Title: Minimalist IR and Fluorescence Probes of Protein Function.

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Abstract: Spectroscopic studies of small proteins and peptides, especially those requiring fine spatial and/or temporal resolution, demand synthetic probes that confer the minimal possible steric and functional change on the native properties. Here we review the recent progress in development of minimally disruptive probes for fluorescence and infrared spectroscopies, as well as the methods to efficiently incorporate them into proteins. Advances in spectroscopy on the one hand result in high specialization of synthetic probes for a particular purpose, but on the other hand allow for the same probes be used for different techniques to gather complementary biochemical information.

Introduction. Semisynthetic and synthetic probes provide invaluable tools for studies of many aspects of protein function, protein folding and protein-protein interactions offering a wealth of biochemical and biophysical information in both *in vitro* and *in vivo* systems. These probes allow for spectroscopic techniques to reach their full potential by either interrogating a particular spot in a macromolecule or by examining the interaction of multiple sites. The rapid progress of spectroscopic techniques we have witnessed over the past decades goes hand in hand with the discovery of new probes. The sheer number of the synthetic probes already developed to date and the versatility of their function is truly impressive! Nonetheless, there is plenty of room for improvement. From a very general standpoint, the “quality” of a probe can be assessed through evaluating three parameters: 1) its inherent utility, i.e. how well it examines the desired function; 2) ease and versatility of introduction of the probe into the protein of interest (ideally both *in vitro* and *in vivo*); 3)

the degree of the native function disruption. In this article we will focus on fluorescence and IR spectroscopies that offer several important advantages for studies of protein function: rapid and straightforward response, low cost and wide availability of the instrumentation, insensitivity to the physical state of the sample (e.g. solution, solid, lipid bilayers), ability to probe many different parameters, possibility of obtaining structural information [1] (Figure 1). This review highlights very recent advances in development of minimally disruptive probes, i.e. the probes that only introduce (or replace) few new atoms compared to the original protein (Figure 2), for fluorescence and IR (including 2D-IR) studies. Such probes are much less likely to produce a major functional change in the protein of interest. While engineered tRNA synthetases [2] have become the gold standard for incorporation of unnatural probes into proteins, they cannot accommodate all the chemical groups of interest. We will review recent work on development of non-canonical auxotrophic strains and semi-synthetic modification of proteins.

Fluorescence probes. Fluorescence spectroscopy is an excellent tool for studying protein–protein interaction, protein dynamics, local environment and protein-membrane interaction. While the ultimate non-disruptive fluorescent probes for such studies would be the intrinsic functionalities found in proteins themselves (tryptophan, tyrosine, cofactors), the experimental challenges associated with often low quantum yields and complex photophysics make the analysis not trivial.

Tryptophan, arguably the most common intrinsic fluorescent probe, has been widely utilized due to its high sensitivity to local environment and the ability to be excited independently of other amino acids. The former is also its Achilles' heel: large shifts of quantum yields and fluorescence maxima positions could potentially change FRET and quenching efficiencies in fluorescence studies and make the data analysis very complicated. The many tryptophan analogs reported throughout the years still possess the same properties [3,4]. Recently we demonstrated that β -(1-azulenyl)-L-alanine (AzAla), a tryptophan mimic, can serve as an excellent alternative to tryptophan [5]. Fluorescence properties of AzAla are not dependent on the local environment and allow for observation of very small effects exerted by the weak native quenchers inherently present in many proteins, such as histidine and methionine. Moreover, AzAla shows simple

monoexponential fluorescence decay and can be excited at 342 nm - well beyond the absorption of typical functional groups found in most proteins.

Replacement of the indole moiety in Trp by azulene results in a very conservative functional change. Incorporation of AzAla into smooth muscle myosin light chain kinase fragment did not perturb its interaction with calmodulin (CaM), moreover weak quenching of AzAla fluorescence by the methionines present in CaM allows for easy and straightforward determination of binding stoichiometry and K_d for this interaction [5]. Substitution of the indole by azulene does not influence the functional properties of antimicrobial peptides: the native and AzAla-substituted melittin show essentially identical CD spectra in the presence and in the absence of lipid bilayer and have very similar MIC values in bacterial inner and outer membrane permeabilization assays [6]. Moreover, electrophysiological characterization of the peptide-lipid bilayer interaction displayed a similar pore forming kinetics for both peptides.

High sensitivity of AzAla to local quenchers together with its insensitivity to local hydrophobicity and functional stability offer great promise for this tryptophan mimic as a chemical biology tool. In a proof-of-concept experiment we tested whether AzAla can help elucidate the mechanism of function of M2 influenza A (M2) proton ion channel, an integral membrane protein that is essential for viral entry into the cell [7]. Introduction of a single AzAla into a transmembrane M2 tetramer resulted in minimal change of the proton flux, suggesting that the mutant peptide associates with the wild type peptide without significant loss of function and a single AzAla residue exhibits no significant detrimental effect on the properties of the M2 ion channel. AzAla's fluorescence quenching by mild intrinsic quenchers allows for direct measurement of the protonation state of the neighboring histidine residue [8]. The pK_a values determined by this method are in excellent agreement with the data obtained by solid state NMR [9], but only required a simple fluorometer (Figure 3).

