Fluorescence study of the RecA-dependent proteolysis of LexA, the repressor of the SOS system in *Escherichia coli*

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The fluorescence of the LexA protein, the common repressor of the SOS system in *Escherichia coli* decreases by about 30% upon incubation with the RecA protein, and its cofactors ATP [or its non-hydrolysable analogue adenosine-5′-O-(3-thiotriphosphate), ATPγS] Mg^{2+} and single-stranded DNA. In the absence of any one of these elements required for the RecA-dependent proteolysis of LexA, this fluorescence change was not observed. The final fluorescence change depends only upon the concentration of LexA regardless of that of RecA. The time course of the fluorescence decrease corresponds well with the kinetics of the decrease of intact LexA protein and the increase of its 2 proteolytic fragments as determined by SDS-polyacrylamide gel electrophoresis. These results allow us to use the fluorescence change as a signal for a detailed kinetic analysis. The velocity of the proteolysis (d[LexA]/dt) is proportional to the concentration of LexA and RecA indicating that the formation of the LexA-RecA complex is the limiting step.

RecA protein *LexA repressor* *SOS system* *Fluorescence* *Proteolysis* *Kinetics*

1. INTRODUCTION

When cellular DNA is damaged or the replication fork is blocked by other means, a number of coordinately regulated responses occurs in *Escherichia coli*, including DNA repair capacity and survival [1–3]. These responses, called induction of the SOS system, are conducted by the proteolysis of the common repressor of these genes, the LexA protein [2]. This proteolysis requires RecA protein in vivo as like that of the repressor of λ phage, which induces the lytic growth. In vitro, purified RecA protein promotes the specific cleavage of LexA and λ repressor in the presence of ATP (or its non-hydrolysable analogue adenosine-5′-*O*-(3-thiotriphosphate), ATPγS), Mg^{2+} and single-stranded DNA [4–6]. The latter could be accumulated in vivo under the conditions of a DNA damaging treatment.

Although a recent study demonstrated that LexA could autocleave itself at the identical specific site as in the presence of RecA [7], RecA stimulates the proteolysis and is required in vivo. Therefore the determination of kinetic parameters would allow the quantitative understanding of the switch on and off of SOS functions. This biologically interesting regulation exhibits a complex aspect including the regulation of LexA [8,9] and RecA synthesis [10] themselves and remains far from complete elucidation.

We demonstrate here that we can follow these kinetics easily and continuously by the measurement of the fluorescence change of LexA protein.

2. MATERIALS AND METHODS

RecA protein was purified principally with the procedure described by Cotterill et al. [11] to which DEAE-cellulose chromatography [12] was added as a final step. The concentration of RecA was determined spectrophotometrically by using $E_{28}^{1\%} = 5.7$ [12]. LexA protein was prepared to homogeneity as described by Schnarr et al. [13] and its concentration was determined using ϵ_{280} =

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7300 $M^{-1} \cdot cm^{-1}$ [14]. As single-stranded DNA, heat-denatured calf thymus DNA (Worthington) was used.

All experiments were carried out in a buffer containing 10 mM Tris, 30 mM NaCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM ATPγS (Boehringer Mannheim) and adjusted with HCl to pH 7.6 at the temperature of the experiment. The reaction was started by the mixing of equal volumes of LexA solution and RecA-DNA solution, preincubated at the experimental temperature for 15 min. The mixing of the 2 solutions was conducted with the aid of a rapid mixing apparatus [15]. The fluorescence was measured at 350 nm (band width, 10 nm) with a Jobin Yvon JY3C spectrofluorimeter using an excitation wavelength of 290 nm (band width, 10 nm).

Electrophoresis was carried out in the presence of SDS and with 15% polyacrylamide gels according to the method of Laemmli [16]. Gels were fixed for 20 min in 20% trichloroacetic acid and stained overnight in freshly prepared 0.3% Page blue 83 (BDH Biochemical), 25% methanol and 7% acetic acid. The amount of each fragment was determined by the spectroscopic measurement of electrically eluted dye from the corresponding band according to Malloy et al. [17]. To facilitate dye elution, the lower edge of glass tubes of an electroelution apparatus was blocked by polyacrylamide gel, instead of a membrane, and the positive charge was applied to the upper chamber.

