

Illuminating proteins with Aladan's lamp

Monica Sundd and Andrew D. Robertson

A new fluorescent amino acid provides opportunities for a better understanding of electrostatics and dynamics in proteins and their role in protein function.

The structure and dynamics of a protein are intimately associated with its function. Exploring relationships between these properties has been challenging because the number of experimental tools is limited. In a recent paper in *Science*, Cohen *et al.*¹ describe an amino acid analog, which they call Aladan, that will be extremely useful for such research. They present both the chemical synthesis of Aladan (Fig. 1a), whose side chain is an environment-sensitive fluorophore, and its incorporation into polypeptides using a variation of *in vitro* translation and standard peptide-synthesis techniques.

The fluorescence of Aladan is highly sensitive to changes in polarity, making it a promising reporter for the physical and chemical environments at specific locations in both globular and membrane proteins. Aladan's properties also make it possible to investigate the dynamics of these environments on femtosecond to nanosecond time scales. These and other applications of Aladan should address major shortcomings in our present understanding of how proteins carry out their functions.

The basic problem

Noncovalent interactions are important for determining both a protein's three-dimensional structure and the extent to which it can interact and react with small ions, water and the thousands of other molecules in the cell. A major goal of many scientists is to learn how, for each protein, these interactions are organized at the molecular level and how their fluctuations are choreographed to bring about highly specific and efficient biochemical functions. This is where Aladan can help.

In principle, a detailed understanding of noncovalent interactions and their fluctuations should follow from knowledge of protein structure in conjunction with computations to calculate energies and dynamics. In practice, the complexity of real proteins still challenges our capacity to understand function accurately on the basis of structure and computation alone.

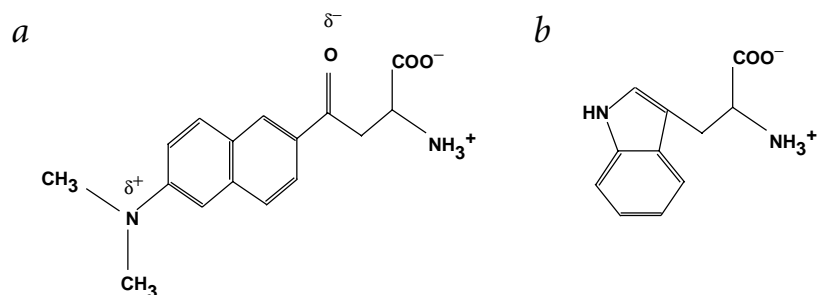


Fig. 1 Chemical structures of **a**, Aladan and **b**, tryptophan. The location of partial positive and negative charges in the UV-excited state of Aladan are indicated on the amino and carbonyl groups, respectively.

Moreover, detailed computational studies at the molecular level cannot be applied to the many interesting proteins of unknown structure, including most membrane proteins. Overall, quantitative experimental data regarding the environment within proteins — such as results that can be obtained using Aladan — are needed to assess and improve computations with proteins of known structure and to provide new information regarding structure, dynamics and function in proteins of unknown structure.

Fluorescence and protein electrostatics

A key application of Aladan is as a probe of protein electrostatics¹. The significance of electrostatic interactions in protein structure and function is well documented^{2–4}. For example, the known structures of ion channels are characterized by highly polar or charged transmembrane pores. The strength, distribution, and fluctuations of the resulting electric fields undoubtedly play an essential role in the rate, selectivity and regulation of ion transport⁵.

The functional significance of protein electrostatics has inspired many computational studies^{6,7}. Given the size of proteins and the number of solvent molecules that must be taken into account, all-atom electrostatics calculations are still an ambitious undertaking. Consequently, the simplest and most common approaches to calculating protein electrostatics treat the

protein as a relatively low-dielectric entity and the surrounding solvent as a high-dielectric environment^{6,7}.

The concept of a dielectric is embodied in Coulomb's Law, in which the free energy of interaction, ΔG , between two charges q_1 and q_2 (unitless residual charge) separated by a distance r (Å), is given by $\Delta G = 332q_1q_2 / (\epsilon r)$ in kcal mol⁻¹. The dielectric constant of the medium, ϵ , in which the charges are immersed, therefore modulates the interaction free energy. Significant questions remain regarding appropriate values of ϵ for the surfaces and interiors of proteins^{6,7}. In fact, proteins are structurally and chemically heterogeneous, so one might expect a wide variation in the dielectric properties and, consequently, the energetics of electrostatic interactions within proteins. In principle, one of the best ways to measure the variation in dielectric within a protein is with fluorescence spectroscopy.

