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Guanosine tetraphosphate-induced dissociation of open complexes at the *Escherichia coli* ribosomal protein promoters *rplJ* and *rpsA* P1: nanosecond depolarization spectroscopic studies

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Abstract

We have measured the fluorescence anisotropy decays of various transcription complexes formed between *Escherichia coli* RNA polymerase (RNAP) and the *rplJ*, *rpsA* P1 and *lacUV5* promoters, where the σ^{70}-subunit of RNAP is covalently labeled with the fluorescent probe 1,5-IAEDANS. The observed changes in the rotational correlation times (ϕ_r) of the σ^{70}-bound probe upon ppGpp or NTP addition to preformed open complexes, were used to directly infer the extent of association of the σ-subunit with these transcription complexes. At the *rplJ* and *rpsA* P1 promoters, the addition of ppGpp (in the absence of heparin and nucleotides), results in the dissociation of RNAP from the binary complex. This is either accompanied by, or leads to the dissociation of a fraction of the holoenzyme-bound σ^{70}. At the *lacUV5* promoter, only a marginal dissociation of RNAP is observed. We propose a model where two types of ppGpp-bound RNAP interact with the ribosomal protein promoters. One is transcription-competent and releases σ^{70} upon elongation, while the other dissociates from the open complex. A fraction of the latter species releases the σ^{70} subunit and is unable to form a transcription-competent holoenzyme. Our data supports the mechanism of open complex-destabilization at stringent promoters by ppGpp.

Keywords: Stringent promoters; ppGpp; Sigma-release; Rotational correlation time

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Abbrevations: RNAP, RNA polymerase; ppGpp, guanosine 3′,5′-bis(pyrophosphate); 1,5-IAEDANS, 5-(2-((iodoacetyl)aminoethyl)ethyl)aminonaphthalene-1-sulfonic acid; σ-AEDANS, σ^{70} covalently-labeled with 1,5-IAEDANS; Eσ-AEDANS, RNA polymerase core enzyme reconstituted with σ-AEDANS; NTPs, nucleoside triphosphates; nt, nucleotide; ϕ_r, rotational correlation time; ns, nanoseconds; NTCB, 2-nitro-5-thiocyanobenzoic acid

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1. Introduction

The response of rapidly growing *Escherichia coli* cells to amino acid and/or primary nutrient deprivation, involves a rapid curtailment of stable RNA synthesis and is mediated by the regulatory nucleotide guanosine 3′,5′-bis(diphosphate) (ppGpp) [1]. The physiological and genetic characterization of various *rpoB* mutants suggested that RNA polymerase (RNAP) is a target for ppGpp and that the effects of this nucleotide are manifest at the level of transcription [2,3].

In vitro transcription assays [4], gel retardation experiments [5], filter-binding [6] and footprinting studies [7] indicate that ppGpp inhibits various steps of transcription initiation; other studies demonstrate that ppGpp causes enhanced pausing in vitro [8–10] and decreasing elongation rates in vivo [11]. Mixed template in vitro transcription assays suggest that ppGpp destabilizes open complexes at rRNA and ribosomal protein promoters [12]. A more definitive treatment of the dissociation reaction comes from filter-binding assays that monitor the fraction of RNAP molecules that remain bound to the promoter. In our previous paper, we demonstrated that ppGpp enhances the rate of dissociation of open complexes at the ribosomal protein promoters *rplJ* and *rpsA* P1 [13]; similar results have been obtained with the *rrnB* P2 and P2F promoters [14]. In these assays, heparin is added to ensure irreversible dissociation, but the presence of this competitor contributes to the decay of open complexes [15] and does not reveal whether the dissociated RNAP molecules are competent to rebind the template, as would be the case in vivo. Hernandez and Cashel have shown that in ppGpp$^+$ and *rpoD* mutant strains, a smaller fraction of the intracellular σ^{70} subunit is associated with core RNAP compared with ppGpp-deficient (ppGpp°) strains, suggesting a ppGpp-dependent weakening of core RNAP-σ^{70} interactions in vivo [16].

In this study, we have investigated the reversible formation and dissociation of binary complexes (in the absence of heparin) between RNAP and the *rplJ, rpsA* P1 and *lacUV5* promoters, using nanosecond depolarization spectroscopy. We covalently labeled the σ^{70} subunit with a fluorescent probe and measured its rotational correlational time (ϕ_r), upon the formation of RNAP holoenzyme and binary complexes. As ϕ_r is a function of molecular size [17], this approach provides a direct, real-time analysis of the effects of ppGpp on the extents of core-σ^{70} and RNAP-promoter interactions. This method has been used in the past to demonstrate the release of the σ^{70} subunit during the transcription cycle [18].

2. Materials and methods

All chemicals and buffers used in this study were of the purest grade available. Nucleotides were from Pharmacia, 1,5-IAEDANS [5-(2-((iodoacetyl)aminoethyl)ethyl)aminonaphthalene-1-sulfonic acid] was purchased from Molecular Probes (Eugene, OR). α^{32}P-UTP was obtained from BARC (Bombay, India); ppGpp was from Sanraku (Japan).

2.1. RNA polymerase and sigma-70 subunit

E. coli RNAP holoenzyme was purified and assayed as described before [13]. The protein was 95% pure as judged by SDS-PAGE analysis. Core RNAP was purified according to Burgess and Travers [19]. The σ^{70} subunit was purified from a super-producing strain under the T7-expression system, following the method of Gribskov and Burgess [20]. This strain was a kind gift from Akira Ishihama, NIG, Japan. Reconstitution of core and σ^{70} subunit was carried out by mixing equimolar amounts of each on ice and gradually increasing the temperature to 37°C over 10 min. Concentrations of holoenzyme, core enzyme and σ^{70} were measured spectrophotometrically using extinction coefficients ($\varepsilon^{1\%}$ at 280 nm) of 6.5, 5.5 and 8.4, respectively [21].

