Assessment of the Exposure and Environments of Tryptophanyl Residues in Ribosomal Protein S1 by Fluorescence Quenching†

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ABSTRACT: The tryptophanyl fluorescence of *Escherichia coli* ribosomal protein S1 is partially quenched by bound nucleic acids, acrylamide, *N*-methylnicotinamide chloride (NMNCl), Cs^+, and I^-. The low molecular weight quenching studies were performed in both the free and polynucleotide-complexed forms of the protein. Polynucleotides with thermolabile secondary structures, such as poly(I), poly(U), and poly(dU), elicit a substantially greater quenching effect than the more stable structures of poly(A) and poly(C) upon binding. Poly(dC) and poly(dA), however, elicit significantly less and virtually no quenching, respectively. The observed differential quenching effects are reconciled in terms of the ease with which their bases form stacked complexes with the indole ring of the tryptophanyl residues and the available number of nucleic acid binding sites on the protein. The fluorescence emission characteristics of native S1 protein imply that the fluorophores are only partially exposed to solvent. The low molecular weight quenching data were analyzed by using both Stern–Volmer [Stern, O., & Volmer, M. (1919) *Phys. Z. 20*, 183–193] and modified Stern–Volmer treatments [Lehrer, S. S. (1971) *Biochemistry 10*, 3254–3263], yielding values for the bimolecular quenching constant, the effective quenching constant, and the fraction of accessible fluorescence. The results reveal that the protein fluorophores are completely accessible to acrylamide, but only 75%, 63%, and 44% accessible to NMNCl, Cs^+, and I^-, respectively. However, in the unfolded form of the protein, NMNCl showed a significant decrease in the quenching constant, implying the disruption of a favorable electrostatic environment(s). The binding of polynucleotides, especially those which bind to both sites of S1 protein, results in a significant reduction in the quenching ability of both acrylamide and NMNCl. There appears to be no indication from the fluorescence studies that polynucleotides induce a major protein conformational change upon binding. These studies provide information about the exposure and environments of the tryptophanyl residues of a protein indispensable in the initiation of protein biosynthesis.

In this paper, we report the results of fluorescence quenching studies of the tryptophanyl residues present in *Escherichia coli* ribosomal protein S1, a protein indispensable for the initiation of protein biosynthesis, using different polynucleotides and the low molecular weight quenchers acrylamide, *N*-methylnicotinamide chloride (NMNCl),[1] Cs^+, and I^-. The low molecular weight quenching studies were performed in both the free and polynucleotide-complexed forms of the protein.

Previous studies by Draper et al. (1977) and Draper & von Hippel (1978a,b) have demonstrated that certain oligonucleotides and polynucleotides are effective quenchers of the tryptophanyl emission in protein S1; such emission is totally quenched by the stacking of the indole ring of a tryptophanyl residue with the nucleic acid base (Mutai et al., 1975; Hélène, 1977). Ligand-induced alterations in protein conformation are another source of fluorophore quantum yield changes (Hélène, 1977). Acrylamide is a neutral molecule which has been shown to be an excellent quencher of tryptophanyl fluorescence (Eftink & Ghiron, 1975, 1976, 1977). Its quenching effect is not strongly influenced by the local electrostatic environments within the protein matrix and is therefore quite suitable for evaluating the exposure of the tryptophanyl residues. NMNCl also quenches tryptophanyl emission efficiently (Holmes & Robbins, 1974). Since this molecule is positively charged, its quenching efficiency is likely to be affected by electrostatic charges surrounding the tryptophanyl residues as are the ionic quenchers Cs^+ and I^- (Lehrer, 1967, 1971).

Materials and Methods

Chemicals. Electrophoretic-grade acrylamide was obtained from Bio-Rad, and NMNCl was from Sigma Chemical Co. Mono-, oligo-, and polynucleotides and *N*-AcTrpNH$_2$ were purchased from P-L Biochemicals and used without further purification. Reagents, such as KI and CsCl, were analytical grade. Concentrations of mono-, oligo-, and polynucleotides were determined spectrophotometrically by using extinction coefficients provided by the manufacturer and are expressed in monomer units.

Buffers. AC buffer contained 50 mM $(HOCH_2)_3CNH_2$-HCl (pH 7.5), 1 mM Na_2EDTA, 0.1 mM DTT, 50 mM NaCl, and 10% (w/v) glycerol. *Phosphate buffer* contained 20 mM Na_2HPO_4 (pH 7.8), 1 mM Na_2EDTA, 0.1 mM DTT, 100 mM NaCl, and 10% (w/v) glycerol. Buffers of higher ionic strength, where required, were prepared by adding the necessary amount of solid salt.

Protein Isolation. The purification procedure for S1 protein from *E. coli* MRE 600 ribosomes has been described in detail (Bear, 1977; Omar, 1979) and is also available upon request. The purification of S1 protein basically involves three main steps: isolation of the ribosome from the bacterial cells and subsequently obtaining a ribosome wash; fractional $(NH_4)_2SO_4$ precipitation of the ribosomal wash to obtain a 35–60% cut;

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[1] Abbreviations used: NMNCl, *N*-methylnicotinamide chloride; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; Gdn·HCl, guanidine hydrochloride; *N*-AcTrpNH$_2$, *N*-acetyltryptophanamide; 5′-AMP, adenosine 5′-monophosphate; 5′-CMP, cytidine 5′-monophosphate; poly(A), poly(riboadenylic acid); poly(dA), poly(deoxyriboadenylic acid); poly(C), poly(ribocytidylic acid); poly(dC), poly(deoxyribocytidylic acid); poly(I), poly(riboinosinic acid); poly(U), poly(ribouridylic acid); poly(dU), poly(deoxyribouridylic acid); CIDNP, chemically induced dynamic nuclear polarization; NMR, nuclear magnetic resonance.

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poly(C)–cellulose affinity chromatography of this fractionated ribosomal wash by using a final eluant of AC buffer containing 1 M NH_4Cl and 3.5 M urea. (The use of 3.5 M urea did not significantly shift the S1 protein tryptophanyl emission wavelength maximum, suggesting that this solvent does not denature or drastically alter the protein conformation, unlike 7 M urea.) S1 protein isolated by our procedure shows a single band on $NaDodSO_4$–polyacrylamide gel electrophoresis which comigrates with bovine serum albumin. Purity was estimated by densitometry to be 95%. The A_{280}/A_{260} was 1.5 ± 0.05 in AC buffer. Protein concentration either was determined spectrophotometrically (0.8 A_{280nm} mg^{-2} mL^{-1}) (Omar, 1979) or was determined by the Coomassie dye method (Bradford, 1976) with bovine serum albumin as a standard. The purified protein (ca. 1 mg/mL) in AC buffer was stored in small aliquots at −70 °C.