In a different approach to create minimalist fluorescence probes, Petersson *et al.* replaced the amide group in the peptide backbone with a thioamide. The incorporation of a single thioamide group is relatively straightforward and the resulting change is functionally conservative. Thioamide group efficiently quenches the native [10] and additionally introduced [11-13] minimal fluorescent probes to provide information about

protein folding and determine the distances between the fluorophore and the thioamide. Gai *et al.* have shown that 4-cyano-L-phenylalanine (PheCN) is quenched by selenomethionine, a naturally occurring analog of methionine, in a distance-dependent fashion [14]. The sensitivity of PheCN's fluorescence to the presence of local quenchers, such as deprotonated N-terminus, can be employed to determine the pK_a of various amino acid side chains in a wide range of values [15].

Quenching studies are not limited to the quenchers inherently present in proteins: the fluorescence of PheCN was effectively quenched by addition of iodide anion in order to determine the conformational heterogeneity of villin headpiece domain HP35 [16].

Synthetically incorporated probes can provide information about protein folding and distance constraints using FRET. Gai *et al.* demonstrated the applicability of PheCN as a FRET donor to 7-azatryptophan and 5-hydroxytryptophan (FRET acceptors) in model systems [17] (Figure 2). Moreover, the PheCN/Tryptophan and PheCN/7-azatryptophan FRET pairs can be used in conjunction with the time-resolved fluorescence decay measurements to investigate conformational heterogeneity of proteins. Analysis of the fluorescence decay provides the donor-acceptor distances in three different protein conformations [18]. Hecht *et al.* demonstrated that 6- and 7-cyanotryptophans, which have substantially higher fluorescence quantum yield as compared to that of Trp, can be used in a similar fashion [19].

IR probes. IR spectroscopy provides several important advantages as described above, but it does require a probe with a distinct IR-active functionality that can be observed in the presence of all other vibrational modes in a protein. Without resorting to isotopic labeling it also leaves only a handful of groups (most commonly those that contain triple bonds, i.e. nitriles, cyanates, thiocyanates, etc., and azides) to be suitable as probes. Fortunately, cyano and azido derivatives of many amino acids are readily available.

PheCN and 4-azido-L-phenylalanine (PheN₃) can be site-specifically introduced into proteins using engineered tRNA synthetases [20]. These probes were shown to be useful reporters of local hydration in proteins [21], however at least in one case new hydrogen bonds were introduced by the probe itself and caution should be used when interpreting the results [22]. The sensitivity and photostability of PheN₃ can be improved by replacing

the azide with a methylazido group (Figure 2) [23]. 5-cyanotryptophan (5-cyanoTrp) provides another probe for studies of local hydration with substantially higher molar absorption coefficient as compared to that of the PheCN [24]. The CN frequency of 5-cyanoTrp depends linearly on the solvent polarity and thus provides a quantitative handle for measuring local environment in proteins by IR spectroscopy [25]. Theoretical studies by Gai *et al.* established that the shift in the C-N stretching frequency of 5-cyanoTrp originates from a combination of the hydrogen bonding of the C-N and N-H groups and the non-specific interactions with the π^* orbital of the ring [26]. Cho *et al.* showed that the lifetime of excited vibrational states of the IR probes can be improved by replacing cyano probes with their thiocyanato and selenocyanato analogs (proline derivative ProSCN and ProSeCN) [27]. IR probes with longer vibrational times are desirable to study protein dynamics occurring on a longer time scales such as hundreds of picoseconds.

PheCN as well as a related azide-containing probe azidohomoalanine (Aha) have been shown to serve as excellent probes for 2D-IR studies of protein dynamics on the ultrafast timescale [28-30]. Measurements of the frequency-frequency correlations over a wide range of time provide information about the local dynamics of an amide and coupling between different modes in a secondary structure. The advantages of 2D-IR experiment over FTIR include better spectral discrimination of the underlying components along with the ability to separate the homogeneous and inhomogeneous contributions. Fayer *et al.* expanded the use of PheCN to investigate the protein dynamics in villin headpiece HP35 [31,32] and in myoglobin through the interaction of the probe with a heme-coordinated CO molecule [33].

Boxer *et al.* used vibrational Stark effect spectroscopy to establish the correlation between the solvatochromic shift in PheCN vibrational frequency and the electrostatic potential inside the protein [34]. Electric field in proteins has been recently shown to be intimately involved in chemical catalysis [35], thus the ability to probe local electrostatic environment is of great use.

Investigation of the protein function by IR is not limited to the probes introduced into amino acid side chains. Gai, DeGrado *et al.* demonstrated that a thioamide group can be used to interrogate the role of backbone-backbone hydrogen bonds in protein dynamics in

a site specific manner [36]. Laser T-jump IR experiments [37] allowed for characterization of backbone hydrogen bonding interactions in a β -hairpin model peptide.

