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A new intrinsic fluorescent probe for proteins

Biosynthetic incorporation of 5-hydroxytryptophan into oncomodulin

Christopher W.V. Hogue, Ingrid Rasquinha, Arthur G. Szabo and John P. MacManus

Division of Biological Science, National Research Council of Canada, Ottawa, Ont.K1A 0R6, Canada

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The tryptophan analog, 5-hydroxytryptophan (5HW), has a significant absorbance between 310–320 nm, which allows it to act as an exclusive fluorescence probe in protein mixtures containing a large number of tryptophan residues. Here for the first time a method is reported for the biosynthetic incorporation of 5HW into an expressed protein, the Y57W mutant of the Ca^{2+} binding protein, oncomodulin. Fluorescence anisotropy and time-resolved fluorescence decay measurements of the interaction between anti-oncomodulin antibodies and the 5HW-incorporated oncomodulin conveniently provide evidence of complex formation and epitope identification that could not be obtained with the natural amino acid. This report demonstrates the significant potential for the use of 5HW as an intrinsic probe in the study of structure and dynamics of protein–protein interactions.

Protein fluorescence; Fluorescence anisotropy; Protein–protein interaction; Alloprotein.

1. INTRODUCTION

Protein–protein interactions are involved in innumerable biochemical control mechanisms. Specific information on the molecular details of these interactions is often difficult to obtain. The intrinsic fluorescence probes, tryptophan (Trp) and tyrosine (Tyr), or extrinsic probes are used to reveal specific features about protein structure and dynamics. Intrinsic Trp fluorescence of a mixture of two proteins presents problems of interpretation when both proteins contain Trp, since their individual fluorescence parameters cannot be resolved. The incorporation of alternate intrinsic fluorophores, in the form of Trp analogs [1] could overcome the limitations of both extrinsic and intrinsic fluorophores. 5-Hydroxytryptophan (5HW) is one such analog, having significant absorbance between 310–320 nm, allowing the exclusive fluorescence excitation of a protein containing a 5HW residue in mixtures with a 'sea' of Trp residues.

The Ca^{2+} binding protein, oncomodulin (OM), lacks Trp, but has two Tyr, at positions 57 and 65. Previous studies [2,3] have shown that the fluorescence of mutants where Trp has been incorporated into different segments of proteins have provided new insights on the structural consequences of metal binding (Ca^{2+} and Mg^{2+}) to the 'EF-hand' binding loops. The mutant, Y57W, was used to assess the incorporation of 5HW and its utility to study protein–protein interactions. It has a Trp in position 57, a site which is position 7 of the CD Ca^{2+} binding loop [4]. The binding of Tb^{3+} to the Ca^{2+} binding sites of this protein allowed a quick, quantitative assay of production and purification of the desired protein [4]. The excitation spectra of bound Tb^{3+} luminescence [4,5] specifically revealed the presence of 5HW in the Y57W protein. Incorporation of 5HW into this particular protein allowed comparative studies with the Y57W mutant, demonstrating the utility of Trp analog incorporation. In addition, this protein was convenient for examining changes in 5HW fluorescence in interactions with anti-oncomodulin antibodies.

2. MATERIALS AND METHODS

2.1. *Expression system*

Auxotrophic strains of bacteria which do not produce Trp are used as hosts for incorporation studies. Since *E. coli* could not be grown in 5HW, an alternative method for cellular incorporation was required. A 'bait and switch' strategy was used. The *E. coli* cell line, W3110/pOxy W57.22, which contains the POxy plasmid (Exogene Corp., Pasadena, CA), was used to express Y57W OM. pOxy W57.22 was constructed by removing a 666 bp fragment from pGem W57.21 (obtained from D. Banville, Biotechnology Research Institute, NRC, Montreal) which contained the 5′ end, coding region and 3′ tail of the coding sequence. This fragment was incorporated into the *Bam*HI/*Sma*I site of the pOxy plasmid containing the OXYPRO oxygen sensitive promoter [6,7]. The final plasmid pOxy W57.22 was then used to transform the Trp auxotroph strain, W3110 Trp A33 [8] (Kindly donated by Dr. E. Li, Washington University, St. Louis, MO)

