Azatryptophans endow proteins with intrinsic blue fluorescence

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Our long-term goal is the *in vivo* expression of intrinsically colored proteins without the need for further posttranslational modification or chemical functionalization by externally added reagents. Biocompatible (Aza)Indoles (Inds)/(Aza)Tryptophans (Trp) as optical probes represent almost ideal isosteric substitutes for natural Trp in cellular proteins. To overcome the limits of the traditionally used (7-Aza)Ind/(7-Aza)Trp, we substituted the single Trp residue in human annexin A5 (anxA5) by (4-Aza)Trp and (5-Aza)Trp in Trp-auxotrophic *Escherichia coli* cells. Both cells and proteins with these fluorophores possess intrinsic blue fluorescence detectable on routine UV irradiations. We identified (4-Aza)Ind as a superior optical probe due to its pronounced Stokes shift of ≈130 nm, its significantly higher quantum yield (QY) in aqueous buffers and its enhanced quenching resistance. Intracellular metabolic transformation of (4-Aza)Ind into (4-Aza)Trp coupled with high yield incorporation into proteins is the most straightforward method for the conversion of naturally colorless proteins and cells into their blue counterparts from amino acid precursors.

expanded genetic code | imaging | optical probes | protein engineering and design | red-shift

An ideal optical probe for the analysis of single proteins or whole proteomes would have the following properties: it is biocompatible, well incorporated into the target protein(s) by the endogenous translational apparatus and does not require posttranslational modifications or extensive host-engineering. Also, this chromophore should be noninvasive, i.e., it introduces minimal structural and functional perturbations into the target(s). The most promising candidates for such a chromophore are Ind analogs. They are basic structures of numerous highly important biomolecules. For example, Ind as part of the side chain of the amino acid Trp is the main source of intrinsic protein fluorescence, and purine bases of nucleic acids are Ind derivatives as well (1).

The canonical amino acid Trp is one of the most suitable targets for protein engineering and design owing to its low abundance in proteins and high relevance for protein stability and function (2). However, due to its complicated photophysics and the unfavorable overlap between nucleic acid and protein fluorescence emission spectra, Trp is not always qualified as a suitable optical probe (3), and alternatives are needed. Trp analogs with their Ind side chains containing a single atom exchange ("atomic mutation") (4) would be highly desirable. These analogs do not perturb the local environment of the substituted target protein(s) but induce considerable spectral changes relative to Trp.

(Aza)Trps meet the above described criteria. In these Trp isosteres, one of the endocyclic, CH, groups of Ind is substituted with nitrogen (Fig. 1*A*). This substitution comprises not only the smallest possible structural alteration of all known Trp analogs but also leads to dramatic changes in the photophysics of the aromatic system. (Aza)Inds resemble nucleic acid purine bases (5) because they contain pyridyl nitrogen with a lone electron pair capable of hydrogen bonding and excited-state tautomerization (6). Consequently, as protein building blocks, (Aza)Trps can furnish proteins with new functions exclusively reserved for nucleic acids, such as charge conductivity (7), a crucial feature for the design of future protein-based optoelectronic data storage devices or molecular wires for information transfer (8).

To date, Trp in bacterial proteins has been replaced by (7-Aza)Trp or with (2-Aza)Trp, either for testing the antimetabolic properties of the Trp isosteres (9) or for collecting information on the local environment (i.e., solvent accessibility) of Trp in proteins with unknown 3D structures (10). However, early reports on (2-Aza)Trp incorporation into proteins could not be confirmed in recent studies (11). Steady-state absorbance and fluorescence of (7-Aza)Trp sufficiently differ from Trp, which facilitates its selective excitation and fluorescence detection (λ_{max} ≈360 nm in hydrophobic environment). However, in aqueous solvents, (7-Aza)Trp fluorescence is strongly quenched, which limits its general use as a fluorophore (12). The identification of other (Aza)Trps with reduced quenching sensitivity and pronounced red-shifts in physiological solutions would overcome this problem.

Recent photophysical studies on free (4-Aza)Ind and (5-Aza)Ind revealed a strong red-shifted fluorescence (>400 nm, "blue fluorescence") when compared with Ind in aqueous solutions (13). Also, they exhibit a diminished quenching sensitivity compared with (7-Aza)Ind [see supporting information (SI) *Text*]. Ind analogs can be condensed with serine by the enzyme Trp synthase (TrpS, EC 4.2.1.20) to produce the corresponding Trp analogs (2, 14), and these substances have been identified by us and others (3, 15) as being superior candidates for the noninvasive design of proteins with significantly improved fluorescence properties.

The auxotrophy-based selective pressure incorporation (SPI) method (11) coupled with intracellular metabolic transformation of (Aza)Ind into (Aza)Trp (16) was successfully used for transmitting their highly desirable spectral features into a complex protein structure. We have chosen recombinant human anxA5 (widely used as apoptotic marker in cell biology) as a model protein (17). It contains a single Trp residue (Trp-187), which is either buried in a hydrophobic cavity at the convex (i.e., membrane binding) side of the molecule (Fig. 1*B*) or exposed to the solvent in the presence of high calcium concentrations or on denaturation (18). Its fluorescence emission (λ_{max} = 318 nm, on excitation at 280 nm) is dominated by the single Trp residue overlapped by that of the 12 Tyr residues in the anxA5 structure.