Fluorescence Quenching Titrations. Titrations were performed with a Perkin-Elmer MPF-2A spectrofluorometer operating in the ratio mode, as previously described (Brown et al., 1977). The spectral bandwidth of the excitation monochromator did not exceed 10 nm, whereas the emission monochromator bandwidth was kept to values below 15 nm. All measurements were recorded at 23–24 °C. Sample absorbance at the excitation wavelength (295 nm) was determined at each stage of polynucleotide titration by using a Beckman ACTA V spectrophotometer, before and after each fluorescence measurement. Emission spectra were recorded in the interval 300–400 nm. A similar titration was performed on a reference solution containing *N*-AcTrpNH_2, as a check for the "inner filter" correction (see below). Relative fluorescence intensities at 340 nm were expressed in arbitrary units, since there was no significant shift in the emission maximum upon quenching. Data were corrected for dilution due to the addition of titrant. Where changes in the absorbance exceeded 0.02 unit, the emission data were corrected for an inner filter effect according to the method of Hêlêne et al. (1969).

Fluorescence quenching induced by nucleic acid, Q, was expressed in terms of fractional intrinsic fluorescence quenching:

$$Q = \frac{F_0 - F_c}{F_0} \tag{1}$$

where F_0 is the initial emission intensity prior to the addition of titrant and F_c is the corrected emission intensity after titrant addition.

For fluorescence quenching with acrylamide, Cs^+, and I^-, sample solutions containing ca. 0.1 mg/mL protein were excited at 295 nm. Quenching titrations with NMNCl were performed by excitation at 300 nm. Corrections for the inner filter effect were made as described above. For acrylamide and NMNCl titrations, phosphate buffer (pH 7.8) contained 0.1 M NaCl, while in the case of titrations with KI and CsCl, buffer solutions contained 0.25 or 0.50 M NaCl. $Na_2S_2O_3$ (0.1 mM) was added to the KI solution to prevent oxidation and formation of I_3^-.

Calculation of the Quenching Data. Corrected fluorescence quenching data were analyzed according to the Stern–Volmer (SV) equation (eq 2) (Stern & Volmer, 1919) and also according to the modified form of the SV equation (eq 3) derived by Lehrer (1971):

$$F_0/F = 1 + K_{SV}[Q] \tag{2}$$

$$F_0/\Delta F = \frac{1}{f_{a(eff)}} + \frac{1}{F_{a(eff)}K_{Q(eff)}[Q]} \tag{3}$$

where F_0 and F are the fluorescence quantum yields in the absence and presence of quencher (Q), respectively ($\Delta F = F_0 - F$), and K_{SV} is the Stern–Volmer quenching constant, obtained from the slope of the plot F_0/F vs. [Q]. ($K_{SV} = \tau_0 k_3$ where τ_0 is the fluorescence lifetime in the absence of quencher and k_3 is the rate constant for bimolecular collision between quencher and fluorophores.) The $f_{a(eff)}$ and $K_{Q(eff)}$ terms in eq 3 (i.e., 1/intercept and intercept/slope for $F_0/\Delta F$ vs. $[Q]^{-1}$ plots, respectively) are the maximum fraction of quenchable fluorescence and the effective quenching constant, respectively, where both parameters are derived from the modified SV plot.

In some cases, the simple Stern–Volmer plots showed considerable positive (upward) curvature, suggesting the presence of a static quenching component. In these cases, data were also plotted according to eq 4 (Eftink & Ghiron, 1976) where

$$F_0/(Fe^{V[Q]}) = 1 + K_{SV}[Q] \tag{4}$$

V is the static quenching parameter. In our calculations, values for the V parameter were arbitrarily chosen to give the best fit to a linear plot when the data were treated according to eq 4.

Fluorescence Lifetime Measurements. The fluorescence lifetime, τ_0, for S1 protein was measured on an ORTEC Model 9200 photon-counting emission spectrometer which was interfaced to a DEC PDP 11/10 minicomputer. A 302-nm interference filter, with a 10-nm bandwidth, was placed on the excitation side, and a Corning 7-51 filter was positioned in front of the emitted light. The lifetime was assigned by comparison of the raw decay data to computer-simulated theoretical curves. The fluorescence decay observed for S1 protein was multiexponential, which was impossible to fit in a satisfactory manner. Therefore, it was "forcefitted" to a single exponential to obtain an average lifetime. Several single exponential measurements were obtained for each sample. The fluorescence lifetime for denatured S1 (in 5.6 M Gdn·HCl) was estimated from its relative fluorescence yield by the procedure of Parker & Rees (1960).

Results

Ribosomal protein S1 exhibits an emission spectrum characteristic of buried and/or partly exposed tryptophanyl residues, with the maximum occurring at 335–337 nm and a half-bandwidth, $\Delta\lambda_{1/2}$, of 53–55 nm. No alteration in the shape of the spectral band occurs with variation of the excitation wavelength from 280 to 296 nm. The absence of a shoulder at 308–310 nm implies that fluorescence contributions from tyrosyl residues are negligible. The emission maximum shifts to ca. 350 nm in the presence of a denaturing solvent such as 6 M urea.

