Intrinsic Fluorescence Properties and Structural Analysis of p13\textsubscript{\textit{suc1}} from \textit{Schizosaccharomyces pombe}\textsuperscript{*}

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\textsuperscript{1}The abbreviations used are: MPP, M phase-promoting factor; DAS, decay-associated spectra; EDAS, excitation decay-associated spectra; GdnHCl, guanidine hydrochloride.

\textbf{p13\textsubscript{\textit{suc1}}} acts in the fission yeast cell division cycle as a component of p34\textsubscript{\textit{cdc2}}. In the present work, structural information contained in the intrinsic fluorescence of p13\textsubscript{\textit{suc1}} has been extracted by steady-state and time-resolved fluorescence techniques. In its native form, the steady-state emission spectrum of p13\textsubscript{\textit{suc1}} is centered at 336 nm. Upon denaturation by guanidine HCl (4.0 M), the emission spectrum is shifted to 355–360 nm and the fluorescence intensity decreases 70%. The same changes are not obtained with p13\textsubscript{\textit{suc1}} at 56 °C or after incubation at 100 °C, and the protein appears to be substantially temperature-stable. The fluorescence decay of p13\textsubscript{\textit{suc1}} is best described by three discrete lifetimes of 0.6 ns (τ\textsubscript{1}), 2.9 ns (τ\textsubscript{2}), and 6.1 ns (τ\textsubscript{3}), with amplitudes that are dependent on the native or unfolded state of the protein. Under native conditions, the two predominant decay-associated spectra, DAS-τ\textsubscript{2} (λ\textsubscript{max} = 332 nm) and DAS-τ\textsubscript{3} (λ\textsubscript{max} = 340 nm), derive from two different excitation DAS. Moreover distinct quenching mechanisms and collisional accessibilities (k(τ\textsubscript{2}) > k(τ\textsubscript{3})) are resolved for each lifetime. An interpretation in terms of specific tryptophan residue (or protein conformer)-lifetime associations is presented. The decay of the fluorescence anisotropy of native p13\textsubscript{\textit{suc1}} is best described by a double exponential decay. The longer correlation time recovered (9 ns \textit{φ} = \textit{g} 15 ns) can be associated with the rotational motion of the protein as a whole and a Stokes radius of 21.2 Å has been calculated for p13\textsubscript{\textit{suc1}}. Anisotropy measurements obtained as a function of temperature indicate that, in solution, the protein exists exclusively as a protate monomer. In 1 M zinc, changes of the anisotropy decay parameters are compatible with subunits oligomerization.

p13\textsubscript{\textit{suc1}} acts in the fission yeast cell division cycle both in G1 and G2 (Forsburg and Nurse, 1991; Reed, 1992). Originally identified in \textit{Schizosaccharomyces pombe} as an extragenic suppressor of certain cdc2 temperature-sensitive mutations (Hayles \textit{et al.}, 1986), in the yeast lysates the product of the \textit{suc1} gene was found associated with the major cell cycle regulator, p34\textsubscript{\textit{cdc2}} (Brizuela \textit{et al.}, 1987; Draetta \textit{et al.}, 1987). The addition of p13\textsubscript{\textit{suc1}} to the kinase assay in vitro was able to rescue the defect in the Cdc2 mutant kinase activity (Booher \textit{et al.}, 1989; Moreno \textit{et al.}, 1989), and the protein was proposed to act as a regulatory component of the p34\textsubscript{\textit{cdc2}} (Brizuela \textit{et al.}, 1987; Draetta, 1990).

The nucleotide sequence of \textit{suc1} has been determined (Hindley \textit{et al.}, 1987), and p13\textsubscript{\textit{suc1}} has been expressed in \textit{Escherichia coli} (Brizuela \textit{et al.}, 1987). From that source p13\textsubscript{\textit{suc1}}-Sepharose beads have been prepared, and their affinity binding to p34\textsubscript{\textit{cdc2}} has been widely used to purify p34\textsubscript{\textit{cdc2}} (Brizuela \textit{et al.}, 1989). The discovery of p34\textsubscript{\textit{cdc2}} homologue in human suggested the universality of cell cycle control mechanisms (Lee and Nurse, 1987). In addition, concurrently with finding a p34\textsubscript{\textit{cdc2}} homologous protein kinase, in the different eucaryotic species investigated, \textit{suc1} gene-related homologues have also been found (Simanis and Nurse, 1986; Draetta \textit{et al.}, 1987; Paris \textit{et al.}, 1990; Elledge and Spottswood, 1991; Hellmich \textit{et al.}, 1992; Lew \textit{et al.}, 1992). In the budding yeast \textit{Saccharomyces cerevisiae}, the CKS1 gene codes for a protein, p18\textsubscript{\textit{CKS1}}, suppressor of Cdc28 mutations (Reed, 1992), while in the early studies on HeLa cells (Draetta \textit{et al.}, 1987), Cdc2 protein kinase was found associated with a 13-kDa polypeptide. More recently, the human homologues of the p13\textsubscript{\textit{suc1}}/p18\textsubscript{\textit{CKS1}} proteins, p6\textsubscript{\textit{CKShs1}}/p6\textsubscript{\textit{CKShs2}}, have been identified as the products of the genes CKS\textsubscript{\textit{hs1}} and CKS\textsubscript{\textit{hs2}}, respectively (Richardson \textit{et al.}, 1990). According to these findings p13\textsubscript{\textit{suc1}} and its homologous proteins appear to be as ubiquitous as the p34 family of kinases, thus suggesting the essential role of p13\textsubscript{\textit{suc1}} as components of the cell cycle control mechanisms.