In this article, we report the successful incorporation of (4-Aza)Trp and (5-Aza)Trp analogs into human anxA5. Both (Aza)-Inds proved to be better optical probes than traditionally used (7-Aza)Ind. Biocompatible synthetic chromophores such as (4-Aza)Ind or (5-Aza)Ind are expected to serve as highly suited intrinsic optical probes for studies of complex biological systems in physiological milieus.

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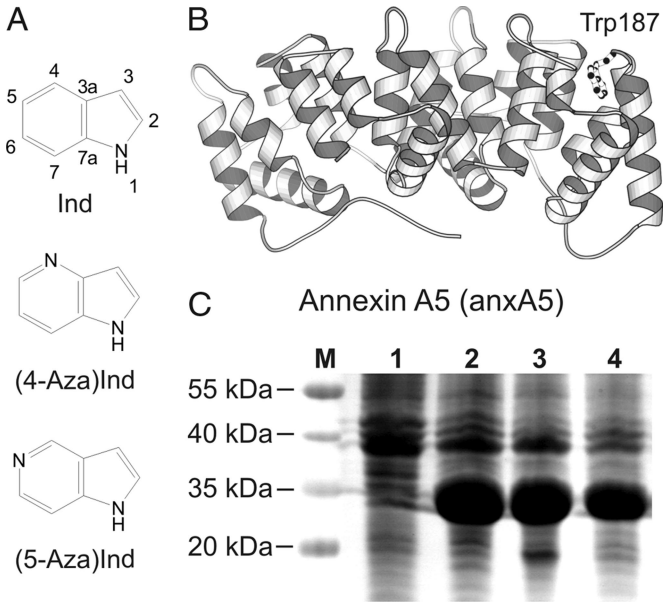

Fig. 1. AnxA5, (Aza)Inds, and bioincorporation experiments. (*A*) Chemical structures of Ind, (4-Aza)Ind, and (5-Aza)Ind. (*B*) Ribbon plot of human anxA5 (side view) with Trp 187 buried in the hydrophobic pocket at the convex side of the molecule. (*C*) Expression profiles of anxA5 in cell lysates of *E. coli* ATCC 49980 grown in minimal medium with Ind and both (Aza)Inds. Note that comparably high expression levels of anxA5 were achieved with all Ind isosteres. (1) Noninduced cell lysate, (2) Trp-anxA5 (wild type-anxA5), (3) (4-Aza)Trp-anxA5, and (4) (5-Aza)Trp-anxA5.

Results and Discussion

Biocompatibility of (Aza)Ind: Blue Fluorescent Bacteria. Before an (Aza)Trp can be incorporated into target proteins, the precursor (Aza)Ind has to be efficiently imported into the cells. Our first observation was that the Trp-auxotrophic *Escherichia coli* strain [American Type Culture Collection (ATCC) 49980] did not grow in synthetic medium with any (Aza)Ind as the sole Ind source. Nevertheless, the same cells nearly reached stationary phase ($OD_{600} \approx 2.0–2.5$) if they were incubated in the presence of limiting amounts of Ind (1–5 μM) in a medium supplemented with 1.3 mM (4-Aza)Ind or (5-Aza)Ind, subsequently. This result indicates that Ind, or more likely Trp, is essential during early growth phases but can be supplemented with (Aza)Inds in the later growth phases. Thus, both substances are sufficiently biocompatible for SPI experiments, which are routinely performed with cell cultures in the midexponential growth phase.

In their pioneering studies in the 1950s, Pardee and Prestidge were able to demonstrate that (7-Aza)Trp incorporation into cellular proteins resulted in inactive enzymes as well as in the inhibition of phage infection of bacteria (19). The most conceivable explanation for the lack of initial growth on Ind analogs is that in the cellular proteome a pool of proteins exists, whose members obligatorily require Ind moieties for their functional and/or structural integrity. Thus, cell growth in synthetic medium is only initiated if minute amounts of the canonical amino acid or its precursor are present.

On excitation with an UV lamp ($\lambda_{Em,max} = 360$ nm, Olympus MT 20 monochromator, Ina-shi), we observed blue fluorescent bacteria when Trp-auxotrophic *E. coli* cells were grown under the above described conditions with (4-Aza)Ind (Fig. S2). In contrast, no fluorescence was detected in the presence of Ind. These observations indicate that (4-Aza)Ind and (5-Aza)Ind were able to be taken up, presumably by means of the Trp/Ind-uptake pathway (20).

Taking into account the high-level expression of the (Aza)Trp variants of anxA5, the blue fluorescence we observed in bacteria most probably originates from the substituted proteins. However, it cannot be ruled out that intracellularly accumulated, free (Aza)Ind adds to the observed fluorescence.