3. RESULTS

When LexA is incubated with RecA and its cofactors, ATPγS, Mg^{2+} and single-stranded DNA, a progressive decrease of fluorescence intensity is observed (fig. 1). Although RecA also gives a fluorescence signal, this is weaker than that of LexA. With low RecA/LexA ratio, the final fluorescence intensity was even smaller than the fluorescence of LexA alone (fig. 1A), showing that this modification was, at least in part, due to LexA. The incubation of LexA alone in the same conditions did not yield a significant fluorescence change in a comparable time scale (fig. 1A). ATP could substitute for ATPγS to obtain this fluorescence decrease (not shown). But the elimination of DNA (fig. 1B) or any one of the

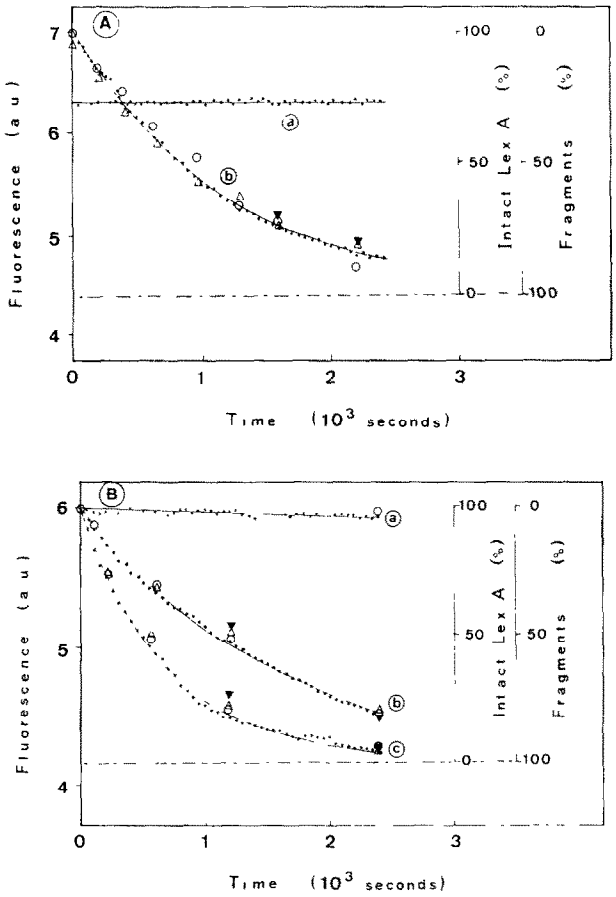

Fig.1. Comparison of the time course of the fluorescence decrease with the cleavage of LexA. The decrease of protein fluorescence (λ_{ex} = 290 nm, λ_{em} = 350 nm) (dotted lines) was compared with the amount of intact LexA (○), and that of N (▼) and C (△) terminal fragments determined as described in the text. To facilitate the comparison, these amounts were normalized and the ordinate for the fragments was inversed. (A) LexA at a concentration of 4 μM was incubated at 20°C in the presence (a) or absence (b) of 1 μM RecA. In both cases Mg^{2+}, ATPγS and single-stranded DNA (5 μM in bases) were present. The difference of initial fluorescence intensity between (a) and (b) corresponds to the fluorescence intensity of 1 μM RecA. In the absence of RecA, there was neither significant fluorescence decrease nor cleavage in this time scale. The solid line corresponds to a monoexpononential decay using k = 9×10^{-4} s^{-1} and ΔF = 25%. (B) LexA (4 μM) was incubated at 37°C with 1 μM RecA and various concentrations of single-stranded DNA: (a) 0 μM, (b), 1.5 μM and (c) 5.0 μM. The solid curves are monoexponential curves with k = 1.3×10^{-3} s^{-1}, ΔF = 32%, and k = 3.0×10^{-3} s^{-1}, ΔF = 32% for (b) and (c), respectively.

elements required for the proteolysis led to the almost complete disappearance of this change. These observations show that the change may be related to the cleavage of LexA. However, we cannot exclude the possibility that it is due to the formation of a stable RecA-LexA complex prior to the cleavage or a slow conformational change of the peptide fragment after the cleavage.

To examine this point, we determined the amount of intact LexA protein and its proteolytic fragments in parallel to the fluorescence measurements. Small aliquots were withdrawn at different times and mixed immediately with SDS solution to stop the reaction. Then the samples were submitted to SDS-polyacrylamide gel electrophoresis. The results were compared with the fluorescence measurements.

