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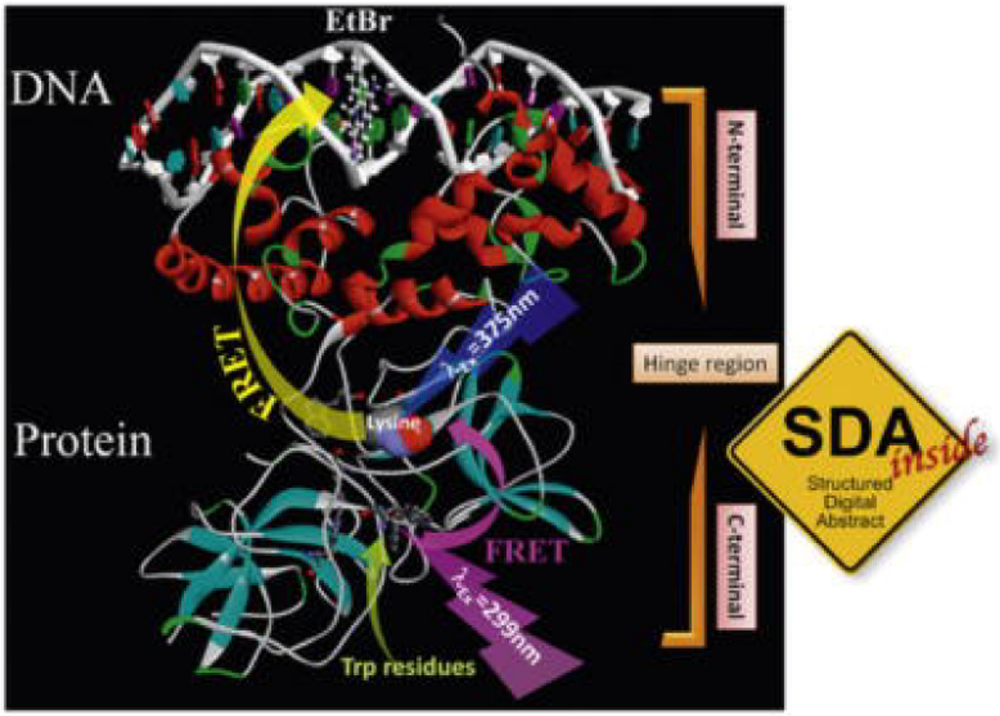

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Recognition of different DNA sequences by a DNA-binding protein alters protein dynamics differentially

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ABSTRACT

λ-Repressor–operator sites interaction, particularly O_R1 and O_R2, is a key component of the λ-genetic switch. FRET from the dansyl bound to the C-terminal domain of the protein, to the intercalated EtBr in the operator DNA indicates that the structure of the protein is more compact in the O_R2 complex than in the O_R1 complex. Fluorescence anisotropy reveals enhanced flexibility of the C-terminal domain of the repressor at fast timescales after complex formation with O_R1. In contrast, O_R2 bound repressor shows no significant enhancement of protein dynamics at these timescales. These differences are shown to be important for correct protein–protein interactions. Altered protein dynamics upon specific DNA sequence recognition may play important roles in assembly of regulatory proteins at the correct positions.

1. Introduction

Flow of essential biological information from DNA to RNA, which is eventually translated to proteins, is the central dogma of molecular biology. This flow of information is tightly regulated by DNA-binding proteins to target DNA sequences with high specificity. Regulatory proteins rarely act alone; the initial recognition is followed by assembly of other molecules of the same or different kinds forming multimeric complexes. Assembly of these complexes involve both protein–DNA and protein–protein interactions [1,2]. However, physico-chemical basis of the specificity of the complex formation is not well understood.

Gene regulatory network of bacteriophage λ is complex. A key element in this complex network is binding of a gene regulatory protein, λ-repressor (CI), to two target DNA sites, O_R1 and O_R2 [3]. These target sites are 17 bp pseudo-palindromic sites bearing close similarity with each other [4]. The binding of repressor dimers to these two DNA sequences is followed by protein–protein interaction between the two dimers, committing the phage to a lysogenic state [5]. It was widely believed that the specificity of formation of this type of complex resides solely in recognition of the DNA sequence by the DNA-binding protein. First inkling that other thermodynamic factors may be involved came from observation that the O_R1 and O_R2 bound repressors are structurally non-equivalent [6]. Recent work with glucocorticoid receptor protein (GRP) suggests that GRP bound to different target sequences have different structures leading to different functional outcomes [7]. However, little is known about protein dynamics when the DNA-binding proteins are bound to different target sites. In this study, we show that O_R1 and O_R2 bound repressors not only have structural differences, but differ significantly in terms of dynamic properties as well.

