Fluorescence Study of Brevin, the M_r 92 000 Actin-Capping and -Fragmenting Protein Isolated from Serum. Effect of Ca^{2+} on Protein Conformation†

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ABSTRACT: The fluorescence characteristics of brevin and the effects of Ca^{2+} on the protein conformation were fully investigated. Brevin contains 18 tryptophans and 27 tyrosines. Analysis of the fluorescence spectra and the accessibility to quenching molecules indicate that the emitting tryptophans are located in a hydrophobic environment (λ_{max} = 324 nm) close to the protein surface. In native brevin, tyrosyl residues do not contribute to the fluorescence emission. Partial quenching of these chromophores has to be attributed to tyrosine → tryptophan resonance energy transfer which is highly efficient. The effect of brevin on actin polymerization has been shown to be Ca^{2+} sensitive [Harris, D. A., & Schwartz, J. H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6798–6802; Thorstensson, R., Utter, G., & Norberg, R. (1982) *Eur. J. Biochem. 126*, 11–16; Wilkins, J. A., Schwartz, J. H., & Harris, D. A. (1983) *Cell Biol. Int. Rep. 7*, 1097–1104; Harris, H. E., & Weeds, A. G. (1983) *Biochemistry 22*, 2728–2741] and brevin binding to hydrophobic matrices to be Ca^{2+} dependent (Z. Soua, personal communication). Ca^{2+} binding to brevin decreases the tryptophan fluorescence polarization degree (without affecting the excited-state lifetime), which suggests a higher chromophore mobility. This effect may be partly related to the slight unshielding of the tryptophan residues observed in fluorescence quenching experiments. Moreover, the reactivity of brevin sulfhydryl groups toward 5,5′-dithiobis(2-nitrobenzoic acid) increases in the presence of Ca^{2+}. On the other hand, fluorescence spectra, quantum yields, excited-state lifetimes, and thermostability remain unchanged. We suggest, therefore, that Ca^{2+} binding to brevin does not induce a bulky conformational change in the protein such as to drastically affect the tryptophan location, but rather local and superficial changes which suffice for brevin interaction with actin. Finally, Ca^{2+} titration of brevin monitored by changes in the fluorescence polarization degree points to the presence of binding sites with dissociation constants of ca. 2×10^{-5} M. These sites appear to be Ca^{2+} specific, since Mg^{2+} up to 10 mM does not induce any change in the fluorescence polarization degree.

Microfilaments perform a wide variety of cellular functions. They are, for example, centrally involved in muscle contraction and other motile phenomena such as changes in cell shape or phagocytosis. In nonmuscle cells, cytoplasmic microfilaments are highly labile and in constant equilibrium with their actin subunits. The molecular basis for this control is of great interest. Many investigators are studying proteins that interact with actin and modify its polymerization properties [for a review, see Schliwa (1981), Craig & Pollard (1982), Korn (1982), and Weeds (1982)]. In particular, several of these proteins can be thought of as a group whose main property is Ca^{2+}-sensitive capping and fragmentation of F-actin.

Brevin, a 92 000-dalton protein isolated from serum (Norberg et al., 1979; Harris & Schwartz, 1981; Harris & Gooch, 1981; Thorstensson et al., 1982; Harris & Weeds, 1983; Wilkins et al., 1983; Lees et al., 1984; Doi et al., 1984), is one of these proteins. Brevin, also termed ADF or plasma gelsolin, increases the initial rate of polymerization and acts on preformed actin filaments by fragmenting the filaments and capping their fast-growing ends. Previous studies suggested that Ca^{2+} is required for filament severing. The protein most closely resembles gelsolin from macrophages (Yin et al., 1981a,b) but is also similar, in many respects, to villin (Bretscher & Weber, 1979, 1980; Glenney et al., 1981), a 90 000-dalton protein from platelets (Bryan & Kurth, 1984; Kurth & Bryan, 1984) or brain (Petrucci et al., 1983), fragmin (Hasegawa et al., 1980; Sugino & Hatano, 1982), and severin (Brown et al., 1982; Yamamoto et al., 1982; Giffard et al., 1984).

Numerous studies have been devoted to brevin's effect on actin polymerization. However, no information is so far available on brevin conformation and Ca^{2+}-induced structural changes. Of the physical techniques for investigating these problems, fluorescence is of particular interest due to its sensitivity and the variety of information on protein conformation it can afford. We therefore undertook a complete investigation of brevin intrinsic fluorescence in the absence and the presence of Ca^{2+}. Results on the tryptophan environment and location in the protein were obtained. In addition, we present evidence that Ca^{2+} does not induce a bulky conformational change affecting the overall protein structure but rather local and superficial changes. Brevin's affinity for Ca^{2+} was also determined.

Materials and Methods

All chemicals were high-grade commercial products. *N*-(2-Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES),[1] EGTA, DTNB, and TNS were purchased from Serva, 9-anthroylcholine was from Molecular Probes, ultrapure guanidinium chloride was from Schwarz/Mann, and acrylamide was from Bio-Rad.

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[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; TNS, 6-(*p*-toluidino)naphthalene-2-sulfonate; 9-AC, 9-anthroylcholine bromide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); SDS, sodium dodecyl sulfate; DTT, dithiothreitol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Ultrapure water (Milli Q instrument from Millipore Corp.), acid- and EGTA-washed plastic ware, and EGTA-treated dialysis tubings were used throughout to minimize calcium contamination. Before use, all buffers and protein samples were Millipore filtered (0.22-μm Millex GV and 0.45-μm HV, respectively).

Protein Purification. Brevin was purified from bovine serum according to the procedure of Harris & Schwartz (1981) modified by Z. Soua (personal communication). Protein purity was checked by SDS–polyacrylamide slab gel electrophoresis (Laemmli, 1970) with a linear 5–20% polyacrylamide gradient. Brevin migrates as a single band of M_r 92000. No proteolytic fragments were detected with Coomassie blue or silver staining. The protein was stored at −80 °C in 10 mM Tris-HCl, 2 mM EGTA, and 0.2 mM DTT, pH 7.5.

