Intrinsic Fluorescence of the Active and the Inactive Functional Forms of Human Thymidylate Synthase

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The observables associated with protein intrinsic fluorescence – spectra, time decays, anisotropies – offer opportunities to monitor in real time and non-invasively a protein’s functional form and its interchange with other forms with different functions. We employed these observables to sketch the fluorometric profiles of two functional forms of human thymidylate synthase (hTS), a homodimeric enzyme crucial for cell proliferation and thus targeted by anticancer drugs. The protein takes an active and an inactive form. Stabilization of the latter by peptides, unlike classical hTS inhibitors, bind it at the monomer/monomer interface offers an alternative inhibition mechanism that promises to avoid the onset of drug resistance in anticancer therapy. The fluorescence features depicted herein can be used as tools to identify and quantify each of the two protein forms in solution, thus making it possible to investigate the kinetic and thermodynamic aspects of the active/inactive conformational interchange. Two examples of fluorometrically monitored interconversion kinetics are provided.

Introduction

While traditional as well as more recent structural techniques, such as X-ray crystallography, NMR spectroscopy and cryo-electron microscopy, can provide detailed information on the structure of a protein in one of its functional states if this can be isolated or made dominant relative to other forms, directly observing such a state in a room-temperature solution while it continuously exchanges with other protein forms remains a challenging task; more so if quantitative information on its relative abundance is desired. Molecular spectroscopies are the elective tools for “seeing” and quantitating molecules. In the case of proteins, while nuclear magnetic resonance spectroscopy wins by far the competition of spatial resolution over electronic spectroscopies, the latter – especially those based on circular dichroism and intrinsic fluorescence – are definitely preferable in terms of ease and rate of operation. In particular such intrinsic steady-state and time-resolved protein fluorescence features as spectra, intensity decays, fluorescence anisotropy and collisional quenching can provide information, both qualitative and quantitative, suitable for exploring the conformational landscape of a protein; and, likely more relevant, for recognizing and quantifying functionally different protein forms, thus making it possible to monitor conformational changes induced by well identified and controllable chemical and physical stimuli. A classical and a more recent reviews on protein intrinsic fluorescence are proposed as refs. [1] and [2].

Thymidylate synthase (TS, EC:2.1.1.45), a 70 kDa homodimeric protein, is a thymidylate-cycle enzyme that catalyzes the methylation reaction of 2-deoxyuridined-5’-monophosphate (dUMP) to 2-deoxothymidined-5’-monophosphate (dTMP) using methylene-tetrahydrofolate as the methylene donor and the reducing agent. [3] This reaction is the only de-novo source of dTMP in humans and its inhibition halts the nucleic acid synthesis and the replication of cells. For this reason, for more than 50 years, TS-directed drugs that mimic either of the enzyme substrates have been important chemotherapeutic agents. [4,5] However, these active-site binding inhibitors impair the self-regulation of the protein, thus causing its over-expression and triggering the often fatal cascade of biochemical events that results in drug resistance. [6] Motivated by the need for TS inhibitors with different mechanisms of action, we have discovered several peptides that, binding at the protein inter-monomer interface rather than at the catalytic pocket, stabilize its inactive conformation and inhibit the growth of cancer cells without inducing over-expression of the enzyme. [7,8]

In the crystal structure of this protein, the loop that includes residues from 181 to 197 can be found in two main conformations connected with each other by a ~180° twist (Figure 1).[9–11] The active conformation is similar to the conformation found with all non-human TSs and features the side chain of C195 inside the protein active site, where it plays an essential role in the catalysis mechanism. In the inactive conformation, C195 lies out of the active site, pointing towards the interface between the two monomers of the protein. This structural peculiarity of the human protein has been associated with the hydrogen bonding ability of R163, a residue specific of the human protein.

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbic.202000722
which has always been found in the active form in crystals, did not exhibit any emission modulation following addition of phosphate ions or dUMP. Protein fluorescence offers a much richer set of observables than just steady-state emission intensity. To put the abundance of information embedded in intrinsic protein fluorescence to good use, an analytical approach certainly has its merits. The natural point of arrival of such a rational approach would amount to recognizing in each overall observable, that is, the protein emission spectrum, quantum yield, time decay etc., the consequences, if existing, of interactions between them, for example, exciton coupling with its spectral consequences or transition-dipole coupling, leading to FRET. However, to prevent such a complex exercise from becoming steriley self-referential, we have chosen, as a guide to our effort, the well-established, though often intricate relationship between protein structure and function on the other. In consideration of the biological and therapeutic relevance of this enzyme, and of its peculiar property of populating two functionally different forms, we have adopted a more synthetic approach in the fluorescence-based exploration of the protein conformational space. While still going through the analysis, whenever appropriate and useful, of the observables in terms of contributions from specific tryptophans, we have however directed our work to collecting and organizing the fluorescence features that identify the two mentioned functional states of this enzyme. This has been done by investigating the fluorescence steady-state spectra, quantum yields, decay profiles and anisotropies in conditions in which either the inactive or the active forms of hTS prevail.

The pronounced change in the overall hTS emission intensity when the protein switches between the inactive and the active conformations is believed to result mainly from W182. According to X-ray diffraction and computational evidence, its indole fluorophore undergoes a large displacement, 5.3 Å (Figure 1), and experiences a strong change in the local environment as the twist of the 181–197 loop occurs. Thus, our effort to provide an at least schematic structural interpretation of the differences observed was boosted by use of the W182A hTS mutant.

