Frictional Resistance to the Local Rotations of Fluorophores in Proteins†

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ABSTRACT: The fluorescence polarization of solutions of various proteins dissolved in 80% glycerol–water was measured in the temperature range of −40 to +20 °C, and the results were analyzed by the plots of reduced anisotropy vs. temperature described in two previous papers [Weber, G., Rholam, M., & Scarlata, S. (1984) *Biochemistry* (first paper of three in this issue); Scarlata, S., Rholam, M., & Weber, G. (1984) *Biochemistry* (second paper of three in this issue)]. The majority of the plots exhibited features similar to those of the peptides: two linear segments joining at a well-defined critical temperature, a common low-temperature slope corresponding to the thermal coefficient of the solvent viscosity, and a characteristic high-temperature slope in the range 0.040–0.015/°C. The plots of lysozyme and diisopropyl fluorophosphonate–chymotrypsin showed three distinct linear regions. It is concluded that the thermal coefficient of the frictional resistance to rotations in proteins is determined, like in peptides, by a small group of amino acid residues, probably less than 10, in close proximity to the fluorophore.

In a previous paper (Scarlata et al., 1984) we studied the frictional coefficient of the local rotations of fluorophores, tyrosine or tryptophan, in small peptides. Briefly, we found that each peptide displays a characteristic critical temperature at which the thermal coefficient of the frictional resistence to rotation changes from a common value, determined by the solvent viscosity, into a smaller one specific to each peptide. The critical temperature corresponds to the solvent viscosity at which the allowed amplitude of the fluorophore rotations becomes limited by internal peptide interactions. From the experimental observations on the peptides we concluded that this limiting condition depends upon the local environmental flexibility.

In the present paper we extend these observations to single-chain proteins of varying complexity and to a small dimer protein in order to derive information on some basic points of protein dynamics.

Materials and Methods

High-purity glycerol, purchased from Aldrich, was stored with a desiccant under vacuum. Bovine serum albumin (BSA),[1] chymotrypsin, chymotryptinogen A, DFP–chymotrypsin, lysozyme, and elastase were from Sigma. Bovine pancreatic trypsin inhibitor and micrococcal nuclease were from Worthington. Tropomyosin was a gift from Dr. G. Phillips; azurin I was from Dr. S. Sligar. Neurophysin II was a gift from Prof. Paul Cohen. BPTI was purified by passage through Sephadex G-75, and the other proteins were purified by passage through a Sephadex G-50 column, to remove aggregates. Inorganic salts, reagent quality, were from Baker. Disulfide bond reduction was carried out by addition of a 300-fold molar excess of β-mercaptoethanol per disulfide.

Fluorescence polarizations and lifetimes of the emission were measured by the methods described in the preceding paper (Scarlata et al., 1984). For BPTI, tropomyosin, and neurophysin the exciting wavelength was 280 nm, for azurin, 295 nm, for chymotrypsinogen, chymotrypsin, and DFP–chymotrypsin, 305 nm, and for the other proteins, 297 nm. The choice of excitation wavelength for each case was determined by two requirements: to excite tryptophan alone in those proteins that exhibit fluorescence from both tyrosine and tryptophan and to do so in a spectral region in which limiting polarization does not change much with wavelength, so that the observed polarization is not unduly sensitive to small shifts in the absorption spectrum with temperature. The exciting light was selected by a monochromator and a Corning 7-54 filter. Tyrosine fluorescence (BPTI, tropomyosin, and neurophysin) was isolated by a Baird Atomic 312-nm interference filter and tryptophan fluorescence by a Schott WG-320 filter.

Results

The proteins were dissolved in 80% glycerol–20% 0.05 M, pH 7.0, phosphate buffer (w/w). The polarization and lifetime of the solutions were determined in the interval −40 to +20 °C. The data were analyzed by employing the relation (Weber et al., 1984)

$$Y = \ln [[A(0)/A(t)] - 1] - \ln (RT\tau/V) = -\ln[\eta(0)] + bt \quad (1)$$

by means of plots of Y vs. the Celsius temperature, as described in the preceding papers.