Previous studies have shown that the binding of certain single-stranded polynucleotides to S1 results in the perturbation of their secondary structure (Bear et al., 1976; Szer et al., 1976), as well as quenching of the intrinsic protein fluorescence (Draper & von Hippel, 1978a,b). Our studies similarly reveal the quenching of intrinsic protein fluorescence induced by bound nucleic acid, but with certain significant differences. Appreciable quenching of S1 fluorescence (40–45%) is induced by the binding of poly(I) and poly(U) as shown in Figure 1. Theoretical binding curves calculated on the basis of two independent polynucleotide binding sites (S. Omar and T. Schleich, unpublished experiments) are indicated by the solid lines. Each binding site is assumed to contribute equally to the quenching. Association constants of ca. 4×10^5 and 6×10^5 M^{-1}, and a Q_{max} equal to 0.45, were calculated for the S1–poly(I) complex in phosphate buffer (pH 7.8) at 0.1 M

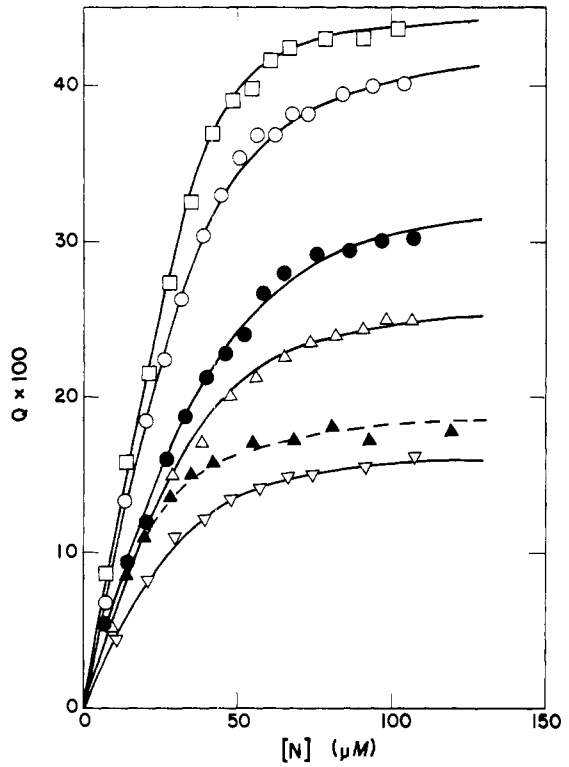

FIGURE 1: Fluorescence quenching titrations of S1 protein by homopolynucleotides: poly(A) (▽); poly(C) (△); poly(dC) (▲); poly(I) (□); poly(U) (○); poly(dU) (●). Solid and dashed lines are calculated quenching curves, assuming two independent binding sites and one binding site, respectively. The S1 protein concentration was ca. 2.0 μM. All titrations were performed in phosphate buffer (pH 7.8) containing 0.10 M NaCl.

NaCl. For the S1–poly(U) complex, association constants of 2×10^5 and 5×10^5 M^{-1} were calculated (Q_{max} = 0.42).

By contrast, poly(A) and poly(C) quench ca. 15 and 25%, respectively, as shown in Figure 1. Association constants of ca. 1×10^5 and 4.5×10^5 M^{-1} (assuming two independent binding sites) were calculated for both polynucleotides. We also observed ca. 30% quenching by poly(dU) (binding constants equal to 1.0×10^5 and 2.5×10^5 M^{-1}; Q_{max} = 0.28), but poly(dC), which binds only to one of the two sites, induced ca. 15% quenching of the protein fluorescence ($K_{assoc} = 1 \times 10^5$ M^{-1}). Thus, polynucleotides with different base moieties induce different degrees of quenching. Similarly, for polynucleotide structures of identical base but different sugar stereochemistry, the polyribonucleotides induce a greater quenching effect than the polydeoxyribonucleotides, even when an equivalent number of binding sites are involved.

However, contrary to previous reports, we did not observe any significant quenching of S1 fluorescence by poly(dA) or the oligomers $p(dA)_4$ and $p(dA)_8$. Furthermore, the absence of a fluorescence quenching effect in the presence of mononucleotides, such as 5′-AMP and 5′-CMP, is consistent with their low binding affinity as demonstrated by equilibrium dialysis studies (Lipecky et al., 1977) and ultrafiltration binding assays (D. Wade and T. Schleich, unpublished experiments).

Acrylamide is an effective quencher of the five tryptophanyl residues contained in the S1 structure (Draper, 1977; S. Omar and T. Schleich, unpublished experiments). A linear Stern–Volmer plot is obtained as shown in Figure 2, implying the predominance of a collisional quenching mechanism. The quenching parameters are summarized in Table I. [Our values for *N*-AcTrpNH$_2$ are in excellent agreement with those previously published (Eftink & Ghiron, 1977).] The value

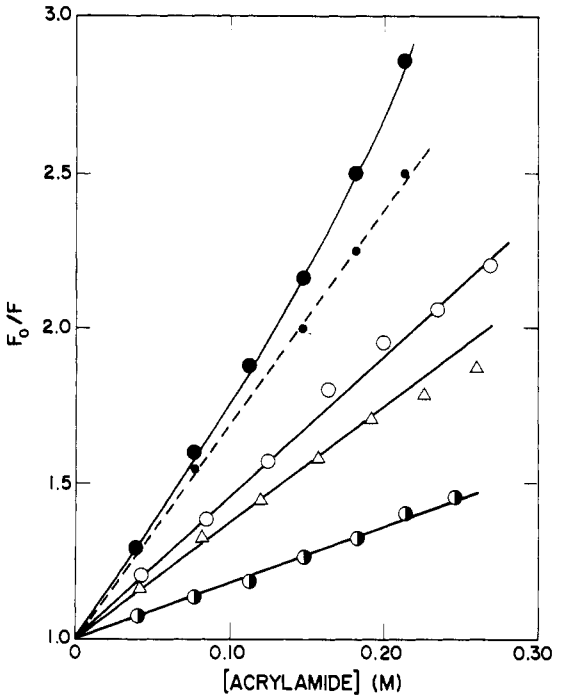

FIGURE 2: Stern–Volmer plots of the fluorescence quenching of S1 protein by acrylamide: native S1 protein in phosphate buffer (pH 7.8) (○); denatured S1 protein in 5.6 M Gdn·HCl (●); S1 protein in 50% (w/v) glycerol phosphate buffer (pH 7.8) (◑); S1 protein + poly(U) (saturating amount) in phosphate buffer (pH 7.8) (△). The dashed line represents denatured S1 protein corrected for static quenching according to eq 4. The phosphate buffer contained 0.1 M NaCl.