The examination was done for different incubation conditions. In all cases we observed a good concordance between the decrease of intact LexA protein and that of fluorescence (fig.1). Both at 20°C (fig.1A) and 37°C (fig.1B), resulting in about a 3-fold difference in cleavage rates, the concordance with the fluorescence measurements was equivalent. Furthermore the appearance of the 2 proteolytic fragments corresponded well with the decrease of fluorescence. With the observation that our electrophoresis gels presented only 4 main bands corresponding to RecA, intact LexA, C- and N-terminal fragments of LexA (not shown), this demonstrates that the observed fluorescence change is due to the specific cleavage.

These results exclude the existence of a significant time lag between the cleavage and the fluorescence change and allow us to use it as a signal for the further quantitative analysis of the cleavage kinetics. These results indicate also that the fluorescence decrease arises mainly or entirely from a modification of LexA. In fact, the final fluorescence change was proportional to only the concentration of LexA regardless of that of RecA, although the concentration of RecA affects the velocity of the fluorescence change (table 1).

The time course of the fluorescence change could be fitted with a monoexponential curve (fig.1) corresponding to $\Delta F = \Delta F_0 \cdot \exp(-kt)$. Supporting this, the determined k values are independent of the initial concentration of LexA for a given RecA concentration (table 1). This monoexponential aspect indicates that the cleavage

Table 1

Rate constants k and intensity changes of protein fluorescence of LexA RecA mixtures at various concentrations

Concentrations		$\Delta F/F^{LexA}$ (%)	k (10^{-3} s^{-1})
LexA (μM)	RecA (μM)		
0.3	1.0	26	3.1
1.0	0.1	30	0.33
1.0	1.0	32	3.3
1.0	2.0	28	6.1
1.0	3.0	25	10.0
2.0	1.0	27	2.8
4.0	1.0	30	3.1

Experiments were conducted at 37°C. In all cases, the concentration of single-stranded DNA (in bases) was 5-times that of RecA. The fluorescence intensity of RecA is about half of that of LexA at equimolar concentrations

velocity ($v = -\mathrm{d}[\mathrm{LexA}]/\mathrm{d}t$) is proportional to the concentration of intact LexA ($v = k \cdot [\mathrm{LexA}]$). On the other hand, the k value is increased proportionally to the concentration of RecA ($k = k' \cdot [\mathrm{RecA}]$) even for a 3-fold excess over LexA (table 1). The velocity is thus proportional to the product of LexA and RecA concentration ($v = k' \cdot [\mathrm{RecA}] \cdot [\mathrm{LexA}]$). This bimolecular aspect of the reaction means certainly that the formation of a LexA-RecA complex is the limiting step in our experimental conditions and would reasonably rule out the model of fast formation of a stable LexA-RecA complex preceding the cleavage.

Furthermore, the cleavage kinetics did not show a significant lag time between the time of mixing and the start of cleavage and the fluorescence intensity extrapolated to zero time is simply the sum of that of RecA and of LexA. These results also did not support the existence of preformation of a stable LexA-RecA complex.

We note that the amount of DNA used in these experiments (5 nucleotides per subunit of RecA) would not be the limiting factor in our experimental conditions. We observed that the maximum rate constant was obtained with 3 or 4 nucleotides of DNA for one subunit of RecA (fig. 1B and other results not shown). This value is somewhat smaller than that observed for the cleavage of λ

phage repressor (5–6 nucleotides per RecA monomer). Higher ratios of DNA/RecA could inhibit the proteolysis of LexA as observed for that of λ repressor [6].

It is interesting to note that the single tryptophan residue of LexA, which is the penultimate amino acid within the C-terminal domain [18,19], changes its fluorescence upon cleavage. This suggests that there may be a contact between N- and C-terminal domains and that this could be important for the interaction with DNA.

Concerning this tryptophanyl residue, it was reported from acrylamide quenching studies that its environment could be heterogeneous [13]. However, all of the LexA molecules were cleaved and the apparent heterogeneity disappears upon cleavage as seen from quenching studies with the purified C-terminal domain (Hurstel and Schnarr, unpublished).

The fluorescence change observed above corresponds well with the fluorescence characters of the purified C-terminal fragment of LexA compared to that of the intact protein, i.e. a decrease of about 25% in intensity and a blue-shift of the emission maximum. This also supports that the fluorescence change observed above arises mainly or entirely from LexA.

Such fluorescence measurements will facilitate a further detailed kinetic study of the RecA-dependent LexA proteolysis. They are much less tedious and more accurate than the electrophoretic determination and allow a continuous recording of the process with time.

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