The relevance of the biological function of p13\textsubscript{\textit{suc1}} has been advanced by several intriguing observations. The p34\textsubscript{\textit{cdc2}}, p13\textsubscript{\textit{suc1}}-Sepharose complex is active as protein kinase (Brizuela \textit{et al.}, 1989). The same matrix has been found to deplete M phase \textit{Xenopus} extracts of the “M phase-promoting factor” (MPP)\textsuperscript{1} (Dunphy \textit{et al.}, 1988), and this evidence has been confirmed among different cell species (Draetta and Beach, 1988; Arion \textit{et al.}, 1988; Draetta \textit{et al.}, 1989; Labbé \textit{et al.}, 1989; Pondaven \textit{et al.}, 1990; Meijer \textit{et al.}, 1989, 1991). In addition, p13\textsubscript{\textit{suc1}} inhibits the entry into mitosis in \textit{Xenopus} extracts (Dunphy \textit{et al.}, 1988) and, microinjected in mammalian oocytes, inhibits the entry into meiosis (Gavin \textit{et al.}, 1992). Moreover, \textit{in vitro} activation of the inactivated precursor of MPP (pre-MPP) from \textit{Xenopus} oocytes results in tyrosine dephosphorylation of the p34\textsubscript{\textit{cdc2}} protein. p13\textsubscript{\textit{suc1}} completely blocks p34\textsubscript{\textit{cdc2}} tyrosine 15 dephosphorylation and kinase activation (Dunphy and Newport, 1989). A model has been proposed in which a Cdc2-specific tyrosine kinase and phosphatase, as well as p13\textsubscript{\textit{suc1}}, might interact to regulate the Cdc2 kinase (Dunphy and Newport, 1989).

Although these data suggest that p13\textsubscript{\textit{suc1}} plays a significant
role in the regulation of p34<sup>calc</sup>, there is no clear agreement about the involvement of the <i>suc1</i> gene product and its homologues in the steps of p34<sup>calc</sup> biochemistry (Dunphy, 1994).

Fluorescence methods provide a useful tool to obtain dynamic and static information on the structure of proteins and macromolecular assemblies (Beechem and Brand, 1985; Eftink, 1991). In addition, these techniques can be used to investigate molecular interactions in the living cell. In this paper, we describe steady-state and time-resolved fluorescence studies on the intrinsic fluorescence of p13<sup>suc1</sup>. A characterization of its photophysics and hydrodynamic properties is presented. These information, combined with the recent characterization of the protein crystal structure (Endicott et al., 1995), will be useful for future studies on p13<sup>suc1</sup> structure/function relationships.

**MATERIALS AND METHODS**

The <i>E. coli</i> [BL21(DE3)] LysS strain expressing the <i>suc1</i> gene product was kindly provided by Dr. Giulio Draetta (Mitotix, Cambridge, MA). Bacterial growth, induction by isopropyl-β-D-thiogalactopyranoside, and purification of the protein were performed following the procedure described by Brizuela et al. (1987). A final gel filtration step on a Sephacryl S-100 HR column (80 x 2 cm) at a flow rate: 0.1 ml/min eluted with a buffer containing 50 mM Tris-HCl, pH 8.0, and 2 mM EDTA was added to obtain full homogeneity of the purified material. Protein concentration was determined by the Lowry method (Lowry et al., 1951) and by a bicinchoninic acid-based method (Smith et al., 1985) using bovine serum albumin as a standard. Routinely, purity of p13<sup>suc1</sup> was confirmed by Endicott et al. (1970) as measured on a Bio-Rad model GS-670 videodensitometer and stored following the instructions of the supplier.

Dialysis against a 70:30, acetonitrile:H<sub>2</sub>O solution). The protein had an apparent Mr of approximately 10,000 (as measured on a Bio-Rad model GS-670 videodensitometer and using the Molecular Analyst software package. Isoelectrofocusing was performed on LKBAmpholine® PAGplates with a LKB apparatus. The measured pl = 5.84 was in excellent agreement with the theoretical value of pl = 5.80 obtained from the SWISS-PROT data base (ExPASy, WWW) of the Geneva University. The NH<sub>2</sub>-terminal region of the protein, blotted onto Pro-Blot (Applied Biosystem), was sequenced and stored following the instructions of the supplier.

Sephadex G-25, G-50, Sepharose CL-6B, and Sephacryl S-100 HR were from Pharmacia Biotech Inc.

**Fluorescence Spectroscopy Measurements**—Technical steady-state fluorescence excitation and emission spectra were obtained with a Jasco FP-550 spectrofluorometer using excitation and emission slit widths of 5 nm each. Fluorescence polarization measurements were performed using two Polacoat dichroic polarizers (Jasco FP-2010) installed in the excitation and the emission paths to record the relative intensities for the four combinations of vertically (v) and horizontally (h) polarized beams (I<sub>vh</sub>, I<sub>hv</sub>, I<sub>vh</sub>, I<sub>hv</sub>). The resulting steady-state emission anisotropy, <i>r</i><sup>ss</sup>, was calculated as follows,

\[
(r) = \left( \frac{I_{vh} - I_{hv}}{I_{vh} + 2I_{hv}} \right) \tag{1}
\]

where 0 = I<sub>vh</sub>/I<sub>hv</sub> is the grating correction factor introduced to normalize for the different sensitivities of the system to detect the horizontally and vertically polarized emission (Azumi and McGlynn, 1962; Paolletti and LePecq, 1969).

Fluorescence quenching measurements of p13<sup>suc1</sup> were performed using acrylamide and potassium iodide as quenchers. With acrylamide, protein samples at increasing concentrations of the quencher were prepared by adding small aliquots from a 8 M stock solution. At the excitation wavelength of 295 nm no corrections for the optical density of the samples were required. With KI, protein samples at increasing concentrations of the quencher were prepared at constant ionic strength using KCl as a counter-ion. Stock solutions of KI (4 M) were freshly prepared in the presence of ~1 x 10<sup>-3</sup> M Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> to avoid I<sub>2</sub> formation (Lehrer, 1971). Fluorescence decay experiments were recorded at increasing quencher concentrations (0–0.42 M). Curves were collected at three emission wavelengths (330, 340, and 350 nm) for each concentration of the quencher, and the data were analyzed by the global procedure. Steady-state and time-resolved fluorescence results were analyzed according to the Stern-Volmer equation (Lehrer, 1971; Lakowicz, 1983),

\[
F_0 \frac{F}{F_0} = 1 + K_{SV} [Q] = \frac{\tau_0}{\tau} \tag{2}
\]

while in the presence of a static contribution and considering the sphere of action model, the complete expression for the quenching ratio is given by Equation 4 (Laws and Contino, 1992),

\[
F_0 \frac{F}{F_0} = \left[ \sum_{i=1}^{n} \frac{f(i)}{[1 + K_{SV} [Q](Q)]} \right]^{-1} \tag{3}
\]

where \( f(i) \) represent the volume of the species-associated interaction sphere.