Table I: Acrylamide Quenching Parameters for Ribosomal Protein S1[a]

fluorophore	K_{SV} (M^{-1})	V (M^{-1})	$K_{Q(eff)}$ (M^{-1})	$f_{a(eff)}$
N-AcTrpNH$_2$	17.40	1.8	18.40	0.99
protein S1	4.50	0.0	4.76	0.98
protein S1 in 5.6 M Gdn·HCl	6.92	0.5	6.89	1.00
protein S1 in 50% (w/v) glycerol buffer	1.92	0.0	1.82	0.99

[a] All measurements were performed in phosphate buffer (pH 7.8) containing 0.10 M NaCl and 5.6 M Gdn·HCl, 50% (w/v) glycerol solutions were made in the same buffer. Errors are 2.4% for K_{SV} and 5.0% for $f_{a(eff)}$ and $K_{Q(eff)}$.

for K_{SV} obtained for the acrylamide quenching of the native S1 protein emission lies within the range of quenching constants of other multi-tryptophanyl-containing proteins (Eftink & Ghiron, 1976). The average lifetime for S1 fluorescence in the absence of quencher is 3.5 ± 0.3 ns. The lifetime and the collisional quenching constant yield a bimolecular rate constant, k_3, equal to 1.3×10^9 M^{-1} s^{-1}.

The linear Stern–Volmer plot implies that the tryptophanyl residues in S1 differ only slightly in their accessibility to acrylamide. The presence of a static factor in acrylamide quenching normally causes upward curvature in the Stern–Volmer plot. However, for a protein such as S1, which contains five tryptophanyl residues, some environmental heterogeneity is expected; thus, some residues may be more readily quenched than others. The static effect of acrylamide quenching most likely is counteracted by selective quenching which tends to make the plot curve downward. In fact, when acrylamide-induced fluorescence quenching was performed with Gdn·HCl-denatured S1, the Stern–Volmer plot displays

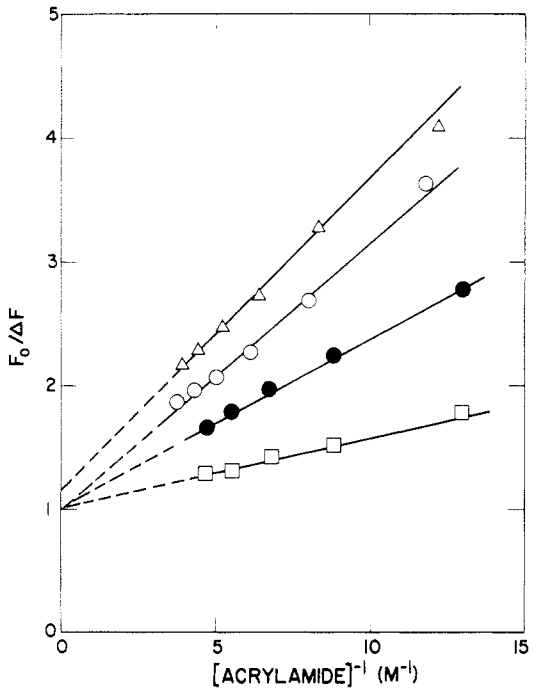

FIGURE 3: Modified Stern–Volmer plots of the fluorescence quenching of S1 protein by acrylamide: native S1 (○); denatured S1 (in Gdn·HCl) (●); S1 + poly(U) (saturating amount) (△); *N*-AcTrpNH$_2$ (□). Experimental conditions are as previously described.

positive deviation (see Figure 2), indicative of a static quenching component. A linear curve fit (eq 4) to the quenching data yields the static quenching parameter (see Table I). An excited-state lifetime of 4.4 ns was obtained for the denatured protein fluorescence. Denaturation of the protein also resulted in a 2-fold increase of the bimolecular rate constant ($k_3 = 2.5 \times 10^9$ M^{-1} s^{-1}) relative to that for the native structure.

Since a molecular collisional mechanism dominates the acrylamide quenching, the rate is expected to be sensitive to alterations in solution viscosity. Quenching studies were performed also in buffer containing 50% (w/v) glycerol. We observed a significant decrease in K_{SV} (see Table I). At this concentration of glycerol, the viscosity of the buffer is ca. 5 times higher than that of the normal buffer which contains only 10% glycerol, resulting in a 2.5-fold decrease in the bimolecular rate constant.

In Figure 3, acrylamide quenching data for native S1 are shown plotted according to the modified Stern–Volmer equation (eq 3), and the quenching parameters are tabulated in Table I. As indicated, the tryptophanyl fluorescence in the native structure of S1 is completely accessible to this quencher.

Acrylamide-induced quenching studies of S1 fluorescence were also performed in the presence of saturating amounts of mono-, oligo-, and homopolynucleotides. Representative plots are shown in Figures 2 and 3. These results are summarized in Table II. We found that a polynucleotide which binds to both S1 sites results in significantly decreased values of the acrylamide quenching parameters. For example, poly(A), poly(C), and poly(U) were found to reduce the collisional quenching constant of acrylamide by 1.2 M^{-1}, whereas the decrease in $K_{Q(eff)}$ was not as great. For the poly(C)–S1 complex, the average excited-state lifetime of the tryptophanyl fluorescence was 3.6 ± 0.5 ns, which implies a k_3 of 0.9×10^9 M^{-1} s^{-1}, a decrease of 30% from the value determined for S1 alone. Furthermore, the fraction of accessible fluorescence was significantly reduced by the binding of these polynucleotides to the protein. By contrast, the effects of polydeoxyribonucleotides, such as poly(dA) and poly(dC), which bind to one site, on the quenching parameters were smaller. However, poly(dU) (binds to both sites) is an exception, in that it induced an effect comparable to that of the polyribonucleotides. We also noted that an oligonucleotide, such as $(Ap)_7A$, has a smaller shielding effect than poly(A). Finally, the effects of mononucleotides, 5′-AMP and 5′-CMP, on acrylamide quenching parameters were found to be negligible.

Table II: Effects of Mono-, Oligo-, and Polynucleotides on Acrylamide Quenching Parameters of Ribosomal Protein S1 Fluorescence[a]

protein S1 +	K_{SV} (M^{-1})	$K_{Q(eff)}$ (M^{-1})	$f_{a(eff)}$
	4.50	4.76	0.98
5′-AMP	4.41	4.75	0.96
5′-CMP	4.41	4.80	0.97
$(Ap)_7A$	3.96	4.35	0.97
$p(dA)_8$	4.17	4.63	0.96
poly(A)	3.24	4.52	0.84
poly(dA)	4.12	4.71	0.97
poly(C)	3.25	4.44	0.86
poly(dC)	3.79	4.38	0.93
poly(U)	3.30	4.75	0.86
poly(dU)	3.48	4.87	0.84

[a] Experimental conditions are as described for Table I. Mono-, oligo-, or polynucleotides were added to S1 protein in saturating amounts (70–90 nucleotide residues per S1 molecule), prior to titrations with acrylamide. Errors are 2.4% for K_{SV} and 4.5% for $f_{a(eff)}$ and $K_{Q(eff)}$.