Structurally λ-repressor is a two domain protein as represented in Fig. 1. The N-terminal domain interacts with the DNA [8], while C-terminal domain is responsible for most protein–protein interactions [9]; these interactions are essential for the co-operative binding [10,11] and for the functioning of the genetic switch [12,13]. In earlier studies, it has been reported [14,15] that the binding of operator DNA to the N-terminal domain, causes a significant conformational change in the C-terminal domain of the protein. One possible role of the conformational change is to couple the specific operator binding information to the protein–protein interactions, which may lead to higher specificity at the required sites [7]. What role protein dynamics plays in these kinds of system is not clear. A deeper understanding would be invaluable in this system in particular and for transcription regulation in general.

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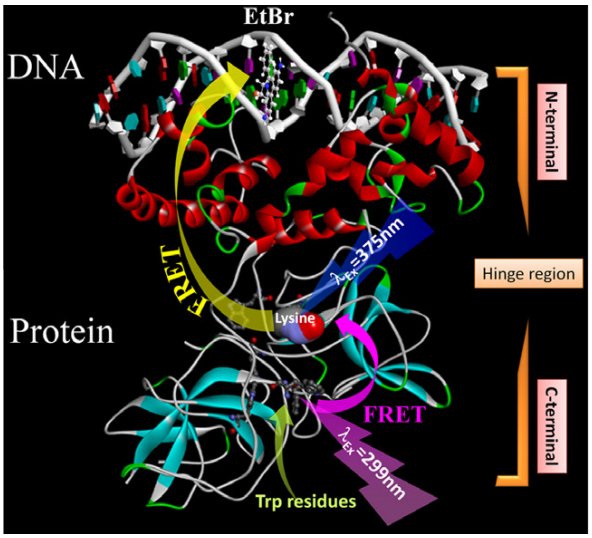

Fig. 1. X-ray crystal structure of λ-repressor bound to operator DNA depicting the simultaneous occurrence of Förster resonance energy transfer (FRET) from tryptophan residues to dansyl, bound to lysine near the C-terminal domain, and from dansyl to EtBr, bound to the operator DNA, is represented schematically. Tryptophan residues were excited at 299 nm and dansyl moiety was excited at 375 nm.

2. Materials and methods

The details of λ-repressor purification and chemical modification with dansyl chloride are described in Supplementary data. The incorporation ratio of dansyl was found to be 1.1 ± 0.2 [15] and the modification procedure essentially attach dansyl chromophore to the lysine residue [16] of the protein. The details of the fluorescence measurements (steady state and time resolved fluorescence spectroscopy) are described separately in the Supplementary data. Analytical Ultracentrifugation was performed using fluorescein tagged operator DNA to explore the protein–protein association as described in the Supplementary data.

3. Results and discussion

Though the λ-repressor has three cysteine residues, they are all unreactive under native conditions and hence are unsuitable for attaching fluorescent probes [17]. Hence, we have used 5-(dimethyl-amino) naphthalene-1-sulfonyl chloride (dansyl chloride) for specific covalent fluorescence labeling of λ-repressor, essentially at the C-terminal region [15] in order to monitor the changes in dynamical flexibility upon complexation with two operator DNA, O_R1 and O_R2. Picosecond resolved Förster resonance energy transfer (FRET) technique has been employed to confirm the location of the dansyl probe with respect to the tryptophan residues of the protein. The tryptophan-dansyl FRET in the protein upon complexation with unlabelled operator DNA has also been employed to monitor significant intra-protein structural rearrangement in the C-terminal domain of the protein. We have also used FRET from dansyl to the intercalated ethidium probe in the operator DNA, which is bound to the N-terminal domain of the λ-repressor, for the reconfirmation of the location of the dansyl probe and to monitor the structural differences between λ-repressor–O_R1/O_R2 DNA complexes (Fig. 1). The polarization gated picosecond resolved fluorescence anisotropy studies on the specific location of the dansyl probe, located at the C-terminal region [15], quantitatively measure the key dynamical time scales associated with the protein–protein and protein–DNA complex.