2.2. *Incorporation of 5-hydroxytryptophan*

Cells were grown in M63 minimal media enriched with 4% glycerol containing 4 μg/ml of L-Trp (1/10th of the normal amount). Cultures

Correspondence address: A.G. Szabo, National Research Council of Canada, Bldg. M54. Montreal Rd., Ottawa, Ont. K1A 0R6, Canada. Fax: (1) (613) 941-4475.

were grown in 20% of the flask volume with vigorous shaking for maximum aeration to produce maximum biomass with minimum activity of the oxygen-sensitive promoter. The stationary phase was reached after 24 h when the added Trp was exhausted. 5HW was then added to a total of 4 μg/ml and the aeration was markedly reduced by combining cultures to fill 75% of the volume in sealed flasks. Cell growth was continued for a further 6 h, after which the cells were harvested. Limited aeration activated the OXYPRO promoter which in turn led to incorporation of 5HW into newly synthesized protein in the existing cells. The amount of mutant Y57(5HW) that was expressed was equivalent to that from previous expression systems [9].

2.3. *Purification of Y57(5HW)*

Y57(5HW) was purified using methods similar to those for other OM mutants [2,9]. The protein ran as a single band on SDS-PAGE. Monitoring the fluorescence excitation spectra across the protein elution profile from the Sephadex G50 gel filtration step showed that a small amount of Y57W was present, a result of promoter leakage [6,7,10]. Attempts to minimize leakage through controlled large scale fermentation conditions were unsuccessful. The desired Y57(5HW) was separated from the Y57W contaminant using HPLC [11] (Fig. 1). An RPSC (C-3) column (4.6mm × 7.5 cm; Beckman; Fullerton, CA) was used. The column was equilibrated and initially isocratically eluted with buffer A (10 mM Tris, 1 mM EGTA, pH 7.2) and followed by a gradient with buffer B (buffer A in *n*-propanol, 2:3 by vol.). Column recycling was confirmed with blank runs ensuring that no residual protein remained on the column after a run. Identification of peak products was achieved using Tb^{3+} additions combined with UV and fluorescence spectra.

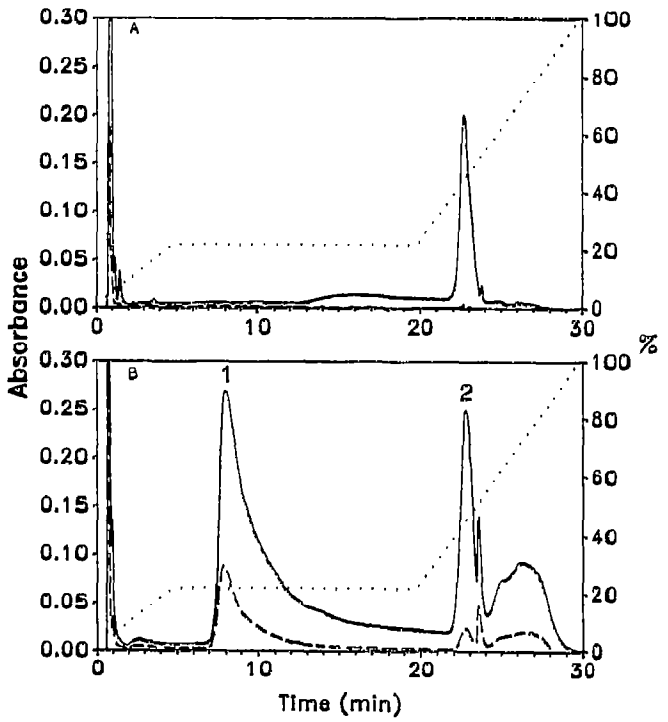

Fig. 1. HPLC elution profiles using non-denaturing reverse-phase HPLC. (A) Elution control run of Y57W. Y57W eluted at 22.7 min, during the second gradient phase, and was detected by absorbance at 280 nm (—); it had no absorbance at 315 nm (- - -). (B) HPLC elution of the partially purified mixture of Y57W and Y57(5HW) from the cell extract. Y57(5HW) was detected by UV absorption at both 315 nm (- - -) and 280 nm (—) and collected between 7–10 min.

