm^{-1}. Complete deuteration has occurred as demonstrated by the lack of any amide II absorption.

One explanation for this low amide I absorption would be that at this pD the proteins have been denatured. However, CD spectra of parvalbumin at each pD (not shown for brevity) were found to be consistent with a predominantly α-helical structure; no evidence of significant proportions of unordered polypeptide segments at elevated pD was found. This indicates that the pD dependence of the FT-IR spectra of parvalbumin and calmodulin is related to the extent of deuteration of the proteins. In agreement with this, spectra of parvalbumin and calmodulin obtained after complete deuteration of the proteins showed no pD dependence. At each pD, spectra exhibited an amide I absorption characteristic of unordered proteins, that is, a band maximum at 1644 cm^{-1}. Such a structure is not supported by NMR spectroscopy (Levine et al., 1983), CD spectroscopy (Closset & Gerard, 1975), or X-ray diffraction (Moews & Kretsinger, 1975; Babu et al., 1985; Herztberg & James, 1985).

The low band frequency seen in ^2H_2O and the CD spectra indicative of α-helical structures requires explanation. One explanation could be that this discrepancy is due to the high concentration required for FT-IR spectroscopy. This is some 2 orders of magnitude larger than that required for CD spectroscopy. Aggregation could occur at the high concentrations required for infrared spectroscopy and induce structural changes within the proteins. Thus, the low amide I frequency may actually reflect denaturation of the protein induced by aggregation. CD and FT-IR spectra were therefore recorded at a range of concentrations in order to assess the effect of protein concentration upon protein structure. No differences were apparent in either CD or FT-IR spectra as the concentration was varied. FT-IR and CD spectra recorded from identical samples still produced apparently contradictory results. Furthermore, aggregation accompanying denaturation always results in the appearance of bands at 1615–1625 cm^{-1} in FT-IR spectra [see, for example, Jackson and Mantsch (1991)], which is not apparent here. The unusual amide I maximum in the infrared spectrum cannot therefore be attributed to aggregation-induced denaturation.

A recent FT-IR spectroscopic (Trewhella et al., 1989) study led the authors to conclude that the low amide I frequency could be due to the helix being abnormal, i.e., a 3_{10}- or α_{II}-helix. The presence of a 3_{10}-helix in parvalbumin (Kumar et al., 1990) and calmodulin (Satushur et al., 1988) has been

demonstrated by X-ray diffraction although α-helices are the main structural motif. Experimental (Yasui et al., 1988; Kennedy et al., 1991) and theoretical (Krimm & Bandekar, 1987) studies indicate that distorted helical structures such as 3_{10}- or α_{II}-helices exhibit amide I frequencies which are higher than that observed for α-helices due to weaker hydrogen bonding, rather than lower as is the case with parvalbumin and calmodulin. Furthermore, if such unusual structures were present, it would be expected that an unusual amide I frequency in H_2O would be observed, which is clearly not the case. It therefore seems unlikely that significant proportions of distorted helices are present.

From the X-ray diffraction data (Kretsinger & Noews, 1986; Babu et al., 1987), EF-hand proteins have in common a high degree of solvent exposure, with the consequence that the peptide bonds are likely to be very highly solvated. In particular, calmodulin and troponin C have a long, highly exposed helical-linking segment. A refined X-ray structure of troponin C found some 20% of peptide groups within the crystal to be hydrogen-bonded to water (Satyshur et al., 1990). With this in mind, spectra of calmodulin and parvalbumin were obtained in the presence of deuterated glycerol to assess the effects of hydration upon the position of the amide I maximum.

Spectra of calmodulin in deuterated glycerol/^2H_2O show a considerable shift in the amide I band maximum (an increase in frequency of 5 cm^{-1}; see Figure 5) compared to spectra of the protein in the absence of glycerol. A similar study has recently been undertaken with adenylate kinase, which is believed to be highly α-helical in nature but shows an amide I band maximum at 1641 cm^{-1} in ^2H_2O (Jackson and Mantsch, unpublished results). Addition of 70% deuterated sucrose resulted in significant shifts to higher frequency. As glycerol and sucrose would be expected to compete with the protein C=O groups to hydrogen-bond to solvent, these results can be interpreted as a decrease in the interaction of the proteins with solvent producing an increase in amide I frequency. Of course, it cannot be ruled out that addition of glycerol produces structural alterations in the proteins. However, CD spectra of parvalbumin and calmodulin obtained in the presence of glycerol are identical with spectra in aqueous solution. Conformational changes induced by glycerol can therefore be discounted.

