Eur Biophys J (2002) 31: 9–13
DOI 10.1007/s002490100182

REVIEW

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Site-specific tryptophan fluorescence spectroscopy as a probe of membrane peptide structure and dynamics

Received: 14 June 2001 / Revised version: 7 August 2001 / Accepted: 8 August 2001 / Published online: 3 October 2001

Abstract The fluorescence from tryptophan contains valuable information about the environment local to the indole side-chain. This environment sensitivity coupled with the ability to synthetically or genetically incorporate a single tryptophan residue at specific sites in a polypeptide sequence has provided the membrane biophysicist with powerful tools for examining the structure and dynamics of membrane peptides and proteins. Here we briefly review the use of site-specific tryptophan fluorescence spectroscopy to probe aspects of peptide orientation, structure, and dynamics in lipid bilayers, focusing on recent developments in the literature.

Keywords Tryptophan · Dynamics · Amphipathic helix · Lipid-protein interactions · Time-resolved fluorescence spectroscopy

Introduction

The full specification of membrane protein structure must include at least two main pieces of information. First, one would like to know the "usual" internal coordinates of the protein, including the Ramachandran polypeptide main-chain backbone dihedral angles and the rotamer angles that specify the orientation of the amino acid side-chains with respect to the main-chain. This information is usually sufficient to characterize the structure of water-soluble proteins in an isotropic medium. However, for membrane proteins and peptides, additional details, such as the orientation with respect to the membrane plane or the degree of penetration in the membrane, are required. The difficulty in addressing the first requirement is evident from the paucity of X-ray crystal structures of membrane proteins in the protein database, despite the high proportion of such proteins encoded in the genome. Fortunately, the questions of orientation and depth of penetration of membrane polypeptides are more easily addressed with the help of spectroscopic techniques (ESR, NMR, infrared, fluorescence, oriented CD), in many cases under conditions that better mimic the physiological environment of the protein, i.e. in a fluid hydrated lipid bilayer.

In the present paper we review the use of one such low-resolution spectroscopic technique, site-specific tryptophan fluorescence spectroscopy, to examine the structure and dynamics of membrane peptides. This method is based on the synthesis or genetic mutation of a series of peptide or protein analogues in which a single tryptophan residue is placed sequentially at specific positions of the peptide. By measuring the environmentally sensitive tryptophan fluorescence in each peptide- or protein-lipid complex as a function of tryptophan sequence position, one can learn about the disposition and dynamics of the peptide in the bilayer.

The format of the paper is as follows. First, we address the use of steady-state fluorescence footprinting measurements to gain insight into the average conformation and orientation of peptides in membranes. This analysis has benefited from the development of aqueous and lipid-resident quenchers which allow the relative transbilayer location of tryptophan residues to be quantitated. We then discuss the use of time-resolved spectroscopy to probe the conformational heterogeneity of the tryptophan residue at specific sites along the polypeptide. Recent studies on both membrane-associated and water-soluble proteins and peptides have established a direct correlation between the intensity decay components, rotamer conformations of the tryptophan residue, and secondary structure local to the indole

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fluorophore. Finally, we address the use of time-resolved anisotropy decay measurements, which probe the rotational diffusion of the indole ring, to map position-dependent protein flexibility in fluid membranes.

Steady-state fluorescence measurements of average peptide conformation and orientation

Transmembrane peptides

The first application of site-specific tryptophan fluorescence to the problem of average peptide orientation in the bilayer used single tryptophan alamethicin analogues to prove the peptide spans the lipid bilayer (Voges et al. 1987). These studies made use of the polarity-sensitive emission wavelength maximum of the tryptophan fluorophore and lipid-resident spin-label quenchers to define the relative transbilayer location of each tryptophan in the sequence. In these studies, the value of the emission wavelength maximum of each peptide was consistent with the expected orientation of the peptide in the bilayer. That is, tryptophans located near the centre of the peptide sequence had emission wavelength maxima consistent with a location within the non-polar hydrocarbon core of the lipid bilayer. The tryptophans near the ends of the sequence had emission maxima consistent with a more polar environment provided by the glycerol backbone/head-group region of the bilayer. This study used fatty-acid-based lipid resident quenchers located at defined depths in the bilayer to reveal the relative transmembrane positions of the tryptophan residues in the peptides. The transmembrane orientation was also observed by oriented CD spectroscopy in lipid multilayers (Vogel et al. 1988).