2.2. Labeling of σ^{70}

Sigma-70 subunit was labeled with the −SH specific probe 1,5-IAEDANS [22]. A 10-fold molar excess of the probe was incubated with σ^{70} in a buffer containing 40 mM HEPES-KOH (pH 7.4), 50 mM KCl, 0.1 mM EDTA and 5% glycerol, at 20°C for 1 h and shifted to 4°C for a further 7 h.

The mixture was then passed through a Sephadex G-10 column equilibrated in the above buffer, to separate free and bound probe and dialysed overnight against 10 mM Tris–Cl (pH 8.0), 100 mM KCl, 0.1 mM EDTA and 5% glycerol, with several changes of buffer. The stoichiometry of binding was estimated from the concentrations of σ^{70} and IAEDANS (ε at 336 nm $= 6.1 \times 10^3$ M^{-1}) [23].

2.3. In vitro transcription

Single-round run-off transcriptions were carried out exactly as described earlier [13]. For the reactions involving ppGpp, the reconstituted and wild-type enzymes were preincubated with ppGpp (final concentration 100 μM) in the standard transcription buffer for 10 min prior to DNA addition. For calculation of percentage inhibition by ppGpp, transcript intensities were first normalized with respect to the recovered intensities of an end labeled dsDNA fragment that was added before precipitation.

2.4. Spectroscopy

All spectroscopic measurements were carried out at 25°C, in Buffer A [40 mM Tris–Cl (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT and 5% glycerol], unless otherwise indicated. Absorption spectra were recorded on a Hitachi 200-20 spectrophotometer, steady-state fluorescence was measured in a Hitachi F-4000 spectrofluorimeter with spectral correction. Fluorescence lifetimes were estimated using a Photon Technology International LS-100 spectrometer where time-resolved excited-state fluorescence decays were obtained in the single photon counting mode. The machine is equipped with a thyratron-gated nanosecond flash-lamp filled with N_2 gas and is run at 22–25 kHz, with a pulse-width of 2 ns. A non-linear least-squares fitting program using an iterative-reconvolution procedure, based on the Marquardt algorithm [24,25] was used for the estimation of amplitudes and lifetimes. Single and double-exponential decay curves were generated using this program and the best-fits were tested by calculating residuals, autocorrelation, χ^2 and Durbin–Watson parameters. A fit was considered good when plots of the weighted residuals and the autocorrelation function fluctuated around zero, with a minimum χ^2 value (between 1.0 and 1.8).

2.5. Theory

For polarized light, the time-dependence of fluorescence may be calculated from the raw data,

$$F(t) = I_{\parallel}(t) - GI_{\perp}(t) \tag{1}$$

where $I_{\parallel}(t)$ is the intensity of light detected with vertical excitation and emission polarizers, $I_{\perp}(t)$ is the intensity detected with a vertical excitation and a horizontal emission polarizer; G is the correction term for the relative throughput of each polarization through the emission optics. Time-dependent anisotropy is defined by;

$$r(t) = \frac{I_{\parallel}(t) - GI_{\perp}(t)}{I_{\parallel}(t) + 2GI_{\perp}(t)} = \frac{d(t)}{F(t)} \tag{2}$$

This function is known to follow a multi-exponential decay law [26];

$$r(t) = \Sigma b_i \exp(-t/\phi_i) + b_\infty \tag{3}$$

Although the sum can run to five terms for completely anisotropic rotational motion, at lower precision levels and with relatively symmetric rotors, Eq. (2) will yield in practice only one or two terms. The b_∞ term refers to the residual anisotropy left after all the transient terms have decayed and is commonly interpreted to imply restricted motion of the rotor. The term ϕ_i denotes the rotational correlational time; it can be solved by the convolution technique.

Noting that,

$$d(t) = r(t)F(t) = I_{\parallel}(t) - GI_{\perp}(t) \tag{4}$$

we obtain,

$$d(t) = \left[\sum_{i=1}^{5} a_i \exp(-t/\tau_i)\right]$$

$$\times\left[\sum_{i=1}^{5} b_j \exp(-t/\phi_j) + b_\infty\right] \quad (5)$$

where a_i and τ_i denote the amplitude and lifetimes of the excited state. Once these terms are solved, they can be used as constants and values for ϕ; b_j can be obtained by reiteration. The time-dependence of total fluorescence decay *F(t)*, is obtained by setting the emission polarizer at 54.7° (magic angle) to the vertical.

3. Results

3.1. Characterisation of σ^{70} labeled with 1,5-IAEDANS

On account of its specificity towards thiol-groups and stability of protein conjugates, 1,5-IAEDANS was chosen for this study. Moreover, proteins labeled with this probe can be excited at 337 nm, the most intense emission line from a N_2-flash lamp, making it suitable for nanosecond spectroscopy. Sigma-70 has three free cysteines at positions 132, 291 and 295 where other fluorescent probes have been attached to monitor the dynamics of the subunit [27].