The steady state absorption and emission spectra of tryptophan in λ-repressor, dansyl bound to λ-repressor and ethidium bound to DNA are shown in Fig. S1, Supplementary data. The absorption spectrum of dansyl in λ-repressor and EtBr bound to operator DNA overlap with the emission spectra of tryptophan and dansyl in λ-repressor, respectively, indicating that these probes can serve as good donor–acceptor (D–A) pairs. The decrease in steady state fluorescence emission of tryptophan in dansyl modified repressor relative to that of unmodified λ-repressor reflects energy transfer from tryptophan to dansyl (Fig. S1, Supplementary data). As evidenced from Fig. 2a, faster fluorescence transient of tryptophan residues detected at 350 nm (excitation 299 nm) in dansyl modified λ-repressor compared to that of unmodified λ-repressor, is an indication of the FRET from tryptophan to dansyl moiety. The temporal decay of the tryptophan residues in dansyl modified repressor shows three time components, including a faster time component of 0.15 ns (45%) unlike unmodified repressor (two longer time components) and as a consequence the average lifetime of tryptophan decreases from 2.8 ns in λ-repressor to 1.37 ns in dansyl modified repressor (Table S1, Supplementary data). The efficiency of energy transfer is found out to be 50%. Both the Förster distance (R_0) and donor (tryptophan)–acceptor (dansyl) distance are estimated to be 14.5 Å.

Upon addition of unlabelled O_R1 and O_R2 DNA to the dansylated repressor, an additional faster component of 20 ps appears in the fluorescence transient of tryptophan detected at 350 nm, which is depicted by Fig. 2a and Fig. 2b respectively. It has to be noted that the control experiment on the tryptophan emission transients from unlabeled repressor with the operator DNA at 350 nm does not show any faster component revealing insignificant possibility of perturbation of tryptophan emission transients in presence of operator DNA due to other non-emissive processes (Fig. S2, Supplementary data). The donor (tryptophan)–acceptor (dansyl) distances in dansyl modified λ-repressor–O_R1/O_R2 DNA complexes are estimated to be 11 and 11.8 Å, respectively. The faster decay of tryptophan in the dansylated λ-repressor in presence of O_R1 and O_R2 DNA clearly reveals a significant structural modification in the C-terminal domain, leading to much shorter dansyl-tryptophan distance in both the complexes. The difference of the tryptophan-dansyl distance in dansylated λ-repressor–O_R1 DNA and dansylated λ-repressor–O_R2 DNA complex is relatively small. However, the difference may arise due to the crucial structural diversity in the C-terminal domain of the repressor bound to O_R1 and O_R2 DNA. We have also explored FRET from dansyl chromophore in the repressor protein to the operator DNA bound EtBr in the protein–DNA complex. The average FRET distance between dansyl and EtBr is estimated assuming one operator DNA contains four ethidium molecules in our experimental condition, obtained from the amplitude of the DNA bound ethidium lifetime as discussed in Supplementary data and Fig. S3. Steady state emission quenching and enhancement of dansyl and ethidium respectively in the complex (Fig. S1, Supplementary data) and the faster fluorescence decay of dansyl in dansyl modified λ-repressor–O_R1/O_R2 DNA–EtBr complexes (Fig. 2c and d, respectively) at 515 nm are the indicatives of the resonance energy transfer. The efficiency of energy transfer from dansyl to EtBr in λ-repressor–O_R2 DNA–EtBr complex (48%) is higher than in λ-repressor–O_R1 DNA–EtBr complex (29%). The dansyl-ethidium distances in λ-repressor–O_R1/O_R2 DNA–EtBr complexes are estimated to be 24 and 20 Å, respectively, indicating the structure of the protein is more compact in the O_R2 complex than in the O_R1 complex as the distance between the DNA, bound to N-terminal domain of the repressor and C-terminal domain of the repressor is considered to be the marker of compactness. A careful analysis of the distances of the lysine residues from the tryptophan residues and the DNA-bound ethidium indicates lysine 134B in the C-terminal region, to be the most potential dansylated