2.4. *Fluorescence spectroscopy*

Solutions of L-tryptophan and L-5-hydroxytryptophan were prepared in 10 mM K_2HPO_4 buffer, 100 mM KCl, pH 7.0. Protein solutions in 10 mM PIPES buffer, 100 mM KCl, pH 6.5. Solutions were diluted to a final absorbance of 0.10 at 280 nm. Fluorescence spectra were corrected for the variation in excitation source intensity and the solvent blank, obtained with a SLM 8000C spectrofluorimeter at 20°C. Excitation spectra were recorded with an excitation bandpass of 4 nm and an emission bandpass of 8 nm. Emission spectra were recorded with emission bandpass of 4 nm. For fluorescence anisotropy and time-resolved fluorescence, protein solutions were prepared in 50 mM $NaHCO_3$ buffer with 100 mM NaCl, 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$, pH 8.5. Fluorescence anisotropy was measured using a T-format detection configuration using Glan–Taylor polarizers. The excitation and emission bandpass were 4 nm. Mixtures of Y57(5HW) and antibodies were made at 1:1 ratios. The Y57(5HW) concentration was determined from quantitative Tb^{3+} titration [4]. Polyclonal antibodies were prepared as previously described [12].

3. RESULTS AND DISCUSSION

Y57W and Y57(5HW) differ only by the 5-hydroxy substituent on the Trp-57 residue. While the Y57(5HW) preparation ran as a single band on SDS-PAGE, it contained contaminating Y57W. These were separated using reverse-phase HPLC (Fig. 1). Y57W was strongly retained by the column (Fig. 1A), even with a longer isocratic elution than shown. Peak 1 of Fig. 1B is Y57(5HW) which eluted at 8 min, during the isocratic phase. Peak 2 contained mainly Y57W, but also had a small amount of another protein which also contained some 5HW as shown by its absorbance at 315 nm. Native OM with Tyr-57 has an elution profile similar to Y57(5HW). This suggests that the hydroxyl group is important in the interaction between the protein and the HPLC column which allows the separation. Mass spectrometry of purified Y57W and HPLC-purified Y57(5HW) gave 12,080.49 (±0.80) and 12,096.94 (±2.55), respectively. Expected masses were 12,079.06 and 12,095.04. The mass difference of 16.45 units was

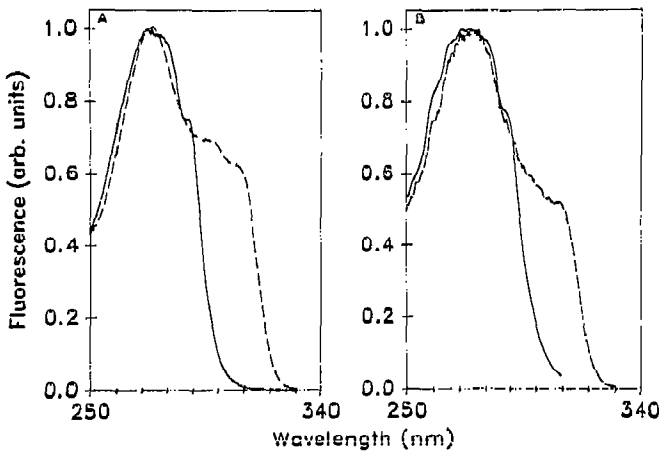

Fig. 2. Fluorescence excitation spectra at 340 nm emission. All spectra were normalized at 280 nm. (A) Trp (—) and 5HW (- - -). (B) purified Y57W (—) and HPLC-purified Y57(5HW) (- · - · -).

in excellent agreement with the expected difference of a single oxygen atom of 16 mass units.

The fluorescence excitation spectra of the amino acids, Trp and 5HW, is shown in Fig. 2A, along with the purified proteins Y57W and Y57(5HW) in Fig 2B. These spectra show the characteristic shoulder of 5HW absorbance between 300–320 nm present in Y57(5HW). Differences between the spectra of the proteins and those of the amino acid solutions are noticeable, particularly the 280/320 nm ratio of the Y57(5HW) spectrum.

