Frictional Resistance to Local Rotations of Aromatic Fluorophores in Some Small Peptides†

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ABSTRACT: We have determined, by observations of the polarization of the fluorescence, the thermal coefficient of the frictional resistance to the rotation of the emitting tyrosine or tryptophan residue in the hormones ocytocin and vasopressin and in other small peptides dissolved in 80% glycerol–water over the temperature range of −40 to +20 °C. The plots of the logarithm of the reduced anisotropy [Weber, G., Scarlata, S., & Rholam, M. (1984) *Biochemistry* (preceding paper in this issue)] vs. temperature consist of two linear regions meeting at a critical temperature, t_c, characteristic for each peptide. In the range $t < t_c$ the slope corresponds to the thermal coefficient of the viscosity of the pure solvent (approximately 0.07/°C). At $t > t_c$ the slope changes abruptly to a lower value that varies from 0.0325 to 0.060 in the different peptides. The low-temperature slope corresponds to a "solvent-limited regime" and the high-temperature slope to a "peptide-limited" regime. In the former regime the increase in the amplitude of the rotations of the fluorophore is determined by the change in the solvent viscosity. The critical temperature signals the attainment of a critical rotational amplitude beyond which rotations cannot increase in a manner consistent with the decrease in solvent viscosity but are instead limited by the immediate peptide environment. A thermodynamic model of the transition between the two regimes is presented.

The fast local motions of amino acid residues in proteins have been the object of much attention [for recent reviews, see Gurd & Rothbeg (1979) and Karplus & McCammon (1981)]. These motions provide a basis for the overall changes in conformation, and there is a general belief that they are necessary to the biological functions of proteins (Careri et al., 1979).

In the preceding paper (Weber et al., 1984) we showed that the temperature dependence of the polarization of the emission of a small fluorophore registered accurately the thermal coefficient of the viscosity of the solvent. We have made similar observations in a series of small peptides containing a single tyrosine or tryptophan residue in order to characterize the thermal coefficient of the frictional resistance of the rotations in the complex molecular environment that surrounds the fluorophore. The choice of solvent (80% glycerol–20% water) was dictated by the necessity of damping the overall motions of the peptide at all temperatures in the employed range without interfering with the local displacements (Gottlieb & Wahl, 1963; Xu & Weber, 1982). In this case we expect that any fluorescence depolarization will result from motions of the fluorescent residue with respect to coordinates rigidily attached to the molecule.

In peptides or proteins with more than one fluorophore the emission may be depolarized by energy transfer among them, a circumstance that renders uncertain the interpretation of the data in otherwise favorable cases [e.g., bovine pancreatic trypsin inhibitor (BPTI) (Kasprszak & Weber, 1982)]. To avoid this latter complication the hormones ocytocin and vasopressin and some related peptides, all containing a single fluorescent residue, either tyrosine or tryptophan, were studied.

Materials and Methods

Vasopressin (lysine), ocytocin, isotocin, vasotocin, somatostatin, and bombesin were from Sigma. Pressinoic and Tocinoic acids were from Peninsula Lab, Inc., and the tripeptide Cys(*S*-methyl)-Tyr-Ile-NH_2 was from Bachem. Their spectroscopic purity was assessed by absorption and fluorescence measurements. Spectral quality glycerol was from Aldrich. Reduction of disulfide bonds was carried out by addition of a 300 molar excess of β-mercaptoethanol. Fluorescence polarization was measured with the apparatus described by Jameson et at. (1976). Polarization values were corrected for solvent background if its contribution to the total intensity exceeded 0.5%. Fluorescence lifetimes were determined by the cross-correlation phase method (Spencer & Weber, 1969) with updated electronics from SLM (SLM Instruments, Urbana, IL). Each quoted value of lifetime is the average of a set of six series of measurements each consisting of 100 samples. For lifetime and polarization determinations excitation was by radiation of 280-nm wavelength (tyrosine) or 300 nm (tryptophan) isolated from the output of a xenon arc lamp by a combination of a monochromator and Corning 7-54 filter. Emission was isolated by Corning 0–53 filter (tyrosine) or Schott WG-320 filter (tryptophan). Temperatures were regulated by a methanol-circulating bath (Neslab LT-50), and optical modules were purged with dry nitrogen to prevent frosting. Viscosity values for glycerol–water mixtures were from Miner & Dalton (1953).