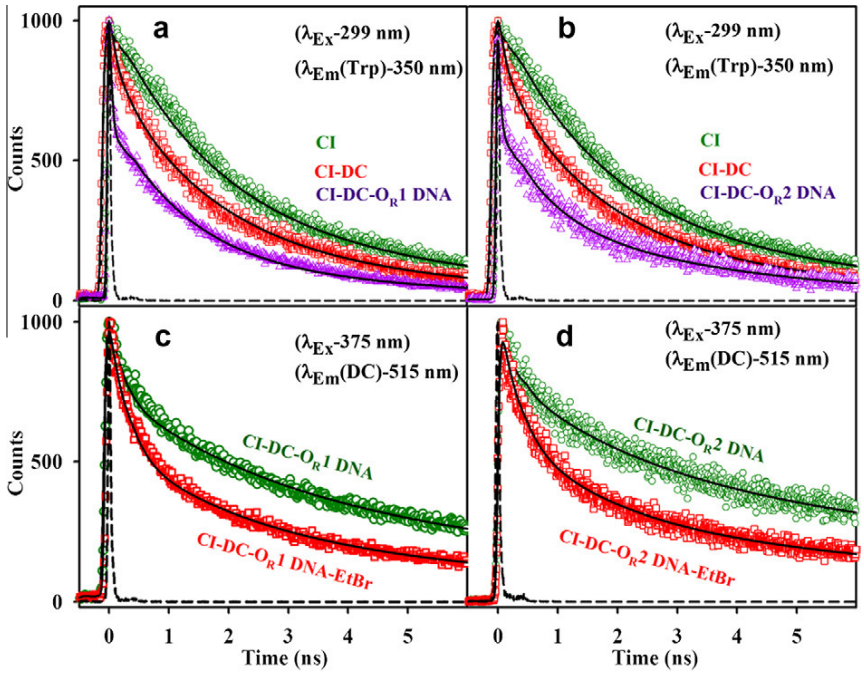

Fig. 2. (a) Picosecond resolved fluorescence transients of tryptophan residues in λ-repressor (CI; green), in dansyl modified λ-repressor (CI-DC; red) and in dansylated λ-repressor–O_R1 DNA complex (CI-DC-O_R1 DNA; violet). Excitation and emission wavelengths were 299 nm (λ_{Ex}-299 nm) and 350 nm (λ_{Em} (Trp)-350 nm) respectively. (b) Picosecond resolved fluorescence transients of tryptophan residues in λ-repressor (CI; green), in dansyl modified λ-repressor (CI-DC; red) and in dansylated λ-repressor–O_R2 DNA complex (CI-DC-O_R2 DNA; violet). Excitation and emission wavelengths were 299 nm (λ_{Ex}-299 nm) and 350 nm (λ_{Em} (Trp)-350 nm) respectively. (c) Picosecond resolved fluorescence transients of dansyl in λ-repressor–O_R1 DNA complex in absence (CI-DC-O_R1 DNA; green) and in presence (CI-DC-O_R1 DNA-EtBr; red) of EtBr. Excitation and emission wavelengths were 375 nm (λ_{Ex}-375 nm) and 515 nm (λ_{Em} (DC)-515 nm) respectively. (d) Picosecond resolved fluorescence transients of dansyl in λ-repressor–O_R2 DNA complex in absence (CI-DC-O_R2 DNA; green) and in presence of EtBr (CI-DC-O_R2 DNA-EtBr; red). Excitation and emission wavelengths were 375 nm (λ_{Ex}-375 nm) and 515 nm (λ_{Em} (DC)-515 nm), respectively.