Moreover, \( K_{SV} \) is equal to \( k_q \tau_0 \) where \( k_q \) is the apparent rate constant for the collisional quenching process and represents a measure of the overall accessibility of the fluorophores. Relative bimolecular collisional quenching constants, \( k_q(\tau) \), can be obtained for each fluorescence lifetime. In this case, a simple modified form of the Stern-Volmer plot of \( 1/\tau \) versus \([Q] \) directly provide the \( k_q(\tau) \) as the slope of the graph.

**Fluorescence Data Analysis**—Fluorescence intensity decay. The decay data were analyzed by nonlinear least square method (Knight and Selinger, 1971; Grinvald and Steinberg, 1974), and decay curves collected at multiple emission wavelengths were simultaneously analyzed.
Fluorescence Spectroscopy of Yeast p13\textit{suc1}

according to the global procedure described by Knutsen et al. (1983). When appropriate, the decay constants were linked across spectral regions. The experimental data (counts/channel > 0.5% of the total counts in the peak) were analyzed assuming that the fluorescence decay follows a multiexponential law,

\[ I(t) = \sum_{i=1}^{n} a_i e^{-\frac{t}{\tau_i}} \quad (\text{Eq. 5}) \]

where the relative amplitudes, \( a_i \), and the decay constants, \( \tau_i \), are the numerical parameters to be recovered. The best fit between the theoretical curve and the data was evaluated from the plot of residuals, the autocorrelation function of the residuals, and the reduced Chi-square (\( \chi^2 \)) (Bevington, 1969). The DAS and the EDAS were obtained by the global procedure (Beecum et al., 1985), and the fluorescence relative intensities at the various wavelengths were expressed as \( a_i \cdot \tau_i \) products. Percent fractional contributions of each decay component to the total emitted fluorescence was, then, calculated as \( a_i \cdot \tau_i / \sum a_i \cdot \tau_i \).

**Fluorescence Anisotropy Decay**—The anisotropy decay can be described by a sum of discrete exponential terms as follows (Wohl, 1969; Tao, 1969),

\[ r(t) = \sum_{i=1}^{n} \beta_i e^{-\frac{t}{\tau_i}} \quad (\text{Eq. 6}) \]

where the sum of the pre-exponential terms \( \beta_i \) is the anisotropy in the absence of rotation, \( \tau_i \), and the \( \phi_i \) values are the rotational correlation times. For a globular protein that approximates the spherical symmetry, the anisotropy decay is reduced to a single exponential. Under this condition the correlation time can be related to the hydrated volume of the rotating protein, \( V \), by the Einstein-Stokes relation, \( \phi = \frac{V \rho k T}{h} \), where \( \eta \) is the solvent viscosity, \( k \) is the Boltzmann constant, and \( T \) is the experimental temperature. Alternatively, complex anisotropy decays suggest deviation from simple spherical symmetry.

The parameters for the decay of anisotropy, \( r(t) \), were recovered from the analysis of the experimental decays of the polarization constants, \( I_{\text{ss}}(t) \) and \( I_{\text{as}}(t) \), by the system analysis approach introduced by Gilbert (1983). According to this method, the fitting functions to obtain \( r(t) \) are the following (Ameloot et al., 1984; Cross and Fleming, 1984),

\[ I_{\text{ss}}(t) = 1/3\beta(t) \cdot (1 + 2r(t)) \quad (\text{Eq. 7}) \]
\[ I_{\text{as}}(t) = 1/3\beta(t) \cdot (1 - r(t)) \quad (\text{Eq. 8}) \]

where \( \beta(t) \) represents the decay of the total fluorescence intensity and does not depend on molecular reorientation. Common parameters were linked, and \( \eta T \) terms were introduced in the analysis of anisotropy decay curves obtained at multiple temperatures.

The variability of the decay parameters was evaluated by determining the joint confidence intervals (Johnson, 1983). All the steady-state and time-resolved fluorescence experiments were run at least twice using different preparations of \( p13\text{suc1} \). The inter-experimental variability was less than 10%.

**Circular Dichroism**—Circular dichroism spectra were recorded on a Jasco J-710 spectropolarimeter. Protein samples (0.15 mg/ml) in 50 mM phosphate buffer, pH 7.4, 2 mM EDTA were measured in a 1-mm path length cell at 0.5-nm interval. Eight spectra were accumulated and averaged to achieve appropriate signal-to-noise ratios. The fractional composition of the secondary structure of \( p13\text{suc1} \) in terms of \( \alpha \)-helix, \( \beta\)-sheet, and random coil was evaluated according to the procedure described by Andrade (1993). According to that procedure, neural network analysis of the protein topological map in the 200–230 nm wavelength range was performed using the k2d,PC software.

**RESULTS**

The isolated protein resulted homogeneously pure as judged by the amino acids sequence of its 2–43 NH\textsubscript{2}-terminal region, by high performance liquid chromatography, by SDS-polyacrylamide gel electrophoresis, and by the measure of its pl (see "Materials and Methods").

**Circular Dichroism**—The CD spectra of \( p13\text{suc1} \) recorded at 20 °C (curve 1), at 56 °C (curve 2), and at 20 °C after incubation at 100 °C for 10 min (curve 3) is presented in Fig. 1. The data were recorded in the 180–300 nm spectral range. The protein was dialyzed overnight against 50 mM phosphate buffer, 2 mM EDTA, at pH 7.4, to remove Tris-HCl. From these data a secondary structure composition of 23% \( \alpha \)-helix, 19% \( \beta \)-sheet, and 58% random coil was obtained for the native protein at 20 °C. At 56 °C, an expected loss of ordered secondary structure is observed. However, when the spectrum is recorded at 20 °C, with a sample previously incubated at 100 °C, a small fraction of secondary structure is recovered and the intensity of the aromatic band (235 nm) is completely restored to the native level.