Table 1. Absorbance and emission maxima (λ_{max}) along with ε_M coefficients of free indole/azaindoles in buffered solution, Trp-anxA5, and related aza-variants

Indoles	$\lambda_{Ex,max}$, nm	ε_M, M^{-1} cm^{-1}	$\lambda_{Em,max}$, nm	RF*	QY
Ind	269	5,264	348	1.00	0.220
(4-Aza)Ind	288	7,262	418	0.30	0.077
(5-Aza)Ind	266	2,663	402	0.16	0.099
Proteins					
Trp-anxA5	278	21,105	318	1.00	—
(4-Aza)Trp-anxA5	278	21,020	423	0.46	—
(5-Aza)Trp-anxA5	278	19,730	414	0.24	—

Experimental conditions are described in *Experimental Procedures*, and related spectra are shown in Figs. 2 and 4 and Fig. S1. All data were recorded in 100 mM Tris·HCl, pH 7.5. RF, relative fluorescence.
*Normalized to the indole intensity at the emission maximum.

Expression and Analytics of (Aza)Trp-anxA5. For the cotranslational incorporation of (Aza)Trps into target proteins, the uptaken (Aza)Inds must be efficiently converted into the related Trp analogs by TrpS. Indeed, the metabolic transformation of various substituted Inds into the related amino acid analogs is described for other bacterial hosts as well (16, 20). Thus, we expected the *in situ* production of (Aza)Trps in the Trp-auxotrophic *E. coli* ATCC 49980 host cells. If the fluorescent Trp analogs accumulate intracellularly up to levels high enough for sufficient activation and charging onto $tRNA^{Trp}$ by the Trp-tRNA synthetase (TrpRS), they will participate in ribosomal translation by recoding the UGG (Trp) coding triplets (11).

For an efficient substitution of the single Trp-187 in anxA5 with (Aza)Trps, the cell fermentation parameters were optimized. Biomass was accumulated by using a defined concentration of Ind (5.5 μM) in new minimal medium (NMM) (4), which allowed robust cell growth to the midexponential phase ($OD_{600} \approx 0.6–0.8$). After Ind depletion, the culture was supplemented with the desired (Aza)Ind (1 mM) for 30 min, followed by the induction of the histidine-tagged anxA5 expression (see *Experimental Procedures* for details). Our model protein, anxA5, was highly expressed by this procedure, regardless of whether Ind, (4-Aza)Ind, or (5-Aza)Ind was provided in the medium (Fig. 1*C*). In general, expression profiles on SDS gels of cells incubated with (4-Aza)Ind or (5-Aza)Ind exhibited prominent fluorescent anxA5 bands at ≈35 kDa on exposure to UV light ($\lambda = 312$ nm). No fluorescence was observed for the parent protein. These results were the first indication for a successful conversion of (4-Aza)Ind or (5-Aza)Ind into the (Aza)Trps and their subsequent highly efficient incorporation into anxA5.

After purification by metal affinity chromatography, the yield of anxA5 purified from cells supplemented with (4-Aza)Ind was higher (≈17 mg per $liter^{-1}$) than that from the Trp-anxA5 culture (≈10–15 mg per $liter^{-1}$), whereas anxA5 in the presence of (5-Aza)Ind was equally well expressed (≈12 mg per $liter^{-1}$). Purified proteins were quantified by Bradford protein assay (Bio-Rad) and their molar extinction coefficients (ε_M) were determined in the wavelength range of 250–320 nm, as shown in Table 1.

Mass spectrometric analyses (MS and MS/MS Q-TOF mass spectrometry) provided further evidence of successful incorporation of (Aza)Trps into anxA5. Although the molecular mass difference between Trp and both (Aza)Trps is only 1 Da, the proof for (Aza)Trp incorporation was furnished by a combined fluorescence/mass determination approach. After tryptic digestion of the protein variants, the resulting peptide fragments were separated by reversed-phase HPLC. The single (Aza)Trp-containing peptide