Figure 1 shows the results obtained with neurophysin II, a dimer of M_r 20K, in which each highly S–S cross-linked monomer of 10 kdaltons contains a single tyrosine. Observations under conditions in which either the dimer or monomer was the prevalent species (Nicolas et al., 1980; Rholam & Nicolas, 1981) gave the same results. The values of t_c (−16 °C) and b(U) (0.035) were similar to those observed in the much smaller peptide ocytocin. The results clearly indicate that the observed quantities are determined by a very limited number of amino acids in the immediate environment of the fluorophore. Figure 2 shows the plots for some proteins containing both tyrosine and tryptophan: azurin and micrococcal nuclease both with a single tryptophan; bovine serum albumin with two. Again, the same general pattern emerged of two slopes joining at a critical temperature. Other proteins, containing several tyrosines and tryptophan residues, were studied, with the results listed in Table I. In almost all of these cases of multiple tryptophan fluorophores we observed only two major slopes joining at a well-defined critical temperature. The same was true of BPTI where energy transfer

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[1] Abbreviations: DFP, diisopropyl fluorophosphonate; BSA, bovine serum albumin; BPTI, bovine pancreatic trypsin inhibitor.

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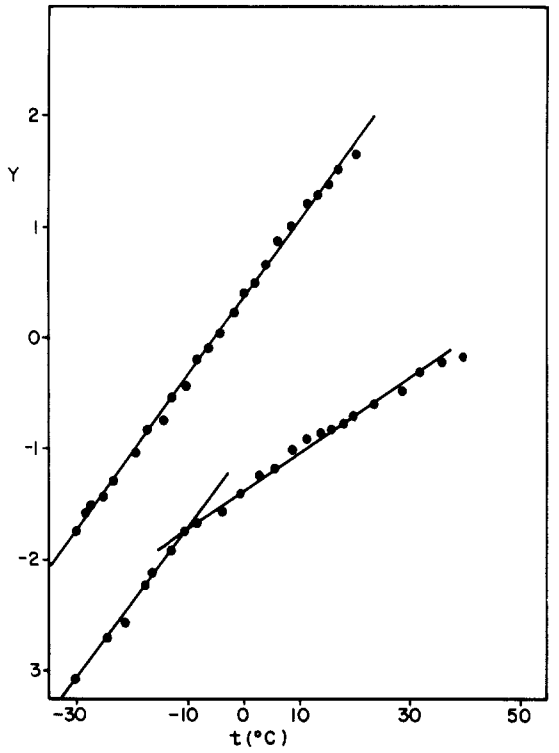

FIGURE 1: Plot of Y vs. t (eq 1) for tyrosine (upper data) and neurophysin (lower data) in 80% glycerol–20% phosphate buffer, pH 7.0. Protein concentration 0.12 mM.

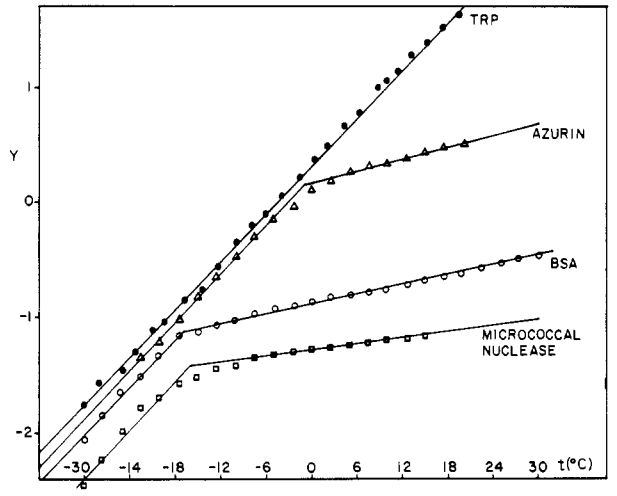

FIGURE 2: Y vs. t for azurin (△), micrococcal nuclease (□), and BSA (○).

among the several tyrosines is thought to be present (Kasprszak & Weber, 1982) and in neurophysin II where the single tyrosine of each subunit does not appear to transfer energy to the other. The only exceptions were lysozyme (Figure 3) and DFP–chymotrypsin. In the plots for these two proteins three slopes were clearly discernible. In the preceding paper (Scarlata et al., 1984) we found that the small peptide ocytocin dissolved in water solutions containing 67–90% glycerol displayed both a constant ratio $b(S)/b(U)$ and a constant rotational amplitude at the critical temperature. Observations in these same solvents were carried out for chymotrypsinogen A excited at 305 nm. This wavelength was chosen in order to excite the tryptophan population residing in the interior of the protein, and therefore out of contact with solvent. The results are presented in Table II. The ratio $b(S)/b(U)$ varied in an unsystematic fashion, but the amplitude at the critical temperature was, like in the peptide, remarkably constant.