Upon removal of free probe by size-exclusion chromatography and extensive dialysis, we observed approx. 1 mol of bound probe per mole of protein, based on absorbances at 280 and 340 nm. We have shown earlier that 2-nitro-5-thiocyanobenzoic acid (NTCB) cleaves σ^{70} at free cysteines generating various discreet fragments of different molecular weights [28]. We observed that when σ^{70} was labeled with 1,5-IAEDANS, no cleavage at the 132-cysteine residue took place, indicating the preferential modification of this residue. On the other hand, both 291 and 295 cysteine residues remained free and NTCB cleavage resulted in fragments of expected size (data not shown). Thus, we conclude that σ^{70} was uniformly labeled with 1,5-IAEDANS at the 132 −SH group.

To ascertain whether any label remained non-covalently attached to σ^{70} even after extensive dialysis, we subjected the σ-AEDANS complex to partial denaturation with urea and followed its fluorescence emission characteristics. Fig. 1A shows that when incremental amounts of urea were added to an equimolar mixture of σ^{70} and free 1,5-IAEDANS there was a monotonous decrease in the fluorophore emission intensity due to dilution. However, when the purified σ-AEDANS complex was treated in a similar way, a progressive blue-shift of the emission maximum was seen, with the occurrence of an isoemissive point at 452 nm (Fig. 1B). This blue shift suggested that the polarity-sensitive probe was progressively moving into a hydrophobic environment on account of its attachment at a specific site on σ^{70}.

3.2. Transcription activity of core enzyme reconstituted with σ-AEDANS

Comparison of the specific activities of core enzyme reconstituted with either σ^{70} or σ-AEDANS, showed that σ-AEDANS was efficient in reconstituting a functional holoenzyme; transcription by purified RNAP holoenzyme acted as a control (Fig. 2A). We then compared the single-round transcription activities of purified holoenzyme and the σ-AEDANS-reconstituted enzyme at the *rplJ, rpsA* P1 and *lacUV5* promoters, in the presence and absence of ppGpp. At all three promoters, the σ-AEDANS-reconstituted enzyme showed lower levels of transcription compared to the holoenzyme (Fig. 2B). The degree of inhibition by ppGpp at *rplJ* and *rpsA* P1 varied between 40 and 60%, while transcription from the *lacUV5* promoter was only marginally reduced.

3.3. Time-resolved anisotropy of DNA–RNA polymerase complexes

The nanosecond emission characteristics of σ-AEDANS can be best fitted by two lifetimes as shown in Fig. 3A. One ranges between 12 and 13 ns and the other between 4 and 5 ns. With corresponding pre-amplitude values, the contribution of both the lifetimes was found to be close to equal in defining the excited state decay. The lifetimes of the probe when reconstituted with core polymerase or in the presence of various

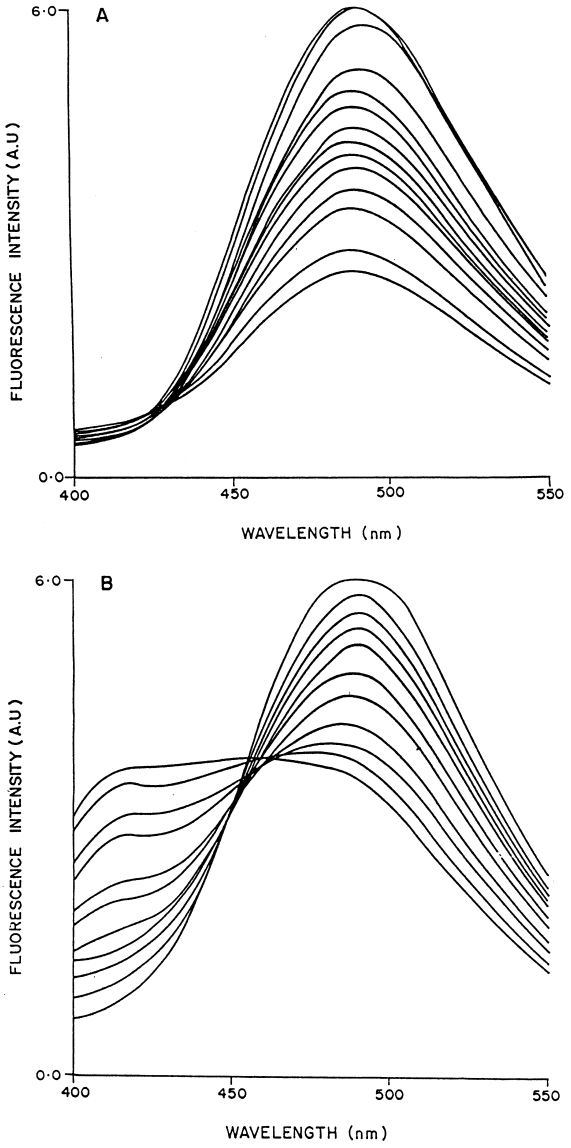

Fig. 1. Fluorescence emission spectra of σ^{70}-1,5-IAEDANS complexes in the presence of urea. Excitation was at 340 nm and emission scanned from 400 to 550 nm with 2.5 nm bandpass. (A) 400 μl σ^{70} (75 nM) and 200 μl 1,5-IAEDANS (150 nM) in buffer A were mixed and incubated at 37°C for 10 min before recording the spectra; 35-μl aliquots of 8 M urea solution were incrementally added and emission spectra recorded after thorough mixing at each step. (B) 600 μl of purified σ-AEDANS (50 nM) was treated with urea as in (A). Fluorescence intensities are not corrected for dilution.