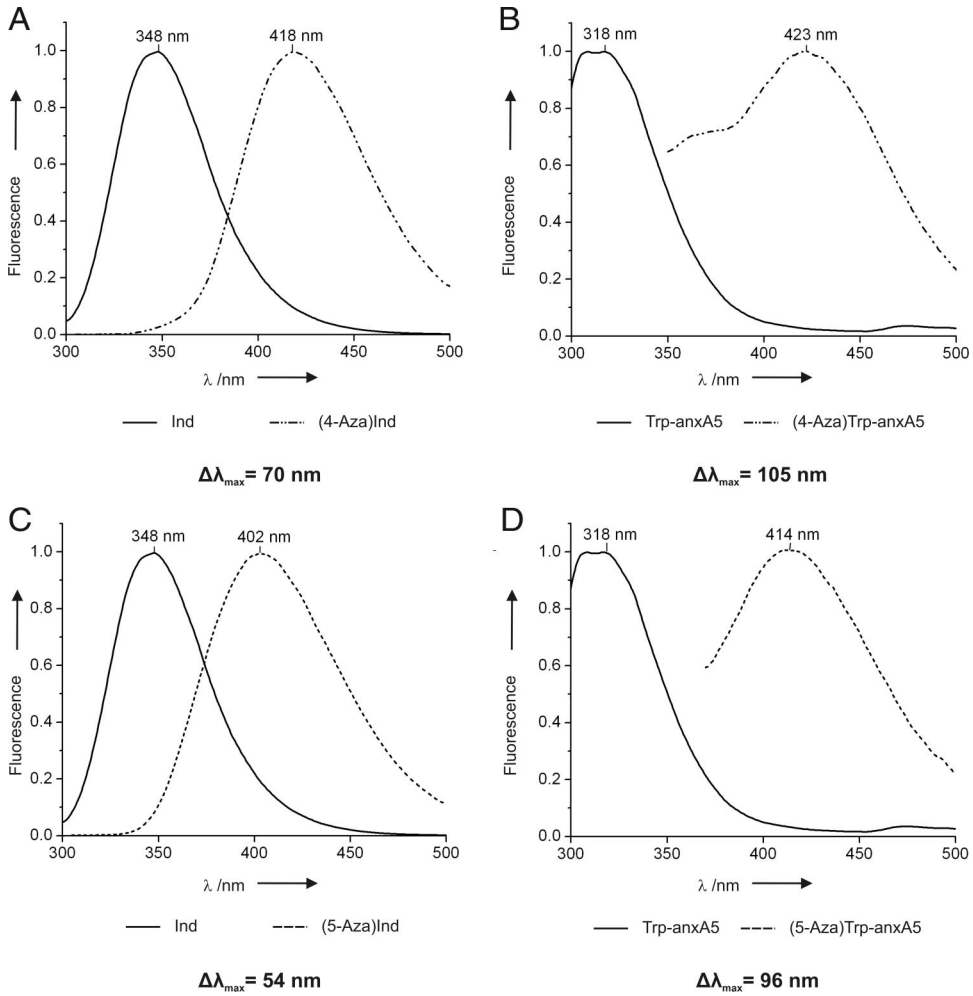

Fig. 2. Normalized fluorescence emission spectra of free Inds and (Aza)Inds in buffered aqueous solutions and of the parent protein and its Aza-variants on excitation at 280 nm. Comparison of the fluorescence spectra of free Ind with free (4-Aza)Ind (*A*) and (5-Aza)Ind (*C*); note the dramatic red-shift in the (4-Aza)Ind emission spectrum (70 nm). The emission maximum of free (5-Aza)Ind is less substantially red-shifted (54 nm). The fluorescence emission maximum of (4-Aza)Trp-anxA5 (*B*) is 107 nm red-shifted and that of (5-Aza)Trp-anxA5 (*D*) is 96 nm red-shifted compared with Trp-anxA5. Note also the spectral shoulder at 365 nm of (4-Aza)Trp-anxA5 (see the text for more details).

[(Aza)WGTDEEK] was identified and collected according to its characteristic fluorescence emission at 400–420 nm (λ_{Ex} = 279 nm). Last, it was analyzed by Q-TOF MS. As expected, the fluorescent peptide revealed a mass of m = 864.36 Da (m/z = 433.19), which agreed with the predicted mass (m = 864.36 Da). In addition, we were able to confirm the amino acid sequence of this peptide by MS/MS (see Figs. S3 and S4).

For the quantitative estimation of the (Aza)Trp incorporation level, amino acid hydrolysis was performed by using thioglycolic acid. The derivatized amino acids were analyzed by HPLC and subsequent mass spectrometry (see *SI Text* for experimental procedure and Figs. S5 and S6). Strikingly, the (4-Aza)Trp-anxA5 variant revealed a substitution of Trp of >90%, and confirmed the high biocompatibility of this small fluorescent probe. The signal of (5-Aza)Trp from the (5-Aza)Trp-anxA5 variant could not be separated from the threonine peak, but we were able to estimate the incorporation of (5-Aza)Trp by comparison of the (5-Aza)Trp-anxA5 profile with that of the parent Trp-anxA5. We calculated an incorporation level of (5-Aza)Trp of ≈70% by means of the reduction of the Trp signal (−2.26%, total 3.2% in the parent protein) and the increase of the threonine peak (+2.26%) in the profile of (5-Aza)Trp-anxA5 compared with Trp-anxA5. We also expressed (7-Aza)Trp-anxA5 for comparative spectroscopic studies, and the level of incorporation was ≈80%. These data were fully confirmed by state-of-the-art Orbitrap mass spectrometric analyses (unpublished data).

Dramatically Red-Shifted Fluorescence. To assess the influence of the nitrogen atom in the Ind ring on the spectral properties of the free Inds or after incorporation into the model protein, fluorescence emission profiles of all Inds and proteins were compared on excitation at their absorbance maxima, as listed in Table 1 and spectra presented in Fig. S7*B*.