The fluorescence emission spectra of Y57(5HW) 280 nm excitation (Fig. 3) shows a maximum at 335 nm, the same as that of 5HW in solution. This indicates that 5HW remains exposed to the solvent in the protein, similar to the exposure of Tyr-57 in native OM, as shown by its crystal structure [13]. The fluorescence of the free amino acid does not begin until 310 nm. This property allows the fluorescence of the single Tyr-65 residue of Y57(5HW) to be seen on the spectra, unobstructed at 300 nm.

The potential of using fluorescence properties of 5HW as an intrinsic probe is succinctly demonstrated in the fluorescence anisotropy experiments with the mixture of Y57(5HW) and anti-oncomodulin antibodies (Fig. 4). The anisotropy at 295 nm of Y57W due to Trp-57 (0.112) and Y57(5HW) due to 5HW-57 (0.105) are essentially identical. At 315 nm excitation, Y57(5HW)/OM has a significantly higher anisotropy (0.188). Anti-OM shows a low anisotropy with excitation at 295 nm (0.135) attributed to the depolarizing effect of multiple Trp fluorophores in each antibody molecule. At 295 nm excitation, the anisotropy of the 1:1 mixture of Y57(5HW) + anti-OM remains low (0.130), owing to the dominant absorbance of the Trp residues in anti-OM antibody at this wavelength. This demonstrates that with 295 nm excitation, fluorescence anisotropy cannot indicate any occurrence of antibody–protein complex formation. On the other hand using 315 nm excitation Y57(5HW) + anti-OM shows an anisotropy of 0.364, close to the theoretical maximum of 0.4. This change indicated a large decrease in the mobility of the 5HW fluorophore that can only be attributed to binding to OM antibody. In the presence of another antibody, anti-PTH (parathyroid hormone), the anisotropy (excitation 315 nm) of the mixture was 0.194, a value virtually identical to that of Y57(5HW) alone. This observation in this latter control experiment indicates that a complex of Y57(5HW) and anti-PTH did not form.

Two conclusions are illustrated in this figure. The most important conclusion is that the binding of the antibody to the protein antigen is demonstrated when 5HW is incorporated into the antigen and the 5HW residue is selectively excited at 315 nm. The fluorescence anisotropy increase of Y57(5HW) in the presence of anti-OM indicates a large decrease in mobility of the fluorophore which was the direct result of antibody binding. Secondly, 5HW is superior to Trp as a fluorescence anisotropy probe, as the theoretical maximum of 0.4 is almost attained in the Y57(5HW)–anti-OM complex. The separation of the ^1L_a and ^1L_b transition in 5-hydroxyindoles [14] allows only the excitation of the ^1L_b transition at 315 nm. In Trp there is insufficient separation of these two electronic transitions. Two absorbing transitions lead to a depolarization of fluorescence because of coupling between the two states, giving lower values of anisotropy.

Time-resolved fluorescence decay measurements [2] with 315 nm excitation, and 340 nm emission, allowed the determination of whether the epitope of Y57(5HW) included the 5HW-57 region. 5HW in aqueous buffer exhibits single exponential decay kinetics with a decay time of 3.60 ns. On the other hand, solutions of Y57(5HW) exhibited double exponential decay kinetics with decay times of 3.11 ns and 0.634 ns, whose pre-exponential values were, respectively, 0.88 and 0.12. The double exponential decay behavior indicates that the 5HW-57 side chain exists in two different conformational states. 5HW-57 must be more conformationally

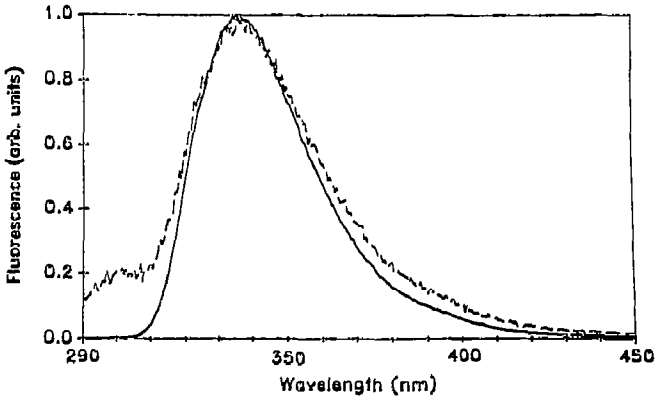

Fig. 3. Fluorescence spectra. 5HW (——) and purified Y57(5HW) (- --) with 280 nm excitation.