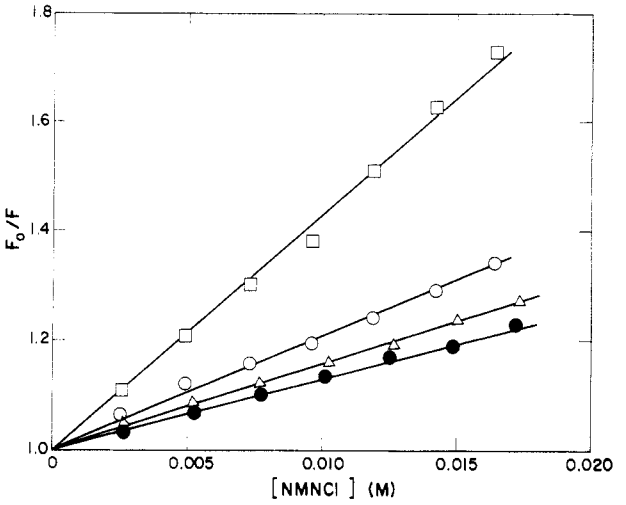

FIGURE 4: Stern–Volmer plots of the fluorescence quenching of S1 protein by NMNCl: native S1 (○); denatured S1 (in Gdn·HCl) (●); S1 + poly(U) (saturating amount) (△); *N*-AcTrpNH$_2$ (□). Experimental conditions are as previously described.

The dominance of the collisional mechanism for NMNCl quenching of S1 tryptophanyl fluorescence is demonstrated by the linearity of the Stern–Volmer plots, as shown in Figure 4. An average Stern–Volmer quenching constant of 20.8 M^{-1}, which implies a bimolecular quenching rate constant of 5.9×10^9 M^{-1} s^{-1}, was obtained. Analysis of the quenching data according to the modified Stern–Volmer formalism (Figure 5) reveals that only a fraction of the protein fluorescence (0.75) was quenched by NMNCl. Thus, in contrast to acrylamide, 25% of the tryptophanyl fluorescence in native S1 is not accessible to this reagent.

The results of NMNCl quenching studies of S1 protein in the presence of mono-, oligo-, and polynucleotides are summarized in Table III. The presence of polyribonucleotides results in ca. a 15% decrease in the Stern–Volmer quenching constant; slightly smaller decreases were observed in the presence of polydeoxyribonucleotides. However, the difference

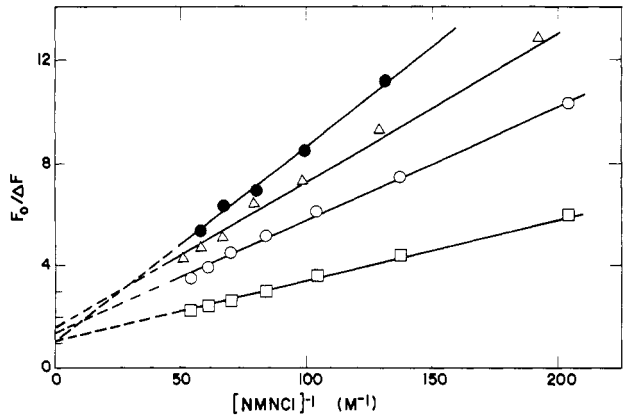

FIGURE 5: Modified Stern–Volmer plots of the fluorescence quenching of S1 protein by NMNCl: native S1 (○); denatured S1 (in Gdn·HCl) (●); S1 + poly(U) (saturating amount) (△); *N*-AcTrpNH$_2$ (□). Experimental conditions are as previously described.

Table III: *N*-Methylnicotinamide Chloride (NMNCl) Quenching Parameters for Ribosomal Protein S1 in the Absence and Presence of Nucleotides[a]

fluorophore	K_{SV} (M^{-1})	$K_{Q(eff)}$ (M^{-1})	$f_{a(eff)}$
N-AcTrpNH$_2$	44.2	44.8	0.96
protein S1	20.8	30.4	0.75
S1/5.6 M Gdn·HCl (denatured S1)	13.8	14.3	0.97
S1 + 5′-AMP	14.7	23.8	0.72
S1 + 5′-CMP	16.0	23.8	0.76
S1 + $(Ap)_7A$	17.6	27.2	0.77
S1 + $p(dA)_8$	19.3	28.3	0.75
S1 + poly(A)	17.8	29.8	0.73
S1 + poly(dA)	19.1	28.7	0.74
S1 + poly(C)	17.0	27.2	0.72
S1 + poly(dC)	18.5	29.7	0.74
S1 + poly(U)	17.3	29.9	0.67
S1 + poly(dU)	18.2	30.3	0.70

[a] Experimental conditions are as previously described. Nucleotide concentrations were approximately 75 residues per S1 molecule. Errors are 2.4% for K_{SV} and 7.0% for $f_{a(eff)}$ and $K_{Q(eff)}$.

between the effect of a polyribonucleotide and the corresponding deoxy form does *not* appear to be significant. We also observed that the effects of the polynucleotides on $K_{Q(eff)}$ were generally negligible (Table III, column 3). This suggests that the environments of the most accessible tryptophanyl residues in S1 are not significantly altered by bound polynucleotides. Very little difference was found between the accessibility of S1 fluorescence in the presence and absence of polynucleotide (cf. $f_{a(eff)}$, Table III, column 4). In contrast, the effects of mononucleotides, such as 5′-AMP and 5′-CMP, are apparently much greater than those of the polynucleotides. A decrease of ca. 30% in K_{SV} and 20% in $K_{Q(eff)}$ is evident for NMNCl quenching of the S1 fluorescence in the presence of these mononucleotides; $f_{a(eff)}$ is not affected.