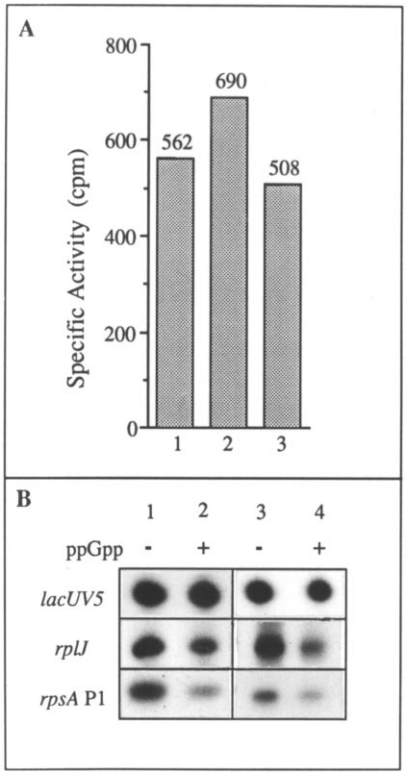

Fig. 2. Transcription profiles of σ-AEDANS-reconstituted holo-RNAP. (A) Specific activities of purified holoenzyme (1), σ^{70}-reconstituted holoenzyme (2) and σ-AEDANS-reconstituted holoenzyme (3). Specific activity is defined as the nanomoles of [^{3}H]UTP incorporated as DE-81 bound RNA/20 min mg^{-1} protein. Assays were carried out as described previously ([13]). (B) Effect of ppGpp addition on single-round run-off transcriptions. Lanes 1 and 2, purified holoenzyme; lanes 3 and 4, σ-AEDANS-reconstituted holoenzyme. Transcript sizes are: *rplJ*, 69 nt; *rpsA* P1, 90 nt; *lacUV5*, 63 nt. Percentage inhibition by ppGpp was calculated with respect to control reactions, without ppGpp.

DNA fragments did not change significantly and are not reported here. Steady-state emission characteristics of labeled σ^{70} in the presence of core polymerase or DNA were not studied further.

Fig. 3B shows the time-dependence of fluorescence anisotropy decay of σ-AEDANS. This decay could be defined with two rotational correlation times (ϕ_r); one was 76 ns and the other component was extremely small (0.9 ns). However, the contribution of the second-term to the total anisotropy decay was only approx. 1% and can therefore be neglected. The observation that σ-AEDANS shows two excited-state lifetimes yet

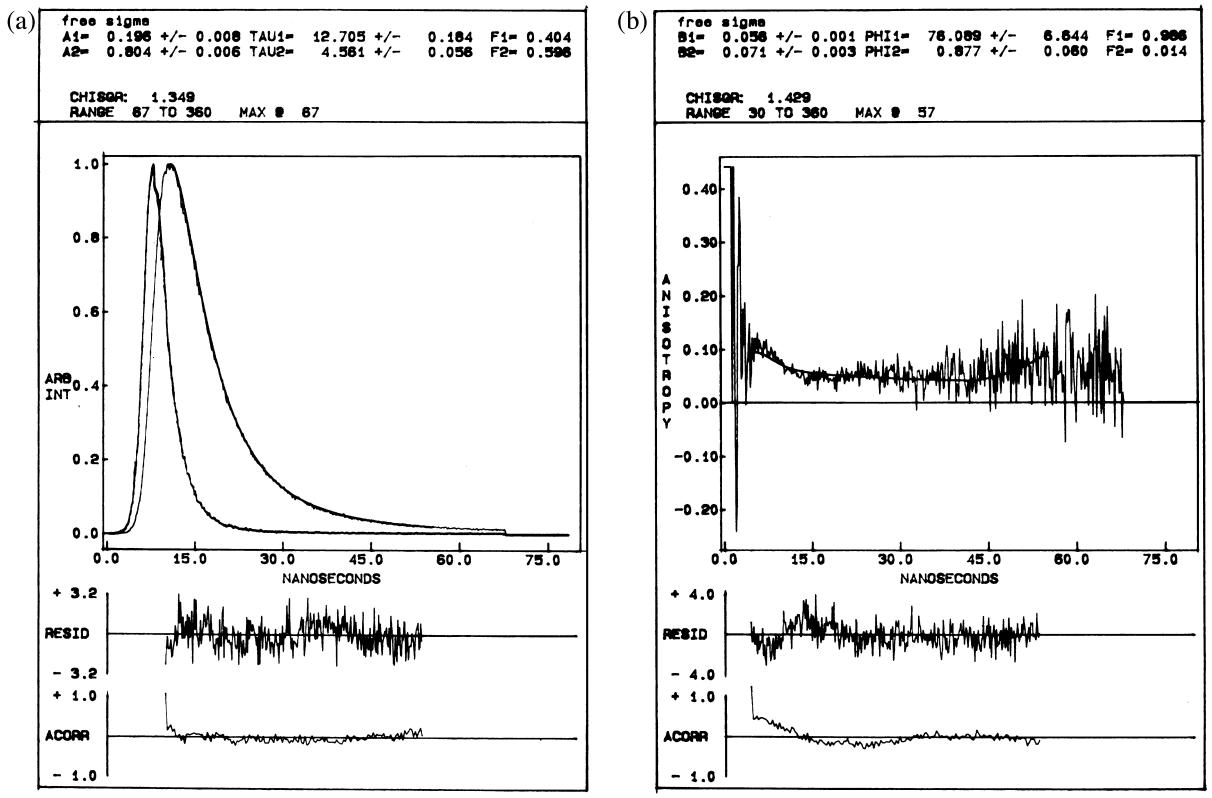

Fig. 3. Nanosecond emission characteristics of σ-AEDANS. (A) Fluorescence lifetime measurement of σ-AEDANS. The lifetimes that define the decay are indicated as 'TAU1' and 'TAU2'. (B) Time-resolved anisotropy decay of σ-AEDANS. The solid line represents the best fit to the obtained data. Rotational correlation times (ϕ_r) obtained are indicated as 'PHI1' and 'PHI2'.

only one major ϕ_r, can be understood from theoretical considerations of anisotropy decay, in the case of randomly labeled spherical molecules [29]. These reveal that the time-dependent anisotropy is independent of the time-dependent excited-state decay. Interestingly, the value of ϕ_r for σ-AEDANS did not change in the presence of excess T7 DNA, indicating again that free σ^{70} does not bind DNA [30].