When compared with Ind, the fluorescence intensity maximum $\lambda_{Em,max}$ of free (4-Aza)Ind in aqueous solution was red-shifted by 70 nm, and that of (5-Aza)Ind was red-shifted by 54 nm (Fig. 2 *A* and *C* and Table 1). The fluorescence characteristics of the dissolved (4-Aza)Ind and (5-Aza)Ind were fully transmitted to the related anxA5 variants, whereas the red-shifts of the (Aza)Trp variants were even more pronounced. In the case of (4-Aza)Trp-anxA5, the red-shift was ≈105 nm, and the (5-Aza)Trp-anxA5 variant exhibited a 96 nm red-shifted emission maximum relative to the parent protein Trp-anxA5 (Fig. 2 *B* and *D* and Table 1.) In Trp-anxA5, the fluorescence emission maximum of the Trp-187 Ind moiety was blue-shifted relative to

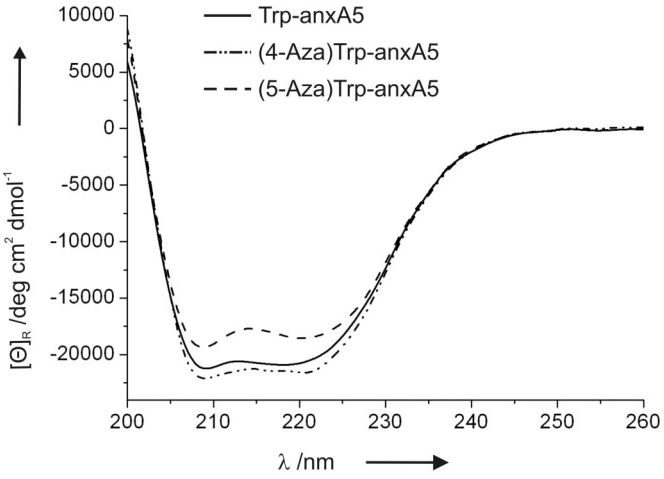

Fig. 3. Secondary structure of (Aza)Trp-anxA5 and the parent Trp-protein. Far-UV CD spectra from 200–260 nm were recorded at 4°C.

free Ind in aqueous solution (Fig. 2 *A* and *B* and Table 1), a consequence of the placement of Trp-187 within a hydrophobic cavity (Fig. 1*B*) of the protein, combined with the spectral contribution of the remaining 12 Tyr residues (4).

The spectral shift of spectral properties of (Aza)Inds into substituted anxA5 was not only successfully transferred but even more pronounced than among free Inds in solution. It is also reasonable that, in the variant proteins, the (Aza)Ind moieties of (4-Aza)Trp and (5-Aza)Trp are not completely buried in the hydrophobic cavity but face the solvent environment. This conjecture was supported by the fluorescence properties of the (Aza)Trp-anxA5 variants, which (on denaturation or Ca^{2+}-titration) resembled those of Trp-anxA5 (see Figs. S8 and S9). Both processes are accompanied by the exposure of Trp-187 to the surrounding solvent (22). The spectral shoulder at $\lambda_{Em,max}$ = 365 nm in the fluorescence emission profile of (4-Aza)Trp-anxA5 (Fig. 2*B*) correlated well with the emission maximum of (4-Aza)Ind in apolar solvents. This shoulder might indicate that at least a portion of (4-Aza)Trp-187 was buried in the hydrophobic cavity of the protein molecule. On denaturation or Ca^{2+}-titration of (4-Aza)Trp-anxA5, this spectral shoulder disappears (see *SI Text*). These observations and considerations strongly indicated the coexistence or an equilibrium between two protein conformations with either a buried or a solvent-exposed (4-Aza)Trp side chain.

Secondary Structure and Folding Cooperativity. Far-UV CD spectroscopy of Trp-anxA5 and its Aza-variants revealed the typical CD profiles of α-helical proteins, with two characteristic minima of similar intensity at 222 nm and 208 nm (Fig. 3). The incorporation of (Aza)Trps had only minor effects on the CD spectra, indicating that the structure of anxA5 remained unchanged. The small differences in the intensity of the CD bands ≈220 nm were most likely due to the contributions of the Trp residues, which were changed by replacing the Ind moiety by an (Aza)Ind moiety. From these profiles, we calculated an α-helical structural content of ≈60% in the secondary structure of anxA5 and its variants. Also, thermally induced denaturation profiles of all three proteins had similar T_m values (± 2°C) and enthalpy of unfolding, indicating cooperative folding (see Fig. S7 *C* and *D* and Table 2). In summary, both (4-Aza)Trp and (5-Aza)Trp, exhibited no significant influence on the structural integrity of anxA5.