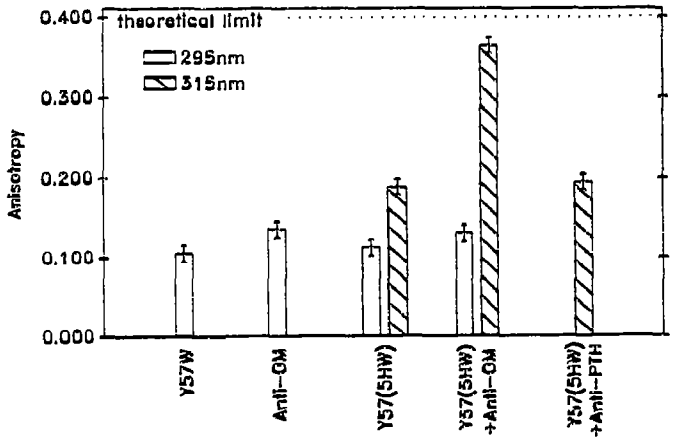

Fig. 4. Steady-state fluorescence anisotropy of Y57W and antibodies with 295 nm excitation; Y57(5HW) with 315 nm excitation.

restricted than either Tyr-57 or Trp-57, whose fluorescence decays with triple exponential kinetics [2], representing three conformational states. In the complex with anti-OM there was hardly any change in the long decay time (3.20 ns) while the shorter decay time changed to 0.953 ns. Both pre-exponential terms changed, to values of 0.74 and 0.26, respectively. The observation of only small changes in these parameters suggest that 5HW-57 is not part of an epitope for the polyclonal anti-OM antibody. The change in the value of the short decay time and its pre-exponential term is probably due to a subtle structural modification in the 5HW-57 locale caused by the binding of the antibodies to another part of the protein, and not to the region near 5HW-57. These latter set of time-resolved fluorescence experiments further demonstrate the potential of obtaining structural information by the selective excitation of the 5HW residue.

Successful biosynthetic incorporation of Trp analogs has been accomplished with non-fluorescent 4-fluorotryptophan [15], 6-fluorotryptophan for 19F NMR [8,16], and 7-azatryptophan [17]. Other Trp analogs have been reported as toxic and unable to sustain cell growth [1]. 7-Azatryptophan has also been proposed as an intrinsic fluorescent probe [17–19] of proteins. 7-Azatryptophan also has appreciable absorbance at wavelengths > 300 nm, however, 5HW has an even higher absorbance above 305 nm, with a well-separated ^1L_b transition. This, together with its longer singlet lifetime (cf. 7-azatrp, ca. 900 ps [19]), makes its more useful as a fluorescence anisotropy probe of protein dynamics.

In this work the incorporation of 5HW into a site-specific protein mutant has been demonstrated for the first time. Another promoter which would not allow 'leakage' would improve the expression system used here. However, the ability to selectively excite the 5HW residue in the expressed protein at 315nm, even in the presence of Trp containing leakage protein impurity, allows one to study the 5HW protein in the presence of proteins which contain large numbers of Trp residues.

The specific incorporation of 5HW into expressed proteins offers significant advantages as a new intrinsic fluorescent probe. They are: (i) specific excitation of 5HW fluorescence is possible in presence of multiple Trp residues in protein–protein complexes; (ii) a higher fluorescence anisotropy of 5HW is observed compared to Trp; (iii) hydrogen bonding in a protein can restrict rotamer populations and manifest itself in the fluorescence decay parameters. The examples shown in this work demonstrate the important potential of incorporating 5HW into proteins for the elucidation of specific local structural features and dynamic parameters in a large number of biochemical studies, such as protein–protein interactions, protein–receptor complexes, and chaperone-mediated protien folding.

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