Upon addition of an equimolar amount of core polymerase to σ-AEDANS, the rotational correlational time of the probe increased to 165 ns, with another very small component as shown in Fig. 4A. This value did not change significantly in the presence of ppGpp (Table 1). Thus it appears that the conformation change in the holoenzyme upon ppGpp binding, if any, cannot be detected by this measurement Addition of a threefold molar excess of *lacUV5* DNA to the reconstituted enzyme, caused a further increase of ϕ_r to 272 ns (Fig. 4B). Such changes are expected, as the formation of a larger molecular complex will slow down the rate of molecular rotation and hence increase the value of ϕ_r. When all four NTPs were added to the σ-AEDANS-core polymerase-*lacUV5* complex, we observed that the ϕ_r value of the probe decreased to that of free σ-AEDANS (Table 1) indicating complete release of the σ-subunit from the complex during transcription. Table 1 shows that the ϕ_r value of the major component of the anisotropy decay of the σ-AEDANS-core polymerase-promoter complex, fluctuates between 252 and 272 ns for the three promoters tested here. The error in estimation of ϕ_r in the case of the *rpsA* P1 promoter, was greater than the others.

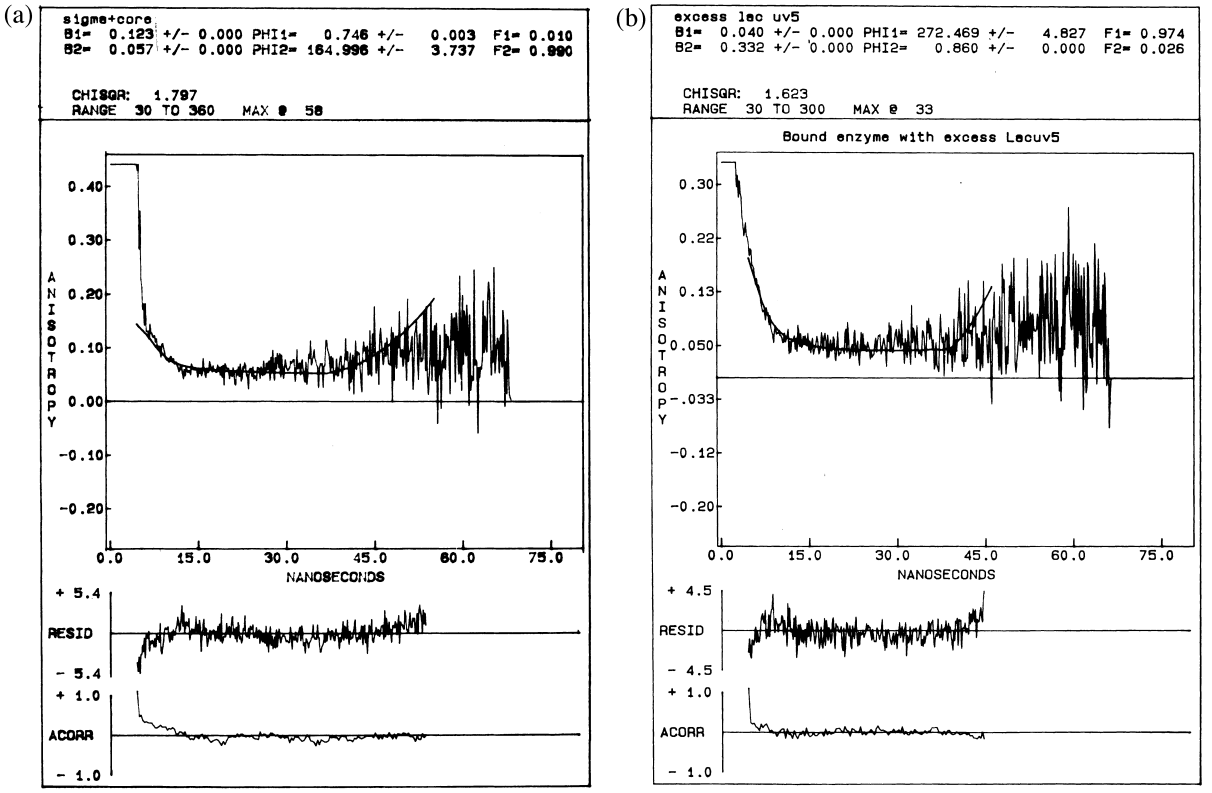

Fig. 4. Time-dependent anisotropy decays of various molecular species containing σ-AEDANS. The decays were fitted to two terms; the corresponding rotational correlation times are indicated as 'PHI1' and 'PHI2', respectively. The weighted residuals and autocorrelation functions for the fits are shown below each decay. (A) Core RNAP reconstituted with σ-AEDANS. Equimolar amounts of σ-AEDANS and core RNAP enzyme were slowly diluted on ice (final concentration 0.1 mM) with 500 μl of Buffer B [10 mM Tris–Cl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 100 μg μl^{-1} BSA and 5% glycerol]. The sample was incubated at 37°C for 10 min prior to measurement. (B) Anisotropy decay of σ-AEDANS-reconstituted-holoenzyme with *lacUV5* DNA; 300 μl of freshly reconstituted σ-AEDANS-holoenzyme [as in (A)] were mixed with 200 μl of 0.56 mM *lacUV5* DNA in Buffer B and incubated at 37°C for 15 min prior to measurement.