Table I: Characteristic Parameters for Proteins with a Single High-Temperature Slope[a]

protein	$b(U)$	crit temp	amplitude	H	$V(1)$, $V(2)$
neurophysin	3.5	−12	13	14	75, 400
azurin	1.7	−1	16	9	71, 970
mic. nuclease	1.0	−17	12	27	82, 5500
BSA	1.4	−17	12	22	52, 2850
BSA-SH	4.7	−20	10	30	42, 265
BPTI	3.5	−12	14	8	80, 490
BPTI–citrate[b]	3.5	−11	14	13	69, 435
tropomyosin	5.2	+9	29	10	200, 480
tropomyosin–acrylamide[c]	5.0	+10	29	12	102, 254
chymotrypsin	2.7	−23	8	15	100, 880
chymo'gen	2.4	−23	8	10	68, 960

[a] $b(U)$ is given in percent decrease per degree, critical temperature in degrees centigrade, amplitude at the critical temperature in degrees of arc, and enthalpy in kilocalories per mole. $V(1)$ and $V(2)$ are apparent volumes of rotation (in mL/mol) as derived from the changing slope of the classical Perrin plot. [b] 0.12 M citrate (Kasprzak & Weber, 1982). [c] 0.13 M acrylamide (Lehrer, 1978).

Table II: Chymotrypsinogen A: Observations in Various Glycerol–Water Mixtures[a]

glycerol (%)	$b(S)$	$b(U)$	$b(S)/b(U)$	crit temp	amplitude
67	0.084	0.028	3.0	−31	13
70	0.104	0.023	4.5	028	12
75	0.082	0.030	2.7	−27	13
80	0.080	0.021	2.9	−23	10
90	0.104	0.043	2.4	−12	13

[a] Excitation was at 305 nm. Units as given in Table I.

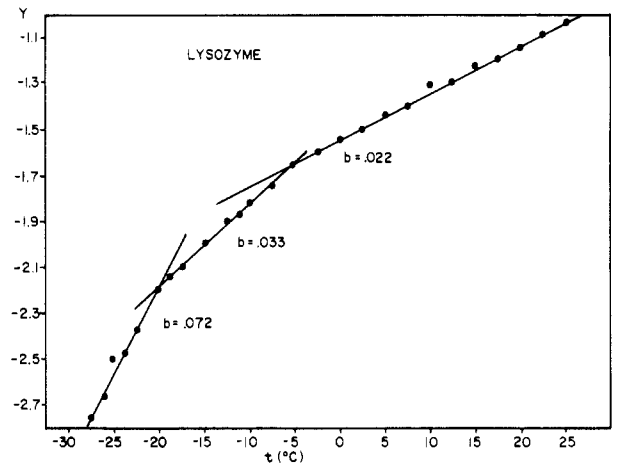

FIGURE 3: Y vs. t for lysozyme.

Discussion

From the observations with simple peptides described in the preceding paper (Scarlata et al., 1984) we expect to observe more than one high-temperature slope in proteins with two or more tryptophan residues with different environments and emitting independently of each other. The polarization addition law (Weber, 1952) may be used to combine the results obtained for two different proteins in order to demonstrate the results that would be expected from two independent emissions. This has been done by combining the polarization data for two pairs: bovine albumin–azurin (Figure 4a) and neurophysin II–tropomyosin (Figure 4b). In these examples the thermal coefficient $b(U)$ appeared to follow the relation

$$\langle b(U) \rangle = f(1)b(U,1) + f(2)b(U,2) \qquad (2)$$

where $f(1)$ and $f(2)$ are the fractions of the fluorescence emission from the two components and $b(U,1)$ and b(U,2) are