We also employed simple ionic quenchers. We found that KI quenched ca. 45% of the intrinsic S1 protein fluorescence with a $K_{Q(eff)}$ of 5.13 M^{-1} (see Table IV). The ionic quencher Cs^+ was also employed. This ion is a much less effective quencher of tryptophanyl fluorescence than I^-. For example, the fluorescence of *N*-AcTrpNH$_2$ is quenched less than 20% of that quenched by I^- (Table IV). Under similar conditions, Cs^+ quenched ca. 60% of the S1 fluorescence (see Table IV) with a quenching constant that is slightly larger than those reported for most proteins (Altekar, 1977). The simple and modified Stern–Volmer plots for Cs^+ and I^- quenching effects are shown in Figures 6 and 7, respectively.

Table IV: Cesium and Iodide Fluorescence Quenching Parameters for Ribosomal Protein S1[a]

quencher	fluorophore	K_{SV} (M^{-1})	$K_{Q(eff)}$ (M^{-1})	$f_{a(eff)}$
CsCl	N-AcTrpNH$_2$	1.70	1.82	0.99
CsCl	S1	0.68	1.19	0.63
KI	N-AcTrpNH$_2$	9.98	10.65	0.97
KI	S1	1.58	5.13	0.44

[a] The phosphate buffer solution contained 0.50 M KCl in place of 0.1 M NaCl. A constant ionic strength (I = 0.5) was maintained. Errors are 4.0% for K_{SV} and 9.7% for $f_{a(eff)}$ and $K_{Q(eff)}$.

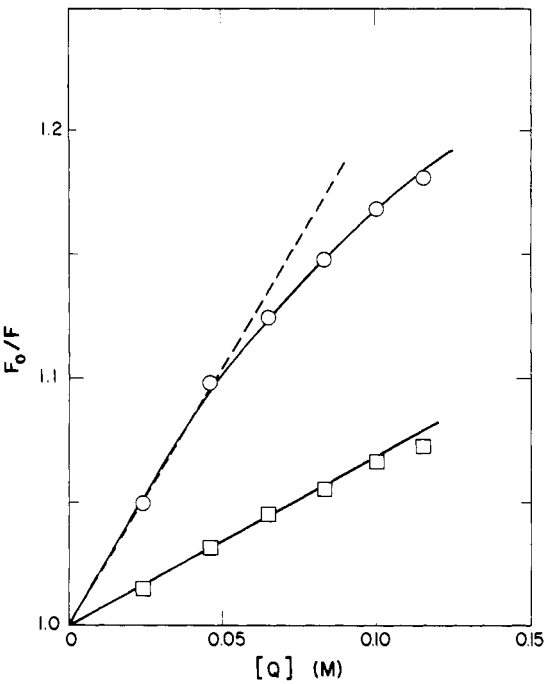

FIGURE 6: Stern–Volmer plots of the fluorescence quenching of S1 plots by KI (○) and CsCl (□). The S1 protein concentration was 1.5 μM in phosphate buffer (pH 7.8) containing 0.50 M KCl instead of NaCl.

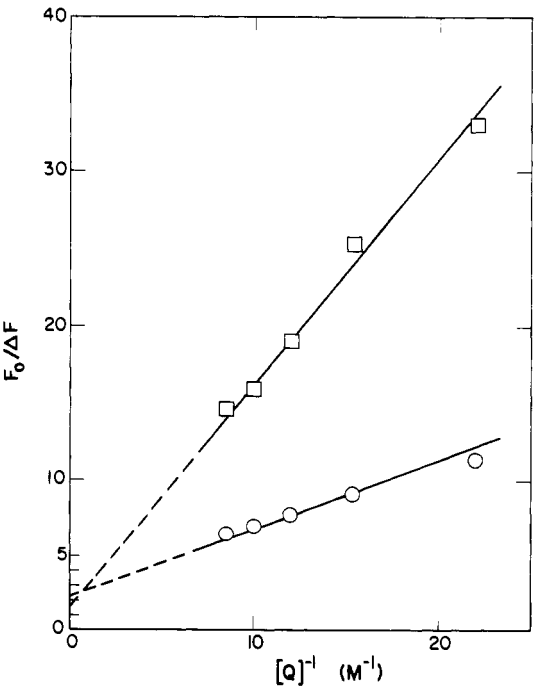

FIGURE 7: Modified Stern–Volmer plots of the fluorescence quenching of S1 protein by KI (○) and CsCl (□). Experimental conditions are as described in Figure 6.

We also attempted to quench S1 fluorescence by use of Cs^+ and I^- ions in the presence of polynucleotides. Unfortunately, such studies had to be abandoned due to the fact that, in the concentration range of 0–0.12 M, these ions only elicited small decreases in the relative fluorescence yield, thus resulting in relatively large experimental errors.

Discussion

The mechanism of protein fluorescence quenching by bound polynucleotide, particularly in the case of multisite proteins, is complex. For nucleic acid quenching of tryptophanyl emission, two dominant mechanisms have been recognized: (i) "direct", quenching that is induced by a stacking interaction between the indole ring of a tryptophanyl residue and a purine or pyrimidine ring; (ii) "indirect", a protein conformational change is induced by ligand binding which in turn modifies the local environment of a fluorophore(s), thereby leading to a change in emission characteristics [see Hélène (1977) and references cited therein]. Other studies (Mutai et al., 1975) have indicated that *total* quenching of tryptophanyl emission occurs upon the stacking of a purine or pyrimidine base with the indole ring. Thus, differential effects dependent on the base are not expected under comparable degrees of stacking.

Hélène and co-workers (Brun et al., 1975; Durand et al., 1975; Maurizot et al., 1978) have proposed a two-step model for the binding of model tryptophanyl-containing peptides to polynucleotides. Application of this model to the S1–polynucleotide interaction results in the following scheme:

$$\text{protein} + \text{poly(N)} \xrightarrow{K_1} \underset{\text{nonstacked}}{\text{complex I}} \xrightarrow{K_2} \underset{\text{stacked}}{\text{complex II}}$$

Complex I arises because of a simple electrostatic interaction between the protein and polynucleotide and does not involve stacking interactions. [Filter binding assays at different ionic strengths confirm the presence of an electrostatic component (S. Omar and T. Schleich, unpublished experiments).] Thus, its fluorescence should remain the same as that of the free protein, provided no changes in protein conformation have occurred. Complex II contains stacked fluorophores and thus would be expected to show at least partial quenching, depending on the fraction of stacked fluorophores, i.e., on the magnitude of K_2. Superimposed on these effects are any ligand-mediated protein conformational changes.