The fluorometric “portraits” here sketched are devised as well characterized, conformation-specific sets of observables able to allow us to identify the two protein forms and monitor their interplay at least in model experiments on the recombinant protein. While a detailed investigation of the kinetic and thermodynamic features of this relevant functional-state interconversion is left for the future, we anticipate here two examples of the use of intrinsic fluorescence for the measurements of the phosphate-ion induced active-to-inactive conversion and of the dUMP-induced inactive-to-active back-conversion.

Results and Discussion

Because the position of the equilibrium between the active and the inactive forms of hTS depends on the concentration of phosphate ions (Pi) and dUMP, we will show and discuss in parallel the fluorescence observables of this protein in solutions where, based on the above reported literature, we expect it to

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Figure 1. Superposition of the XRD structures of one of the WT hTS monomers in the active (PDB ID: 1HVY, white ribbon) and inactive (PDB ID: 1HW3, transparent gray cartoon) protein conformations. The 181–197 loop is shown in dark blue and orange for the inactive and active forms, respectively. The tryptophan side chains are shown in blue (inactive form) and yellow (active form). Trp 109 is not resolved in the inactive XRD structure. Cys 195 and Arg 163 are from the active conformation. Some residues at the monomer–monomer interface from the active conformation of the twin monomer (K47-F59-Q61-R175-R176-I178-R215-S216-T251) are shown as gray dots. Inset: enlarged picture of the Trp 182 region, with the distance between the Trp 182 indoles in the two protein forms.

While sulfate or phosphate anions favor crystallization of the inactive form of hTS, the active form is obtained when crystals are produced in the presence of dUMP or some of its analogues.[9,12] On the other hand, the active—inactive interconversion of hTS, with its dynamic and thermodynamic features, remains elusive. According to a computational simulation of such a transition, the 181–197 loops of the two monomers twist one after the other, and the catalytic C195 can either flip across the loop or undergo a torsion outside it.[13] Attempts to produce such a transition in the crystalline phase by soaking the inactive protein crystals with ligands that stabilize the active form have failed.[9] In solution, evidence that both forms of hTS are populated and interchange has been obtained indirectly from enzyme activity and inhibition experiments[10,12] and, more directly but at a preliminary level, from steady-state intrinsic fluorescence.[14] Phan et al. report that the tryptophan emission intensity of hTS increased following addition of phosphate ions, that favor the inactive form, and markedly decreased when dUMP, that favors the active form, was subsequently added. Consistent with the interpretation of the observed intensity changes as associated with the active—inactive interconversion, Escherichia coli TS, which has always been found in the active form in crystals, did not exhibit any emission modulation following addition of phosphate ions or dUMP.

However, for a structural investigation of a protein, intrinsic protein fluorescence offers a much richer set of observables than just steady-state emission intensity. To put the abundance of information embedded in intrinsic protein fluorescence to good use, an analytical approach certainly has its merits. The natural point of arrival of such a rational approach would amount to recognizing in each overall observable, that is, the protein emission spectrum, quantum yield, time decay etc., the consequences, if existing, of interactions between them, for example, exciton coupling with its spectral consequences or transition-dipole coupling, leading to FRET. However, to prevent such a complex exercise from becoming steriley self-referential, we have chosen, as a guide to our effort, the well-established, though often intricate relationship between protein structure and function on the other. In consideration of the biological and therapeutic relevance of this enzyme, and of its peculiar property of populating two functionally different forms, we have adopted a more synthetic approach in the fluorescence-based exploration of the protein conformational space. While still going through the analysis, whenever appropriate and useful, of the observables in terms of contributions from specific tryptophans, we have however directed our work to collecting and organizing the fluorescence features that identify the two mentioned functional states of this enzyme. This has been done by investigating the fluorescence steady-state spectra, quantum yields, decay profiles and anisotropies in conditions in which either the inactive or the active forms of hTS prevail.

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Results and Discussion

Because the position of the equilibrium between the active and the inactive forms of hTS depends on the concentration of phosphate ions (Pi) and dUMP, we will show and discuss in parallel the fluorescence observables of this protein in solutions where, based on the above reported literature, we expect it to
be mainly in the inactive conformation ([Pi] = 140 mM, [dUMP] = 0), in the active conformation ([Pi] = 140 mM, [dUMP] = 200 μM) and in a mixture of the two ([Pi] = 20 mM, [dUMP] = 0).

**Steady-state emission**

Addition to an hTS solution in PBS ([Pi] = 20 mM) of phosphate ions to a 140 mM final concentration causes a small tryptophan emission enhancement (λ\text{ex} = 295 nm, +15\% at the emission maximum, Figure 2); subsequent addition of 200 μM dUMP is accompanied by a marked intensity decrease (--45\%), as well as a small blue shift: the band centroid moves from 353.5 to 354.5 and back to 352.8 nm. Following the addition of 200 μM dUMP to a PBS solution ([Pi] = 20 mM) of hTS, the emission efficiency measured by exciting at 295 nm decreases by a factor 1.7 ± 0.2. However, much smaller dUMP concentrations can produce well observable fluorimetric changes (Figure S1 in the Supporting Information). To account for the decrease in emission yield following dUMP addition, inner filter effects can be ruled out because dUMP can absorb neither excitation light nor light emitted by the protein (Figure S1). Also, the higher S0–S1 excitation energy of dUMP relative to tryptophan makes tryptophan-to-dUMP excitation energy transfer energetically unfeasible. On the other hand, the standard potential for reduction of uridine to its radical monoanion, −1.1 V\text{[15]} would be suitable for the occurrence of quenching of tryptophan emission by excited tryptophan-to-dUMP electron-transfer\text{[16]}.