If the increased solvation of calmodulin and parvalbumin compared to other water-soluble proteins is the cause of the unusual spectroscopic behavior, then it may be expected that an abnormally low amide I maximum would be seen in H_2O as well as in ^2H_2O, which is not the case. With simple amides such as propionamide, the position of the amide I maximum is markedly dependent upon the water content when water/dioxane mixtures are used as solvent, due to hydrogen bonding of water to the amide groups (Kobayashi & Kobayashi, 1980). Similarly, a marked dependence upon ^2H_2O content was seen when ^2H_2O/dioxane mixtures were used as solvent for the deuterated amide. However, for similar $H_2O/^2H_2O$ contents, the magnitude of the shift was always some 50% greater when the mixture contained ^2H_2O, a result taken to indicate that the hydrating effect of ^2H_2O is greater than that of H_2O. This terminology is perhaps misleading, suggesting that the amide interacts with more ^2H_2O molecules than H_2O, which is unlikely. We suggest that this difference is due rather to the difference in the strength of the deuterium and hydrogen bond. This difference has recently been demonstrated with films of melittin in lipid (Jackson and Mantsch, unpublished results). Hydration of dry films with H_2O vapor resulted in a shift of the amide I maximum by some 3 cm^{-1}, while hydration of the fully deuterated peptide with ^2H_2O vapor produced a 6 cm^{-1} shift. This result can be interpreted as reflecting a greater strength of the deuterium bond.

The unusual amide I frequency of EF-hand proteins in ^2H_2O may therefore arise from an increased interaction of these proteins with solvent compared to other helical proteins due to their unusually open structure, the low frequency only observed in ^2H_2O due to a stronger interaction of this solvent with the peptide C=O groups compared to H_2O.

Elevation of the temperature of solutions of parvalbumin and calmodulin to 70 °C in the absence of Ca^{2+} produced drastic changes in the FT-IR spectra of these proteins. Two intense, narrow bands of the type generally associated with β-sheet structures are apparent at 1615 and 1685 cm^{-1}. However, comparison of these spectra with those of predominantly β-sheet proteins such as ribonucleases A and S (Haris et al., 1986) and concanavalin A (Alvarez et al., 1987) shows the new band at 1615 cm^{-1} in the spectra of EF-hand proteins to occur at a frequency much lower than that normally assigned to β-sheet secondary structures. We (Jackson, Haris, and Chapman, unpublished observations) and others (Clark et al., 1981) have recently observed this to be a general phenomenon in thermally denatured proteins. We propose that these new bands are due to the unfolding of helical polypeptide chains with the concomitant formation of intermolecular hydrogen bonds. Addition of Ca^{2+} ions to parvalbumin and calmodulin prevents these molecular rearrangements of the protein by stabilizing the α-helices.

ACKNOWLEDGMENTS

We thank Dr. Peter Bayley and Dr. Stephen Martin of the Department of Biochemistry, NIMR, Mill Hill, London, for performing CD measurements and the late Dr. Gabriella Sarzala, who was involved in the initial stages of this work and generously supplied us with calmodulin.

Registry No. Ca, 7440-70-2; D, 7782-39-0.

REFERENCES

Alvarez, J., Haris, P. I., Lee, D. C., & Chapman, D. (1987) *Biochim. Biophys. Acta 916*, 5.

Babu, Y. S., Sack, J. S., Greenhough, T. J., Bugg, C. E., Means, A. R., & Cook, W. J. (1985) *Nature 315*, 37.

Babu, Y. S., Bugg, C. E., & Cook, W. J. (1987) *Methods Enzymol. 139*, 632.

Chang, C. T. C., Wu, C.-S. C., & Yang, J. T. (1978) *Anal. Biochem. 91*, 13.

Clark, A. H., Saunderson, D. H. P., & Sugget, A. (1981) *Int. J. Pept. Protein Res. 17*, 353.

Closset, J., & Gerday, C. (1975) *Biochim. Biophys. Acta 405*, 228.

Haris, P. I., Lee, D. C., & Chapman, D. (1986) *Biochim. Biophys. Acta 874*, 255.

Haris, P. I., Lee, D. C., Sarzala, G., & Chapman, D. (1987) British Biophysical Society Meeting, Cambridge, Abstr. 4.

Heidorn, D. B., & Trewhella, J. (1988) *Biochemistry 27*, 909.

Hertzberg, O., & James, M. N. G. (1985) *Nature 313*, 653.

Jackson, M., & Mantsch, H. H. (1991) *Biochim. Biophys. Acta* (in press).

Jackson, M., Haris, P. I., & Chapman, D. (1989) *J. Mol. Struct. 214*, 329.

Kennedy, D. F., Crisma, M., Toniolo, C., & Chapman, D. (1991) *Biochemistry* (in press).

Kobayashi, M., & Kobayashi, M. (1980) *J. Phys. Chem. 84*, 781.

Kretsinger, R. H., & Nockolds, C. E. (1973) *J. Biol. Chem. 248*, 3313.