**Steady-state Intrinsic Fluorescence**—The steady-state emission spectra of \( p13\text{suc1} \) recorded under native and denaturing conditions are presented in Fig. 2. Under native conditions, emission spectra were obtained exciting the protein in both the tryptophan, 295 nm (curve 1), and the tyrosine, 275 nm (not shown), absorption bands. No differences of the steady-state emission spectra profiles were observed, suggesting a very poor contribution of the tyrosine residues. The maximum of the fluorescence intensity was centered at 336 nm, as expected for chromophores partially buried inside the protein. In 4.0 M GdnHCl, curve 2, the emission spectrum of \( p13\text{suc1} \) was shifted to 355–360 nm, and its intensity was decreased of approximately 70%. Higher guanidinium concentrations (6.0 M) did not cause further effects on the fluorescence spectrum. Emission spectra were also recorded in the absence of GdnHCl, at 56 °C, curve 3, at 20 °C, by cooling the same sample, curve 4; and finally at 20 °C, with a sample previously incubated at 100 °C for 10 min, curve 5. These data suggest that, up to 56 °C, the structural transitions induced on \( p13\text{suc1} \) by heat are mostly reversible and even more severe temperature treatments do not denature the protein completely.

**Time-resolved Intrinsic Fluorescence**—To obtain dynamic information on \( p13\text{suc1} \), the intrinsic fluorescence decay of the protein has been resolved in the nanosecond time scale. The decay of the fluorescence intensity was measured exciting the samples at 295 nm. A typical result of the analysis of the experimental data is presented in Fig. 3, together with the statistical parameters used to judge the quality of the fitting. The fluorescence decay of \( p13\text{suc1} \) is well described by three discrete decay components of 0.6 ns (\( \tau_1 \)), 2.9 ns (\( \tau_2 \)), and 6.1 ns (\( \tau_3 \)); with the longer lifetime accounting for 84%, the medium lifetime for 32%, and the shortest lifetime for 4% of the total emission, respectively. The decay parameters of the native
protein are presented in Table I together with the results obtained with \( p13^{\text{Suc1}} \) at 56°C and in the presence of 4.0 M GdnHCl. In all the cases, the statistical quality for biexponential fittings was not adequate. It is shown that the decrease of the steady-state fluorescence intensity observed upon denaturation is due to a significant increase of the contributions of the shortest lifetime (16% versus 4%), accompanied by a dramatic decrease of the contribution of the long lifetime (35% versus 64%), with smaller effects from the contribution of the medium lifetime (49% versus 32%). Upon guanidine denaturation the fluorescence lifetimes were modestly affected, whereas at 56°C the decay parameters recovered indicate that the intensity decrease of the steady-state spectrum (Fig. 2, curve 3) is mainly due to a collisional mechanism with only minor effect on the relative weight of each decay constant.

In order to monitor the photophysical changes associated with the unfolding of \( p13^{\text{Suc1}} \), fluorescence decay curves have been collected at increasing GdnHCl concentrations (Eftink, 1994). The recovered amplitudes are presented in Fig. 4. In the same figure the values obtained after overnight dialysis against GdnHCl-free buffer of the sample prepared at 4.0 M GdnHCl is also shown. From these data it is derived that unfolding of the native protein is induced by GdnHCl concentrations higher than 0.5 M and that this process can be reversed by removal of the denaturing reagent.

Resolution of DAS provides useful information to assign conformation (Ross et al., 1981; Knutson et al., 1982; Neyroz et al., 1987; Royer et al., 1990). The DAS of \( p13^{\text{Suc1}} \) obtained at 20°C, in 50 mM Tris-HCl, pH 8.0, and 2 mM EDTA, exciting at 295 nm are presented in Fig. 5A. Three lifetime DAS were recovered. As expected from the data presented in Table I, the spectrum associated with the long lifetime (6 ns) showed the major contribution with the maximal intensity at 340 nm, the spectrum associated with the medium lifetime (3 ns) showed an intermediate contribution with the maximal intensity at 340 nm, and finally the shorter lifetime (0.6 ns) showed a minor contribution with the maximal intensity at 325 nm. In the same figure the DAS obtained in the presence of 4.0 M GdnHCl are also shown. Besides to the expected red-shift of all the spectra, it appears that even under denaturing condition the each decay constant retains distinct spectral profiles. The resolved spectral distributions suggest the association of the two major lifetimes with the emission of the tryptophan residues (\( \lambda_{\text{max}} \approx 330-340 \text{ nm} \)). In addition, the resolved EDAS, presented in Fig. 5B, indicate the existence of ground-state heterogeneity. This finding further support the assignment of each decay component with individual tryptophan residues or conformer.

The assignment of the shorter lifetime (\( \lambda_{\text{max}} \approx 325 \text{ nm} \)) with a specific residue’s emission is more problematic. It might originate from tyrosinate emission, or more likely, it might represent the contribution of some collisionally quenched tryptophan rotamer (\( p13^{\text{Suc1}} \) conformer) (Szabo and Rayner, 1980).

Fluorescence Quenching Measurements—To test the physical association of lifetimes with individual fluorophores in different sites, the distinct accessibility of the \( p13^{\text{Suc1}} \) fluorescence decay constants to quencher molecules (KI and acrylamide) have been evaluated (Lehrer, 1971; Ross et al., 1981; Knutson et al., 1982, 1983). Stern-Volmer plots (\( 1/\tau_i \) versus [Q]) of the results obtained by time-resolved quenching measurements are presented in Fig. 6 (center and right panels). In addition, in the
Time-resolved fluorescence intensity parameters of p13\textsuperscript{suc1} recovered under native and denaturing conditions

Experiments were carried out in 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, at 20 °C (native), at 56 °C, and in the presence of 4.0 M GdnHCl (denatured). The excitation wavelength was 295 nm, and fluorescence decay curves were collected at three emission wavelengths (325, 335, and 345 nm for native at 20 and 56 °C, 345, 355, and 365 nm for denatured). The data were simultaneously analyzed by global analysis; the reported amplitudes refer to the decay observed at 335 nm (native and 56 °C) and 355 nm (denatured). The reported χ² are the global reduced.

