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Molecular characterisation of recombinant green fluorescent protein by fluorescence correlation microscopy

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The *c*DNA for the green fluorescent protein (GFP) of *Aequorea victoria* has been expressed in transformed cells of *Saccharomyces cerevisiae* and the recombinant GFP isolated. Protonation and deprotonation of the cloned and purified GFP produced major effects on its spectral absorption characteristics with an increase in pH enhancing the fluorescence emission of the GFP more than twofold. Finally, molecular characterisation of GFP by fluorescence correlation microscopy in a minimal target volume of 1 fL yielded a translational diffusion coefficient (D_T) of 8.7×10^{-7} $cm^2.sec^{-1}$, equivalent to a Stokes radius of 2.82nm for a monodisperse globular protein of 27kDa. © 1995 Academic Press, Inc.

Green fluorescent protein (GFP) from *Aequorea victoria* is a powerful fluorophore formed autocatalytically by internal cyclization and oxidation of the Ser-Tyr-Gly sequence at positions 65-67 within the 238 amino-acid polypeptide [1]. Following the cloning of the *c*DNA for GFP [2], there is growing interest in the expression of GFP and of GFP-fused proteins as unique fluorophores to monitor a wide range of intracellular processes, including gene expression [3,4], microtubule formation [5], the assembly of functional ion-channels [6], and the identification and localisation of specific proteins [7,8]. To optimise the utilisation of GFP for this purpose requires a sensitive technique, not only for the detection and resolution of the endogenous label, which may be expressed at low abundance, but one which can also measure the molecular dynamics of GFP-labelled target molecules in intracellular compartments of restricted dimensions and differing structural complexity. The technique of fluorescence correlation microscopy (FCM) is ideal for this purpose, combining as it does the high resolution of

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confocal optics with the precision of fluorescence correlation spectroscopy for monitoring molecular interactions in a sample probe volume of only 1fL [9,10].

In this report we describe the expression and isolation of recombinant GFP from *Saccharomyces cerevisiae* together with its molecular characterisation by FCM *in vitro* and the effects of deprotonation in enhancing the fluorescence emission intensity of the cloned GFP molecule.

Methods

Expression and isolation of GFP

Appropriate oligonucleotides were synthesised and used for PCR amplification of the *c*DNA encoded by pGFP10.1 [2]. The coding sequence for six histidine residues was fused to the 3' end of GFP. The amplified DNA fragment was cloned between the *Bam*HI and *Sac*I sites of the multicopy yeast plasmid pVT103-U, placing the gene between the promoter and 3' sequence of the *ADH1* gene [11]. Transformed *Saccharomyces cerevisiae* cells MGLD-4a (a, *leu2*, *ura3*, *his3*, *trp1*, *lys2*) produced highly green fluorescent, but mainly sectored colonies. A low percentage of the colonies were unsectored and remained brightly fluorescent in the absence of selection. These were presumed to contain a chromosomally integrated copy of the plasmid, and such a yeast transformant was used for protein purification.

GFP-expressing yeast cells were grown to saturation in 500ml YEPD media over 2 days at 303 K. Extended growth ensured maximal yield of the post-translationally modified fluorescent protein. The cells were harvested by centrifugation at 3500g for 10 minutes, resuspended in 20 ml of 50mM Tris-HCl pH 8.0, 1M sorbitol, 50mM DTT, and incubated at 303 K for 10 minutes. The cells were then pelleted, resuspended in 20 ml of the same buffer with 2.5mg/ml zymolyase, and incubated at 303 K for 2 hours with gentle shaking. Cells were harvested by centrifugation, resuspended in 10 ml 0.1% NP40, and lysed with 20 strokes of a Dounce homogeniser. The homogenate was cleared by centrifugation at 10,000g, and the supernatant was filtered through a 0.45 micron cellulose acetate filter. The filtered yeast extract was made to 50 mM sodium phosphate pH 7.5, 500 mM NaCl, 5 mM imidazole, and loaded onto a Ni-chelate sepharose column (Pro-Bond, Invitrogen). The column was washed with the same buffer containing 30 mM imidazole, and eluted with buffer containing 150 mM imidazole. Elution was monitored using a long-wavelength UV lamp, and green fluorescent fractions were collected. The eluted protein was washed and concentrated in 20mM Tris-HCl pH 7.5, 1mM EDTA by centrifugation in Centricon-10 (Amicon) concentrator units. Purification was monitored by SDS gel electrophoresis. The yield of GFP was 15mg from 500 ml of cultured *Saccharomyces* cells.