(4-Aza)Ind Vs. Traditionally Used (Aza)Inds. Recently, Twine and Szabo reported (6-Aza)Ind as novel Ind-based chromophore

Table 2. Thermodynamic parameters of Trp-anxA5 and its aza-variants

anxA5 variant	T_m, K	ΔT_m, K	ΔH_m, kJ mol^{-1}	$\Delta\Delta H_m$, kJ mol^{-1}
Trp	325.35 ± 0.07	—	−650.34 ± 30.79	—
(4-Aza)Trp	323.25 ± 0.04	−2.10	−698.19 ± 20.34	47.85
(5-Aza)Trp	323.39 ± 0.01	−1.96	−640.26 ± 06.04	−10.08

Experimental conditions and calculation methods for all unfolding profiles are described in *Experimental Procedures*, and related curves are shown in Fig. S3. ΔT_m *and* $\Delta\Delta H_m$ are the differences between T_m *and* ΔH_m of the parent and substituted proteins.

with intriguing spectral properties (23). Whereas (6-Aza)Ind has a strong emission signal and large red-shift in hydrophilic environments, its fluorescence was markedly quenched in aprotic solvents (and most probably in the protein core as well). Unfortunately, our attempts to generate fully substituted (6-Aza)Trp-anxA5 by using the same conditions as for (4-Aza)Ind and (5-Aza)Ind were unsuccessful; most probably because the biocompatibility of (6-Aza)Ind is not as good as in case of the other tested (Aza)Inds like (4-Aza)Ind and (5-Aza)Ind. When compared with (5-Aza)Ind, (4-Aza)Ind proved to be a better optical probe, exhibiting significantly higher biocompatibility and a larger red-shifted fluorescence maximum. Also, it features higher fluorescence intensity, ε_M, and a comparable quantum yield (QY) (Table 1). In addition, (4-Aza)Ind in anxA5 induced similar structuring of the protein interior when compared with the parent protein and its (5-Aza)Ind-containing counterpart (Fig. 3).

As mentioned above, the main drawback of the traditionally used (7-Aza)Ind for protein design is that its exposure to aqueous solvents leads to dramatic quenching (12). Our fluorescence measurements of free (7-Aza)Ind in aqueous physiological buffers fully confirmed these observations (Fig. 4*A*). Also, we measured an ≈5-fold difference in QYs between (7-Aza)Ind (0.017) and newly examined (5-Aza)Ind and (4-Aza)Ind (Table 1).

To compare (7-Aza)Ind vs. (4-Aza)Ind in protein structure, related anxA5 variants were also expressed and purified. As expected, (7-Aza)Trp-anxA5 exhibited less fluorescence intensity and a diminished red-shift of the fluorescence maximum (Fig. 4*B*). Also, on denaturation of (7-Aza)Trp-anxA5, the fluorescence intensity of the unfolded protein was almost fully quenched, reflecting full solvent accessibility of (7-Aza)Trp-187. In contrast, the fluorescence intensity of the denatured or calcium-bound (4-Aza)Trp- and (5-Aza)Trp-anxA5 variants remained largely unchanged (see *SI Text*). Last, it has often been claimed that the main advantage of (7-Aza)Ind in protein structures is its selective excitability (3). However, this feature also exists in the absorbance profile of (4-Aza)Ind, which has a broad absorbance band of ≈310 nm (Fig. S1).

Attributes like dramatically improved spectral properties (Stokes shift of ≈130 nm, a significantly higher QY, and lower quenching tendencies) along with excellent incorporation yields into proteins and good biocompatibility in living cells, offer promising prospects for (4-Aza)Ind as a very attractive new intrinsic optical probe for proteins.

Toward New Intrinsically Colored Proteins and Cells. In the last decade, we and others reported the translation of various Aza-, hydroxy-, amino-, halogen-, and chalcogen-containing Trp analogs and surrogates into different target protein sequences (15). The power of this protein engineering approach is best illustrated by the design of a gold fluorescent protein (24) with a Stokes shift of ≈100 nm achieved by direct introduction of 4-aminoTrp (25) into the complex chromophore of the parent cyan fluorescent