| p13\textsuperscript{suc1} | α₁ | τ₁ | α₂ | τ₂ | α₃ | τ₃ | χ² |
|--------------------------|----|----|----|----|----|----|----|
| Lower bound\textsuperscript{a} | 0.19 | 0.45 | 0.30 | 2.39 | 0.24 | 5.79 |
| Native | 0.22 | 0.61 | 0.34 | 2.93 | 0.33 | 6.09 | 1.16 |
| Upper bound | 0.25 | 0.83 | 0.38 | 3.68 | 0.40 | 6.50 |
| Lower bound | 0.45 | 0.49 | 0.51 | 2.07 | 0.11 | 5.70 |
| Denatured | 0.75 | 0.53 | 0.56 | 2.17 | 0.15 | 5.90 | 1.08 |
| Upper bound | 1.02 | 0.59 | 0.61 | 2.59 | 0.17 | 6.12 |
| Lower bound | 0.05 | 0.16 | 0.09 | 1.27 | 0.11 | 3.36 |
| 56 °C | 0.07 | 0.30 | 0.10 | 1.49 | 0.12 | 3.44 | 1.24 |
| Upper bound | 0.09 | 0.46 | 0.11 | 1.74 | 0.13 | 3.53 |

\textsuperscript{a} The lower and the upper bound values refer to the joint-confidence interval calculated for a confidence-level probability of 68% (Johnson, 1983).

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Fluorescence Spectroscopy of Yeast p13\textsuperscript{suc1}

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**FIG. 4. Lifetimes amplitudes as a function of denaturant concentration.** Relationship between the pre-exponential terms (α₁, α₂, τ₁, τ₂, α₃, τ₃) recovered from the analysis of p13\textsuperscript{suc1} fluorescence decay data obtained at increasing GdnHCl (0–4 M) concentrations. The experimental conditions were the same of Fig. 3. On the separated vertical right axis individual symbols indicate the results obtained after removal of the denaturant.

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It is shown that a kₜ(τ₂) twice as higher as kₜ(τ₃) has been measured with both the quenchers, suggesting that the medium lifetime is associated with a more exposed fluorophore than the longer lifetime. In addition, in Table II are reported the fluorescence intensity specific quenching constant/species, K\textsubscript{SV}($τ_i$), the average lifetime Stern-Volmer constant, K\textsubscript{SV}($τ_m$), calculated for the collisional process, and the steady-state constant, K\textsubscript{SV}($τ_s$), obtained from the linear regression of the steady-state fluorescence intensity quenching data presented in Fig. 6 (left panel). It can be seen that K\textsubscript{SV}($τ₂$) is higher than K\textsubscript{SV}($τ₃$) and that K\textsubscript{SV}($τ_m$) is significantly lower than K\textsubscript{SV}($τ_s$). The latter result and the appearance of an upward curvature of the steady-state Stern-Volmer plot obtained with acrylamide suggest the existence of a static quenching contribution. In fact, a notable blue-shift of the emission spectrum has been revealed at high quencher concentrations. According to the resolved DAS, this feature implies that the overall quenching (static and collisional) of the τ₃ species is higher than that of the τ₂ species. Since resolution of the collisional process (lifetime quenching measurements) have provided K\textsubscript{SV}($τ₂$) < K\textsubscript{SV}($τ₃$), it follows that the effect observed originates from a specific static quenching of the τ₃ species. To substantiate this conclusion, the DAS obtained at 0.23 M KI are presented in Fig. 7. In the upper right inset of the figure are shown the normalized steady-state emission spectra recorded in the absence and the presence of 0.23 M KI. In comparison with the DAS presented in Fig. 5A, a decrease of the relative contribution of the longer lifetime DAS is apparent, which is strongly consistent with the shift from 336 to 334 nm of the steady-state emission spectrum.

**Time-resolved Fluorescence Anisotropy—** The decay of the fluorescence anisotropy of p13\textsuperscript{suc1} has been measured to define its rotational hydrodynamic properties. Measurements were performed at multiple experimental temperatures, and the data collected were then analyzed both separately (a single temperature experiment at the time) and simultaneously (all the temperature experiments in a single global analysis run). In the latter case, φ₂ terms were linked across the entire temperature range and the relative η/7 solvent viscosity correction factors were introduced in the analysis model. Fig. 8 shows the fluorescence anisotropy decay profiles (which are convolved with the exciting light pulse) of p13\textsuperscript{suc1} in buffer at 21 °C and in the presence of 4.0 M GdnHCl. The parameters recovered are reported in Table III. Two correlation times were required to describe accurately the decay of the emission anisotropy of native p13\textsuperscript{suc1}. A fast correlation time, φ₁ < 1 ns, can be associated with the rapid flexibility of the indole ring, and it reports the dynamics of the microenvironment surrounding the tryptophan residues. On the other hand, the long correlation time, φ₂ = 9–15 ns, can be associated with the rotational diffusion of the protein as a whole, and it reports the hydrodynamic properties of p13\textsuperscript{suc1}. The plot of the correlation times as a function of the solvent viscosity and temperature is presented in Fig. 9. In the figure it is shown that, within the experimental error, the long correlation time is purely dependent on the solvent viscosity, with no evidence of changes of the molecular shape or dimension of p13\textsuperscript{suc1} (i.e. self-association) as function of the experimental temperature. In addition, the absence of significant changes of the short correlation time support its assignment to the dynamics of the indole ring, which is scarcely affected in this temperature range.