However, the contact nature of this process, the distances between the center of the dUMP uridine and the centers of the tryptophan indoles in the crystallographic structure of the hTS-dUMP complex (in the PDB 1HVY structure, 7.7 Å for Trp 109, 12.8 Å for Trp 182 and larger for the other tryptophans), the presence of other portions of protein between the two putative partners and, thus, the need for large conformational fluctuation to put the two partners in van der Waals contact (see, e.g., ref. [18]) make contact electron transfer extremely unlikely. Although electron tunneling through covalent and hydrogen bonds makes long-range electron transfer possible within proteins,\text{[19]} the electron tunneling times reported for several Ru proteins are longer or much longer than the tryptophan emission lifetime range, that is, 0.1–10 ns. We thus share the previously proposed explanation of these changes in intrinsic protein fluorescence in terms of interconversion of WT hTS from the inactive form at tens of millimolar Pi to the active one at tens of micromolar dUMP.\text{[14]} In the presence of 200 μM dUMP, we could not revert the dUMP-induced inactive-to-active conversion by adding Pi to solutions of hTS up to Pi concentrations of the order of 300 mM.

To help interpret the centroid spectral shift in Figure 2, as well as other fluorescence changes that will be reported later, we now focus on the contributions to the WT hTS emission of Trp 182, a residue that, being included in the 181–197 loop, is directly involved in the conformational active—inactive interconversion and may thus act as a local fluorometric reporter of such a structural/functional change. The W182A mutation causes an overall blue shift of the protein emission (Figure 3). The maximum moves from 338 to 333.5 nm when both tyrosines and tryptophans are excited at 280 nm (Figure 3a), and from 341 to 337 nm upon selective tryptophan excitation at 295 nm. Similarly, the mutation causes the centroids of the emission spectra to move from 353.55 (WT hTS) to 349.64 nm (W182A hTS). The emission spectrum of the W182A mutant was...
fitted as a single gaussian band with maximum at 334 nm and width 4800 cm⁻¹; the WT emission spectrum as the combination of two gaussian bands with maxima at 337 and 361 nm and widths 4100 and 5000 cm⁻¹ (Figures 2b, c). Together, these findings suggest that W182 is responsible for the latter redshifted band, that is, that this residue experiences a more polar environment than, on the average, the other emitting tryptophans. For protein tryptophans, exposure to water correlates with accessibility to water-soluble quenchers. Indeed, 0.3 M iodide (KI) quenched WT hTS more than W182A hTS, ~80% and ~50%, respectively. Preferential quenching of more exposed tryptophans was confirmed, in both cases, by a 5 nm emission blue shift observed following iodide addition (Figure S2).

The emission spectra in Figure 3a were obtained from WT and W182A mutant samples having the same absorbance at the excitation wavelength, and show that the WT protein has a stronger emission than the W182A mutant. This is quantitatively confirmed by the W182A/WT relative emission quantum yields in Table 1. These are always lower than unity and decrease when the excitation wavelength is moved towards the red edge of the absorption spectrum, where tyrosines can no more be excited. We see no reason to think of different absorption contributions by the five tryptophans of hTS. So, if we could assume that the W182A mutation does not largely affect emission from the surviving tryptophans, we would conclude that Trp 182 contributes to the overall hTS emission with a quantum yield about 4.4 times the average of the quantum yields of the other four tryptophans (a derivation of this estimate is provided in the Supporting Information). The assumption and the associated conclusion are supported by both the time-resolved data described in the next paragraph and the observation that the single W182A mutation does not cause significant changes in the far-UV circular dichroism (CD) spectrum of hTS (Figure S3), hence in the secondary structure of the protein.

At variance with the WT protein, we observed only minor changes in the steady-state emission of the W182A mutant following the addition of phosphate ions up to 140 mM or of dUMP up to 200 μM (Figure S4). This definitely rules out the possibility that dUMP may quench the four tryptophans other than Trp 182 of the two proteins. So, when Trp 182 is replaced by alanine, either the Pi- or dUMP-induced active—inactive interchange should be hampered; or, if any conformational change occurred, it would not significantly affect the observable fluorescence properties of the remaining four tryptophans. As we will see in the next paragraph, the fluorescence lifetime profiles of the two proteins, together with CD evidence, support the latter hypothesis. Hence, the Trp 182 residue assumes a pivotal role as a unique fluorometric reporter of this functional interconversion.

### Table 1. W182A/WT hTS relative emission quantum yields at different excitation wavelengths. Solvent: phosphate buffer saline (PBS, [Pi]=20 mM, pH 7.5). Estimated uncertainties: ± 0.05.

| λex (nm) | Φf W182A/Φf WT |
|---------|----------------|
| 270     | 0.75           |
| 280     | 0.80           |
| 290     | 0.65           |
| 295     | 0.60           |

### Emission decay

The decays of the tryptophan emission ($\lambda_{ex}=296$ nm) of the WT and W182A hTS proteins in the PBS buffer ([Pi]=20 mM, pH 7.5) were best deconvolved as the sum of four exponential functions ($\chi^2<1.2$). An example is shown in Figure S5. A fifth component, with a lifetime in the tens of ns/few microsecond range, was usually employed to fit the tail of the time profiles but had overall abundances of 1% or less and is thus not further considered.