Results

This study was begun by measuring the fluorescence polarization of the tripeptide Cys(*S*-methyl)-Tyr-Ile-NH_2 in 80% glycerol–20% phosphate buffer (0.05 M, pH 7.0) over the temperature range −40 to +25 °C. The data were analyzed by a plot of the logarithm of the reduced anisotropy of emission vs. temperature as described in the preceding paper (Weber et al., 1984). Unlike the free probes (tyrosine, tryptophan, or typical organic fluorophores) two slopes were seen (Figure 1): one at a lower temperatures ($t < -8$ °C) of 0.07/°C, equal to that observed with tyrosine and a distinctly different one, 0.055/°C, at higher temperatures.

Figure 2 shows similar plots for vasopressin and ocytocin dissolved in the same solvent and observed over a similar temperature range. The plots are typical of the findings with the other peptides: Over a low-temperature range the slope

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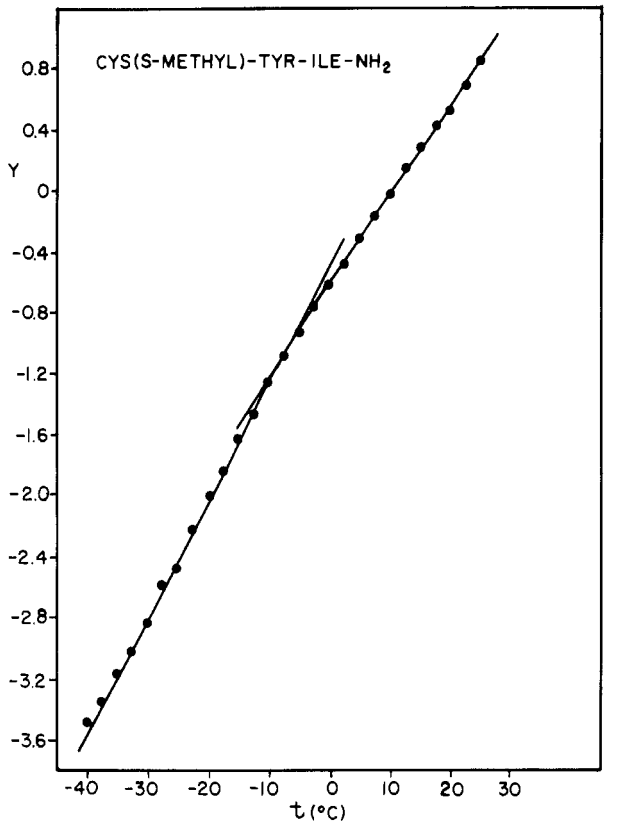

FIGURE 1: Plot of Y vs. Celsius temperature for the tripeptide Cys-(*S*-methyl)-Tyr-Ile-NH_2 in 80% glycerol–20% 0.05 M phosphate buffer, pH 7.0 (eq 8). Phase-measured lifetimes varied from 2.5 to 5 ns.