Absorption and Fluorescence excitation and emission spectra

Samples of purified GFP were diluted to approximately 4.5 μM in buffer solution containing 10 mM each of glycine, sodium citrate, sodium phosphate, and 5 mM Tris-HCl. Individual samples were adjusted over the range pH 4.0 to 11.9 with 1M NaOH or HCl. The spectrophotometer and fluorimeter(Hitachi F-2000) used to characterise the GFP samples were calibrated with a fluorescein standard of high purity

(F-1300, Molecular Probes), which was diluted to a range of optical densities comparable to those of the GFP samples, the same buffer system being used for each fluorophore. The fluorescein standard used has the following properties: $\varepsilon=9x10^6$ $m^{-1}.M^{-1}$, Q=0.92, and excitation and emission maxima at 489 and 513 nm, respectively, all at pH 9.0 (Haugland R., pers. comm.). The extinction coefficient of the recombinant GFP, was taken to be $2.1x10^6.m^{-1}.M^{-1}$ at 392nm [12].

Fluorescence Correlation Microscopy (FCM)

The Gaussian illumination intensity profile essential for FCM was provided by an ion-argon laser (Omnichrome 532-AP) emitting at 488nm and focussed, *via* an optical fibre, within a sample volume of 1 fL as defined by a confocal aperture fitted to a Zeiss UPL inverted microscope modified for epi-illumination (Fig.1). Fluorescence signals passing through a 510 nm (±5 nm FWHM) interference filter were detected by a photomultiplier tube-discriminator unit (Thorn/EMI 9863 KB100) and the signal output analysed by a Malvern K7032 multi-8 correlator. Normalised autocorrelation functions for fluorescence fluctuations detected in a defined sample volume were well-described by the two-dimensional approximation, $G_{1,1}(t) = \gamma\beta(1+t/\tau_r)^{-1}$. A single species of fluorescent particles therefore generates an intensity correlation function with a time constant (τ_r) which, together with the radius ω_0 of the focussed beam waist in the sample, defines the translational diffusion coefficient (D_T) of the fluorophore in the plane normal to the optical axis of the FCM. The amplitude of the normalised function at t=0 , *i.e.*, G(0), is inversely proportional to the number density (<N>) of mobile fluorescent particles within the sample volume; γ is an instrumental constant embodying the spatial profiles of illumination intensity and optical collection efficiency for all points within the observed volume. The two instrumental constants, γ and ω_0, required for calculation of

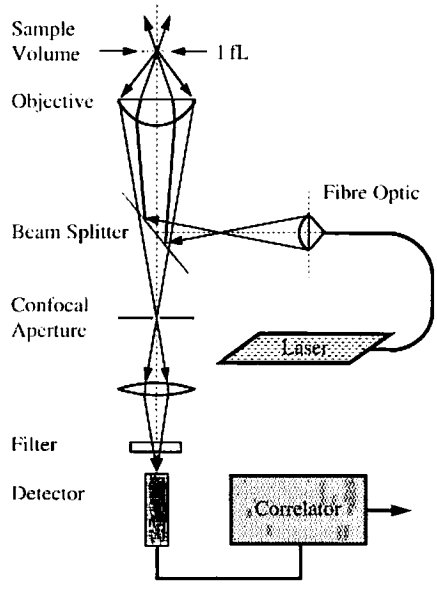

Fig. 1. Fluorescence correlation microscopy: diagram of the confocal laser-based microscopic system used for molecular characterisation of recombinant GFP by fluorescence correlation spectroscopy.