The addition of ppGpp to the holoenzyme-*lacUV5* promoter complex in the absence of NTPs, causes a decrease in ϕ_r to 215 ns, indicating that a fraction of the binary complex has dissociated. Similar measurements at the *rplJ* and *rpsA* P1 promoters, showed a decrease in ϕ_r to $\sim$130 ns. As these values lie between the determined ϕ_r values for free σ-AEDANS (76 ns) and core + σ-AEDANS (165 ns), we infer that upon ppGpp addition, dissociation of the binary complex occurs, with a fraction of the dissociated enzyme remaining as core and free σ-AEDANS. At this point, it is not clear whether the release of σ-AEDANS occurs from the binary complex, or from the dissociated holoenzyme. No significant dissociation of σ-AEDANS was observed when ppGpp is incubated with the reconstituted holoenzyme alone (data not shown). We attempted to titrate the free σ-AEDANS by adding a twofold excess of core enzyme to the sample containing the *rplJ* promoter complexes, but found no significant increase in ϕ_r (146 ± 10 ns). When all four NTPs were added to the sample containing *rpsA* P1 complexes and ppGpp, complete release of σ-AEDANS was observed, indicating that even in the presence of ppGpp, a fraction of enzyme molecules remained competent to carry out transcription.

Table 1
Rotational correlation times (ϕ_r) of various molecular species

System[a]	ϕ_r (ns)
Free σ-AEDANS	76 ± 6
σ-AEDANS + core RNAP (Eσ-AEDANS)	165 ± 4
Eσ-AEDANS + ppGpp	176 ± 8
Eσ-AEDANS + *lacUV5*	272 ± 5
Eσ-AEDANS + *lacUV5* + ppGpp	215 ± 0.1
Eσ-AEDANS + *lacUV5* + NTPs	78 ± 0.1
Eσ-AEDANS + *rplJ*	252 ± 10
Eσ-AEDANS + *rplJ* + ppGpp	136 ± 10
Eσ-AEDANS + *rplJ* + ppGpp + core RNAP	146 ± 10
Eσ-AEDANS + *rpsA* P1	260 ± 40
Eσ-AEDANS + *rpsA* P1 + ppGpp	132 ± 6
Eσ-AEDANS + *rpsA* P1 + ppGpp + NTPs	69 ± 0.1

[a] Concentrations of DNA templates and Eσ-AEDANS were as indicated in Fig. 4. In all cases, ppGpp and NTPs were added to final concentrations of 1 mM and 50 μM, respectively.

3.4. Estimation of the fractions of free σ-AEDANS and free / DNA-bound holoenzyme

For a system containing transcription complex-bound, core enzyme-bound and free σ-AEDANS, the parallel and perpendicular components of the fluorescence decay according to Weber's law of addition of polarization [31], are given by:

$$I_{\parallel}(t) = \frac{F(t)}{3}[f_a\{1 + 2A_o \exp(-t/\phi_a)\} + f_b\{1 + 2A_o \exp(-t/\phi_b)\} + f_c\{1 + 2A_o \exp(-t/\phi_c)\}] \quad (6)$$

$$I_{\perp}(t) = \frac{F(t)}{3}[f_a\{1 - A_o \exp(-t/\phi_a)\} + f_b\{1 - A_o \exp(-t/\phi_b)\} + f_c\{1 - A_o \exp(-t/\phi_c)\}] \quad (7)$$

where ϕ_a, ϕ_b, ϕ_c and f_a, f_b, f_c are the rotational correlation times, and fractions of transcription complex-bound, core enzyme-bound and free σ-AEDANS, respectively. Substituting these in Eq. (4) we obtain,

$$r(t) = f_a\{A_o \exp(-t/\phi_a)\} + f_b\{A_o \exp(-t/\phi_b)\} + f_c\{A_o \exp(-t/\phi_c)\} \quad (8)$$

Substituting $\phi_a = 270$, 260 or 252 ns, $\phi_b = 165$ ns and $\phi_c = 76$ ns in Eq. (8), we constructed theoretical anisotropy decay curves for fixed values of f_a, f_b and f_c at each promoter (Fig. 5). The best fit of *r(t)* for various systems containing ppGpp to the theoretical curves, gave the f_a, f_b and f_c values for that system. It should be emphasized here that ϕ_a and ϕ_b represent rotational correlation times of complete binary complex and free enzyme, respectively and have no contribution from free labeled σ^{70}.

For the *lacUV5* + ppGpp system, only 5% of the total σ-AEDANS was present as free species and the observed ϕ value of 215 ns in the presence of ppGpp corresponds to 70 and 25% of the total σ-AEDANS being associated with promoter-polymerase complexes and free holoenzyme, respectively (Table 2). In contrast the ϕ value of 136 ns for the *rplJ* + ppGpp system corresponds to 25% free, 65% core enzyme-bound and 10% transcription complex bound σ-AEDANS. The proportion of free σ-AEDANS decreased marginally to 20% when the excess core enzyme was added, while the proportion associated with DNA-polymerase complexes was 15%. We suggest that even though the free σ-AEDANS can associate with core RNAP and bind to the promoter, these complexes dissociate in presence of ppGpp, yielding no significant change in the proportions of free and bound σ-AEDANS. At the *rpsA* P1 promoter, only 10% of the total σ-AEDANS is associated with promoter-polymerase complexes, while 30% is present as the free species.