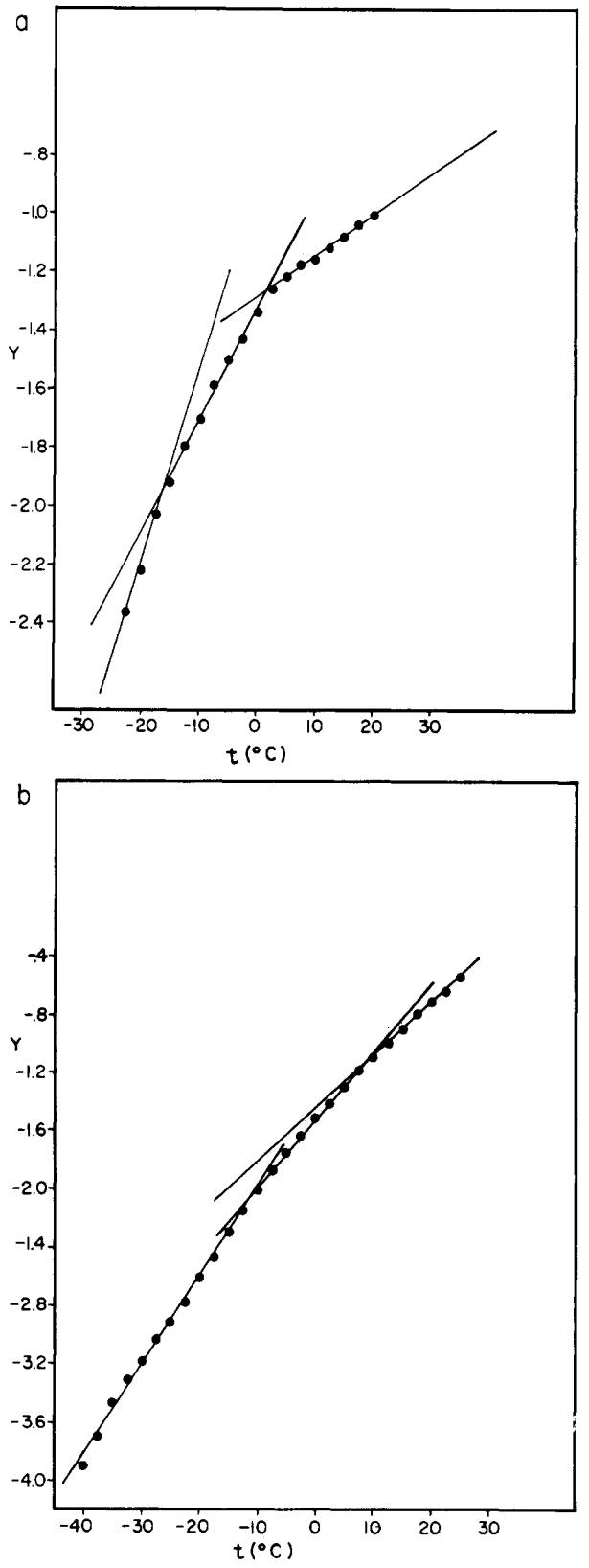

FIGURE 4: Synthesized plots of Y vs. t employing eq 1 and 2 and the experimental data for pairs of proteins, as described in the text. (a) BSA–azurin; (b) neurophysin II–tropomyosin.

the corresponding high-temperature slopes.

Rigorously, a linear combination of fluorescence anisotropy values that individually obey eq 1 yields a complex function and will only follow relation 2 if limiting anisotropies and high-temperature slopes are sufficiently close to each other. Heterogeneous chromophores will be clearly resolved only if the independent high-temperature slopes, b(U,1) and b(U,2),

Table III: Characteristic Parameters for Proteins Exhibiting Two High-Temperature Slopes, A and B[a]

	slope	b(U)	crit temp	amplitude	ΔH
lysozyme	A	3.2	−20	10	10
	B	2.14	−5	19	9
lysozyme (reduced)	A	4.0	−26	9	7
	B	1.76	+3	22	5
DFP–chymotrypsin	A	2.74	−23	8	15
	B	0.85	+10	28	32

[a] Units as given in Table I. V(1) and V(2) were not calculated.

are both small in comparison to the common low-temperature slope and the corresponding critical temperatures differ by more than 10 °C. This situation applies evidently to lysozyme. It is known that approximately 90% of the fluorescence of lysozyme is due to the two tryptophan residues at positions 62 and 108 (Imoto et al., 1971). The existence of three slopes indicates that their environments must be very different with regard to the resistance that they offer to the tryptophan local rotations. In a recent molecular dynamics study (Ichiye & Karplus, 1983), it was found that at room temperature—"in vacuo"—one of these tryptophans does not appreciably rotate during the 25 ps of the simulation while the other undergoes strongly depolarizing motions in the same interval. Our experimental observations concern rotations in a solvent with a viscosity of about 2 P and that take place in a time interval approximately 100 times longer than the time interval explored in the molecular dynamics calculations. Nevertheless, experiments and calculations agree in demonstrating a large difference in the local rotational properties of the two relevant tryptophans. The small fast motions of the protein fluorophores appear to be strongly coupled to the solvent only for solvent viscosities of the order of several poises. At viscosities of the order of 1 cP we expect the amplitude of the local rotations to be largely determined by the surrounding residues which thus make up the fluorophore "solvent". This reasonably clear conclusion provides experimental justification for the disregard of the solvent in the calculation of fast protein motions by the methods of molecular dynamics (Karplus & McCammon, 1981).