The four component lifetimes and relative abundances are plotted in Figure 4 for WT and W182A hTS at four emission wavelengths, from 320 to 380 nm, to allow for a direct
comparison between the fluorescence decay profiles of the WT and the mutant proteins in spectral regions representative of emissions from tryptophan indoles experiencing increasingly polar environments. The widths of these components obtained from a non-extensive lifetime distribution decay analysis at 340 nm are reported in Figure 5. In all cases, short (0.1–0.25 ns) components appear with low abundances that further decrease when moving from 320 nm to higher emission wavelengths. Thus, in both proteins, the shortest-lived components are attributable to tryptophan indoles lying in low-polarity, likely hydrophobic regions. On the other extreme, the abundance of the longest component of WT hTS with lifetime 8.0 ± 0.3 ns increases from 30% at 320 nm to 70–75% at 360 and 380 nm. Thus, the most water-exposed tryptophans are among the ones with the longest-lived decays, that is, the ones least affected by intrinsic protein quenchers. The width of this component in the lifetime distribution (black or gray curves in Figure 5) is remarkably small and suggests these residues to lie similarly out of reach for intrinsic quenchers. For the W182A hTS mutant protein, the 8 ns component disappears, leaving a longer-lifetime component (10 ± 0.8 ns) with abundances from 10% at 320 nm to 25% at 360–380 nm. This implies that in the WT hTS conformations that are populated in the PBS buffer Trp 182 largely contributes to the 8 ns component and, in keeping with previously described steady-state results (Table 1 and Figure 3), that it is least quenched by intramolecular protein quenchers. Also, its longer lifetime and larger water exposure than, on average, the other tryptophans offer a simple explanation for the more pronounced quenching of WT than W182A hTS by iodide ions.

The two remaining lifetimes, 1.7 ± 0.3 ns and between about 4 and 5 ns, feature relatively broad distribution components, especially the latter (Figure 5). The shorter one has an abundance that decreases when the emission wavelength is shifted to larger values and is likely attributable to tryptophan indoles lying in low-polarity environments. On the other hand, the abundance of the 4–5 ns component is around 25% at all emission wavelengths. So, this component should correspond to a variety of conformations that are characterized by a rather wide range of polarities of the indole microenvironment.

Apart from the appearance of the 10 ns component with significant abundances in the low-energy emission region, the W-to-A mutation does not have a strong impact on either the other three lifetime values or their relative abundances. This finding, combined with the similar far-UV CD spectra (Figure S3), confirms that the relative W182A/WT emission quantum yield (roughly 6/10) reported previously results from a 4.4 times higher quantum yield of Trp 182 relative to the average of the other four tryptophans.

The effects of the additions of Pi, hence of conversion of the residual protein molecules in the active form to the inactive form, and of dUMP, hence of the opposite conversion of all protein molecules, on the emission decay profiles of WT hTS are synthesized in Figure 5 (for economy of presentation, data are only reported for emission at 340 nm but very similar results were obtained at 360 nm, see Table S1). Again, small changes, essentially a further narrowing of the 8 ns distribution component, are found with WT hTS following addition of Pi to a 140 mM final concentration starting from a 0.1 mM solution, thus causing complete conversion to the inactive form (from gray to black circles and lines in Figure 5). More marked changes follow dUMP addition up to 200 μM with the corresponding conversion to the active form (red circles and lines in Figure 5). As for the best fitting parameters, a significant shortening is observed for the second-longest lifetime, from 4.5 to 3.5 ns, with no change in the already large distribution width. This finding supports the idea that such a component represents a collection of conformations around one or more tryptophan residues that is broad and modifiable. However, the main changes brought about by the dUMP-induced conversion to the active protein conformation concern the longest-lived component. Its lifetime shortens from 8 ± 0.3 ns to 7.0 ± 0.6 and the corresponding distribution component broadens dramatically. Its relative abundance drops from 35 to 6% at 320 nm, from 55 to 15% at 340 nm, from 65 to 20% at 360 nm. As an obvious consequence, the relative abundances of the other components increase while their relative proportions are roughly maintained, apart from a slight increase in the relative abundance of the 3.5 ns component in the red portion of the emission spectrum. The lifetime profile of the very little active W182A mutant (red circles in Figure 4) exhibits no sensitivity to dUMP addition (Figure S6), in keeping with the previously mentioned indifference of the mutant steady-state emission to Pi or dUMP addition (Figure S4).

Figure 5. Effect of Pi and dUMP additions on the fluorescence lifetimes and corresponding relative abundances (circles) and on the positions and widths of the four components of the lifetime non-extensive distributions (lines) of WT hTS. Solvent: PBS buffer; [Pi] = 20 mM (gray), 140 mM (black); [Pi] = 140 mM and [dUMP] = 200 μM (red). Each quartet of circles was obtained from a different emission decay experiment. For the sake of presentation, we selected three lifetime distributions representative of the many obtained under the three experimental conditions. Each of the lifetime distribution components was normalized to the same maximum to allow for a direct visual comparison. The two arrowed curves underline the change in position and width of the two longest-lifetime distribution components associated with addition of 200 μM dUMP to a WT hTS in 140 millimolar Pi solution. Aem = 340 nm.
Fluorescence anisotropy and indole rotational mobility

If for simplicity one assumes a single fluorescence lifetime and a single “effective” rotational diffusion mechanism for fluorescence depolarization, the well-known Perrin equation:

\[ r = \frac{r'}{1 + \tau_c/\tau} \]

establishes a relationship among \( r \), the steady-state anisotropy, \( r' \), the limiting anisotropy in the absence of depolarization, for which we take 0.35, \( \tau \), the emission lifetime and \( \tau_c \), the rotational correlation time.\(^{[21]}\) Changes in the protein conformation such as those occurring in the hTS active/inactive interconversion may affect the emission decay rate, here synthetically represented by \( \tau \), and the local rotational mobility of the tryptophan indole fluorophore, here synthetically represented by \( \tau_c \), thus causing changes in \( r \). The average steady-state excitation anisotropies in Figure 6 and Table S2, together with the excitation anisotropy spectra in Figure S7, show that the W182A mutation results in a slight increase in anisotropies (compare the results for the two proteins in PBS, that is, at [Pi] = 20 mM, circled in blue in Figure 6). Although small if compared with the estimated uncertainty on anisotropy data (0.005), this increase is consistently found at all tested excitation/emission wavelength pairs and is connected with the overall increase in the emission decay rate caused by the mutation.