We have shown that polynucleotides such as poly(I) and poly(U) induce a substantially greater quenching effect of S1 fluorescence than either poly(A) or poly(C). The differential quenching effects of these polynucleotides may be reconciled by taking into account their different conformational structures, and the ease with which their bases form stacked complexes (i.e., complex II) with the indole ring of the tryptophanyl residues. Poly(U) with a T_m of 4 °C is in a random coil form at room temperature. Similarly, the structure of poly(I), which is very dependent upon concentration and ionic strength, is also in a random coil form under our conditions (Sarkar & Yang, 1965). The absence of an ordered secondary structure in these polynucleotides most likely enables the adoption of a conformation which permits optimal interactions with functional groups of the protein, thereby facilitating the formation of a stacked and consequently quenched complex. By contrast, at neutral pH, poly(A) and poly(C) form single-stranded stacked helical structures which exhibit broad melting transitions with T_m values of ca. 45 and 51 °C, respectively (Felsenfeld & Miles, 1967). Thus, we conclude that polynucleotides with thermostable secondary structures cannot readily adopt conformations which permit maximum stacking interactions, and hence, their interactions with S1 do not elicit as much quenching.

Lipecky et al. (1977) have demonstrated that the interactions between S1 and polynucleotides such as poly(I) and poly(U) are much stronger than those of poly(A) and poly(C). Furthermore, poly(I) and poly(U) inhibited more than 80% of the reaction between S1 and *N*-ethylmaleimide, while the inhibition afforded by poly(A) and poly(C) was much less. Our fluorescence quenching results are consistent with those of the *N*-ethylmaleimide binding assays.

The preference for pyrimidine over purine bases suggested for S1 binding (Miller et al., 1974; Carmichael, 1975) is not manifested in both of these studies. Our model reduces the base specificity of S1 binding to the flexibility of the polynucleotide structure.

We have fitted theoretical binding isotherms to the polynucleotide-induced quenching data shown in Figure 1 by assuming simple noncooperative equilibrium binding of nucleic acid to one and two S1 protein binding sites. The hyperbolic shape of the experimental quenching curve suggests that the binding process is essentially noncooperative. To take into account protein overlap of nucleic acid bases, we introduced the site size as an empirical adjustment parameter which was kept constant for a particular S1 protein–nucleic acid system. In all of the theoretical binding isotherms shown in Figure 1, the designated number of binding sites (either 1 or 2) used in the calculation gave a substantially better fit than the other. The details of binding isotherm calculation, its extension to *N*-bromosuccinimide-modified S1 protein, and confirmation by filter binding assays will be described in a forthcoming publication (S. Omar and T. Schleich, unpublished experiments). The polynucleotide-induced quenching results and the binding isotherm calculations support the findings of Draper et al. (1977) and Draper & von Hippel (1978a,b), who report the presence of two polyribonucleotide binding sites, with *one* of these sites able to bind polydeoxyribonucleotide, per S1 protein molecule.

Among the polydeoxyribonucleotides used in our quenching studies, we find that poly(dU) (which binds to either of the two S1 binding sites) is a better quencher compared to either poly(A) or poly(C), although the calculated binding constants do not indicate overall tighter binding. The high quenching efficiency of poly(dU) can be explained in terms of the structural flexibility model; i.e., this polydeoxyribonucleotide does not have an ordered secondary structure. We have observed that the quenching effect of poly(dC) is less than that of poly(C) and that the quenching effect of poly(dA) is much less than that of poly(A). Poly(dC) and poly(dA) bind to only one of the two reported S1 protein nucleic acid binding sites (Draper & von Hippel, 1978a). There is no significant difference in thermostability between these polynucleotide pairs; thus, their smaller quenching effect presumably arises from one less available binding site.

The possibility of ligand-induced protein conformational changes must be considered. We have no evidence to support such a phenomenon. The fluorescence spectral parameters (λ_{max}, $\Delta\lambda_{1/2}$, and τ) in both the free and complexed states are essentially the same. Thus, it would appear that in the complexed state the observed fluorescence changes can be accounted for in terms of two types of fluorophores, unperturbed and totally quenched.

Applying Burstein's spectral rules (Burstein et al., 1973) to the S1 protein, we conclude that the fluorescence of native S1 arises from the tryptophanyl residues which are only partially exposed to solvent. The maximum wavelength of the

emission is shifted to 350 nm upon unfolding and is accompanied by a broadening to a $\Delta\lambda_{1/2}$ of ca. 60 nm, indicating complete exposure of the fluorophores; the increase in K_{SV} for acrylamide quenching upon denaturation is consistent with the enhanced fluorophore exposure. Such exposure also leads to a positive deviation (upward curvature) in the Stern–Volmer plot, indicative of a static quenching contribution.

We have performed quenching titrations in the presence of 50% glycerol and find that the quenching constant is considerably reduced (Table I) but with no change in the overall accessibility of the fluorophores, implying that the environments of the tryptophanyl residues are not affected by the glycerol medium used to produce high viscosity. We conclude that the rate constant for quenching is largely limited by the diffusion coefficient of acrylamide in the buffer solution, rather than by diffusion through a semi-fluid-like protein matrix.

NMNCl is also found to quench S1 protein tryptophanyl fluorescence quite efficiently. Two mechanisms are possible in this quenching process. NMNCl contains an electron-deficient ring which can form a nonfluorescent charge-transfer complex with a fully exposed indole ring face (Coan et al., 1975). The formation of such a complex can be detected by the appearance of an absorption band at 350 nm (Deranleau et al., 1969; Bradshaw & Deranleau, 1970). This ground-state complex was not detected in the interaction of S1 with NMNCl.