Amino Acid Analysis. Protein samples (0.3 nmol) were hydrolyzed for 24 h at 110 °C in 6 M hydrochloric acid containing 0.02% 2-mercaptoethanol in tubes sealed under nitrogen and using norleucine as internal standard. After evaporation, the amino acids were analyzed with a Durrum D500 analyzer (sensitivity: maximum absorption 0.5–2.0). Cysteine was determined as carboxymethylcysteine after carboxymethylation of the protein sample, followed by 6 M hydrochloric acid hydrolysis. Brevin (0.4 nmol) was carboxymethylated under conditions derived from the general method of Crestfield et al. (1963): the protein was first denatured in 8 M urea in the presence of 0.02 M dithiothreitol. Then a 50-fold molar excess of iodoacetic acid over the thiol groups was added to the reaction mixture, and alkylation was carried out under nitrogen, in the dark, at room temperature for 15 min. Reagent was added 3 more times, and the reaction was continued each time for 15 min. Excess iodoacetic acid was destroyed with 2-mercaptoethanol. The solution was then dialyzed against 0.01% NH_4OH, vacuum-dried, and processed for hydrochloric acid hydrolysis as indicated above. Tryptophan was determined after a 24-h, 3 M methanesulfonic acid hydrolysis (Liu & Chang, 1971). The solution was then diluted with 100 μL of 0.2 M sodium citrate buffer, pH 2.2. Aliquots were analyzed with a Durrum D500 analyzer.

UV Absorption Spectra. UV absorption spectra were recorded on a Cary 219 spectrophotometer at 25 °C in quartz cuvettes with a light path of 1 cm and at a speed of 0.2 nm/s. Correction for scattered light was made according to Gérard et al. (1975).

The extinction coefficient of brevin was determined by taking M_r = 92000 and measuring the protein concentration of a given brevin solution by amino acid analysis. Subsequently, the protein concentration was routinely measured by UV spectroscopy, taking a molar extinction coefficient at 280 nm of ϵ_M^{280nm} = 150000 M^{-1} cm^{-1}.

Fluorescence was measured with an absolute spectrofluorometer (FICA 55) on air-equilibrated solutions in quartz vessels placed in a thermostated metallic holder which could be heated or cooled by fluid circulation. The temperature within the cuvette was measured with a thermocouple (accuracy ±0.5 °C). The intrinsic fluorescence quantum yield of brevin (ϕ) was determined as previously described (Gérard et al., 1975), taking L-tryptophan in water (ϕ = 0.14) as reference. Protein solutions (absorbance at 280 nm <0.12) were excited either at 295 nm (selective excitation of tryptophans) or at 280 nm (excitation of both tryptophanyl and tyrosyl residues).

Lifetimes (τ) were measured with the single photoelectron technique (Gérard et al., 1975). Excitation was performed at 295 ± 5 nm, and fluorescence was observed at 342 ± 5 nm. Excitation and emission wavelengths were respectively selected with a Jobin Yvon monochromator (H10) and an interference filter with a band-pass of 8 ± 0.5 nm. Fluorescence decay profiles were analyzed with an iterative reconvolution nonlinear least-squares method (Grinwald & Steinberg, 1974; Grinwald, 1976). Precision of fit was evaluated from the reduced χ^2 and the residual autocorrelation curve.

Tryptophan fluorescence quenching experiments were carried out by adding aliquots of an acrylamide stock solution (8.6 M) to brevin in the absence or presence of Ca^{2+}. The excitation wavelength was set at 295 nm. As acrylamide did not alter the overall shape of the fluorescence spectrum (no shift in the maximum, no spectrum enlargment), changes in the fluorescence intensity at 340 nm were recorded. Data were corrected not only for dilution but also for the screening effect due to the absorption of acrylamide at 295 nm, according to the equation (Hélène et al., 1971)

$$I_p = I_m \frac{(d_p + d_s)(1 - 10^{-d_p})}{d_p[1 - 10^{-(d_p+d_s)}]}$$

where I_m represents the fluorescence intensity measured at 340 nm, I_p the fluorescence intensity corrected for light scattering and the screening effect of acrylamide, d_p the protein absorbance, and d_s the absorbance due to the light scattering of the protein molecules and to acrylamide absorption.

Linear Fluorescence Polarization Measurements. Fluorescence polarization is characterized by the degree of polarization:

$$p = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp}$$

where $I_\parallel$ and $I_\perp$ are respectively the parallel and perpendicular components of the fluorescence relative to the direction of the polarized excitation. Measurements were carried out at 25 °C under continuous illumination using a T-format device (SLM 8000 SC spectrofluoropolarimeter). With this apparatus, $I_\parallel$ and $I_\perp$ are detected simultaneously on two separate channels with vertically polarized excitation. The two channels are balanced by using horizontally polarized excitation. The excitation wavelength was set at 295 nm (monochromator) and the emission at 342 nm (interference filter with a band-pass of 8 ± 0.5 nm).

Relaxation times (ρ) were deduced from the fluorescence polarization values using Perrin's relation:

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho}\right)$$

where ρ is the relaxation time of the unit carrying the tryptophan residue, τ is the fluorescence lifetime, and p and p_0 are the measured and fundamental polarization degrees, respectively.

The relaxation time for a spherical protein can be calculated from

$$\rho_0 = \frac{4\pi\eta r^3}{kT} \quad (1)$$

where η is the viscosity of the medium, k the Boltzmann constant, T the absolute temperature, and r the radius of the spherical protein.