Nearer to the core of this work, that is, the effects of the active—inactive interconversion, the anisotropy measured at an emission wavelength of 310 nm (light gray bars in Figure 6), thus mainly assignable to tyrosines and buried tryptophans, holds 0.12 ± 0.01 and is essentially independent of the Pi concentration, hence on the relative populations of the two protein forms. On the other hand, the anisotropies measured at 340 nm, that may be taken as an average emission wavelength for the tryptophans of hTS, and at 360 nm, representative of the most water-exposed tryptophans, are always lower than 0.1 and decrease as the Pi concentration is increased, that is, as population of the inactive conformation is progressively favored (green curved arrow in Figure 6). The decrease is most pronounced for the tryptophans with most red-shifted emissions, that is, the most exposed ones, including Trp 182 (\( \nu_{\text{ex}} = 360 \text{ nm}, \) black bars in Figure 6). Consistently, we find that, following dUMP addition, the anisotropies measured at 340 and 360 nm increase (brown ovals and curved arrow in Figure 6), with the two values more similar to each other than in 140 mM Pi before nucleotide addition.

The time decay of anisotropy, measured at 340 nm under 296 nm excitation for WT hTS in 20 mM Pi, can be fitted by a bi-exponential decay with characteristic times 1.0 ± 0.2 and 8 ± 1 ns. The former likely represents an average indole rotational correlation time associated with local torsional mobility of this fluorophore; the latter characterizes an average depolarization contribution due to overall protein tumbling. For the inactive form of hTS, in the red side of hTS emission, from \( \tau = 8 \text{ ns} \) and \( r = 0.035 \) we estimate \( \tau_c = 0.9 \text{ ns} \), consistent with the shorter anisotropy decay time; in the blue side, from \( \tau = 0.1-0.25 \text{ ns} \) and \( r = 0.14 \), we estimate \( \tau_c = 0.07-0.17 \text{ ns} \). So, local indole motion, rather than overall protein tumbling, is the thermal process responsible for most of the fluorescence depolarization found. Such a local reorientation is slower, implying higher hindrance by the surroundings, for indoles lying in more polar environments than for buried tryptophans. This and the narrow lifetime distribution components in Figure 4 are consistent with the average \( \beta \) (temperature) factor of the indole atoms of Trp 182 being the smallest one among the hTS tryptophans in the X-ray structure of the inactive form (Table S3). Somehow consistently with the much broader 7 ns lifetime distribution component (Figure 5), the average \( \beta \) factor of this indole becomes the largest one, though comparable with those of Trp 90, Trp 109 and Trp 139, in the active form.

Overall, these observations are consistent with the idea that the inactive form, that prevails at high Pi concentrations in the absence of dUMP, features a relatively long-lived and red-shifted emission that we have mainly attributed to Trp 182 and is responsible for the lower anisotropy, especially when measured at 360 nm, than the anisotropy measured at low Pi concentrations where the active form, with its shortened 3.5 and 7.0 ns components, is more abundant or in the presence of dUMP where it becomes dominant.

Concerning changes in the local environment of the tryptophan side chains that would result in changes in their

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**Figure 6.** Steady-state fluorescence excitation anisotropies averaged between 260 and 285 nm, \( r_{\text{ex}} \) (260–285 nm), of WT hTS at the indicated Pi concentrations (0.1–140 mM), without and with 200 \( \mu \text{M} \) dUMP, and of W182A hTS at [Pi] = 20 mM. The anisotropies were measured at emission wavelengths 310 nm (light gray), 340 nm (gray) and 360 nm (black). The green curved arrow highlights the dependence of the WT hTS anisotropy on the Pi concentration. The blue ovals help compare the WT and W182A results at [Pi] = 20 mM. The brown ovals and curved arrow guide to a direct comparison between the anisotropies of WT hTS in the inactive [Pi] of 140 mM and active (+ dUMP) forms. The results at 310 nm were corrected for a small water Raman contribution centered at 280 nm. Typical estimated uncertainties were ± 0.01 at 310 nm and ± 0.005 at the other two emission wavelengths.
local angular mobility hence in the rotational correlation times, the CD spectra of WT hTS in the near UV (the 250–310 nm region, where absorption by the chromophoric aminoacidic side chains occurs) show that above 290 nm, where only tryptophans absorb, the spectra are similar at low and high Pi concentrations, in the absence of dUMP and at 200 μM dUMP (Figure S3). Because the intensity of the near-UV CD induced in this achiral chromophore is crucially related with the rotational mobility of the side-chain indole,[22] we deduce that the conformational changes associated with addition of Pi and dUMP do not involve any marked mobility change of the tryptophan indoles.

Assigning fluorescence signatures to hTS tryptophans

To associate one or more of the five tryptophans of hTS (Figure 1) to a protein fluorescence feature, we will use, for steady-state spectra, the direct relationship between the maximum emission wavelength and the polarity of the indole microenvironment indicated by the hydrophobic/hydrophilic characters of the residues lying around it and, for the emission decay, the proximity of that tryptophan fluorophore with its intrinsic quenchers, namely, electron acceptors such as the carbonyl C atom of a peptidic function or a carboxylic side chain, or proton donors like protonated amine side chains.[1,17,23] Although, on a larger distance scale, Förster coupling with other intrinsic indoles may give rise to changes in the emission time decay,[24] assessing the relevance of this modulation of the decay rate of a given tryptophan residue requires experimental information from suitably mutated proteins (typically with only two tryptophans at a time) and/or support from reliable quantum mechanical calculations; given the complexity of this problem for a protein with ten tryptophans per dimer like hTS, we will not consider these effects in this work.