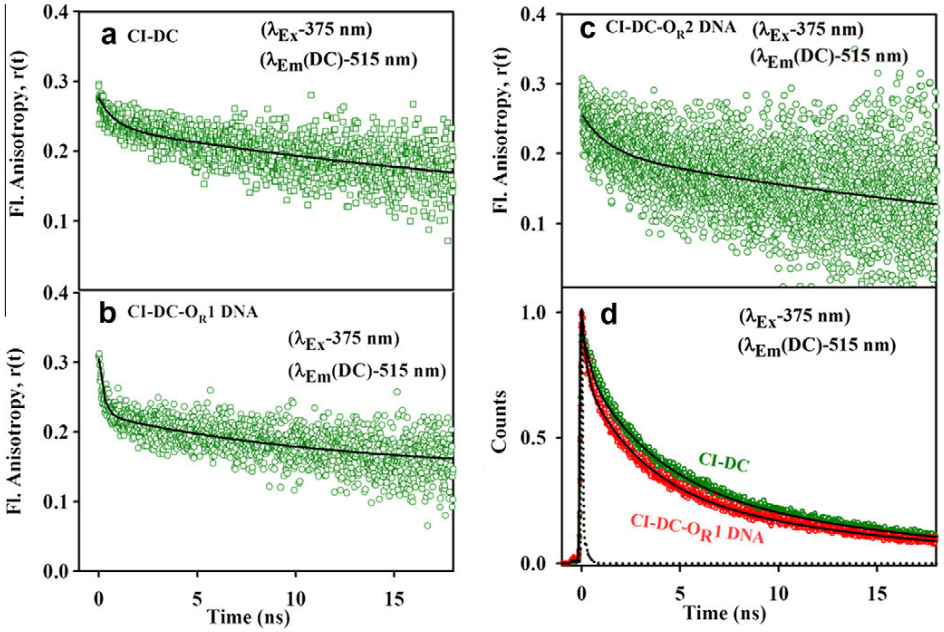

Fig. 3. Fluorescence anisotropy, $r(t)$ of (a) dansyl in λ-repressor (CI-DC; green). (b) dansyl in λ-repressor–O_R1 DNA complex (CI-DC-O_R1 DNA; green). (c) Dansyl in λ-repressor–O_R2 DNA complex (CI-DC-O_R2 DNA; green). (d) Picosecond resolved fluorescence transients of dansyl in λ-repressor (CI-DC; green) and in λ-repressor–O_R1 DNA complex (CI-DC-O_R1 DNA; red). Excitation and emission wavelengths were 375 nm (λ_{Ex}-375 nm) and 515 nm (λ_{Em} (DC)-515 nm) for all the systems, respectively.

site (Fig. 1) having average distance of 12 Å from the tryptophan residues and 28 Å from the all possible EtBr binding sites in the operator DNA due to the arch conformation of the DNA (using VIEWER LITE software). Our observation of those distances 11

and 24 Å (for O_R1 complex using FRET technique) are consistent with the estimated values. The corresponding time constants are tabulated in Table S1, Supplementary data.

Polarization gated fluorescence anisotropy decay profiles of dansyl in λ-repressor and in λ-repressor–operator DNA complexes are shown in Fig. 3. The rotational flexibility of the protein before and after complexation with operator DNA is investigated by time-resolved fluorescence anisotropy studies. The fluorescence anisotropy, $r(t)$, which can decay in time due to the rotational motion of the molecules and consequently leads to depolarization of the fluorescence is fitted to bi-exponential decay function (Table S2, Supplementary data). The faster time constant indicates the local/inertial motion of the probe and the slower one reveals the overall tumbling motion of the entire protein [18]. The estimated rotational time constant (τ) for the repressor associated with the overall λ-repressor tumbling is estimated to be 27 ns using Stokes–Einstein–Debye (SED) equation [19]. In our numerical fitting of the fluorescence anisotropy decay transient (Fig. 3a), we have fixed the longer time constant to be 27 ns. In the case of λ-repressor, we have obtained the faster time constant to be 800 ps, a quantitative measure of the flexibility of the lysine bound dansyl in the C-terminal domain. Upon complexation with the O_R1 and O_R2 DNA, the faster time components become 370 ps and 1.3 ns, respectively. The observation clearly indicates that the flexibility in the C-terminal domain of the protein upon complexation with the O_R1 DNA is much higher compared to that of the O_R2 DNA (Fig. 3b and c). The faster fluorescence decay of dansyl probe in λ-repressor upon interaction with the O_R1 DNA is evident from Fig. 3d. The flexibility of the dansyl probe leading to its exposure to the aqueous environment is evident from the time-resolved decay (faster decay due to TICT process [20]) of the probe upon interaction with the O_R1 DNA (Fig. 3d) and as a consequence, the average lifetime of dansyl decreases from 5.56 ns in dansyl modified λ-repressor to 3.48 ns in dansyl modified repressor–O_R1 DNA complex. On the contrary, the dansyl in the λ-repressor–O_R2 DNA complex shows insignificant change (data not shown) in the fluorescence decay compared to that of the free protein indicating less flexibility in the C-terminal domain, which is consistent with the anisotropy study. The corresponding time constants are tabulated in Table S1, Supplementary data.