<N> and D_T , respectively, were measured using monodisperse R-phycoerythrin (Molecular Probes), as described elsewhere [9,13]. Correlation functions of GFP were obtained at pH 11.9, to maximise the quantum yield from the protein at 488 nm. Dilutions were made over the range 100 to 400 nM and all experiments were carried out at ambient temperature (298 K)

Results and Discussion

The absorption characteristics of the recombinant GFP confirmed its authenticity, absorption maxima of 392 and 475nm (Fig. 2) coinciding exactly with those of native GFP [14,15]. Furthermore we have confirmed that protonation and deprotonation of recombinant GFP, like that of wild-type GFP, gives a shift in the magnitude but not the wavelength of the absorption peaks (Fig. 2). This change in magnitude of the characteristic absorption spectrum of GFP was reflected in an enhanced fluorescence emission intensity at the higher pH values (Fig. 3). It is unlikely that these pH-mediated changes in the optical properties of recombinant GFP are the result of structural damage to the molecule because native GFP is known to be conformationally extremely stable and only degraded in harsh environments which largely destroy its fluorescence [16]. Moreover, whereas a decrease in pH increased the magnitude of the 392 nm peak it decreased correspondingly that of the 475nm peak. The converse occurred with an increase in pH, the 392 nm peak being decreased and the 475nm peak increased. It is much more likely therefore that an increase in pH over the pH range 5.0 to 11. 9 leads to progressive deprotonation of an essential ionisable group in the fluorogenic core of GFP.

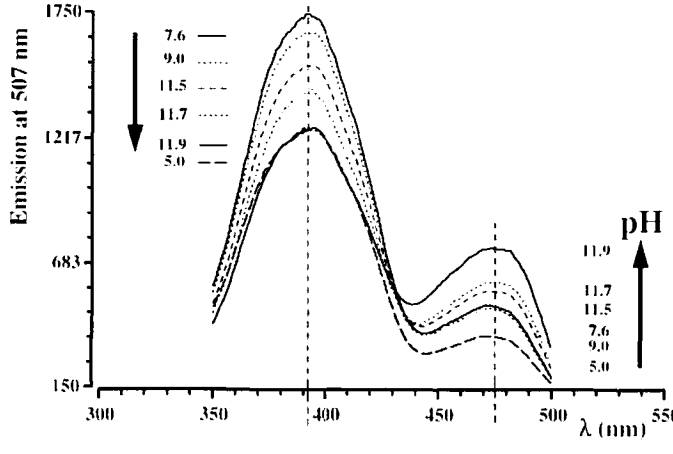

Fig. 2. Fluorescence excitation spectrum of recombinant GFP. Dependence of fluorescence emission at 507nm on pH over the range 5.0 to 11.9.

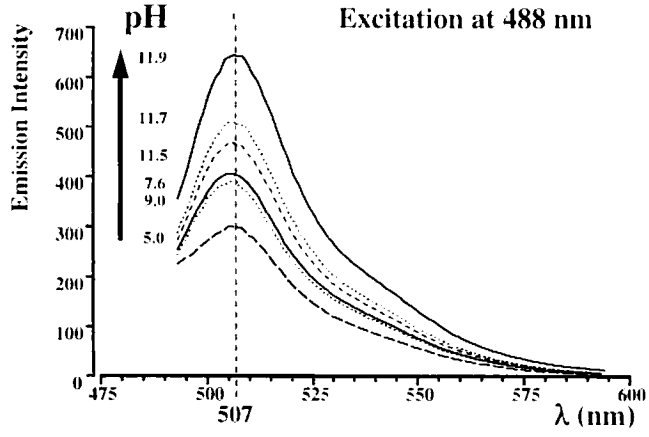

Fig. 3. Fluorescence emission spectrum of recombinant GFP with excitation at 488nm. Dependence of emission intensity with pH over the range 5.0 to 11.9.