Ca^{2+} Removal from Brevin. Ca^{2+} was removed by extensive dialysis against buffer plus 2 mM EGTA followed by the appropriate buffer in the presence of 10^{-4} M EGTA. Ca^{2+} contamination was measured by atomic absorption spectrophotometry with a Varian 1150 apparatus. In all cases, residual free Ca^{2+} was <0.1 mol/mol of protein. The presence

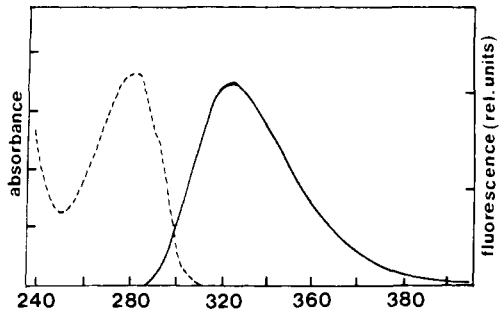

FIGURE 1: Absorption (---) and fluorescence (—) spectra of brevin. Native brevin with fluorescence spectra obtained upon excitation at 280 and 295 nm in the absence or presence of 10^{-4} M free Ca^{2+}. Buffer conditions: 50 mM HEPES, 150 mM NaCl, and 10^{-4} M EGTA, pH 7.5.

of EGTA does not affect the absorption or fluorescence characteristics of the protein.

Fluorometric Titrations. Titration of metal-free brevin with Ca^{2+} was performed by adding aliquots of $CaCl_2$ stock solutions and correcting for dilution. The experiments were performed in the presence of EGTA, and the free Ca^{2+} concentration was calculated from the Ca^{2+} added by using the binding constants for EGTA determined previously (Haiech et al., 1979). The pH was kept constant throughout the experiment.

Curve Fitting. Ca^{2+} titration curves were fitted according to the equation:

$$y = \frac{(x/c)^n}{1 + (x/c)^n}(a - b) + b \qquad (2)$$

where y stands for p (the polarization degree) at a given concentration (x) of Ca^{2+}, n represents the Hill coefficient, c the association constant between Ca^{2+} and brevin, b the polarization degree when $x = 0$, and a the polarization degree when $x = \infty$.

Titration of Brevin Sulfhydryl Groups. The sulfhydryl groups were titrated with Ellman's procedure (1959). The reaction of sulfhydryl groups with DTNB was monitored by recording the increase in absorbance at 412 nm which corresponds to the production of free 5-nitro-2-thiobenzoate. An extinction coefficient of 13 600 M^{-1} cm^{-1} was taken at 412 nm to determine the number of –SH groups readily titratable in the protein. This extinction coefficient was the same in buffer and in 5 M guanidinium chloride.

RESULTS

The UV absorption spectrum of brevin in 50 mM HEPES and 10^{-4} M EGTA, pH 7.5 (Figure 1), presents the characteristics of tryptophan absorption with a maximum at 282 nm and a shoulder at 291 nm. This spectrum is not affected by the addition of 150 mM KCl or NaCl, or of 10^{-4} M free Ca^{2+}. Brevin denaturation by guanidinium chloride (data not shown) induces a slight blue shift in the absorption spectrum with the maximum located at 280 nm and the shoulder at 290 nm.

The molar extinction coefficient of native brevin at 280 nm was found to be 150 000 ± 5000 M^{-1} cm^{-1}. This is close to the theoretical value, $\epsilon_M^{280} \simeq 141\,000$ M^{-1} cm^{-1}, calculated for a protein containing 18 tryptophanyl and 27 tyrosyl residues (which is the aromatic amino acid composition of brevin as shown in Table I) and assuming average values for the molar extinction coefficients of tryptophanyl and tyrosyl residues at 280 nm ($\epsilon_{M,Trp}^{280} = 5850$ M^{-1} cm^{-1} and $\epsilon_{M,Tyr}^{280} = 1340$ M^{-1} cm^{-1}; Magne et al., 1977).

Fluorescence Spectra. The native brevin fluorescence spectrum (Figure 1) is independent of the excitation wavelength. Excitation at 280 nm (where both tyrosines and tryptophans absorb) or at 295 nm (selective excitation of tryptophans) gives a fluorescence spectrum with a short-wavelength maximum at 324 ± 1 nm and a spectrum width ($\Delta\lambda$), determined at half-maximal intensity, of 50 ± 0.5 nm. Normalization of the two emission spectra at 380 nm, where fluorescence originates only from tryptophanyl residues, gives rise to superimposable spectra, which suggests that the fluorescence of tyrosyl residues in native brevin is not detectable. These spectral characteristics of brevin are not affected by adding Ca^{2+} to the protein solution.

Table I: Amino Acid Composition of Brevin from Triplicate Analyses after 24-h Hydrolysis

amino acid	residues/mol[a]	amino acid	residues/mol[a]
Asx	79	Ile	22
Thr	44	Leu	67
Ser	60	Tyr	27
Glx	79	Phe	39
Pro	51	Trp[c]	18
Gly	82	His	15
Ala	85	Lys	47
Cys[b]	7	Arg	58
Val	57		total 847
Met	10		

[a] Values were calculated on the basis of M_r 92 000 and rounded to the nearest integer number. [b] Cys determined as carboxymethylcysteine after carboxymethylation and acid hydrolysis. [c] Trp evaluated after hydrolysis with methanesulfonic acid.

Denaturation drastically alters the fluorescence parameters, shifting the maximum toward 348 nm and enlarging the spectrum, with a half-maximum width ($\Delta\lambda$) of 62 nm for excitation at 295 nm.

Fluorescence Quantum Yield and Decay Times. Emission quantum yields of native and denatured brevin (Table II) were determined for excitation at 280 nm (ϕ_P^{280}), where both tyrosyl and tryptophanyl residues absorb, and at 295 nm (ϕ_P^{295}), corresponds to a selective excitation of tryptophanyl residues. Like the fluorescence spectra, the quantum yields of native brevin were not altered by the presence of Ca^{2+}.