That the main Trp 182 contribution to the protein steady-state emission spectrum lies prevalently in the bathochromic region of the latter, thus suggesting a polar average microenvironment for its indole, stems naturally from the overall blue shift of the emission spectrum resulting from the W182A mutation (Figure 3a). It is also consistent with the observation that the 8 ns component, whose weight in the decay of WT hTS increases as the emission wavelength is shifted towards the high-wavelength tail of the spectrum (Figure 4), is replaced by a 10 ns component with much a lower abundance in the decay of the W182A mutant.

From Figure 1 we can see that, among Trp 81, Trp 90, Trp 139 and Trp 182 (Trp 109 could not be resolved in the structure of the inactive form of the protein), only Trp 182 undergoes a marked displacement, 5.3 Å, with a likely associated change in the local environment and in emission properties when interconversion between the inactive form of the protein, where it protrudes towards the other monomer, and the active one, where it lies inside its own monomer, takes place. Both polar and hydrophobic side chains, or part of them are found within 5 Å from Trp 182 in both protein forms by X-ray crystallography (Figures S8 and S9), but three crystallization water O atoms are found in the same 5-Å-radius sphere in the inactive form, one in the active one. Thus, probably because of its exposure towards the inter-monomer interface and the presence therein of several ordered water molecules, Trp 182 experiences a polar microenvironment in the inactive protein form, consistently with the mentioned observations.

When addition of dUMP promotes conversion of WT hTS to the active protein form the change in the decay component profile (Figure 5) suggests that Trp 182 be somewhat quenched, with the 8 ns lifetime slightly shortened to 7 and, possibly, 3.5 ns. Assuming, as usually done, an invariant radiative decay probability, a lifetime decrease from 8 to 3.5 ns would be consistent with the 1.7 times lower emission quantum yield of the active relative to the inactive forms of hTS. The lifetime shortening suggests that quenching of the Trp182 indole emission by electron or proton transfer occurs with a slightly enhanced efficiency as a result of a subtle change in structure or dynamics occurring in this region with the inactive-to-active conversion. While lack of backbone carbonyl C atoms near the Trp 182 indole in both protein forms (Figures S8 and S9) is consistent with the relatively long lifetimes attributed to this residue, we cannot extract clear evidence of this subtle change from the X-ray crystal structures.

At the other lifetime distribution extreme, the abundance of the few-hundred-ps component decreases as the emission wavelength is shifted to larger values, from 320 to 380 nm (Figure 5). Therefore, the indole fluorophores responsible for this emission preferentially lie in low-polarity environments and experience quenching by, likely, some electron acceptor or proton donor lying a few ångströms from them. Based on the XRD structures of the active and inactive forms of WT hTS we tentatively assign this blue-shifted and short-lived component mainly to Trp 139. In both active and inactive hTS, within a 5-Å-radius sphere centered on the indole group of this amino acid, we can find, in toto or in part, ten amino acid side chains (Figure S9), all of which except two tyrosines and one cysteine are hydrophobic and clearly vouch for the low polarity of the microenvironemnt of the Trp 139 indole. In the same, quite crowded sphere around this indole, we can spot six backbone carbonyl C atoms some of which likely lie at electron transfer distance from the indole of Trp 139. The latter, however, likely applies to the Trp90 indole too. In fact, in both forms of the protein six backbone carbonyls lie within 5 Å from it (Figure S9). However, at variance with Trp 139, and inconsistently with the low-wavelength emission of the shortest-lived emission component of hTS, the environment of the Trp 90 indole is quite polar. In the 5-Å-radius sphere centered in this fluorophore we find charged, polar and hydrophobic side chains (Figure S9) together with two ordered water molecules. It seems therefore reasonable to expect this indole to be quenched by electron transfer with residual, short-lived emission in the intermediate-to-low energy spectral region. This might be the source of the weak few-hundred-ps decay component detected at emission wavelengths from 340 to 380 nm (Figure 4).

The obvious main suspects for the 1.6 ns lifetime emission and the relatively broad lifetime distribution band centered around 4 ns are, in addition to partially quenched emission
from Trp 182, the remaining two tryptophans, Trp 81 and Trp 109. The relative abundance of the former, shorter-lifetime component decreases by moving to the low-energy side of the emission spectrum (Figure 5). Thus, it should come from indoles that, compared with the indole fluorophores responsible for the sub-nanosecond lifetime emission, lie in microenvironments with similarly low polarities but are less efficiently quenched. On the contrary, the 4–5 ns component abundance is quite independent of the emission wavelength, from 320 to 380 nm, for both the inactive form of WT hTS and the W182A mutant (Figure 5). This and the larger width of its lifetime distribution band suggest this “component” to be associated with indoles experiencing a broad distribution of microenvironment polarities and characterized by quenching phenomena with low to intermediate efficiencies.

The environment polarities experienced by the indole of Trp 81 does not change much when the protein switches between the active and the inactive forms (we cannot extend this statement to Trp 109 because it was not resolved in the crystal structure of the latter form). The indoles of both tryptophans lie near only one backbone carbonyl, a finding that may be consistent with the relatively long lifetimes of these components that indicate little efficient emission quenching. From the vast majority of hydrophobic side chains and the absence of ordered water molecules in the 5-Å-radius sphere around it (Figure S9), we consider Trp 109 the main responsible for the 1.6 ns component. Finally, the Trp 81 indole microenvironment features a rather ill-defined polarity as it includes, on one hand, eight hydrophobic side chains and, on the other, a charged side chain (Lys 82) and four ordered water molecules. We propose its emission to contribute to the 4–5 ns component together with other tryptophans in less frequently visited points in the protein conformational space.