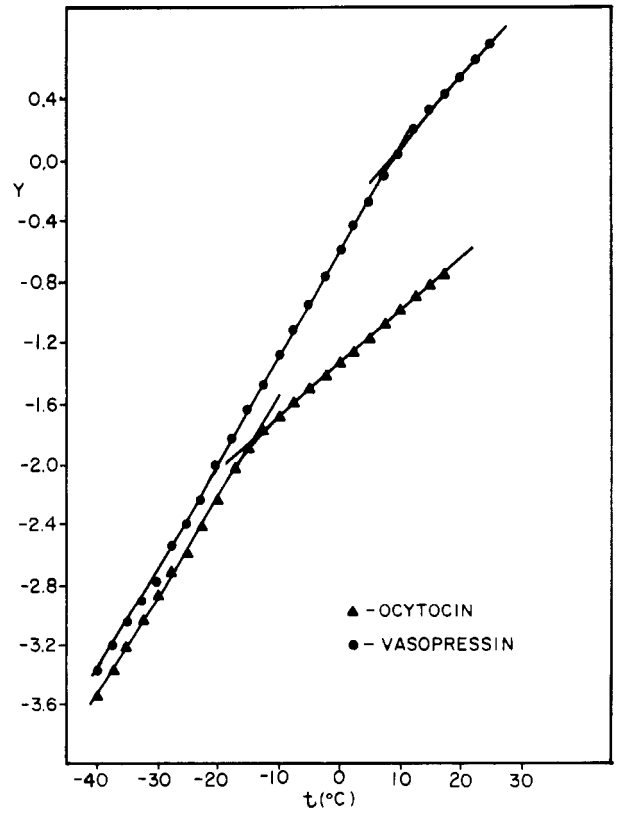

FIGURE 2: Y vs. Celsius temperature for ocytocin (▲) and vasopressin (●). Conditions are the same as given in Figure 1.

is very close to that observed over the whole temperature range for the free probes, approximately 7%. In the high-temperature range the slopes observed for vasopressin and ocytocin

Table I: Covalent Structures and Data for Parameters[a]

species	covalent structure
tripeptide	Cys(*S*-methyl)-Tyr-IleNH_2
ocytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH_2
tocinoic acid	Cys-Tyr-Ile-Gln-Asn-Cys
isotocin	Cys-Tyr-Ile-Ser-Asn-Cys-Pro-Ile-GlyNH_2
vasopressin	Cys-Tyr-Ile-Ser-Asn-Cys-Pro-Lys-GlyNH_2
pressinoic acid	Cys-Tyr-Phe-Gln-Asn-Cys
vasotocin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-GlyNH_2
bombesin	*p*-Glu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
somatostatin	NH_2-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-CysOH

species	b(U)	t_c	amplitude	ΔH
tripeptide	5.5	−7	15.5	20
ocytocin	3.5	−16	12	7
ocytocin-SH	5.8	−7	16	8
tocinoic acid	5.5	−12	12–13	10
isotocin	4.6	−14	12	10
vasopressin	5.0	+7	26–27	35
vasopressin-SH	6.0	−10	14	4
pressinoic acid	5.3	+7.5	26–27	5
vasotocin	5.45	−16	12	23
bombesin	4.9	−14	12	11
somatostatin	4.5	+6	18	9
somatostatin-SH	3.2	−12	12	30

[a] b(U) given as percent decrease per degree, critical temperature in degrees centigrade, amplitude in degrees of arc, and ΔH in kilocalories per mol.

Table II: Data for Ocytocin in Various Glycerol–Water Mixtures[a]

glycerol (%)	b(S)	b(U)	b(S)/b(U)	amplitude
67	7.7	4.0	1.9	12
70	10.0	5.0	2.0	12
75	8.0	4.0	2.0	12
80	7.0	3.5	2.0	12
90	10.0	5.0	2.0	11–12

[a] Units as in Table I. The irregular change in frictional coefficient of the viscosity with percent glycerol is discussed in Weber et al. (1984).

differ markedly: 0.035/°C for ocytocin and 0.050/°C for vasopressin. The critical temperatures, that is, the temperature at which the two slopes meet, are also very different: −16 for ocytocin and +7 for vasopressin. Similar experiments were carried out with other peptides, and the same pattern was uniformly observed.