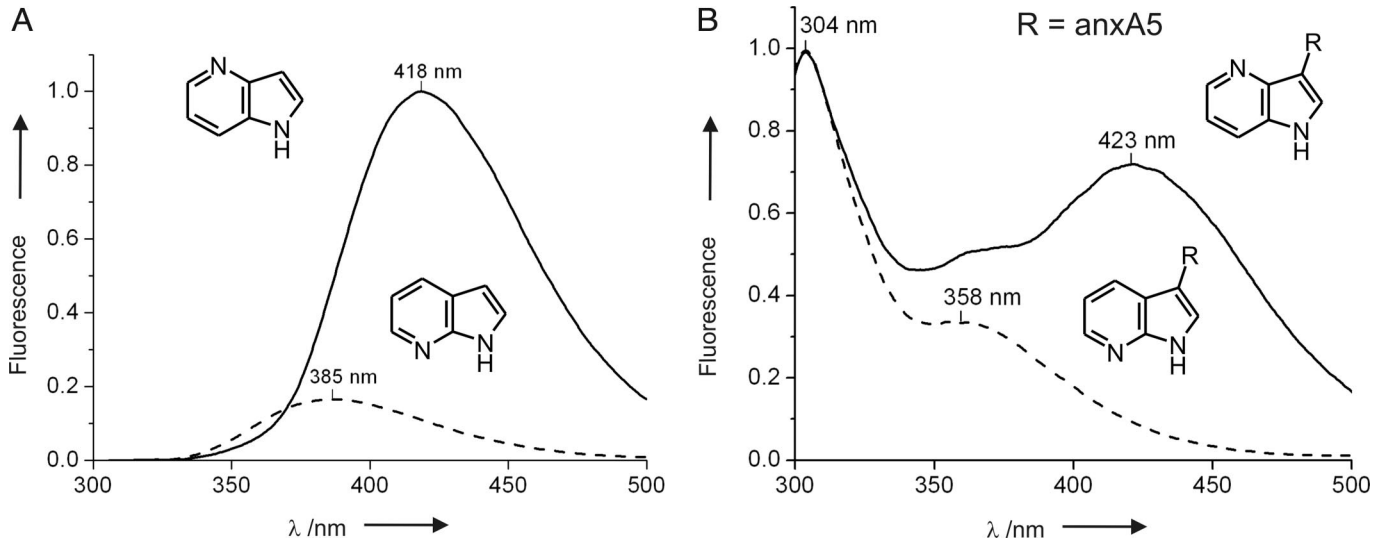

Fig. 4. Fluorescence profiles of (4-Aza)Ind and (7-Aza)Ind and related proteins in aqueous buffered solution. (*A*) Fluorescence spectra of (4-Aza)Ind (solid line) and (7-Aza)Ind (dashed line) on excitation at the respective absorbance maximum. Note the strong red-shift (33 nm) in the profile of (4-Aza)Ind ($\lambda_{max,em}$ = 418 nm) when compared with (7-Aza)Ind ($\lambda_{max,em}$ = 385 nm) along with markedly higher fluorescence intensity of (4-Aza)Ind. (*B*) Emission profiles (normalized to protein fluorescence) of (4-Aza)Trp-anxA5 (solid line) and (7-Aza)Trp-anxA5 (dashed line) on excitation at 280 nm. Note that the emission maximum $\lambda_{max,em}$ of (4-Aza)Trp-anxA5 ($\lambda_{max,em}$ = 423 nm) is 65 nm more red-shifted than of (7-Aza)Ind ($\lambda_{max,em}$ = 358 nm). The spectral shoulder of (4-Aza)Trp-anxA5 in the range between 350 and 370 nm is discussed in the text.

protein. However, this dramatic red-shift is the result of two successive steps. First, the synthetic fluorescent amino acid is translated into the target protein sequence, and second, this residue is posttranslationally integrated into the chromophore structure.

Classical site-directed mutagenesis by using the standard amino acid repertoire is the most commonly used approach in contemporary protein design. However, in the majority of naturally chromophore-free proteins, the exchange of Trp for other standard aromatic amino acids does not yield novel spectral properties. For example, Trp→Tyr/Phe/His replacements often bring about significant chemical and steric alterations and can be associated with unpredictable negative effects on the functional and structural integrity of the protein. Apart from bulky fluorescent protein fusion tags, even small fluorophores like FlAsH or coumarins, although they are cell-permeable, are useful only within limits, because they require either the tailoring of the target gene sequences before labeling or bioorthogonal conjugation reactions, respectively (26, 27).

The incorporation of (4-Aza)Ind into the model protein anxA5 generated intensively blue fluorescent *E. coli* cells, and is an important step in our efforts to create intrinsically colored proteins. However, for much wider applications in cell biology, novel intrinsic fluorophores with excitation maxima far above the range of 300–350 nm are required. Thus, intrinsically green fluorescent optical probes that can be excited with blue light would represent a major advance. This "green area" is currently dominated by the green fluorescent proteins. However, these bulky fluorescence tags might become redundant if synthetic chemistry provides the next generation of translationally active intrinsic optical probes that feature high QYs and far red-shifted fluorescence covering a spectral window between "green" and "red."

Experimental Procedures

Chemicals, Fermentation, Analog Incorporation, and Protein Purification. All chemicals were purchased from Sigma–Aldrich, unless stated otherwise; (4-Aza)Ind and (5-Aza)Ind were purchased from Molekula and Biosynth. Protein expression was performed by using the Trp auxotrophic *E. coli* strain ATCC 49980 with the genotype WP2 (trp-, uvrA-, and malB-) (28, 29). Cells were transformed with the pQE80 vector (Qiagen) carrying the anxA5 gene with an N-terminal His-Tag. Transformants were grown in NMM (30), containing 5.5 μM Ind as natural substrate until depletion of Ind at OD_{600} = 0.6–0.8. As already described in the literature, we expressed and purified TrpS, which catalyzes the conversion of Ind to Trp and of Ind derivatives to the appropriate Trp derivatives (31). We incubated (Aza)Inds with L-serine and TrpS in Tris·HCl buffer, pH 7.5, and added the mixture to the cell culture. This procedure was applied to reduce the stress to the cells by supporting the conversion of (Aza)Ind to (Aza)Trp. (Aza)Inds were added to the medium at 1 mM final concentration, and 30 min later target protein expression was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Protein expression was performed over night at 30°C with vigorous shaking. After cell harvest, cells were lysed by osmotic shock (32) and the lysate cleared by high speed centrifugation (40,000 × *g*, 4°C, 40 min). Target proteins were purified from the supernatant by using a Ni-NTA column (GE Healthcare) equilibrated with sodium dihydrogen phosphate buffer (50 mM/20 mM imidazole/300 mM NaCl, pH 8.0). Proteins were eluted by means of imidazole gradient (20–250 mM), concentrated and purity was analyzed by SDS/PAGE and Coomassie staining.