4. Discussion

Several properties of the fluorescent probe 1,5-IAEDANS have enabled its use in this study. Among these, the specificity of covalent linkage at cysteine residues, the stability of the bound probe over long time periods, appreciable quantum yields (0.27 in water and 0.67 in ethanol) and the retention of enzyme activity upon labeling, are critical for our studies. The complex formed between σ^{70} and 1,5-IAEDANS showed a characteristic blue shift in the fluorescence emission

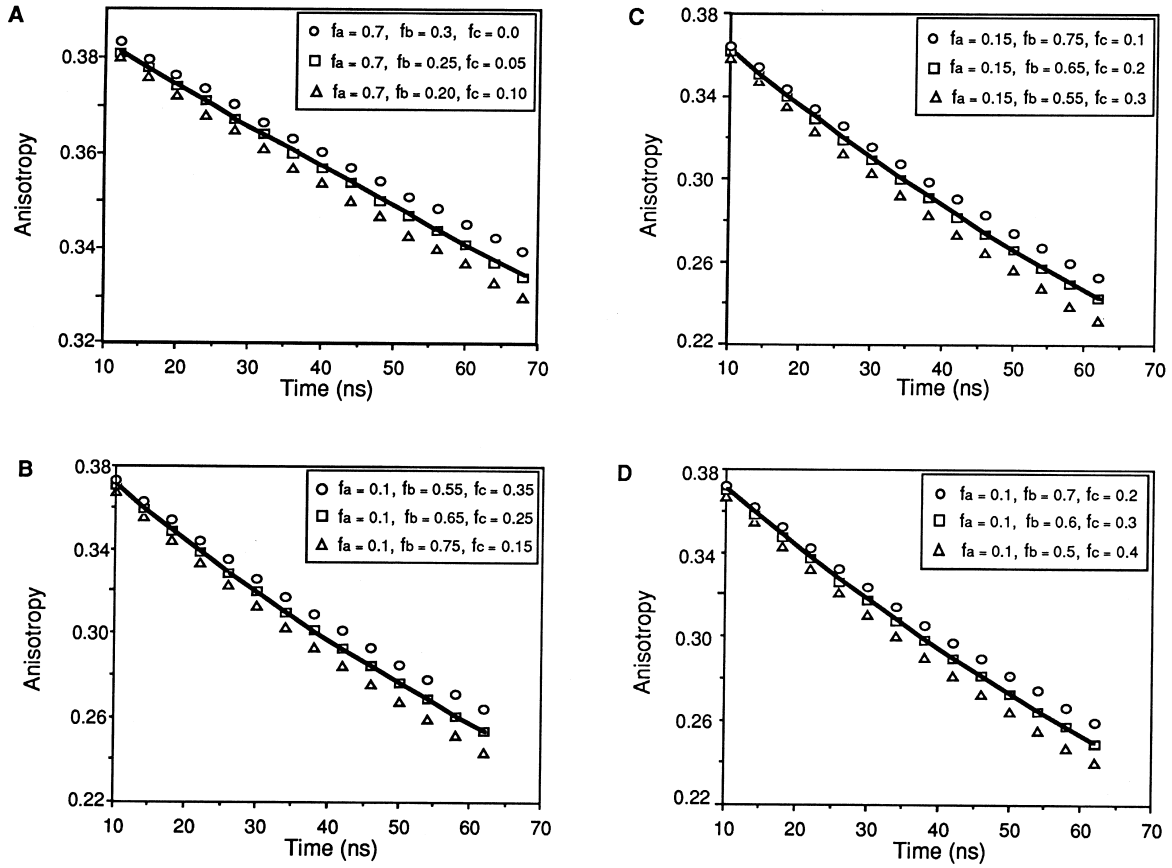

Fig. 5. Estimation of f_a, f_b and f_c for various systems containing ppGpp. Theoretical curves for fixed values of f_a, f_b and f_c were obtained as described in the text. The solid lines represent the decays of samples containing ppGpp, using the experimentally determined ϕ_r values (Table 1). In all cases, A_o was taken as 0.4. (A) *lacUV5* + ppGpp (B) *rplJ* + ppGpp (C) *rplJ* + ppGpp + core RNAP and (D) *rpsA* P1 + ppGpp. f_a, f_b and f_c were determined from the best-fit of these decays to the theoretical curves.

maximum in the presence of urea, from which we infer the formation of a covalent complexes. Blue-shifts in the emission maximum of this probe have been previously observed [32,33] and are

Table 2
Percentage of the total σ-AEDANS for various systems existing as binary complex-bound, holoenzyme associated, or as free σ-AEDANS

System	Binary-complex Bound[a]	Holoenzyme Associated[a]	Free σ-AEDANS[a]
Eσ-AEDANS + *lacUV5* + ppGpp	70	25	5
Eσ-AEDANS + *lacUV5* + NTPs	0	0	100
Eσ-AEDANS + *rplJ* + ppGpp	10	65	25
Eσ-AEDANS + *rplJ* + ppGpp + core RNAP	15	65	20
Eσ-AEDANS + *rpsA* P1 + ppGpp	10	60	30
Eσ-AEDANS + *rpsA* P1 + ppGpp + NTPs	0	0	100

[a] Values shown are obtained from the best-fit to the theoretical curves (Fig. 5).

attributed to large changes in macromolecular conformation.