The slope of approximately 0.07 observed in the low-temperature range in all the peptides admits a simple interpretation: the increase in rotational amplitude with temperature in this range is virtually controlled by the thermal coefficient of the solvent viscosity; that is, the increasing angular motions in this range are made possible by the increase in free volume with temperature that the solvent will allow. The specific characteristics of the peptides show themselves in the differences in the critical temperature and the high-temperature slope, b(U). Table I contains the values of slopes, critical temperatures, and some other derived quantities for all the cases studied. The vasopressin and ocytocin values of t_c and b(U) almost bracket the observed range of these quantities. To further determine the influence of the solvent, observations were made in ocytocin dissolved in a series of glycerol–water mixtures ranging from 67 to 90% glycerol. The results (Table II) show that in all cases the low-temperature slope, b(S), corresponded to the thermal coefficient of the viscosity of the solvent. As discussed in the preceding paper (Weber et al., 1984) the thermal coefficient of the viscosity is a very irregular

function of the glycerol content over this mixture range. While the low-temperature slopes followed this course, the high-temperature slopes changes in such a fashion that the ratio $b(S)/b(U)$ remained constant, indicating that the high-temperature slope is determined by a compromise of solvent and peptide properties. In other words, the effective resistance to rotation offered by the immediate environment of the peptide in this thermal region is not solely dependent upon the peptide composition but is also influenced by the solvent. The critical temperature increased systematically—over some 16 deg—as the glycerol content went from 67 to 90%. This behavior clearly suggests that the onset of the peptide-limited regime is determined by the attainment of a critical amplitude which is reached at higher temperature as the viscosity of the solvent is increased. From the values of the anisotropy of emission at the critical temperature $[A(t_c)]$ and the limiting anisotropy $[A(0)]$, the average cosine square of the rotational angle, θ, may be determine by means of the relation (Perrin, 1936)

$$\langle \cos^2 \theta \rangle = [1 + 2A(t_c)/A(0)]/3 \quad (1)$$

The angle corresponding to this cosine squared is shown in Table II to be a constant 12 deg of arc, independent of glycerol content. The critical amplitudes of the different peptides, derived from their study in 80% glycerol, range from 9 to 27 deg of arc.

Chemical Equilibrium Model. Evidently a detailed explanation of the rotational differences among the different peptides shown in Table I could be given, if at all, by the kind of microscopic analysis employed by the method of molecular dynamics. We can nevertheless attempt a description in terms of parameters that have some physical significance for the case. As the change of thermal coefficient occurs abruptly, over a range of at most very few degrees, we expect it to result from the replacement of one kind of fluorophore environment by another. At the lower temperatures the peptide domain surrounding the fluorophore may be considered as interacting strongly with solvent (S state). At the higher temperatures we expect the interactions with solvent to be much weaker, and the peptide may be considered as unsolvated, relative to the low-temperature condition (U state). We shall assume that the observed thermal coefficient results from contributions of these two states in proportions that are determined by a chemical equilibrium between the S and U states. A solvation free energy ΔG corresponds then to the reaction

$$\text{U} + \text{solvent} \rightarrow \text{S} \quad (2)$$

The average thermal coefficient $b(T)$ is given by

$$b(T) = b(S)f(S) + b(U)f(U) \quad (3)$$

and $f(S)$ and $f(U)$, the fractional contributions of the two states, are set by the thermodynamic relations

$$f(S) = 1/(1 + K) \qquad f(U) = K/(1 + K) \quad (4)$$

with

$$K = \exp[-\Delta G/(RT)] \quad (5)$$

The apparent viscosity of the medium at temperature T is

$$\eta(T) = \eta(0) \exp[b(T)[T - T(0)]] \quad (6)$$

$T(0)$ is a characteristic kelvin temperature chosen for convenience to be close to the center of the temperature range explored. We found it convenient to set $T(0) = 273.1$ K, the Celsius zero, so that $T - T(0) = t$, the Celsius temperature. $\eta(0)$ equals the apparent viscosity of the environment at zero centigrade; for 80% glycerol–water it is close to 2.5 P. At the critical Celsius temperature, t_c, the viscosity is