With the only purpose to schematically summarize the many details provided in this paragraph, we provide a pictorial sketch of the proposed association between tryptophan indoles, their microenvironments (Figure 7).

Kinetics of phosphate-ion induced active-to-inactive conversion and dUMP-induced inactive-to-active back-conversion measured by using intrinsic fluorescence

As examples of the use of the reported difference in intrinsic protein fluorescence between the two functional forms of hTS to monitor their interchange, we show the results of preliminary experiments in which emission at 345 nm is employed to follow the kinetics of the active-to-inactive and of the inactive-to-active conversions induced, respectively, by phosphate ions and dUMP (Figure 8). As expected, an addition of phosphate ions corresponding to a final concentration 140 mM induces conversion to the inactive form of the small fraction of active enzyme molecules present at the initial 20 mM Pi concentration; this results in a small, but well measurable, emission increase. On the other hand, the subsequent addition of dUMP to a final concentration 52 μM induces back-conversion to the active form that appears as a 42% decrease in emission intensity. From our lifetime profiles and the steady-state results reported in ref. [14] and confirmed by us, at [Pi] = 140 mM the equilibrium is almost completely shifted towards the inactive form at [dUMP] = 0 and to the active one at [dUMP] = 52 μM. So, if the two limit intensity values at high Pi concentration at [dUMP] = 0 and 52 μM, I° and I°', are known, the emission intensity measured in intermediate experimental conditions, Ii, will provide a direct measurement of the mole fractions, fi, of the two protein forms:

\[ f_i = \frac{I_i([\text{dUMP}] = 0) - I_i([\text{dUMP}] = 52 \mu M)}{I_i'}([\text{dUMP}] = 0) - I_i'([\text{dUMP}] = 52 \mu M)] \]

\[ I = A \text{ equilibration kinetics, where } I \text{ and } A \text{ represent hTS in the} \]

![Figure 7. Schematic association between hTS tryptophan indoles, their emission spectral regions (top, inactive form, λem = 280 nm) and lifetime components (bottom, inactive form, λem = 360 nm).](image)

![Figure 8. Time evolutions of the hTS intrinsic emission intensities at 345 nm following the addition of phosphate ion (left) and dUMP (right). The intensities have been normalized to their values before ligand addition (I°'). Black curves fit the results according to Equation (1). T = 13 ± 1°C. Other details are provided in the text.](image)
inactive and active forms bound to Pi in the first experiment and to dUMP in the second one. At any time, the observed emission intensity is the sum of the contributions from the two protein forms, \( I_t = I'_t + I''_t \). After straightforward passages, we obtain:

\[
\frac{I_t}{I'_t} = \frac{I''_t}{I'_t} + \left( \frac{I''_t}{I'_t} \right) e^{-kt}
\]

where \( I'_t \) and \( I''_t \) are, respectively, the emission intensities at equilibrium and before ligand addition, and \( k = k_{\text{a}} + k_{\text{d}} \) is the sum of the rate constants (or pseudo-rate constants) for the inactive-to-active and the reverse conversions. From the first experiment we obtained \( k(\text{Pi}) = 0.145 \pm 0.015 \text{ s}^{-1} \); from the second, \( k(\text{dUMP}) = 3.4 \pm 0.3 \text{ s}^{-1} \). 0.145 s\(^{-1}\) represents an estimate of \( k_{\text{d}} \) for Pi-bound hTS in the absence of dUMP, where the equilibrium is shifted towards the inactive form and \( k_{\text{a}} < k_{\text{d}} \). 3.4 s\(^{-1}\) is instead a lower limit for \( k_{\text{a}} \) for dUMP-bound hTS. In fact, while the clear difference between the two results indicates that, likely, the slower kinetics with the smallest \( k \) value is not limited by diffusion and reflects the intrinsic kinetics, to conclude the same for the faster conversion we need results at a higher time-resolution. Forthcoming experiments performed using a stopped-flow equipment with fluorescence detection will take advantage of an improved time resolution and will aim at a full kinetic investigation by focusing on the effects of temperature and of the concentrations of the protein, the phosphate ions and dUMP.

**Conclusion**

The experimental work herein reported shows how intrinsic fluorescence of hTS could offer a set of observables useful to assemble the fluorometric profiles of two functionally relevant regions in the conformational space of this protein, namely, those favored by addition of Pi and dUMP, the so-called inactive and active forms, respectively. A synthetic, pictorial representation of the differences between the explored fluorescence observables in the two protein forms is proposed in Figure 9.

In particular, we have shown that out of the five tryptophans per monomeric unit, it is Trp 182 that produces the most evident fluorometric changes associated with the active/inactive conformational interconversion. In the inactive form of the protein, where the catalytic loop to which it belongs is twisted out, facing the other monomer, it features an unquenched (8 ns) and red-shifted emission, properties that are likely associated with its exposure to an aqueous (or otherwise polar) environment and the absence of nearby intrinsic quenchers. Transition to the active form produces a well measurable quenching, to 7 and 3.5 ns of the 8 ns lifetime contribution and a 45% decrease in the steady-state emission intensity, with a concomitant slight blue shift of the emission spectrum. It also causes a marked broadening of the longest-lifetime band in the lifetime distribution and an increase in fluorescence anisotropy; these features indicate a somewhat increased conformational dynamics/disorder in the protein region that contains this tryptophan and is most involved in the structural change associated with the active—inactive interconversion.