Fluorescence spectra analysis (Figure 1) suggested that tyrosyl residues in native brevin were not involved in the fluorescence emission. So the fractional yield ϕ_P^{280}(Trp) relative to the protein tryptophan emission upon excitation at 280 nm is 0.12, i.e., equal to the total protein quantum yield. This value of ϕ_P^{280}(Trp) was used in the following section to calculate tyrosine → tryptophan energy transfer.

Fluorescence lifetimes of brevin are shown in Table II. Mathematical analysis of the decay curves gave two lifetimes which have no physical meaning in the case of a protein containing 18 tryptophans. Since the results we shall discuss averaged out from the various chromophores, we calculated the mean lifetime as $\langle\tau\rangle = \sum_i \beta_i \tau_i$ where τ_i is the ith decay constant and β_i its associated percent weight. The native brevin lifetime is shorter than that of model compounds, which indicates the existence of quenching processes (see Discussion). Its value is not affected by adding Ca^{2+}. However, after denaturation, the brevin lifetime becomes comparable to that of most denatured proteins and similar to that of *N*-acetyl-L-tryptophanamide in water.

Tyrosine → Tryptophan Energy Transfer. For a clearer insight into the mechanism behind tyrosine fluorescence quenching in brevin, we investigated the tyrosine → tryptophan energy-transfer process according to the method previously described (Gérard et al., 1975). The efficiency (η) of this process is given by

$$\phi_P^{280}(\text{Trp}) = \phi_P^{295}(f_{Trp}^{280} + \eta f_{Tyr}^{280})$$

Table II: Fluorescence Characteristics of Brevin and Model Compounds[a]

conditions	λ_{ex} (nm)	λ_{max} (nm)	$\Delta\lambda$ (nm)	ϕ_P	ϕ_{Trp}	τ (ns)	$\langle\tau\rangle$ (ns)	$\phi/\langle\tau\rangle$ (s^{-1})	ω
native brevin – Ca^{2+}	280	324 ± 1	50 ± 1	0.12 ± 0.009					
	295	324 ± 1	50 ± 1	0.13 ± 0.009		0.5 ± 0.15 (19%), 2.9 ± 0.15 (81%)	2.5 ± 0.15	5.2×10^7	0.22
native brevin + Ca^{2+}	280	324 ± 1	50 ± 1	0.12 ± 0.006					
	295	324 ± 1	50 ± 1	0.13 ± 0.007		0.6 ± 0.15 (18%), 2.9 ± 0.15 (82%)	2.5 ± 0.15	5.2×10^7	0.22
brevin denatured in 4.6 M guanidinium chloride	280	348 ± 1	66 ± 1	0.097 ± 0.006					
	295	348 ± 1	62 ± 1	0.12 ± 0.005		0.29 ± 0.15 (9%), 3.27 ± 0.15 (91%)	3 ± 0.15	4×10^7	0.14
N-acetyl-L-tryptophanamide in water		352 ± 1	60 ± 1		0.14	3		4.7×10^7	
N-acetyl-L-tryptophanamide in dioxane		325 ± 1	48 ± 1		0.30	4.5		6.7×10^7	

[a] $\Delta\lambda$ is the spectrum width determined at half-maximal fluorescence intensity, ϕ_P the protein fluorescence quantum yield, τ the fluorescence lifetime, $\langle\tau\rangle$ the average lifetime, and ω the efficiency of the static quenching process. $-Ca^{2+}$ and $+Ca^{2+}$ correspond respectively to Ca^{2+} concentrations $<10^{-8}$ M and equal to 10^{-4} M.

As brevin contains 18 tryptophanyl and 27 tyrosyl residues (Table I), f_{Trp}^{280}, the fractional absorption of tryptophanyl residues at 280 nm, was calculated to be 0.74 for native brevin. The value of $\phi_P^{280}(Trp)$ has been calculated previously, and that of ϕ_P^{295} is shown in Table II. Resonance energy transfer between tyrosyl and tryptophanyl residues therefore occurs with an efficiency around 0.7 in native protein (whether Ca^{2+} is present or not). This high efficiency of energy transfer shows that in native brevin most of the energy absorbed by tyrosines is transferred to tryptophans.

Fluorescence Quenching by Acrylamide. Adding quencher molecules (O_2 or acrylamide) or ions (I^-, Cs^+, or NO_3^-) to protein solutions induces fluorescence quenching which gives information on the fluorophores exposure in protein. Quenching, which involves physical contact between the quencher and the excited fluorophore, is described by the modified Stern–Volmer relationship (Birks, 1970; Eftink & Ghiron, 1976b):

$$I_0/I = (1 + K_{sv}[Q])e^{V[Q]} \quad (3)$$

In this equation, the quenching reaction kinetic is operationally dissected into a collisional component (characterized by K_{sv}) and a static one (characterized by V); I_0 and I are the fluorescence intensities at an appropriate emission wavelength in the absence and presence of a given quencher concentration [Q], K_{sv} is the collisional quenching constant which is the product of the bimolecular quenching rate constant (k_q) and the mean lifetime of the protein in the absence of quencher ($\langle\tau_0\rangle$), and V is the static quenching parameter related to the probability of finding a quencher molecule close enough to an excited chromophore to quench it immediately or statically.

Quenching of brevin fluorescence by acrylamide is shown in Figure 2, where I_0/I (with I corrected as indicated under Materials and Methods) is plotted as a function of added acrylamide concentration. In the absence of Ca^{2+}, a straight line was observed which, according to eq 3, corresponds to $K_{sv} = 2.72 \pm 0.07$ M^{-1} with $k_q = (1.09 \pm 0.03) \times 10^9$ M^{-1} s^{-1} and $V = 0$. In the presence of Ca^{2+}, the curve is at first linear and then curves slightly upward, pointing to a small static quenching contribution. Under these conditions, the best fit for eq 3 was obtained for $K_{sv} = 2.8 \pm 0.3$ M^{-1} with $k_q = (1.12 \pm 0.1) \times 10^9$ M^{-1} s^{-1} and $V = 0.2 \pm 0.1$ M^{-1}. Therefore, adding Ca^{2+} to brevin slightly affects tryptophan fluorescence quenching which suggests a change in the location of tryptophan residues.