$$\eta(t_c) = \eta(0) \exp[t_c[b(S) + b(U)]/2] \quad (7)$$

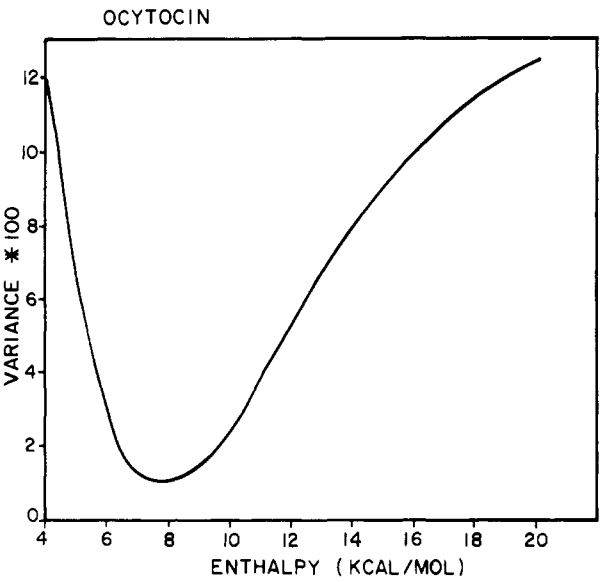

FIGURE 3: Plot of the variance between Y calculated as a function of ΔH by means of eq 3–8 and the experimental values of Y for ocytocin, shown in Figure 2.

The change in fluorescence anisotropy, $A(t)$, with temperature is determined by the equation (Weber et al., 1984)

$$Y(t) = \ln [[A(t)/A(0)] - 1] - \ln (RT\tau/V) = -\ln [\eta(0)] + tb(T) \quad (8)$$

Here $A(0)$ is the anisotropy corresponding to the motionless fluorophore and $b(T)$ is given by eq 3. The temperature dependence of $b(T)$ is in turn determined by that of ΔG. From the thermodynamic relation

$$\Delta G = \Delta H - T\Delta S \quad (9)$$

where ΔH and ΔS are the standard enthalpy and entropy changes of reaction 1 and the condition that at $t = t_c$ $\Delta G = 0$, we derive the relations

$$\Delta G = \Delta H(1 - T/T_c) \quad (10)$$

$$t_c = \Delta H/\Delta S \quad (11)$$

The critical temperature t_c and the slopes in the high- and low-temperature regimes are unambiguously determined from plots of $Y(t)$ vs. t, so that the single adjustable parameter ΔH determines the shape of the plot described by (8). As ΔH increases in absolute value, the transition from one slope, $b(S)$, to another, $b(U)$, becomes more abrupt. The curvatures of the experimental plots in the region close to t_c permit an estimate of ΔH, which for the cases studied (Table I) was in the interval 4–35 kcal/mol. Lower values of the enthalpy correspond to smooth continuous variations rather than abrupt transitions, and distinction among values of ΔH greater than 40 kcal is not possible because of the finite precision of both temperature and anisotropy measurements. The precision in the assigned ΔH values may be judged from Figure 3 which presents a plot of the variance between the observed values of $Y(t)$ and those calculated as a function of ΔH by fitting the data of ocytocin shown in Figure 2 to eq 3–8.

Discussion

At low temperatures, the small, fast motions of the chromophore are dictated by the frictional resistance of the solvent, and any increase in temperature (kinetic energy) results in an increase in rotational amplitude as allowed by the change in solvent viscosity. At the critical temperature this amplitude is such that further increases become limited by the local