The difference among the anisotropies measured before and after dUMP addition might not be, if considered alone, large enough to make this a reliable observable to recognize and quantify the active and inactive forms of WT hTS. Similarly, a comparison only based on emission intensities requires a constant, sometimes complex alignment of such experimental parameters as the protein optical density at the excitation wavelength, the extent of light scattering due, for example, to protein aggregates, the significance of inner filter effects in hTS samples that were treated differently in order to produce interconversion between the two forms. In particular, because of the need to avoid inner filter effects on such an UV-absorbing and -emitting fluorophore as tryptophan, use of intrinsic emission intensity to screen compounds designed to stabilize the inactive form of the protein would be characterized by an exceedingly severe limitation of the chemical space that could be sampled. However, through the two examples of active-to-inactive and inactive-to-active kinetics, we have shown how, whenever spectral interferences can be avoided, emission intensity can be employed to get access to useful mechanistic information concerning this relevant conformational change.
With respect to emission intensities, the mentioned alignment requirements are probably less severe for such intrinsic fluorometric reporters of protein conformational changes as spectra, lifetimes and anisotropies. Therefore, it is probably a suitably devised combination of these observables, what we might call a "fluorometric portrait", possibly enriched by additional observables, for example, the accessibility to collisional quenchers of tryptophan emission, that may offer a toolset useful to recognize and quantify the two main functionally relevant hTS conformational collections and to gain insight into structural, dynamic and thermodynamic features of their interchange.

Experimental Section

The WT and W182A hTS proteins were prepared and purified as reported in ref. [26]. Briefly, they were expressed in E. coli BL21(DE3) cells using 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) as the inducer. The proteins were purified in a phosphate buffer (20 mM KH₂PO₄, 50 mM NaCl, PBS) at pH 7.5 using His-tag affinity purification followed by size-exclusion chromatography. Protein purity was confirmed to be at least 90% by SDS-PAGE gel. The mutant was analyzed, following trypsin digestion, by both MALDI (4800 Plus MALDI TOF/TOF, Applied Biosystems) and nanoLC-EASI-Q-Tof (6520 Accurate-Mass Q-Tof, Agilent Technologies) mass spectrometries. MS and MS/MS results showed the peptide containing the mutation. When low phosphate ion (Pi) concentrations were required, WT hTS was transferred from PBS to Tris-HCl buffer (pH 7.5) by dialysis. dUMP, urea and all inorganic salts were purchased from Sigma-Aldrich.

Absorption spectra were recorded with a Cary100 UV/Vis spectrophotometer with 1 cm × 1 cm quartz cells. Steady-state fluorescence measurements were carried out with a Horiba Jobin-Yvon FluoroMax-3 spectrophotometer. The excitation and emission spectra were corrected for the lamp emission spectrum and the detector response spectral sensitivity. In order to minimize protein aggregation and inner-filter effects, absorbances at 295 nm were typically 0.15, corresponding to protein concentrations 3–4 μM (ε₂₉₅ ~ 40,000 M⁻¹ cm⁻¹). Because at 25 °C the equilibrium constants for the dissociation of the protein dimers to monomers are 5 × 10⁻⁴ and 47 × 10⁻⁴ M for WT and W182A hTS, respectively, at these concentrations, the WT protein is essentially fully dimeric, while about half of the mutant dimers are dissociated to monomers. Emission band centroids, λₑ, were computed within a Microsoft Excel sheet according to the band centroid definition: λₑ = ΣλᵢIᵢ/ΣFᵢ, where λᵢ is a wavelength, F the corresponding emission intensity and i runs across the entire band. Relative quantum yields were determined using samples with equal absorbances at each of the several different excitation wavelengths used. The relative yields were calculated as ratios of the areas of the corrected emission bands. The steady-state anisotropy was measured with the Horiba Jobin Yvon dedicated software using quartz polarizers in the FluoroMax3 fluorometer. Fluorescence decays were measured with a Horiba FluoroMax4 equipment, employing a 296 nm LED excitation source. The instrument response function was approximately 1 ns and was reduced to about 0.1 ns after deconvolution. The peak channel in the decays contained 10⁶ counts and at least two, but more often 4–6 decays were measured at each emission wavelength on different samples. The fluorescence decays were fitted either to a sum of exponentials or in terms of a non-extensive lifetime distribution [26] using 4 or 5 exponential components. Accepted fittings had χ² values lower than 1.20. Measurements were performed at 20 ± 2 °C in PBS or, when low Pi concentrations were required, in Tris-HCl buffer added with Pi.

For the kinetic active-to-inactive conversion experiments, the emission intensity of 1.6 μM of hTS in PBS (Pi = 20 mM) was measured at 345 nm before and after addition of 100 μL of a 2 M KH₂PO₄ water solution, corresponding to a final Pi concentration 140 mM. Then, the inactive-to-active back-conversion kinetics was followed, again by measuring the emission at 345 nm, before and after addition of 10 μL of a 9 mM dUMP solution in water, corresponding to a final concentration 52 μM. The solution was magnetically stirred to accelerate reactant mixing and, with the aim of reducing the conversion rates, it was cooled to 13 ± 1 °C, a temperature at which fogging of the cuvette walls was still acceptable. Emission intensities were measured every 0.2 s.

Acknowledgement

This work has been supported by the Italian Association for Cancer Research (AIRC), projects IG 10474 and IG 16977.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: fluorescence · intrinsic protein fluorescence · protein functional forms · proteins

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Manuscript received: October 19, 2020
Revised manuscript received: February 4, 2021
Accepted manuscript online: February 7, 2021
Version of record online: March 26, 2021