Mass Spectrometry. (Aza)Trp-anxA5 was digested overnight at 37°C by modified trypsin (Promega), according to instructions of the supplier. The resulting peptide mixture was separated by means of C_{18}-RP-HPLC (Waters Alliance 2695 with photodiode array detector 996 and fluorescence detector 2475; column, Waters Xterra RP C_{18} 3.5 μm [2.1 × 100 mm]; Waters) and the fluorescence of the eluate was recorded on-line after excitation at 279 nm. Eluted fraction showing the characteristic (Aza)Ind fluorescence (λ_{Ex} = 400–420 nm) was collected and further characterized by off-line-nano-MS by using a Q-TOF Ultima (Waters) spectrometer in MS and MS/MS mode.

Estimation of the Protein Concentration by Bradford Protein Assay. For calculation of the ε_M of wt-anxA5 and variants, the protein concentrations were determined by using the Bradford Protein Assay (Bio-Rad). According to instructions of the supplier, the protein concentration was calculated by comparison of the OD_{595} values with those of a BSA calibration curve. As a quality control, the ε_M of Trp-anxA5 obtained by Bradford assay favorably compares with its coefficient calculated by the software ProtParam provided by the Expasy Proteomics Server (www.expasy.ch) (33).

UV-Absorbance and Fluorescence Measurements. We acquired ε_M of Inds by UV_{280} measurement with the UV/VIS spectrometer lambda 19 (PerkinElmer and Life Sciences) by using sample concentrations of 4 μM in PBS (15 mM $Na_2HPO_4 \cdot 2H_2O$/1.8 mM KH_2PO_4/140 mM NaCl/2.7 mM KCl, pH 7.4). Fluorescence spectra of the proteins were measured by using a luminescence spectrometer LS50B (PerkinElmer and Life Sciences) at 20°C in Tris·HCl buffer

(100 mM, pH 7.5). The protein concentration was 0.5 μM. Spectra were recorded from 300–500 nm with an excitation wavelength of 280 nm (slit 10/10). Fluorescence spectra of Ind and (Aza)Ind were recorded at 20°C on excitation at the following maximum excitation wavelengths: Ind 269 nm, (4-Aza)Ind 288 nm, (5-Aza)Ind 266 nm, and (7-Aza)Ind 288 nm (excitation/emission slit 6/6 nm). Samples were dissolved in Tris·HCl buffer (100 mM, pH 7.5) or isopropanol.

Determination of QYs. QY of (Aza)Inds were recorded in Tris·HCl buffer (100 mM, pH 7.5). The absorbance maximum was determined by using the UV/VIS spectrometer lambda 19 (PerkinElmer and Life Sciences). Fluorescence spectra were recorded with the luminescence spectrometer LS50B (PerkinElmer and Life Sciences) at 20°C at the determined maximum absorbance wavelengths as follows and emission/excitation slits of 5/5: Ind 269 nm, (4-Aza)Ind 288 nm, (5-Aza)Ind 266 nm, and (7-Aza)Ind 288 nm. Excitation spectra were corrected automatically by the instrument for artifacts originating from the xenon lamp (energy output is wavelength and time-dependent) and from the excitation monochromator (efficiency is wavelength-dependent). Emission spectra were corrected with respect to artifacts originating from the emission monochromator (efficiency is wavelength-dependent) and the sample multiplier (sensitivity is wavelength-dependent). Correction files for emission spectra are yearly updated and provided by the manufacturer of the instrument (PerkinElmer and Life Sciences). All measurements were made with constant detector current. Fluorescence spectrum integration was performed with Origin 6.1G (OriginLab). QYs were calculated according to Lakowitz (1) with Trp as standard (QY = 0.14 in H_2O).

CD Spectra, Melting Curves, and Determination of Thermodynamic Parameters. CD spectra and melting curves of Trp-anxA5 and the (Aza)Trp variants were measured in PBS at protein concentrations of 0.2 mg per mL^{-1} by using the Circular Dichroism Spectropolarimeter JASCO J-715 (JASCO International). The measurement was performed in a 110-QS Hellma quartz cell (optical path-length: 0.1 cm) by using temperature control by a Peltier type FDCD attachment (model PFD-350S/350L; JASCO International). Ellipticity changes were recorded between 200 nm and 260 nm at 4°C for CD spectra. The α-helix proportion was calculated with CDNN CD spectra deconvolution (Version 2.1, Martin Luther University Halle-Wittenberg, Halle, Germany). Melting curves were measured at 222 nm as a function of temperature from 4°C to 95°C, gradient: 30°C h^{-1}. The fraction of unfolded protein was calculated from the corresponding ellipticity data. T_m value and van't Hoff enthalpy (ΔH_m) were determined as described (20).

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