The values of ϕ_r for bound and free σ^{70} reported here are different from those reported earlier. This may be due to the different nature of the probes used in the two studies. Moreover, we have used a non-linear regression analysis to calculate the time constants of emission and anisotropy decay, whereas Wu et al. [18], approximated the decay as a linear curve.

4.1. A general model for transcription initiation in the presence of ppGpp

We have investigated the reversible formation of various promoter-polymerase complexes in the presence of ppGpp by following the rotational correlation times of 1,5-IAEDANS bound to the σ^{70} subunit. We interpret the changes in ϕ_r values as being due to changes in the relative populations of various intermediate species and we propose a model to account for the formation of these species (Fig. 6). This model incorporates the findings of various studies that ascribe the inhibitory effects of ppGpp to different steps of initiation [6,7,12,13]. An important outcome of this model is that promoter-specific effects of ppGpp reflect differences in the relative rates of conversion of the molecular species described.

We propose that two types of binary complexes are formed in the presence of ppGpp. The first type (designated RP_o; Fig. 6) is stable and RNAP can escape from the promoter to form RP_e, upon NTP addition, with the release of σ^{70}. Evidence for this species comes from the ϕ_r measurements at the *rpsA* P1 promoter, where even in the presence of ppGpp, NTP addition shows a complete release of the σ-subunit. The other type of binary complex (designated 'A'), dissociates and a fraction of the released holoenzyme (designated 'B'), undergoes further dissociation to yield free σ^{70} and core RNAP (species 'C'; Fig. 6). At the stringent *rplJ* and *rpsA* P1 promoters, this type of binary complex is predominantly formed, evidenced by the fact that 90% of σ-AEDANS is not associated with the binary complex (Table 2). The fraction of undissociated holoenzyme molecules (1-'B') and the re-associated species 'C' can rebind to the template, but will eventually form the ppGpp-sensitive species 'A'. This accounts for the observation that ϕ_r at *rplJ* does not significantly change when an excess of core enzyme is added. The binary species 'A' may be identical to, or may convert to intermediate species in the pathway to

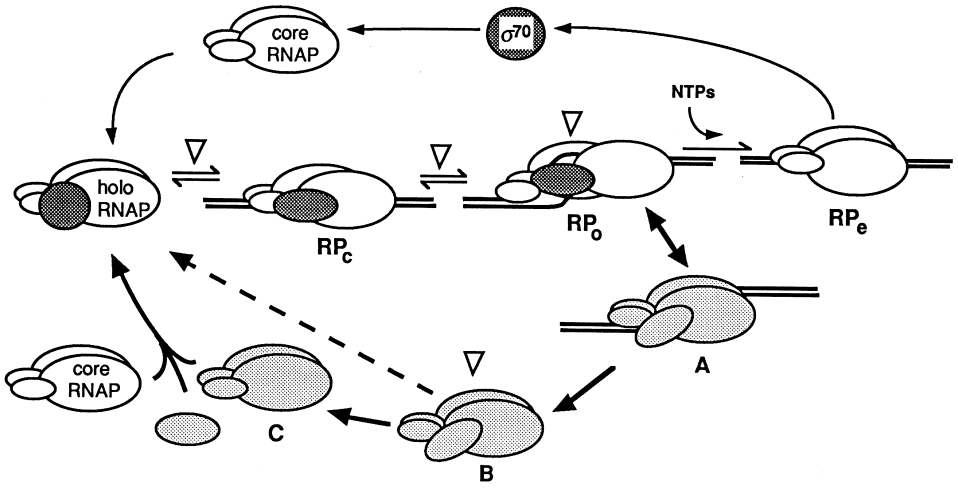

Fig. 6. General model for the ppGpp-induced dissociation of open complexes. RP_c, RP_o and RP_e refer to closed, open and elongating complexes, respectively. The ppGpp-sensitive open complex, released and dissociated holoenzyme are designated 'A', 'B' and 'C', respectively and are dotted to indicate that they are proposed intermediate species. The possible sites of ppGpp action are indicated by ∇. The dashed line indicates that a fraction of the species B does not dissociate and can rebind the template.

RP_o formation, and this may account for earlier observations [13,14] that RP_c to RP_o formation is rate-limiting in the presence of ppGpp.

Circular dichroism studies of RNAP holoenzyme [34] have indicated large conformational changes upon ppGpp binding. Given the predominant contribution of the σ^{70} subunit to the overall spectrum [35] and the negligible changes observed with the core enzyme, it is likely that perturbation of the σ^{70} secondary structure is an important outcome of ppGpp binding. However, the release of the σ^{70} subunit from the holoenzyme is a promoter-dependent effect and we suggest that ppGpp-binding induces an altered interaction of the σ^{70} subunit with the rest of the binary complex 'A', that distinguishes this species from the stable RP_o. A fraction of the dissociated holoenzyme (species 'B') would also have an altered core–σ^{70} interaction, leading to the formation of free σ^{70} and core RNAP (Fig. 6).

Our model accounts for the ppGpp-induced dissociation of σ^{70} at the ribosomal protein promoters observed by nanosecond fluorescence depolarization studies. We stress that the release of σ^{70} is a promoter-dependent phenomenon and that the inhibition of RP_o formation by ppGpp at other promoters may involve mechanisms that differ in the extents of formation of the intermediate species described here.

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