From the long correlation times recovered at each temperature and using the Stokes-Einstein relationship, nearly related Stokes radii have been obtained for p13\textsuperscript{suc1}. The values presented in Table III provide an apparent mean Stokes radius of 21.2 Å. This result is in excellent agreement with the radius recently obtained for p13\textsuperscript{suc1} monomer (21.2 Å) using small-angle x-ray scattering (Birck et al., 1995).
In Table III the results of the analysis of the anisotropy decay of $p_{13}^{sucl}$ in the presence of 4.0 M GdnHCl and 1 mM zinc acetate are also shown. Under denaturing conditions the fluorescence anisotropy transient is reduced to a rapid single exponential decay (Fig. 8) as expected for an unfolded protein. More important, the changes of the anisotropy decay parameters obtained in the presence of zinc ions are compatible with a change of the molecular size of $p_{13}^{sucl}$. In particular, the increase of the short correlation time, from the subnanosecond range to 5 ns, together with the long correlation time recovered, 14 ns, are consistent with a reorientational dynamics, which reflects rotations along the short and the long symmetry axis of a larger molecular species (i.e. $p_{13}^{sucl}$ homodimer). However, because of the typical $\tau_d$ ratio of the intrinsic emission of proteins, the precision and the accuracy of the data was not adequate to fully characterize the zinc-induced oligomerization transitions. In this regard, extrinsic fluorescence represents the best tool. Extrinsically labeled $p_{13}^{sucl}$ conjugates have been prepared and the results obtained to define the effect of zinc on protein oligomerization will be published elsewhere.

**DISCUSSION**

The biochemical dissection of complex biological processes is of great value for the comprehension of their significance in vivo. *S. pombe* $p_{13}^{sucl}$, as well as its human homologous $p_{9}^{CKShs1}$, is the product of an essential gene in the regulation of eukaryotes cell cycle. Molecular biology techniques have allowed purification of $p_{13}^{sucl}$ in sufficient amount to perform physical and biochemical studies. In fact, preparation of $p_{13}^{sucl}$ crystals has been recently obtained, and the protein structure has been solved to 2.7-Å resolution (Endicot et al., 1995). Interestingly, these authors have found $p_{13}^{sucl}$ to crystallize as a dimer in the presence of zinc and the potential role, in vivo, of this property has been reasonably addressed. Moreover, in a preceding report (Dunphy et al., 1988) it was noticed that treatment at 100 °C had no effect on the inhibitory activity of $p_{13}^{sucl}$ to antagonize mitotic conversion. Thus, it was concluded that $p_{13}^{sucl}$ is a thermostable protein.

To define the actual biological relevance of structure/function relationships, solution studies with the isolated protein add crucial information to the data collected in the crystal state and
in cultured cells. In this respect, the multidimensional character of fluorescence techniques provide adequate sensitivity and an ideal ensemble of different experimental approaches. The present work was aimed to obtain structural information on p13<sup>suc1</sup> by fluorescence spectroscopy methods and to provide useful “markers” for biological assays.

In solution, the secondary structure composition of the native protein obtained from the analysis of the CD spectrum is compatible with a compact globular conformation, in substantial agreement with the crystal structure described by Endicott et al. (1995). Interestingly, even harsh temperature treatment does not seem to affect the aromatic residues environment.

The intrinsic fluorescence of the protein is dominated by the emission of the tryptophan residues (Trp-71 and Trp-82). The position of the maximal fluorescence intensity suggests a poor interaction of these residues with the surrounding solvent. Indeed, the red-shift observed upon full denaturation by 4.0 M GdnHCl is consistent with their exposure at the water-to-protein interface. Yet, with unfolding a severe decrease of the fluorescence intensity is detected. These changes of the intrinsic fluorescence of p13<sup>suc1</sup> reproduce well known motifs in the study of unfolding transitions in proteins (Benfenati et al., 1990; Eftink, 1994). In particular, they suggest that the native conformational arrangement of Trp-71 and Trp-82 is disrupted by guanidinium denaturation.

Interestingly, the same fluorescence changes are not present with p13<sup>suc1</sup> at 56 °C. Actually, the lower quenching observed arises predominantly from the collisional temperature effect and is obtained in the absence of any significant shift of the emission spectrum. Thus, our data provide evidence for a thermostable protein core that may be related to the thermostable inhibitory activity of p13<sup>suc1</sup> described by Dumphy et al. (1988).

In fact, it is suggested that the tryptophan residues are part of this domain and may be involved with this function of p13<sup>suc1</sup>. While steady-state fluorescence measurements can provide an intensity and time-averaged description of the fluorophore environment, nanosecond time-resolved fluorescence spectroscopy is the tool of choice to monitor relevant biological events occurring in this time domain.

The transition from the native state, N, to the unfolded state, U, has been further investigated by time-resolved techniques. While the exponential mode of the fluorescence decay of p13<sup>suc1</sup> is not changed upon denaturation, and three decay constants are required to obtain the best fit in both the states N and U (Table I), the evaluation of the relative contributions of each lifetime to the total emitted fluorescence reveals, in fact, significant changes (Fig. 4). In particular, with the transition from the state N to the state U, the weight to the total fluorescence intensity of the short lifetime increases, whereas the weight of the long lifetime decreases significantly. Altogether the results presented demonstrate the ability of the fluorescence kinetic parameters to monitor changes of the structural state of p13<sup>suc1</sup>. In particular, it is shown that the significant increase of the short lifetime amplitude (α<sub>1</sub>), concomitant with the decrease of the long lifetime amplitude (α<sub>3</sub>), is a reliable “marker” of the protein unfolding.

Our data are in excellent agreement with the intensity decays behavior versus denaturant concentration examined by Eftink (1994) with staphylococcal nuclease A and indicate that the pre-exponentials associated with the τ values can be related to the fraction of molecules in the native and unfolded states. In a similar study on toxins, Dahms and Szabo (1995) have correlated the shift in the relative contribution of each fluorescence decay time to the disappearance of the β-sheet secondary structural features. They have used fluorescence to