Fluorescence Polarization. Steady-state fluorescence polarization measurements were performed to obtain information on tryptophan motion in brevin and, consequently, on the protein dynamics. A wavelength of 295 nm, rather than 280 nm, was chosen to avoid depolarization due to tyrosine → tryptophan energy transfer as well as to internal transition between the two oscillators ^1L_a and ^1L_b (^1L_b being the lowest energy oscillator from which fluorescence stems). At 295 nm, selective excitation of tryptophan, and more presicely of its ^1L_b state, occurs.

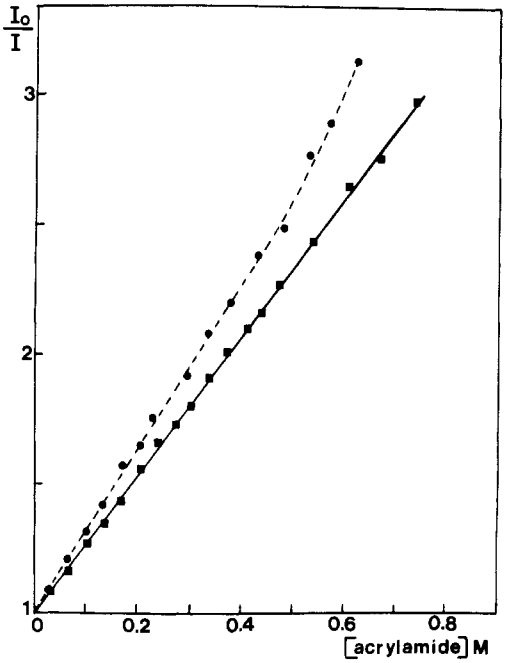

FIGURE 2: Stern–Volmer plots for acrylamide quenching of brevin. Brevin fluorescence was quenched by successive addition of stock acrylamide solution (8.6 M). Fluorescence intensities recorded at 340 nm (λ_{ex} 295 nm) were corrected as indicated under Materials and Methods. Conditions: brevin (8×10^{-7} M) in 50 mM HEPES, 150 mM KCl, and 10^{-4} M EGTA, pH 7.5, in the absence (■) or presence (●) of 10^{-4} M free Ca^{2+}.

Polarization values (p) of multi-tryptophan-containing proteins represent a weighted average over the various tryptophan mobilities. They nevertheless allow the evaluation of the overall variation in the mobility of the emitting residues and hence afford some information as to their location.

Fluorescence polarization degrees are shown in Table III. Calcium binding to brevin induces a 15% decrease in p without any change in the excited-state lifetime. In guanidinium chloride, p is even lower, in agreement with a looser protein structure after denaturation.

Information on brevin tryptophan mobility was obtained from relaxation times (Table III) calculated according to Perrin's equation (see Materials and Methods). If the fluorophores were rigidly attached to the protein, their relaxation time would be identical with that of brevin which, assuming

Table III: Fluorescence Polarization Data for Brevin[a]

	polarization degree, p	av lifetime, $\langle\tau\rangle$ (ns)	relaxation time, ρ (ns)
native brevin − Ca^{2+}	0.200 ± 0.003	2.5 ± 0.15	8.2 ± 0.7
native brevin + Ca^{2+}	0.170 ± 0.005	2.5 ● 0.15	5.9 ± 0.7
brevin denatured in 4.6 M guanidinium chloride	0.094 ± 0.002	3 ± 0.15	2.8 ± 0.22

[a] Excitation and emission wavelengths were respectively set at 295 and 342 nm. p represents the polarization degree, $\langle\tau\rangle$ the average fluorescence lifetime, and ρ the fluorophore relaxation time. For the calculation of ρ, 0.36 was taken as the fundamental polarization degree of tryptophanyl residues excited at 295 nm.

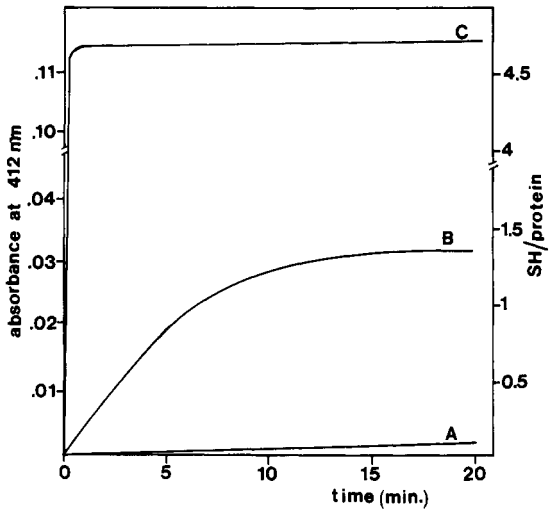

FIGURE 3: Titration of brevin sulfhydryl groups with DTNB in the absence (curve A) or presence of 10^{-4} M free Ca^{2+} (curve B) and in the presence of 5.5 M guanidinium chloride (curve C). The reaction was started by DTNB and was monitored by the absorption at 412 nm. Curves were continuous chart recordings. Buffer conditions for native brevin: 50 mM HEPES, 150 mM NaCl, and 10^{-4} M EGTA, pH 7.45.

brevin is globular with a radius of 44 Å, i.e., Stokes radius (Z. Soua, personal communication), was calculated as 260 ns (see eq 1). Data in Table III therefore indicate that brevin tryptophan rotation occurs independently of the protein matrix and that Ca^{2+} binding to brevin slightly enhances the fluorophore mobility.