London and co-workers (Ren et al. 1997) have generated a series of single tryptophan transmembrane peptides which has provided an important source of information concerning the quantitative relationship between tryptophan emission wavelength and tryptophan depth in phospholipid bilayers. The tryptophan depth in C-18 bilayers was quantitated using the parallax method and a series of phospholipid-based lipid resident quenchers. As expected for transmembrane peptides, tryptophans located near the centre of the peptide sequence were located furthest from the membrane surface and exhibited the lowest emission maxima (318 nm). As the tryptophan residue was moved progressively closer to the membrane surface, an increase in maximum emission wavelength was observed. A plateau in emission wavelength to ca. 337 nm was observed for tryptophans located closest to the bilayer surface and in the head-group region. London's laboratory have exploited this relationship to determine the equilibrium constant for formation of non-transmembrane peptide orientations induced through changing properties of the lipid bilayer such as cholesterol content or acyl-chain length. The results of this study provided direct evidence that the steady-state fluorescence spectrum of tryptophan in transmembrane peptides could be used to quantitate the relative position of a solvent-exposed tryptophan residue in lipid bilayers (see Fig. 1).

In-plane helices

Chung et al. (1992) used the approach of O'Neil et al. (1987) to study the zero-applied voltage state of model ion channels based on the ideally amphipathic sequence $(LSSLLSL)_3$. In this study the properties of the tryptophan fluorescence were correlated with the location of the residue with respect to the polar or non-polar face of the helix i.e. along a plane perpendicular to the long axis of the helix. The periodicity (3.8 residues per turn) of the tryptophan emission wavelength maxima and the collisional quenching by an aqueous quencher cesium ion showed that these peptides were on average α-helical and oriented with the α-helical axis parallel to the bilayer surface. Analysis of the lipid-based quenching showed that the non-polar face of the helix could lie within the region of the phospholipid acyl chains while that of the polar face was located within the polar head-group region of the phospholipid. On the basis of these results the formation of an ion-channel conducting state was hypothesized to involve the re-orientation of the helices from their initial parallel orientation on the lipid surface to a transmembrane orientation (see Fig. 2).

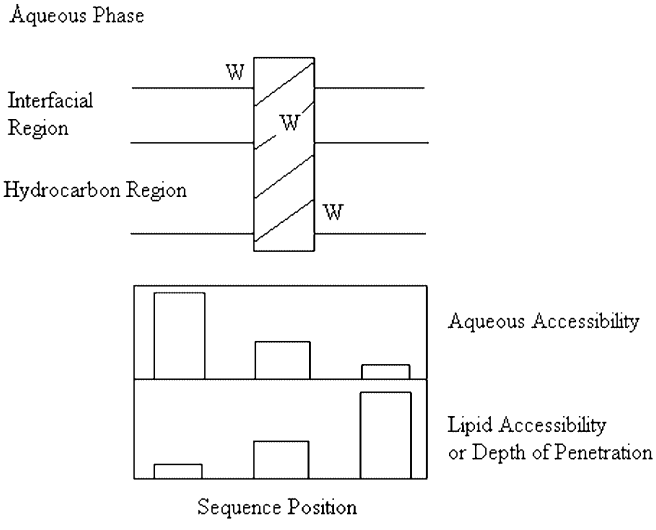

Fig. 1 *Top*: model of a transmembrane peptide with single tryptophan substitutions (denoted by *W*) at various positions along the peptide sequence and exposed to the aqueous phase, to the interfacial region, and to the hydrocarbon region of a lipid bilayer (inner leaflet not shown). *Bottom*: accessibility profile as a function of tryptophan position for the transmembrane peptide. Note the dependence of the aqueous and lipid accessibilities on sequence position. The aqueous accessibilities can be measured using water-soluble quenchers or using tryptophan emission maxima (corrected for sequence induced effects; see Ren et al. 1997). The lipid accessibilities or depth of penetration of the tryptophan residues are measured using quenching by spin-labelled lipids (Voges et al. 1987; Ren et al. 1997; Ladokhin 1999a)