Protein fluorescence has long been recognized as a potential measure of dielectric constants within proteins⁸, and Aladan should help realize this potential. Fluorescence is emitted upon relaxation of electronic excited states to the ground state. The energy difference between the excited and ground states is reflected in the wavelength of emission — larger energy differences mean shorter wavelengths. Because the dipole moment (that is, the separation between positive and negative charges) in the excited state is different from that in the ground state,

the energy difference between the excited and ground states (and, consequently, the wavelength of maximum emission, λ_{\max}) is sensitive to the protein dielectric at the fluorophore. Nonpolar environments (that is, small values of ϵ) increase the energy difference between excited and ground states, and the fluorescence maximum undergoes a blue shift, whereas more polar environments lead to a red shift in λ_{\max} . To estimate ϵ in a protein by fluorescence, λ_{\max} of the fluorophore in the protein is compared to λ_{\max} for the isolated fluorophore in a series of solvents with different values of ϵ .

Aladan has several key advantages over other fluorophores that have previously provided glimpses of the electrostatic environment within proteins^{1,8}. First, the fluorescent moiety in Aladan, DAN (6-dimethylamino-2-acylnaphthalene), is more sensitive to solvent polarity than are the intrinsic fluorophores, tyrosine and tryptophan. For example, the λ_{\max} in DAN decreases by over 100 nm going from water to cyclohexane⁹. In comparison, λ_{\max} for tryptophan decreases by ~40 nm⁸; λ_{\max} for tyrosine is insensitive to solvent polarity⁸. Second, the fluorescence of Aladan in proteins is easily resolved because the emission wavelengths of Aladan do not overlap with those of tyrosine and tryptophan. Third, the charges responsible for the excited state dipole of Aladan are localized to the amino and carbonyl substituents on the naphthalene ring (Fig. 1a), regardless of the environment around the fluorophore. In contrast, tyrosine and tryptophan each have two excited states that are similar in energy but in which the dipoles are nearly perpendicular to each other. The environment within proteins can lead to complex contributions from these various excited states of tyrosine and tryptophan⁸, and the simpler behavior with Aladan will facilitate interpretation about the structural basis for the observed fluorescence. Finally, and perhaps most importantly, Aladan can be incorporated into nearly any position in a protein using *in vitro*

translation or standard solid-state peptide synthesis, and its fluorophore is relatively close to the peptide backbone. These two factors decrease the uncertainty in positioning the fluorophore relative to the rest of the protein and leave little or no uncertainty in labeling efficiency compared to chemical modification of cysteine or lysine residues with fluorophores.

Dielectric constants in proteins have been estimated using a variety of experimental and theoretical approaches^{6,7}. Recent studies of pK values of buried ionizable groups in *Staphylococcal* nuclease offer a particularly well characterized set of estimates for the protein interior^{10,11}. It will be interesting to see the extent to which the dielectric constants in nuclease, and previous estimates for other proteins, agree with results for the same proteins in which Aladan has been incorporated. Looking beyond comparisons with previous studies, Aladan is likely to yield new information about electrostatics in more complex systems such as membrane proteins¹.

Fluorescence and protein dynamics

Aladan is likely to contribute significant new insights into motions in and around proteins. In general, time-resolved fluorescence spectroscopy is a rich source of information about motions within proteins and in the surrounding solvent^{8,12,13}. UV-excitation of a fluorophore occurs on the femtosecond time scale or faster, prior to any resulting protein or solvent rearrangements. The initial excited states are thus higher in energy and have shorter emission wavelengths than excited states populated at longer times because the surroundings have not yet relaxed in response to the new charge separation in the fluorophore. Consequently, the time between excitation and subsequent nanosecond-scale emission of fluorescence offers opportunities to follow the dynamics of protein and solvent motions. The results of Cohen *et al.*¹ for Aladan-containing variants of the B1 domain of protein G (GB1) demonstrate that Aladan is indeed a promising probe for such dynamics studies.

Possible limitations

While Aladan shows promise as a probe of protein electrostatics, dynamics and interaction, the large size of its side chain relative to tryptophan, the largest naturally-occurring amino acid (Fig. 1) may limit its application. As a test of Aladan's potential, Cohen *et al.*¹ demonstrate that incorporation of Aladan into two different potassium channels does not impair their function. Similarly, Aladan has been substituted into four different positions, two buried and two exposed, in the B1 domain of protein G and the variant proteins are folded. However, inspection of the supplementary material shows that substitutions at one exposed position and one buried position lead to substantial decreases in protein stability. Nevertheless, the substantial advantages of Aladan over other fluorophores and, more generally, over other probes of electrostatics and dynamics, are likely to make it a very useful and popular tool for exploring the relationship between structure and function in proteins.

Monica Sundd and Andrew D. Robertson are in the Department of Biochemistry, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242 USA. Correspondence should be addressed to A.D.R. email: andy-robertson@uiowa.edu

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