Krimm, S., & Bandekar, J. (1986) *Adv. Protein Chem. 38*, 181.
Kumar, V. D., Lee, L., & Edwards, B. F. P. (1990) *Biochemistry 29*, 1404.
Lee, D. C., Hayward, J. A., Restall, C. J., & Chapman, D. (1985) *Biochemistry 24*, 4364.
Lee, D. C., Haris, P. I., Chapman, D., & Mitchell, R. C. (1990) *Biochemistry 29*, 9185.
Levine, B. A., Delgarno, D. C., Esnouf, M. P., Klevit, R. E., Scott, G. M. M., & Williams, R. J. P. (1983) *Ciba Found. Symp. 93*, 72-97.
Martin, S. M., & Bayley, P. (1986) *Biochem. J. 238*, 485.
Moews, P. C., & Kretsinger, R. H. (1975) *J. Mol. Biol. 91*, 201.
Satyshur, K. A., Rao, S. T., Pyzalska, D., Drendel, W., Greaser, M., & Sundaralingam, M. (1988) *J. Biol. Chem. 263*, 1628.
Surewicz, W. K., & Mantsch, H. H. (1988) *Biochim. Biophys. Acta 952*, 115.
Trewhella, J., Liddle, W. K., Heidorn, D. B., & Strynadka, N. (1989) *Biochemistry 28*, 1294.
Yasui, S. C., Keiderling, T. A., Bonora, G. M., & Toniolo, C. (1986) *Biopolymers 25*, 79.

Extracting Hydrophobic Free Energies from Experimental Data: Relationship to Protein Folding and Theoretical Models†

Kim A. Sharp, Anthony Nicholls, Richard Friedman, and Barry Honig*

Department of Biochemistry and Molecular Biophysics, Columbia University, 630 West 168 Street, New York, New York 10032

Received March 27, 1991; Revised Manuscript Received June 26, 1991

ABSTRACT: Solubility and vapor pressure measurements of hydrocarbons in water are generally thought to provide estimates of the strength of the hydrophobic effect in the range 20–30 cal/(mol·Å^2). Our reassessment of the solubility data on the basis of new developments in solution thermodynamics suggests that the hydrophobic surface free energy for hydrocarbon solutes is 46–47 cal/(mol·Å^2), although the actual value depends strongly on curvature effects [Nicholls et al. (1991) *Proteins* (in press); Sharp et al. (1991) *Science 252*, 106–109]. The arguments to support such a significant increase in the estimate of the hydrophobic effect stem partly from theoretical considerations and partly from the experimental results of De Young and Dill [(1990) *J. Phys. Chem. 94*, 801–809] on benzene partition between water and alkane solvents. Previous estimates of the hydrophobic effect derive from an analysis of solute partition data, which does not fully account for changes in volume entropy. We show here how the ideal gas equations, combined with experimental molar volumes, can account for such changes. Revised solubility scales for the 20 amino acids, based on cyclohexane to water and octanol to water transfer energies, are derived. The agreement between these scales, particularly the octanol scale, and mutant protein stability measurements from Kellis et al. [(1989) *Biochemistry 28*, 4914–4922] and Shortle et al. [(1990) *Biochemistry 29*, 8033–8041] is good. The increased strength of the hydrophobic interaction has implications for the energetics of protein folding, substrate binding, and nucleic acid base stacking and the interpretation of computer simulations.

The hydrophobic effect has provided a major unifying concept in understanding the structure and function of biological systems. Hydrophobicity can be defined phenomenologically in terms of the low solubility of nonpolar molecules in water. The underlying physical interactions that are responsible for this effect are also thought to yield the attractive forces that cause nonpolar molecules to aggregate in an aqueous medium, producing, for example, what is certainly a major driving force in protein folding (Dill, 1990). A distinction is sometimes made between hydrophobicity as measured from solubilities and from intermolecular association (Wood & Thompson, 1990), but it is likely that they represent different manifestations of the same physical phenomenon: the energetically costly disruption of the hydrogen-bonding network of water by solute molecules that cannot themselves form hydrogen bonds.

A quantitative measure of the hydrophobic effect is usually obtained from the solubilities of nonpolar molecules, particularly hydrocarbons, in water. In this paper it is argued that solvation free energies that have been extracted from such experimental data have been evaluated incorrectly, which has led to a serious underestimate of the magnitude of the effect. The standard form of the expression used to relate hydrophobic free energies to observed concentrations is $\Delta G = -RT \ln K$, where K is a partition coefficient. The problem is that this expression is correct only under special circumstances. As we show in this paper, when the solute and solvent molecules are of different sizes, an additional term [that is widely used in polymer theory (Flory, 1941; Huggins, 1941)] enters into the analysis. This leads to much larger transfer free energies. This finding has a number of important implications, including a requirement for a new hydrophobicity scale, the need to reevaluate the extent of agreement between experiment and computer simulations, and the need to revise the parameters used in protein folding algorithms and in the evaluation of binding energies.

†Support from the NSF (PCM88-05434), ONR (N00014-90-J-1713), and NIH (GM-30518 and GM-41371) is gratefully acknowledged.
* To whom correspondence should be addressed.

0006-2960/91/0430-9686$02.50/0