The implications of differentially altered dynamics of the protein bound to different DNA sequences raise the issue of its functional importance. Two λ-repressor dimers, bound to O_R1 and O_R2 on the same piece of DNA interact to produce a tetrameric looped complex (microloop), which is critical for the regulatory network. We have thus attempted to elucidate if this differential dynamics is important for protein–protein interaction between two λ-repressor dimers bound to O_R1 and O_R2. The interaction was studied by sedimentation equilibrium (Fig. 4). We have first attempted to determine the interaction of O_R1 bound repressor dimer with an O_R2 bound repressor dimer by sedimentation equilibrium. In this experiment, O_R1 was end labeled with fluorescein and a labeled O_R1/repressor complex was formed. This was mixed with an excess of unlabeled O_R2/repressor complex and sedimentation equilibrium run was performed. Fitting of the concentration profile to appropriate equations (Supplementary data) yields dissociation constant of 4.4 μM and 41.6 μM, for O_R1/repressor–O_R2/repressor and O_R1/repressor–O_R1/repressor, respectively. Clearly, the structural and dynamical differences are important for the correct protein–protein interaction even in trans.

The time scales at which the protein dynamics is significant (sub-nanosecond) suggest that the difference may lie in motion of small protein segments. The protein–protein interaction interfaces in general are tightly packed and may require plasticity for formation. Given the dynamics dependent protein–protein interactions strength reported here, we suggest that the formation of correct DNA–protein complexes may be regulated by the DNA sequences through modulation of static and dynamic changes of the bound proteins. In conclusion, we demonstrate that binding of transcription factors to specific DNA sequences alters the dynamical properties of the bound protein in a DNA-sequence dependent manner and the dynamical difference contributes to the formation of correct regulatory complex.

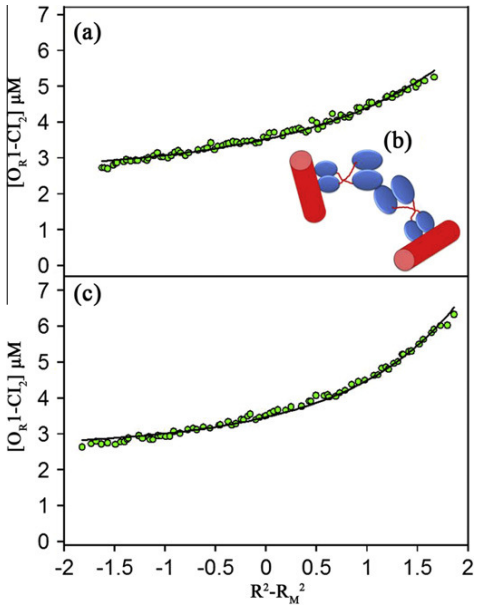

Fig. 4. Determination of dimer–tetramer association by sedimentation equilibrium. The inset cartoon figure (b) shows the type of association that is being studied here. The red rod represents the operator DNA, while the blue balls represent the protein. (a) Self association of O_R1–repressor complex and (c) association between O_R1–repressor complex and the O_R2–repressor complex. An oligonucleotide duplex containing O_R1 sequence and C6 amino link at 5'end was labeled with fluorescein and stoichiometric complex was formed with repressor. It was either run alone at 2.7 μM or with additional 5 μM unlabeled O_R2/repressor complex. The concentration gradient was monitored at 495 nm.

Acknowledgments

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Appendix A. Supplementary data

Materials and methods, isolation and chemical modification of λ-repressor, details of steady state, time-resolved spectroscopy and analytical ultracentrifugation, and supplementary figures and tables. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.12.032.

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