The high-temperature slopes observed with the proteins were sometimes smaller than those seen in the small peptides (e.g., BSA, micrococcal nuclease), but in a number of proteins they did not differ much from the peptide values. It is thus safe to conclude that this parameter is determined in the proteins by the interactions of the fluorophore with the few amino acids in the immediate vicinity. This conclusion is strengthened by examination of the data on proteins collected in Tables I and III: Neither the critical temperatures, the corresponding rotational amplitudes, nor the calculated enthalpy changes in the transition between the S and U states (Scarlata et al., 1984) differ greatly from those observed in the peptides.

It is interesting to compare the information obtained from the Perrin plots with that available from plots of the data according to eq 1. From analysis of the Perrin plots one can extract some information as to the effective volume of the rotating unit and the apparent amplitude of its motion, but the selection of the portions of the plot that can be thus analyzed (Kasprszak & Weber, 1982) is, at best, arbitrary. Table I summarizes the effective volumes V(1) and V(2) determined by this procedure: It seems clear that no systematic conclusion of interest can be derived from these values. The plot that we have introduced does not involve the subjective appraisal necessary to derive effective rotational volumes by means of the Perrin plot, and the analysis yields characteristic average properties of the surroundings rather than those of

the kinetic unit itself: the thermal coefficient of the frictional resistence that the immediate environment opposes to the rotation of the fluorophore and the amplitude of the fluorophore rotations at which its interaction with the protein environment becomes important. It demonstrates unequivocally the existence in peptides and proteins of fluorophore environments that differ greatly in these properties. These quantitative differences cannot be presently interpreted in terms of the motions associated to particular conformations of the molecules but do furnish data for the evaluation of future detailed calculations of the dynamics of proteins.

Registry No. Nuclease, 9026-81-7; chymotrypsin, 9004-07-3; tyrosine, 60-18-4; tryptophan, 73-22-3; lysozyme, 9001-63-2.

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Raman Spectroscopy of Homologous Plant Toxins: Crambin and α_1- and β-Purothionin Secondary Structures, Disulfide Conformation, and Tyrosine Environment†

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ABSTRACT: The Raman spectrum of crambin crystals is different from the spectrum of crambin in solution. The amide I spectrum of crambin in solution is not different from the solution spectra of proteins homologous (>40%) with crambin, α_1- and β-purothionins. We have two interpretations of these results. One is that helical segments in crambin and the purothionins in solution are more irregular than those in crystalline crambin. Comparative analyses of amide I and amide III spectra, and of the conformational preferences of the amino acid sequences of these proteins, are consistent with this interpretation. The other is that, due to the way helical segments in crambin are stacked end on end along the same axis in the crystal, transition dipole coupling along the axis of these extended helixes enhances the amide I intensity of helical residues. On the basis of a combined Raman and sequence conformational analysis, we propose that the structure of the purothionins is the same as that of crambin in solution and that residues 7–12 in crystalline crambin are somewhat more regular and ordered than they are in solutions.

In this paper we describe the measurement and analysis of as small structural differences in proteins in crystals and solutions and with highly conserved amino acid sequences. We combine a quantitative Raman measurement of structure with an analysis of the secondary structure preferences of the protein sequences for crambin and two toxins, α_1- and β-purothionin. The crystal structure of crambin is known to a very high resolution by X-ray diffraction (Teeter & Hendrickson, 1979; Hendrickson & Teeter, 1981) and by neutron diffraction (Teeter & Kossiakoff, 1982). Therefore, we have also used this knowledge to make inferences about the structure of crambin and of the toxins in solution, which are compatible with the differences in the Raman spectra.

An analysis of how different amino acid sequences determine similar three-dimensional protein structures in the globins has been done by Lesk & Chothia (1980), but the effects of sequence differences on secondary structure were not emphasized. The conformational properties of amino acids in proteins have been analyzed [for example, see Chou & Fasman (1974), Robson & Suzuki (1976), and Levitt (1978)], but this important work has not lead to consistently accurate structure predictions.

One analysis of the accuracy of predictive methods shows that in four structure-type predictions based on sequence alone only 49% of the residue conformations are assigned correctly (Garnier et al., 1978). However, this same study concludes that if secondary structure content is known, 63% of all residues can be predicted correctly. The approach we take here combines accurate secondary structure estimates, an analysis of sequences, and a comparison with the known structure of crambin.

A method has been developed to quantitatively analyze the Raman amide I spectrum of proteins to obtain estimates of secondary structure content (Williams et al., 1980; Williams

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