Reactivity of -SH Groups. We investigated the reactivity of brevin -SH groups to Ellman's reagent (DTNB) in the absence and presence of Ca^{2+} and in the presence of guanidinium chloride. For the native protein in the absence of Ca^{2+}, the rate and extent of reaction with DNTB were very low (Figure 3), which suggests that -SH groups are not readily accessible to the reagent. In the presence of Ca^{2+}, the reactivity toward DTNB increases and after 18 min reaches a plateau corresponding to 1.3 mol of titratable -SH groups per mole of protein. Brevin denaturation exposes -SH groups which react instantaneously with DTNB. Data collected for 20 min indicate that this fast reaction accounts for 4.6 -SH groups per brevin. Since brevin contains seven cysteinyl residues (Table I), it might be postulated from the foregoing data that brevin has one disulfide bond plus five free -SH groups which are strongly masked in the native protein.

Temperature Effect on Brevin Fluorescence. Figure 4 represents the temperature dependence of brevin fluorescence quantum yield in the absence and presence of Ca^{2+}. Fusion curves obtained under both conditions are akin, which suggests that temperature-induced changes in the fluorescence characteristics of brevin were Ca^{2+} independent. Up to 45–47 °C, the fluorescence quantum yield decreased almost linearly, with

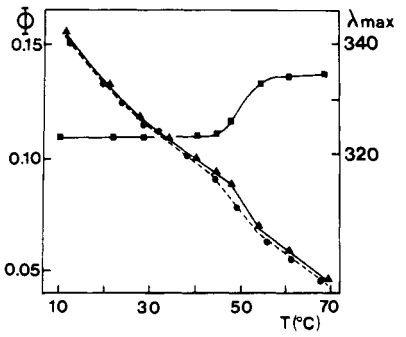

FIGURE 4: Temperature effect on brevin fluorescence parameters. Variation in the fluorescence quantum yield upon heating a brevin solution (10^{-6} M) in 50 mM HEPES, 150 mM KCl, and 10^{-4} M EGTA, pH 7.5, in the absence (▲) or presence (●) of 10^{-4} M free Ca^{2+}. (■) Change in the fluorescence maximum, λ_{ex} 295 nm.

the maximum of emission and the half-maximal width respectively clamped at 324 and 60 nm. The linear drop in fluorescence quantum yield as a function of temperature is not to be related to a change in the protein structure but to an increased efficiency of the radiationless deactivation of the fluorophores. Between 47 and 53 °C, the slope of the fusion curve changed drastically. In addition, fluorescence spectra at these temperatures showed a shift of the maximum toward 335 nm, an enlargment of the half-maximal width to 65 nm, and a sharp increase of the Rayleigh scattering intensity. These spectral changes were totally irreversible and persisted after 55 °C. At 70 °C, the protein precipitated. Brevin thus appears to be heat stable up to about 45 °C. Above this temperature, an irreversible conformational change takes place in which tryptophanyl residues become globally more exposed to solvent (as indicated by the shift in λ_{max} and the increase in half-maximal width) and the protein tends to aggregate.

Hydrophobic Properties of Brevin Studied with Fluorescent Probes (TNS or 9-AC). Brevin purification involves Ca^{2+}-dependent hydrophobic chromatography on phenyl-Sepharose. To test the possibility of Ca^{2+}-induced exposure of a hydrophobic region(s), we looked at the interaction between fluorescent hydrophobic probes such as TNS or 9-AC and brevin in the absence and in the presence of Ca^{2+}. When TNS, which is weakly fluorescent in aqueous media, binds to hydrophobic sites in proteins, a sharp increase in its fluorescence intensity with a blue shift in the maximum, is observed (McClure & Edelman, 1966). With brevin, no significant TNS fluorescence change is observed either in the absence of in the presence of Ca^{2+} (data not shown). The same observation was made with 9-AC.

The results strongly suggest that brevin has no hydrophobic domains and that Ca^{2+}, although it triggers brevin binding to phenyl-Sepharose, does not expose a hydrophobic patch(es). These properties are at variance with those obtained for proteins such as calmodulin (Laporte et al., 1980; Follenius & Gérard, 1984) or S100 proteins (Baudier & Gérard, 1983) but are similar to those obtained for calelectrins (Südhof, 1984).

Ca^{2+} titration of brevin was carried out by using the change in the fluorescence polarization degree (p) as a function of added Ca^{2+} (Figure 5). In the absence of Ca^{2+} (50 mM HEPES, 150 mM KCl, and 10^{-4} M EGTA, pH 7.5), the brevin polarization degree was 0.195. Adding Ca^{2+} reduced p by 15% to give 0.169 at the titration plateau. A mathematical fit of the experimental data according to eq 2 (see Materials and Methods) gave a Ca^{2+} binding association constant of (0.48 ± 0.07) × 10^5 M^{-1} (K_D = 2 × 10^{-5} M), with a Hill coefficient (n) equal to 2 ± 0.5. The same values were

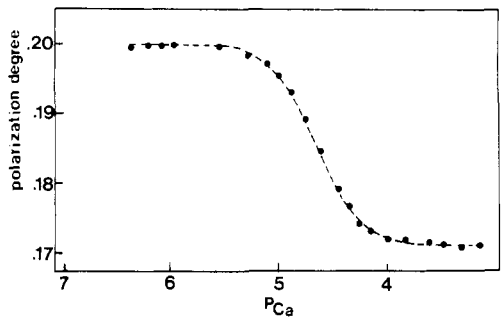

FIGURE 5: Variation in brevin tryptophan fluorescence polarization degree as a function of Ca^{2+} addition (●). Ca^{2+} was removed from brevin (1.2×10^{-6} M) as indicated under Materials and Methods. Buffer conditions: 50 mM HEPES, 150 mM KCl, and 10^{-4} M EGTA, pH 7.5; λ_{ex} 295 nm, λ_{em} 342 nm. Data points were fitted according to eq 2.

obtained when KCl was replaced by NaCl.