Advances in incorporation of synthetic probes into proteins. Despite much effort by several research groups, introduction of some synthetic probes, including AzAla, into proteins using engineered amber codon tRNA synthetase was not successful prompting search for alternative methodologies. Some of the difficulties encountered with this approach can be due to the relatively restrictive biological machinery of *E. coli*. On the other hand, auxotrophic bacterial strains, which were historically the first tools for *in vivo* production of synthetically labeled proteins, are efficient and inexpensive [38], but suffer from fairly limited substrate scope.

Broos *et al.* created a tryptophan auxotroph (PA1002) of the gram-positive bacterium *Lactococcus lactis* [39] that co-expresses *L. lactis* tryptophanyl-tRNA synthetase (lacTrpRS) under a tightly controlled nisin promoter [40]. This strain is an attractive host for recombinant production of proteins, including membrane proteins. The resulting expression system is the most versatile Trp analog expression system known: it allows for incorporation of Trp analogs such as 7-azatryptophan, 5-hydroxytryptophan, 5-fluorotryptophan and 5-methyltryptophan [39] as well as AzAla [41], the latter cannot be introduced by any other means with high yield and incorporation efficiency.

Various (semi)synthetic methods for incorporation of thioamides into the protein backbone were devised by Petersson *et al.*, who employed native chemical ligation at either cysteine [13] or methionine [42] sites to fuse short, synthetically prepared thioamide fragments with recombinantly expressed proteins. Alternatively, thioamides may be introduced N-terminally using an aminoacyl transferase [43] or chemically activated thioamide-containing amino acid [44].

Cysteine alkylation is by far the most common method of introducing various functional groups into proteins. Gai, DeGrado *et al.* used this method to create a 4-cyanobenzyl derivative of cysteine [45] and Boxer *et al.* converted a cysteine to Cys-S-CN [46] to probe local environment using IR. Recent work by Pentelute, Buchwald *et al.*, which showed that

cysteines can be modified using organometallic palladium reagents [47], will undoubtedly further expand the repertoire of the synthetic probes for IR and fluorescence.

Conclusions. Potential interference of the unnatural probe introduced into a protein with protein's native function is always a major concern in every biochemical and biophysical technique. Studies of small proteins and peptides, especially those requiring fine spatial and/or temporal resolution, demand probes that confer the minimal possible steric and functional change on the native function. Moreover, the probes of choice must have useful spectroscopic features and paths for straightforward introduction into proteins. Thus finding functional groups to satisfy all these requirements is not an easy feat. It is hardly a surprise that a lot of synthetic probes reported to date are quite specialized and technique-driven. Indeed, if IR spectroscopy requires a distinct band in a fairly narrow range of available wavelengths, how many small functional groups can provide it? But as we showed above, even fairly limited repertoire of molecules gives a lot of useful biochemical information. Moreover, new features are being discovered in already known probes as a result of ongoing development of spectroscopic techniques. Based on analysis of the properties of IR and fluorescence probes presented above, it is apparent that the same reporter molecule introduced into a protein can provide additional information by simply applying a different technique (e.g. local environment by IR and conformation heterogeneity by fluorescence). Recent advances in methods for incorporation of synthetic probes are likely to result in new opportunities to investigate protein properties.

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Figure 1. Approaches to introduce IR and fluorescence probes into proteins and the types of biochemical information these probes provide.

Figure 2. Chemical structures of the probes discussed in this paper and their natural analogs. Natural amino acids have been modified to create minimally disruptive fluorescence and IR probes that: have high fluorescence quantum yield (7-azaTrp, 5-fluoroTrp, 6-cyanoTrp, 7-cyanoTrp); can be excited independently of Trp (7-azaTrp, 5-hydroxyTrp, 5-fluoroTrp, AzAla); are not sensitive to the local environment (AzAla, PheCN, 5-hydroxyTrp); have favorable photophysics (AzAla, 5-fluoroTrp); are suitable for FRET (donor: PheCN, acceptors 7-azaTrp, 5-hydroxyTrp, and 5-cyanoTrp); have strong vibrational signature (5-cyanoTrp, PheCN, 4-cyanobenzylCys, Cys-S-CN, 4-methylazidoPhe, PheN₃, Aha); have high photostability (4-methylazidoPhe); possess increased vibrational lifetime (ProSCN, ProSeCN); can be used for ultrafast measurements (Aha, PheCN).

Figure 3. Determination of pK_a of a histidine in a functional ion channel using AzAla fluorescence. a) A functional tetramer was assembled using three M2 transmembrane (TM) peptides and one Trp41AzAla M2 TM peptide. A model of a tetramer of M2 transmembrane domain containing a single AzAla residue (blue spheres). Side chains of residues other than His37 and Trp41 are omitted for clarity; waters are shown as red spheres. b) Fluorescence pH dependence of AzAla-containing M2 tetramer ($\lambda_{\text{ex}} = 342 \text{ nm}$, $\lambda_{\text{em}} = 382 \text{ nm}$). The fit of the fluorescence intensity to a multi-protonation state model provides pK_a values of proton-conducting histidines. Adapted from ref. 8.

Figure 1

Figure 2

NATIVE STRUCTURES

IR ACTIVE or FLUORESCENT ANALOG

Figure 3

a

b