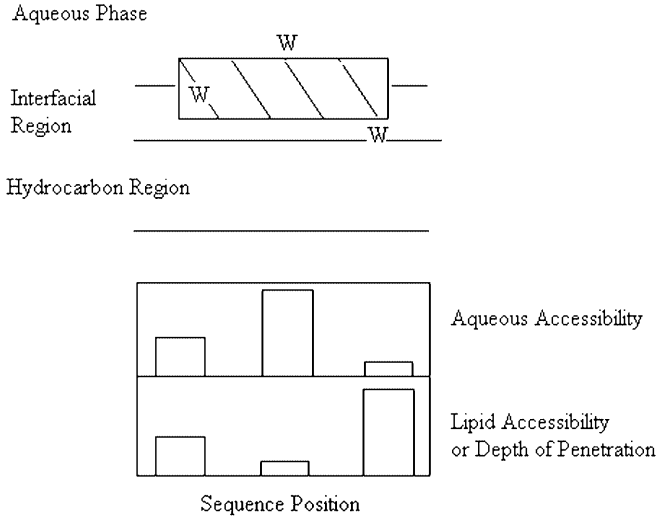

Fig. 2 *Top*: model of an in-plane peptide on the outer leaflet of a lipid bilayer with single tryptophan substitutions (denoted by *W*) in the interfacial region, the aqueous phase, and in the hydrocarbon region (inner leaflet not shown). *Bottom*: accessibility profile as a function of tryptophan position for the surface-associating peptide. Note the dependence of the aqueous and lipid accessibilities on sequence position; in general the periodicity of this function is related to the secondary structure of the peptide (see Chung et al. 1992; Clayton and Sawyer 1999a). The aqueous accessibilities can be measured using water-soluble quenchers or using tryptophan emission maxima (corrected for sequence induced effects; see Ren et al. 1997). The lipid accessibilities or depth of penetration of the tryptophan residues are measured using quenching by spin-labelled lipids (Voges et al. 1987; Ren et al. 1997; Ladokhin 1999a)

Clayton and Sawyer (1999a) used the tryptophan emission wavelength maximum, acrylamide aqueous-phase quenching and lipid-resident quenching to prove that class A amphipathic helices align predominantly parallel to the lipid surface. The position-dependant tryptophan fluorescence parameters were not consistent with a transmembrane orientation or a surface orientation with an average periodicity of 2, 3 or 4.4 residues. However, a surface associating structure with a periodicity of 3.6 residues per turn could account for the data suggesting an α-helix. Moreover, the values of the emission wavelength maxima of the tryptophans located in the non-polar face of the helix suggested that the helices penetrated no deeper than the carbonyl region of the phospholipid ester groups. These results were corroborated by oriented CD and X-ray diffraction measurements in fluid multilayers (Hristova et al. 1999).

According to current theories of lipoprotein structure, the helices of apolipoproteins are either organized parallel to the lipid surface in a belt arrangement around the lipid particle or perpendicular to the monolayer surface and parallel to the acyl chains of the phospholipid. Maiorano and Davidson (2000) employed site-specific tryptophan fluorescence to determine the orientation and depth of penetration of a helix derived from apolipoprotein A-I in reconstituted high-density lipoproteins. Two tryptophan mutant proteins were expressed, each with a single tryptophan located two helical turns distant in the primary sequence. Using parallax analysis of lipid-resident quenching the authors showed that the mutated tryptophan residues in helix 4 of apolipoprotein were located about 6 Å from the centre of the bilayer and parallel to the membrane surface. Their analysis was in agreement with the belt model for lipoprotein structure and provided one of the first experimental measurements of the location of an apoA-I helix with respect to the bilayer edge.

Membrane topology

Wimley and White (2000) have developed a novel method of determining the membrane topology of tryptophan-containing peptides. They synthesized a lyso-phospholipid-based quencher that utilizes a methyl-coumarin chromophore attached to the head-group of lyso-phosphoethanolamine. The coumarin derivative quenches tryptophan fluorescence via dipole-dipole Forster energy transfer. The quencher characteristics are such that only tryptophans on the same bilayer leaflet as the quencher are quenched. The membrane topology of a number of tryptophan peptides was obtained by comparing the degree of quenching in symmetric (both leaflets labelled) and asymmetric (outer leaflet labelled) lyso-quencher-labelled vesicles. This technique should be of use in assessing whether a given peptide can equilibrate freely across the bilayer, form a stable transmembrane structure or remain only on one surface. In combination with site-specific tryptophan fluorescence, this lipid quencher could, in principle, be used to determine the direction of insertion of transmembrane peptides.

Evaluation of lipid exposure of tryptophan residues

Ladokhin (1999a) has presented a unified formalism for determining the degree of tryptophan penetration in a membrane using lipid-resident quenchers, and utilized the new approach to study temperature-dependent conformational changes in tryptophan mutants of a membrane anchoring non-polar peptide of cytochrome b_5.

Time-resolved fluorescence spectroscopy of tryptophan conformation in membrane peptides

In proteins and peptides, tryptophan fluorescence usually displays a complex decay profile which has been attributed to excited-state reactions (Hudson et al. 1999; Ladokhin 1999b), a distribution of sub-states (Alcala et al. 1987; Alcala 1995) or multiple micro conformations of the tryptophan residue (Szabo and Rayner 1980; Chen et al. 1991; Millar 1996; Sillen et al. 2000). In the latter case, the ground-state conformers have been identified as rotamers of tryptophan that arise from rotation of the indole ring about the C_α-C_β and/or the

C_β-C_γ bond, the interconversion between rotamers being slow relative to the fluorescence time-scale. Recent studies of water-soluble peptides and proteins have established a correlation between the tryptophan rotamer population distribution, the proportion of different lifetime components in the intensity decay and the secondary structure local to the indole chromophore (Millar 1996). In certain cases the prediction of the actual fluorescence lifetime values of each of the tryptophan conformers has been possible, based on spectroscopic data and molecular dynamics simulations (Sillen et al. 2000). This raises the interesting possibility that time-resolved tryptophan spectroscopy might be useful in examining the tryptophan conformations of membrane peptides and proteins.