No change in p was observed after adding Mg^{2+} (up to 10^{-2} M) to Ca^{2+}-free brevin, which indicates that Mg^{2+} cannot replace Ca^{2+} in changing tryptophan mobility.

Discussion

State of Tryptophanyl Residues in Brevin As Revealed by Intrinsic Fluorescence. Brevin contains 18 tryptophanyl and 27 tyrosyl residues (Table I). However, analysis of the fluorescence spectrum obtained after excitation at 280 nm, where both tyrosines and tryptophans absorb, indicates that tryptophans are the only detectable emitting residues in native brevin. The native brevin fluorescence spectrum has a short-wavelength maximum at 324 nm, with a width (determined at half-maximal intensity) of 50 nm. These fluorescence characteristics are similar to those of the model compound *N*-acetyl-L-tryptophanamide in dioxane, which suggests that tryptophanyl residues in brevin are buried within the hydrophobic core of the protein.

Further information on the location and exposure of the tryptophan residues in brevin was obtained by acrylamide fluorescence quenching studies. In multi-tryptophan proteins, heterogeneous fluorescence and the difference in accessibility of the residues to quencher molecules render interpretation of Stern–Volmer plots somewhat tricky. Although brevin contains numerous tryptophans, its fluorescence maximum, at 324 nm, strongly suggests that the emission and the location of the tryptophans are fairly homogeneous. The linearity of the Stern–Volmer plot for brevin in the absence of Ca^{2+} points toward the absence of static quenching due to acrylamide molecules which are in steady-state contact with the chromophore. Eftink & Ghiron (1976a) have suggested that the more an indole ring is shielded by segments of a protein, the less it will be subject to static quenching. Collisional quenching of such chromophores requires acrylamide to penetrate the surrounding matrix, and the quenching rate constant, k_q, in this case constitutes a measure of the fluorophore burial depth. According to Eftink & Ghiron (1976a), k_q values for brevin in the absence of Ca^{2+} correspond to intermediate topographical positioning of the tryptophan residues; i.e., they are not buried deep inside the protein nor are they completely exposed to the solvent. On the basis of quenching data, the emitting tryptophans might be near the protein surface (cf. k_q) but sterically shielded by the protein matrix and loosely accessible to the aqueous medium (cf. blue fluorescence and linearity of the Stern–Volmer plot). Ca^{2+} binding does not affect the position of the tryptophan residues with respect to the protein surface (k_q unchanged) but does slightly reduce the shielding of the chromophores, allowing static quenching to take place. The latter effect is low and only detected at acrylamide concentrations above 0.6 M. Superficial location of the tryptophan residues is corroborated by the observation that Ca^{2+} binding affects fluorescence polarization (which mostly reflects local changes) whereas it does not induce any (or only minor) modification in the other fluorescence parameters. Moreover, the decrease in the fluorescence polarization degree induced by Ca^{2+} may well be related to partial removal of steric hindrance brought about by protein segments around the tryptophans, allowing the chromophores to rotate more freely.

Quenching Mechanism in Brevin. In view of results on the location and environment of tryptophans in brevin, *N*-acetyl-L-tryptophanamide in dioxane and water is taken as respective models for native and denatured brevin. Only tryptophan data obtained upon excitation at 295 nm (selective excitation of tryptophanyl residues) will be discussed.

Comparative analysis of the brevin quantum yield (ϕ) and average decay time ($\langle\tau\rangle$) with those of model compounds (Table I) indicates that both dynamic and static quenching processes occur in the protein.

Dynamic quenching, due to a diffusion-controlled encounter between the excited fluorophores and the quenching groups of the protein, is revealed by lower lifetimes than those of model compounds. Dynamic quenching seems to disappear after protein denaturation.

Static quenching, related to permanent contact between the chromophore and the quenching group, can be deduced from ϕ/τ ratios, since it decreases quantum yields without affecting decay times. The efficiency (ω) of this process can be evaluated from the ϕ/τ ratios of the protein and the monomer model system, since the fraction, ω, of the fluorescent residues subjected to this process is given by

$$\omega = 1 - \frac{(\phi/\tau)_p}{(\phi/\tau)_m}$$

where the subscript p refers to the residues and m to the corresponding model system. Table II shows that the probability of tryptophans being quenched by a static process is higher in the native ($\omega = 0.22$) than in the denatured ($\omega = 0.14$) protein. This observation is easily explained by the fact that static quenching is due not only to residues close to the chromophores in the primary structure but also to groups which become adjacent to the indole rings after folding of the polypeptide chain.

Structural Changes Induced by Ca^{2+} Binding to Brevin. Brevin's effect on actin polymerization has been reported to be Ca^{2+} dependent (Harris & Schwartz, 1981; Thorstensson et al., 1982; Wilkins et al., 1983). In addition, Ca^{2+} changes the behavior of brevin during hydrophobic chromatography. We therefore applied fluorescence methods to gain clearer insight into the structural changes accompanying Ca^{2+} binding to brevin. Since most of the tryptophanyl residues lie close to the protein surface, in a hydrophobic environment, a large-scale structural rearrangement induced by Ca^{2+} binding would probably involve the regions where at least some tryptophans are located. If the environment of tryptophans changes, this will be reflected by more or less profound changes in the fluorescence spectrum (shape, position of the maximum, half-maximum width).

While one would not expect a tremendous change in fluorescence because of the high tryptophan content of brevin, analysis of the fluorescence spectra, quantum yields, and decay times has shown that these different parameters remain unchanged within the limits of experimental error. Only slight

unshielding of the chromophores and an increase in their overall mobility were revealed by fluorescence polarization and quenching studies. The results demonstrate with a high probability that if Ca^{2+} binding to brevin does produce structural changes capable of modifying the protein activity in actin polymerization, they are of very local character and produce no major changes in the polypeptide chain. A similar lack of gross protein structure changes has been described for enzymes and, particularly, (Na^+, K^+)-dependent adenosinetriphosphatase during its mechanochemical working cycle (Chetverin et al., 1980).