### Table II

| Time-resolved bimolecular quenching rates, k<sub>q</sub>(i), and fluorescence intensity quenching constants, K<sub>SV</sub>(i), of the intrinsic fluorescence of p13<sup>suc1</sup> |  |
|---|---|---|---|---|
| k<sub>k</sub>(τ<sub>2</sub>) | 0.52 | 0.59 | 0.58 | 0.56 |
| k<sub>k</sub>(τ<sub>3</sub>) | 0.33 | 0.26 | 0.25 | 0.23 |
| k<sub>k</sub>(τ<sub>4</sub>) | 0.43 | 0.38 | 0.37 | 0.35 |
| K<sub>SV</sub>(τ<sub>2</sub>) | 2.38 | 1.71 | 1.69 | 1.66 |
| K<sub>SV</sub>(τ<sub>3</sub>) | 1.98 | 1.56 | 1.55 | 1.53 |
| K<sub>SV</sub>(τ<sub>4</sub>) | 2.09 | 1.84 | 1.82 | 1.80 |
| K<sub>SV</sub>(ss) | 3.99 | 7.17 | 7.15 | 7.13 |

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Fluorescence Spectroscopy of Yeast p13<sup>suc1</sup>

**TABLE III**

| Temperature (°C) | β<sub>1</sub> | φ<sub>1</sub> ns | β<sub>2</sub> | φ<sub>2</sub> ns | φ<sub>3</sub> | Stokes radius Å |
|-----------------|-------------|-------------|-------------|-------------|-------------|---------------|
| Native          |             |             |             |             |             |               |
| 6.0             | 0.062       | 0.54        | 0.068       | 15.6 (14.9) | 21.4        |
| 10.0            | 0.055       | 0.33        | 0.051       | 12.3 (13.0) | 20.6        |
| 14.0            | 0.076       | 0.49        | 0.057       | 11.5 (11.5) | 21.0        |
| 19.0            | 0.101       | 0.37        | 0.059       | 9.3 (9.9)   | 20.6        |
| 21.0            | 0.061       | 0.64        | 0.059       | 10.5 (9.4)  | 21.8        |
| 25.0            | 0.038       | 0.71        | 0.041       | 9.3 (8.4)   | 21.7        |
| GdnHCl (4.0 M)  |             |             |             |             |             |               |
| 21.0            | 0.169       | 0.28        |             |             |             |               |
| Zn<sup>2+</sup> (1 mM) | 0.068 | 5.50 | 0.052 | 14.2 |

Fig. 9. Rotational correlation times of native p13<sup>suc1</sup> as a function of temperature. The same data reported in Table III are plotted with respect to ηT. The filled circle symbols indicate the long correlation time (φ<sub>2</sub>), the open triangle symbols indicate the short correlation time (φ<sub>1</sub>). The open diamond symbols indicate the correlation times as recovered by the global analysis, where the φ<sub>2</sub> parameter was linked over the entire decay data set obtained as a function of temperature; —, linear fits correspond to the global analysis results. The fit of the linked φ<sub>2</sub> versus ηT (linear correlation coefficient r = 1) is displayed for purposes of linearity evaluation. (The viscosity of water was taken from CRC Handbook of Chemistry and Physics, 70th Ed., CRC Press, Boca Raton, FL.)

**method** has been used to further investigate the protein structure.

Based on the evidence that even single tryptophan containing proteins show multiplexponential decay kinetics (Beechem and Brand, 1985), attempts have been made to correlate lifetime components with the existence of rotamer populations (Szabo and Rayner, 1980), their distribution and their stability in the excited state. According to this representation, multieponential decays can be explained in terms of the existence of different ground-state conformers/rotamers of the protein, each with different fluorescence lifetimes. In alternative, a more direct model has been successfully tested by Brand and coworkers on horse liver alcohol dehydrogenase (Ross 1981; Knutson et al., 1982), a typical protein with two tryptophans, which exhibits a double exponential decay. In this case, the association of lifetimes with specific quenching constants and the results of energy transfer studies have been used to assign each decay constant to a single tryptophan residue.

In general, the benefit of association and overdetermination techniques developed for time resolved data (Knutson et al., 1992, 1983; Beechem et al., 1985; Lakowicz, 1992) to discriminate between diverse physical models is widely recognized.

In this work, the three lifetimes of p13<sup>suc1</sup> have been associated with distinct emission spectral distributions (DAS) (Fig. 5a). In addition, the two major spectra (DAS–τ<sub>2</sub> and DAS–τ<sub>3</sub>), which represent 96% of the total emitted fluorescence, have their origin from apparently different excitation spectra (EDAS) (Fig. 5b). Based on these findings the more realistic explanation of the multi-exponential decay of p13<sup>suc1</sup> is a simple two-compartment heterogeneity model. Finally, it has been shown that different DASs are recovered in 4.0 M GdnHCl. Assuming that the two tryptophan residues still experience different environments under denaturing conditions, this result may further validate the above description.

Quenching experiments have provided useful information. The overall quenching of the intrinsic fluorescence of p13<sup>suc1</sup> has been separated into a collisional and a static contribution. From the different approaches used (time-resolved and steady-state quenching measurements, steady-state emission spectra, and DAS resolution at high [Q]), it appears that the two mechanisms can be associated to different lifetime species. In particular, two decay intensity components with characteristic diffusional accessibility have been resolved. The component associated with τ<sub>2</sub> is twice as much accessible as the component associated with τ<sub>3</sub>, k(τ<sub>2</sub>) ≫ k(τ<sub>3</sub>) (Fig. 6, Table II). The resolution of this feature of p13<sup>suc1</sup> fluorescence emission is pertinent for two reasons. First, the association of a physical property (accessibility) with a fluorescence decay parameter

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...probe local secondary structure in peptides. With larger proteins such as p13<sup>suc1</sup> a quantitative estimation of the changes of the secondary structure with denaturation is not straightforward even by dynamic fluorescence spectroscopy. Nonetheless, very useful qualitative information on the protein structure can be obtained. For example, using this approach we have found that unfolding is triggered by a guanidinium concentration typical χ<sup>2</sup> ranged from 1.1 to 1.3. The values of φ<sub>2</sub> recovered from a global analysis are indicated in parentheses, where all the decay data collected as a function of temperature were simultaneously analyzed with linking the long correlation times and introducing factors. The global not reduced equation (see Materials and Methods); typical χ<sup>2</sup> ranged from 1.1 to 1.3. The values of φ<sub>2</sub> recovered from a global analysis are indicated in parentheses, where all the decay data collected as a function of temperature were simultaneously analyzed with linking the long correlation times and introducing factors.