A study of the time-resolved tryptophan fluorescence from five single-tryptophan analogues of the class A amphipathic helix has shown a correlation between the proportions of different lifetime components in the intensity decay and the tryptophan rotamer distributions in the protein database (Clayton and Sawyer 1999b). In aqueous solution, the peptides were unstructured and a triple exponential function was required to fit the decay data. The average pre-exponential factors of the three lifetime forms of the peptides matched closely to the average $\chi 1$ distributions (reflecting rotation about the C_α-C_β bond) of tryptophan residues located in unstructured regions of native proteins as determined by X-ray crystallography. Association of the peptides with small unilamellar vesicles composed of egg phosphatidylcholine induced a helical conformation in the peptides and reduced the complexity of the tryptophan intensity decay to a double-exponential function. There was striking similarity between the proportions of the lifetime forms in the intensity decay of peptide-lipid complexes and the rotamer distributions for tryptophan residues found within α-helical regions of globular proteins. The conclusion reached from the average time-resolved fluorescence concerning the structure of the peptide at the lipid surface was in accord with that based on the steady-state fluorescence analysis. Analysis of the rotamer distributions as a function of tryptophan position revealed a secondary correlation between the population amplitude of the short lifetime component fluorescence and the disposition of the tryptophan residue relative to the lipid and aqueous phases. This correlation suggests that tertiary interactions with the lipid influence the rotamer distributions so as to decrease the frequency of the less populated rotamer. This conclusion is in accord with the analysis of side-chain distributions and tertiary interactions in the crystal database of proteins.

Soulages and Arrese (2000) studied the interaction of five single-tryptophan mutants of the apolipoprotein apolipophrin-III with a lipid surface. Each mutant contained a tryptophan residue in the non-polar face of one of five different amphipathic α-helical domains. Interestingly, the intensity decays of these helical mutants when bound to lipid were described by a double exponential function with the average population of the lifetime forms similar to those expected for tryptophans in helical regions of proteins.

Time-resolved anisotropy decay and tryptophan dynamics in membrane peptides

Time-resolved fluorescence anisotropy studies of membrane proteins and peptides are capable of providing direct information on structural fluctuations occurring during the excited state lifetime of the fluorophore. In the case of tryptophan, the orientation of the indole side-chain can be altered by changes in the χ_1 and χ_2 angles, due to rotations about C_α-C_β and C_β-C_γ bonds, by changes in the Ramachandran angles φ and ψ of the local peptide backbone or by whole body tumbling.

A particularly interesting application of fluorescence anisotropy decay to membrane peptides is provided by the work of Vogel et al. (1988), who mapped the site-specific tryptophan motional dynamics at different sequence positions along the length of a transmembrane peptide. The dynamics of the tryptophans was greater near the ends of the peptide rather than at the centre, despite there being a bilayer microviscosity gradient in the opposite direction. These data showed the existence of rapid sub-nanosecond internal fluctuations superimposed on hindered helical orientational fluctuations of the order of 10 ns.

Clayton and Sawyer (2000) have employed similar methodologies to study the site-specific tryptophan dynamics in class A amphipathic peptides at a phospholipid bilayer interface.

At least two molecular motions of the indole ring were resolved from the anisotropy decays. A short correlation time of 3 ns, which was associated with the short lifetime component in the intensity decay, and a long correlation time of 30 ns, which was associated with the long lifetime component in the intensity decay. The amplitude of the short correlation time was dependent on the position of the tryptophan on the hydrophobic or hydrophilic side of the helix, with the tryptophan facing the aqueous phase showing a greater degree of depolarization than the tryptophans facing the lipid. The short lifetime/short correlation time was attributed to a solvent-exposed rotamer, which reorients with respect to the polypeptide backbone via librations or rotation about the C_β-C_γ and/or C_α-C_β bonds.

The long correlation time of 30 ns was of the order of magnitude expected for rigid body precession or rigid rod tilting motions in the lipid bilayer. The amplitude of this motion correlated with the position of the tryptophan in the sequence and revealed a decreased contribution as the tryptophan was moved progressively from the N-terminus to the C-terminus of the peptide. Interestingly, the increase in indole dynamics with sequence position correlated with the increase in level of polypeptide disorder derived from X-ray diffraction studies (Hristova 1999).

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