Ca^{2+} binding to brevin allows it to bind to hydrophobic matrices such as phenyl-Sepharose without exposing hydrophobic patches on the protein surface (cf. the interaction with TNS). This particular behavior is still not clear although it suggests that the presence of a real hydrophobic region is not a prerequisite for binding to phenyl-Sepharose. Small changes, of hydrophobic character, may be enough for the interaction with phenyl groups.

Brevin Ca^{2+} Binding Sites. Ca^{2+}-induced changes in the fluorescence polarization degree seem to be specific for Ca^{2+}, since they are not triggered by other divalent cations such as Mg^{2+} or Zn^{2+} (data not shown). The number of calcium binding sites and their affinity for the cation have not yet been determined for brevin. However, reports on gelsolin, a closely related protein, indicate the existence of two Ca^{2+} binding sites with $K_D \simeq 10^{-6}$ M (Yin & Stossel, 1980; Bryan & Kurth, 1984). However, further experiments are required to determine the similarity between gelsolin and brevin Ca^{2+} binding sites and to establish the correlation between the binding sites titrated by fluorescence polarization, those detected by direct binding measurements (equilibrium or flow dialysis), and the functional binding sites involved in brevin's effect on actin polymerization.

Concluding Remarks. Fluorescence did not reveal any considerable protein structure change in brevin after Ca^{2+} binding. This result is striking in light of the widespread view that the functioning of Ca^{2+} binding proteins is accompanied by drastic changes in the conformation of the protein molecules. However, the possibility of more important conformational changes at the level of interprotein interactions cannot be ruled out.

Brevin is akin to gelsolin, an intracellular M_r 90 000 actin binding protein present in a variety of muscle and nonmuscle cells (Yin et al., 1980, 1981a,b; Kurth et al., 1983; Rouayrenc et al., 1984; Kurth & Bryan, 1984; Olomucki et al., 1984), where it is thought to play a role in cell motility. Both proteins have common properties, namely, the ability to fragment actin filaments in a Ca^{2+}-dependent manner and to cap the preferred growing end of the filament. Although very close to each other, brevin and gelsolin have recently been shown to be different molecular entities, with brevin being secreted into the extracellular medium (Yin et al., 1984), where it is supposed to have a scavenging function involved in keeping the bloodstream free from cytoskeletal remnants of damaged cells (Thorstensson et al., 1979; Van Baelen et al., 1980; Harris & Schwartz, 1981). As regards the Ca^{2+} concentration in blood (ca. 1 mM), brevin Ca^{2+} binding sites will always be saturated by the cation. These sites, therefore, cannot be considered as regulatory sites, and their physiological significance, if any, remains unknown.

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Registry No. Ca, 7440-70-2; *N*-acetyl-L-tryptophanamide, 2382-79-8; acrylamide, 79-06-1.

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Photolytic Interruptions of the Bacteriorhodopsin Photocycle Examined by Time-Resolved Resonance Raman Spectroscopy†

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ABSTRACT: An investigation of the photolytic conditions used to initiate and spectroscopically monitor the bacteriorhodopsin (BR) photocycle utilizing time-resolved resonance Raman (TR^3) spectroscopy has revealed and characterized two photoinduced reactions that interrupt the thermal pathway. One reaction involves the photolytic interconversion of M-412 and M′, and the other involves the direct photolytic conversion of the BR-570/K-590 photostationary mixture either to M-412 and M′ or to M-like intermediates within 10 ns. The photolytic threshold conditions describing both reactions have been quantitatively measured and are discussed in terms of experimental parameters.

Bacteriorhodopsin (BR)[1] has been identified as a protein complex containing the chromophore retinal in the purple membrane (PM)[1] of *Halobacterium halobium*. It functions in the PM to convert absorbed light into the energy required to translocate protons across the membrane and to perform ATP[1] synthesis. These fundamental biochemical activities were first reported by Oesterhelt & Stoeckenius (1973) and have been substantiated subsequently by many workers [for review, see Stoeckenius et al. (1979)].

The maximum absorption of the BR complex occurs at 570 nm, which has led to the designation BR-570. The optical excitation of BR-570 initiates a chemical cycle involving several distinct intermediates [including K-590, L-550, M-412, and O-640 (Stoeckenius et al., 1979)]. Although these intermediates have been only partially characterized, several important features of the molecular bonding and conformation of the retinal chromophore have been elucidated by transient spectroscopies. The early studies of the BR photocycle were derived primarily from transient absorption spectroscopy, while recently, more structurally sensitive data have been obtained from spectroscopies based on vibrational Raman scattering (Lewis et al., 1974; Aton et al., 1977; Marcus & Lewis, 1978; Terner et al., 1979; Stockburger et al., 1979; Braiman & Mathies, 1980; Atkinson, 1981). Vibrational resonance Raman (RR)[1] spectroscopy has been especially useful for examining the bonding and conformations of molecules containing retinal. Specific regions of the RR spectra of the retinal chromophore have been associated with a variety of structural parameters via studies of both biochemically active compounds and synthetically prepared model systems (Eyring et al., 1980a,b; Alshuth & Stockburger, 1981; Massig et al., 1982). These data provide the essential information required to identify the structure of retinal, even in a complex biochemical system such as BR. RR spectroscopy, therefore, is particularly well suited to examine the species that comprise the BR-570 photocycle.

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[1] Abbreviations: BR, bacteriorhodopsin; PM, purple membrane; ATP, adenosine 5′-triphosphate; RR, resonance Raman; TR^3, time-resolved resonance Raman; cw, continuous wave; SDS, sodium dodecyl sulfate; fwhm, full width at half-maximum.

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