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Fluorescence Spectroscopy of Yeast p13\textsuperscript{suc1}

(lifetime) is strongly consistent with the existence of distinct ground-state species that retain distinct excited-state signature. Second, it provides support to the one-to-one assignment of lifetimes and tryptophan residues. The importance of local charges in “tunnelling” ionic quenchers to preferential sites of proteins has been first inferred by Lehrer (Lehrer, 1971) measuring the iodide quenching constants for poly(Lys\textsuperscript{+}-Trp\textsuperscript{−}) and poly(Glu\textsuperscript{−}-Trp\textsuperscript{+}) at neutral pH. In the p13\textsuperscript{suc1}, Trp-82 is part of the positively charged cluster revealed by the crystal structure (Endicott, 1995), and the higher accessibility of τ\textsubscript{2} has been probed using the negatively charged I\textsuperscript{−} ions. In addition, a strong static contribution has been shown to affect preferentially the τ\textsubscript{3} species. Thus, based on these evidences, with p13\textsuperscript{suc1} it appears reasonable to associate Trp-82 with τ\textsubscript{2} and Trp-71 with τ\textsubscript{3} where Trp-82 is preferentially quenched by a collisional mechanism, whereas Trp-71 is significantly quenched by a static mechanism.

In view of this model, the unfolding data discussed above can be explained in terms of a conformational transition that is more effective on the structural environment of Trp-71 than on the environment of the “exposed” Trp-82. Accordingly, the very short lifetime, τ\textsubscript{1}, could be assign to a quenched rotamer of Trp-71 whose concentration is very small in the native state and increases significantly in the unfolded state.

It is important to notice that time resolution of the intrinsic fluorescence intensity of p13\textsuperscript{suc1} and the proposed lifetime tryptophan assignment provide a useful marker to probe the functional role of a striking surface feature of p13\textsuperscript{suc1} monomer, namely the cluster of positively charged residues surrounding Trp-82.

The fluorescence anisotropy decay parameters represent a more direct measure of the overall tertiary and quaternary structure of proteins than do the fluorescence intensity decay parameters. In fact, they report the dynamics of local and global rotational movements of proteins and can provide a close estimate of their shape and size. Here the technique has been used to determine the dimension of native p13\textsuperscript{suc1} in solution and to examine the potential ability of its subunits to associate into dimers or higher oligomeric forms.

The distribution of conserved side chains on the surface of p13\textsuperscript{suc1} provides an “hydrophobic patch” that has been indicated as a notable candidate for a site of protein-protein interaction (Endicott, 1995). Thus, experiments have been carried out in a range of different temperatures (6–25°C) to search for hydrophobic bonding between p13\textsuperscript{suc1} subunits.

With the native protein two correlation times have been recovered from the analysis of the anisotropy decay data (Table III). A very rapidly relaxing component, φ\textsubscript{1} (<1.0 ns), can be ascribed to subnanosecond torsional vibrations of the indole ring or other local dynamic events of the emitting residues. These processes cause a very fast depolarization and are not appreciably influenced by solvent viscosity (Fig. 9). Conversely, a longer lasting relaxation component in the anisotropy decay of p13\textsuperscript{suc1}, φ\textsubscript{2} (>9 ns), corresponds to the frictional diffusion of the rotating particle. Accordingly, this process is dependent on solvent viscosity (Fig. 9). The change from a double to a single (φ = 0.3 ns) exponential anisotropy decay observed upon demutation further supports the assignment of the long correlation time to the rotational movements of the whole protein. The correlation time of 9.4 ns, at 21°C, recovered by the global analysis of the anisotropy decay data is longer than that expected for an anhydrous 13 kDa globular protein (~5 ns). Nonetheless, this a very common observation in polarization studies of proteins (Lakowicz, 1983). The discrepancy has been accounted for by the hydration of the protein, which yields a much larger rotating particle, and by the shape of the protein, which might deviate from the spherical symmetry (i.e. prolate ellipsoid). Moreover, the Stokes radius of 21.2 Å obtained with p13\textsuperscript{suc1} by fluorescence techniques is in striking agreement with the result obtained with p13\textsuperscript{suc1} monomer by scattering techniques (Birk et al., 1995). These results together with the biochemical information obtained with the purification procedure (gel filtration on Sephacryl S-100 HR and pi) indicate that p13\textsuperscript{suc1} in buffer solution exists as a monomer with an elongated shape.

The power and sensitivity of anisotropy decay analysis to investigate protein-protein association at the nanomolar-micromolar concentrations have been described in preceding reports (Beechem and Brand, 1985; Neyroz et al., 1987). In Table III and in Fig. 9 other important information is presented, the shape and the dimension of p13\textsuperscript{suc1} do not change with increasing the temperature and solvent viscosity. Both the results obtained by the “single temperature experiment” and by the “global temperature experiments” analysis reveal that the recovered φ\textsubscript{2} values are purely function of the fluid viscosity. The plot of φ\textsubscript{2} versus ρ\textsuperscript{f} is linear, and the calculated Stokes-radius is constant within the experimental error. Thus, in the evaluated range of temperatures, there is no evidence for major conformational changes of the protein driven by hydrophobic forces (i.e. subunit self-association).

Finally, the intrinsic anisotropy decay of p13\textsuperscript{suc1} has been measured in the presence of zinc. Given the limitations implicit in the method used, our data are consistent with the original observation of Endicott et al. (1995), which found p13\textsuperscript{suc1} to crystallize as a dimer in the presence of 1 mM zinc. Extrinsic fluorescence studies with longer decaying fluorescent probes will be crucial to define this potential and intriguing structure/function relationship of p13\textsuperscript{suc1}.

In summary, the results obtained by steady-state and time-resolved fluorescence techniques on p13\textsuperscript{suc1} have revealed significant structural information. Future studies should provide further insight into the physical nature of its fluorescence properties, into specific structural changes that occur during self-association or protein-protein interaction, into the effect of pH, ionic strength, and specific ions (i.e. zinc) on its conformation, and into the functional role of the p13\textsuperscript{suc1}-p34\textsuperscript{cd2} interaction.

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