peptide environment. In this higher temperature regime the rotational amplitude is a function of both the external solvent and the amino acid domain surrounding the fluorophore. The changes in the coupling of the fluorophore motions with those of its peptide environment, at the critical temperature, take place with a systematic increase in both enthalpy and entropy. These arise from two distinct effects: First, bonds between solvent and amino acid residues are broken with an increase in heat content; second, disappearance of these bonds permits additional dispositions of solvent and amino acids and thus results in a systematic increase in entropy with the increase in enthalpy. This behavior is uniformly encountered in the thermal dissociation of simple molecular complexes [e.g., see Spencer & Weber (1972) and Visser et al. (1977)]. The abrupt change that leads the system from one characteristic thermal coefficient to another depends only on the total value of the enthalpy and therefore upon the number of bonds formed by the solvent with the fluorophore domain that disappears in the transition of the S to the U state. The sharpness of the transition from the solvent regime to the peptide regime depends upon the strength of the interactions between solvent, on one hand, and the fluorophore and its immediate surroundings, on the other hand, and is reflected in the magnitude of the enthalpy change. The broader observed transitions take place over a temperature span of more than 4 deg and yield lower enthalpies than those occurring in a narrower temperature span. The enthalpy change calculated for any given case depends also upon the value of the slope in the "peptide regime", lower values of b(U) corresponding to apparently larger enthalpies. The larger values (20–35 kcal/mol) of the enthalpy of the S $\rightarrow$ U transition require comment: If the underlying physical model is at all applicable, a rearrangement of solvent and peptide must take place in which a number of degrees of freedom change simultaneously. Perhaps the simplest hypothesis to explain the sudden onset of these changes is that a particular dominant interaction (e.g., tyrosine–phenylalanine in vasopressin) serves as a nucleus or seed for the appearance of a new peptide conformation when the temperature is lowered. It is reasonable to suppose that the fluorophores tyrosine or tryptophan, being much more polarizable than the surrounding nonaromatic amino acids, are likely to take part in every case in the dominant peptide interaction.

Ocytocin, tocinoic acid, and all the linear peptides have similar critical temperatures. Vasopressin, pressinoic acid, and somatostatin yield much higher values. In comparing the data for these three peptides with those for the reduced form, or with other linear structures (Table I), we conclude that both the neighboring phenylalanine and the disulfide bond result in damping the coupled motions of fluorophore and environment at lower solvent viscosity as a consequence of a more rigid peptide structure. The data of Table I are sufficient to conclude that tertiary structure is more important than amino acid sequence in determining the critical temperature. In examining the values of b(U) in Table I we see no direct correlation between b(U) and chain length, cyclic character, or amino acid composition. Disulfide reduction increases b(U) by 0.015 in ocytocin but decreases it in somatostatin by 0.012. The linear peptides have b(U) in the range 0.049–0.060 per deg with the exception of reduced somatostatin. We conclude that the value of b(U) is controlled by the immediate interactions of the fluorophore: The restriction by the disulfide bonds can help to realize a low value of b(U), but this can also be present in the absence of S–S bonds if the interaction of nearby amino acid residues with the fluorophore is sufficiently strong. The thermal coefficient of the frictional effects of the immediate amino acid environment is uniformly lower than that of the environment provided by 80% glycerol. Butanol viscosity has a thermal coefficient of about 4%/deg, reflecting the smaller contribution of hydrogen bonding and the larger contribution of van der Waals forces to the viscosity in comparison with glycerol or glycerol–water mixtures. van der Waals forces do in fact provide the main interactions between tyrosine or tryptophan and the nearby amino acid residues in these peptides and in proteins. Thus, the two temperature ranges uniformly observed can be also characterized as those in which hydrogen bonds and van der Waals forces determine respectively the thermal coefficient of the frictional resistance to the local fluorophore rotations.

Registry No. Cys(*S*-methyl)-Tyr-Ile-NH_2, 82668-39-1; ocytocin, 50-56-6; tocinoic acid, 34330-23-9; isotocin, 550-21-0; vasopressin (8-lysine), 50-57-7; pressinoic acid, 35748-51-7; vasotocin (8-arginine), 113-80-4; bombesin, 31362-50-2; somatostatin, 38916-34-6; tyrosine, 60-18-4; tryptophan, 73-22-3.

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