The second mechanism of NMNCl quenching involves an excited-state energy transfer from the fluorophore to the quencher accompanying a molecular collision. This mechanism appears to be the dominant one in NMNCl quenching of S1 protein fluorescence. From the results of the quenching reactions between S1 and NMNCl, we can also obtain some information about the accessibility and environments of the tryptophanyl side chains. Only ca. 75% of the S1 fluorescence is quenched with an effective quenching constant of 30.4 M^{-1}, which is larger than that obtained for the denatured protein (see below). Such a high quenching constant may be attributed to an attractive electrostatic force arising from negatively charged groups surrounding some or all of the protein tryptophanyl residues. Furthermore, the unquenched fluorescence arises from residues inaccessible to NMNCl due to their unfavorable environments, such as poor steric exposure (but still accessible to acrylamide), or by being surrounded by positively charged basic amino acid residues. The role of electrostatic effects in NMNCl quenching has previously been demonstrated (Holmes & Robbins, 1974).

Denaturation of the protein in 5.6 M Gdn·HCl exposes the tryptophanyl residues and is thus expected to increase the bimolecular quenching constant and the fraction of quenchable fluorescence. This was observed in the acrylamide quenching of S1 fluorescence performed under denaturing conditions. Surprisingly, similar quenching titrations with NMNCl resulted in a 2-fold *decrease* of the Stern–Volmer constant compared to the value obtained for the native structure. The fluorescence of the denatured protein was totally quenched by NMNCl, implying that all tryptophanyl residues are accessible. The reason for the decrease in the quenching constant is not entirely clear. However, it is possible that negatively charged fluorophore environments are destroyed as a result of protein unfolding. This in conjunction with the high ionic strength of the denaturing solvent leads to the elimination of the favorable electrostatic interaction between the fluorophores and the quencher, which is most likely responsible for the high rate constant of NMNCl quenching of the native S1 fluorescence.

The apparent higher accessibility of the S1 fluorescence to Cs^+, relative to I^-, may be attributed to the influence of the electrostatic environments found in the protein, i.e., a greater fraction of S1 fluorescence arises from fluorophores located in negatively charged environments, and therefore would be expected to be more effectively quenched by Cs^+ than by I^-. Effects of electrostatic factors in ionic quenching reactions are significant and have been thoroughly discussed elsewhere (Lehrer, 1971; Altekar, 1977).

In this context, it is interesting to note that photo-CIDNP 360-MHz NMR studies of native S1 protein using a negatively charged lumiflavin dye have failed to detect accessible tryptophanyl residues (T. Schleich and R. Kaptein, unpublished experiments). This approach is based upon the interaction of an excited-state dye with tryptophanyl, histidyl, and tyrosyl residues and has been found quite useful in probing the surface topography of proteins (Kaptein, 1978).

Draper (1977) reported a value of 9.9 M^{-1} for $K_{Q(eff)}$ in the quenching of S1 fluorescence by I^-, whereas we report in this study a value equal to 5.5 M^{-1}. Draper's value appears too high for I^- quenching by a collisional mechanism, and he interpreted this anomaly in terms of weak anionic binding and did not consider the possibility of selective quenching. In fact, the simple Stern–Volmer plot for I^- quenching shows downward curvature (see Figure 6), suggesting the occurrence of selective quenching of S1 fluorescence. Protein heterogeneity, in turn leading to conformational variability, does not appear to be a realistic explanation since the protein used in these studies is fully competent to bind nucleic acid as evidenced by the employment of poly(C)–cellulose affinity chromatography with elution achieved by use of a nondenaturing buffer.

We have observed that the presence of polynucleotides, particularly those which bind strongly to both sites of S1, results in a significant decrease of the effective quenching ability of both acrylamide and NMNCl. A polynucleotide bound to a protein can reduce the accessibility of fluorophores to quenchers by steric blockage, by the induction of a conformational change, or by the provision of an unfavorable electrostatic environment. Some of the tryptophanyl residues in the S1 protein are expected to become sterically more shielded by the polynucleotide and, hence, quenched at a slower rate, concomitant with a decrease in the fraction of accessible fluorescence. It appears that polynucleotides which bind to both sites of S1 [poly(A), poly(C), poly(U), and poly(dU) (Draper & von Hippel, 1978a,b; S. Omar and T. Schleich, unpublished experiments)] rather than to one site [poly(dA) and poly(dC)] induce a greater effect on the quenching parameters $f_{a(eff)}$ and K_{SV}. This is most apparent in the quenching effects of the neutral acrylamide quencher. In addition, the effects of mononucleotides and oligonucleotides on the acrylamide quenching parameters are small. Surprisingly, there is only a small indication that polynucleotides induce an effect on the *effective* quenching constants of acrylamide.

The same effects of polynucleotides on the NMNCl quenching parameters of S1 protein fluorescence were observed, although to a significantly lesser degree. The difference between the effects induced by polyribonucleotides and polydeoxyribonucleotides on the quenching parameters is also much less. However, we found that the effects of mononucleotides (5′-AMP and 5′-CMP) on NMNCl quenching parameters are greater than those of the polynucleotides. This finding is inconsistent with the fact that mononucleotides have a much lower binding affinity to S1 than do the polynucleotides (Lipecky et al., 1977). The only plausible explanation is that the NMNCl ring interacts with the base rings to form a

charge-transfer complex. Thus, the quenching efficiency of NMNCl would be significantly reduced, and this is reflected in the values of the effective quenching constants. In fact, we observed that when NMNCl is excited at 300 nm a weak emission at 383 nm is apparent. This emission increases in intensity by 35% when 5'-AMP or ApA is added to NMNCl in aqueous solution. This phenomenon was not observed upon the addition of oligo- or polynucleotides under comparable conditions.

In summary, these studies have provided information on the exposure of the tryptophanyl residues in ribosomal protein S1. These residues are completely accessible to acrylamide, although this reagent most likely quenches the emission of individual fluorophores with slightly different rates. Since acrylamide is a neutral molecule, its quenching effect is sensitive only to the degree of exposure of the fluorophores in the protein, but not to their electrostatic environments. However, due to the electrostatic heterogeneity of the tryptophanyl environments in S1, charged reagents, such as NMNCl, I^-, and Cs^+, do not completely quench the protein fluorescence. The binding of polynucleotide to S1 protein confers an additional shielding of the tryptophanyl residues and thus reduces their accessibility to low molecular weight quenchers. There is no indication from the fluorescence studies that polynucleotides induce major conformational changes in the protein upon binding, since the relevant fluorescence spectral parameters are not affected. Furthermore, the negligible effect of polynucleotides on the effective quenching constants of acrylamide and NMNCl implies that the environments of the most accessible residues are not altered by the binding of these nucleic acid structures.

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