It has been suggested recently that the 475nm peak is attributable to deprotonation of Tyr-66 and formation of the anionic species of the phenolic moiety of tyrosine at the key tripeptide sequence of GFP [17]. Our results are entirely consistent with this possibility which would also account for the enhanced fluorescence emission (>2x) seen at high pH (ie., 11.9) with excitation at 488nm (Fig. 3). Whatever the precise molecular basis for the pH-sensitivity of the GFP fluorescence signal, this observation opens up the important possibility that, in addition to yielding measurements of particle mobility and number density by FCM (see below), recombinant forms of GFP may also be capable of sensing local environmental changes in pH, although whether this is possible within the physiological range intracellularly remains to be determined.

For measurement of the molecular hydrodynamic properties of GFP by FCM we have increased the pH to optimise the quantum yield at the 488 nm excitation wavelength available from our argon-ion laser. The correlation functions in Fig. 4 were obtained from the fluorescent fluctuations for two concentrations of GFP equivalent to 120 and 240 molecules per fL, approximately the volume defined by the optical arrangement of our FCM. These are upper values for molecular number and are based on the assumption that those molecules which absorb maximally at 392 nm also fluoresce over the pH range 5.0 to 11.9 when excited at 488 nm. Measured values of <N> for the two concentrations of GFP are given in Fig. 4.

From the time constant of the correlation function (τ) the measured D_T was 8.7 x 10^{-7}

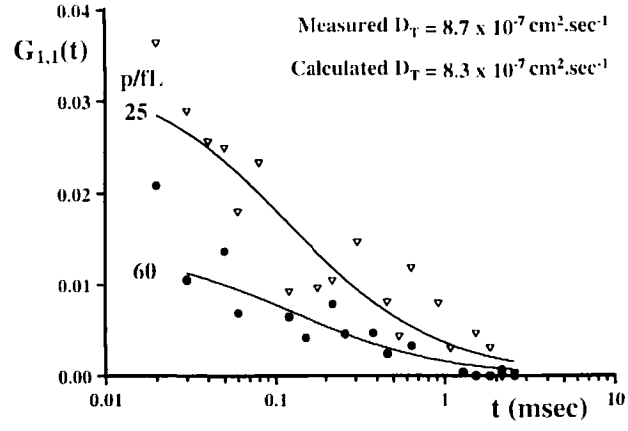

Fig. 4. Correlation functions for emission of fluorescence from recombinant GFP at two different molecular concentrations in an optically defined target volume of 1fL. The correlation sample time interval was 10μsec and the pH 11.9. Note that the amplitude of the normalised correlation function is inversely proportional to the molecular number density (<N>) of mobile fluorescent particles within the 1fL sample volume (see Methods).

$cm^2.sec^{-1}$, equivalent to a Stokes radius of 2.82 nm for a monodisperse globular macromolecule. Comparisons on a molecular weight basis (GFP; 27 kDa) with BSA (65 kDa), a well-characterised globular protein molecule, gave a calculated D_T for GFP of 8.3 x 10^{-7} $cm^2.sec^{-1}$, in close agreement with the measured value.

In these experiments we have determined the D_T of cloned GFP from an ensemble average (<N>) of individual fluorescent molecules freely diffusing across a quasi-cylindrical probe volume of 1fL, a volume equivalent, for example, to approximately 1/1500 that of a typical pancreatic islet β-cell of 20μm diameter [18]. The relatively high fluorophore concentrations we have used, and measurement times extending to several minutes, were dictated by the technical requirements of our present instrumentation. However, the measurements we have made are unlikely to represent an absolute detection limit of molecular number density since an increase in detector sensitivity, together with improved reduction in background noise, will allow lower concentrations of fluorophore to be characterised over much shorter time periods. With such improvements [see. 19], in conjunction with the development of simple point mutations in recombinant GFP to produce a shift in its absorption maximum for excitation from 392 to 488 nm [12,20], the technique of FCM should be capable of even greater refinement and allow the molecular dynamics of endogenously-labelled GFP to be defined with <1 target molecule of GFP per fL.

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