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Time-resolved EPR immersion depth studies of a transmembrane peptide incorporated into bicelles

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ABSTRACT

The reduction in EPR signal intensity of nitroxide spin-labels by ascorbic acid has been measured as a function of time to investigate the immersion depth of the spin-labeled M2δ AChR peptide incorporated into a bicelle system utilizing EPR spectroscopy. The corresponding decay curves of *n*-DSA (n = 5, 7, 12, and 16) EPR signals have been used to (1) calibrate the depth of the bicelle membrane and (2) establish a calibration curve for measuring the depth of spin-labeled transmembrane peptides. The kinetic EPR data of CLS, *n*-DSA (n = 5, 7, 12, and 16), and M2δ AChR peptide spin-labeled at Glu-1 and Ala-12 revealed excellent exponential and linear fits. For a model M2δ AChR peptide, the depth of immersion was calculated to be 5.8 Å and 3 Å for Glu-1, and 21.7 Å and 19 Å for Ala-12 in the gel-phase (298 K) and L_α-phases (318 K), respectively. The immersion depth values are consistent with the pitch of an α-helix and the structural model of M2δ AChR incorporated into the bicelle system is in a good agreement with previous studies. Therefore, this EPR time-resolved kinetic technique provides a new reliable method to determine the immersion depth of membrane-bound peptides, as well as, explore the structural characteristics of the M2δ AChR peptide.

1. Introduction

Membrane-bound proteins are known to play important roles in controlling physiological function in organisms. Immersion depth studies are critical to our understanding of the three dimensional structure of membrane proteins and peptides and, thus, provide important insights into the function of these molecules [1]. Membrane immersion depth is commonly determined by neutron diffraction techniques, nuclear magnetic resonance (NMR) spectroscopy, fluorescence resonance energy transfer (FRET) spectroscopy, parallax analysis of fluorescence quenching by spin-labels, and collision gradient and rotational correlation time methods utilizing electron paramagnetic resonance (EPR) spectroscopy [1–11]. In this paper, an alternative approach has been used to explore the immersion depth of membrane-bound proteins utilizing EPR spectroscopy. A reliable new bicelle technique is presented to determine the membrane immersion depth of single site spin-labeled peptide or proteins by EPR spectroscopy. To demonstrate the applicability of this method a highly studied transmembrane peptide M2δ, of the pentameric transmembrane protein ($\alpha_2\beta\gamma\delta$) AChR was used [12–14]. The method is based on kinetic analysis of the reaction of the ascorbic acid molecule with the paramagnetic nitroxide group of a spin probe incorporated into phospholipid bilayers. The structures of various spin-labels, cholestane (CLS) and *n*-doxylstearic acids (*n*-DSA; *n* = 5, 7, 12, or 16), were used in this study and the relative locations of their nitroxide moieties within the phospholipid bilayers are characterized in Fig. 1. Ascorbic acid permeates into the phospholipid bilayers subsequent to its addition into the membrane sample and gradually reduces the bilayer embedded paramagnetic nitroxides into diamagnetic hydroxylamines [15,16]. Direct analysis of the kinetics of reduction of these spin-labels and the corresponding rate constants is exploited to establish a calibration curve to probe the immersion depth of specific residues of spin-labeled transmembrane M2δ AChR peptide.

At physiological pH value, taking into account the pK_1 and pK_2 values of ascorbic acid, this study reveals that the oxidation–reduction reaction between the nitroxide group and ascorbate ion in the bicelle system obey a first order rate law. If the reaction is simple and limited by diffusion of ascorbate from the aqueous partition to the inner part of the membrane, with the nitroxide localized in the later, its kinetics will

Abbreviations: L_α-phase, liquid–crystalline phase; M2δ, M2 segment of the δ subunit; AChR, nicotinic acetylcholine receptor; EPR spectroscopy, electron paramagnetic resonance spectroscopy; CLS, cholestane (3β-doxyl-5α-cholestane); *n*-DSA (*n* = 5, 7, 12, and 16), 5, 7, 12, and 16-doxylstearic acids; DMPC, 1, 2-Dimyristoyl-*sn*-glycerol-3-phosphocholine; DHPC, 1, 2-Dihexanoyl-*sn*-glycerol-3-phosphocholine; Δχ, magnetic susceptibility anisotropy tensor; [1], amino acid residue 1; [12], amino acid residue 12; SL, spin-labeled; [1] SL-M2δ AChR, M2δ AChR peptide subunit spin-labeled at amino acid residue 1; [12] SL-M2δ AChR, M2δ AChR peptide subunit spin-labeled at amino acid residue 12; NMR spectroscopy, nuclear magnetic resonance spectroscopy; FRET, fluorescence resonance energy transfer; MTSSL, 1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl-methanethiosulfonate.

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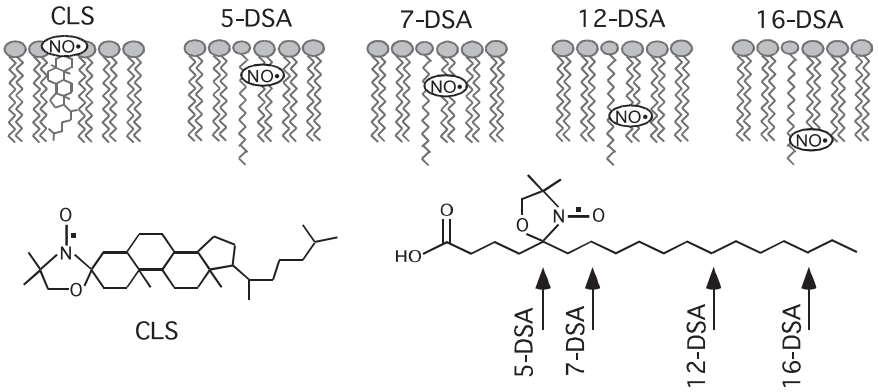

Fig. 1. Chemical structures of CLS, 5-DSA, 7-DSA, 12-DSA, and 16-DSA spin-labels used in this study and the relative locations of their nitroxide moieties (NO•) within the phospholipid bilayers. The arrow indicates the position of the doxyl labels attached to the acyl chain of the stearic acid.

be zero order with respect to the nitroxide. The data analysis of this rate law is not always straightforward, as the reaction is also known to be an overall second order reaction in other studies. The order of chemical reaction is complicated by several factors such as, permeability, composition of the membrane system, pH, type of nitroxide, mode of preparation, and temperature [17].

In this report, we examine the immersion depth of membrane-bound rigid spin-labeled M2δ AChR utilizing kinetic measurement X-band EPR spectroscopy, in order to explore the structural characteristics of the transmembrane segment of the receptor into the bicelle system. The kinetic method employed in this work provides a novel and accurate method to determine the immersion depth of site-specific residues of membrane-bound peptides. The kinetic measurement EPR bicelle method is pertinent due to the good agreement between the proposed structural model of M2δ AChR using different biophysical methods.

2. Methods

2.1. Materials

Fmoc amino acids and other chemicals for peptide synthesis were purchased from Applied Biosystems Inc. (Forster City, CA), 1, 2-Dimyristoyl-*sn*-glycerol-3-phosphocholine (DMPC), 1, 2-Dihexanoyl-*sn*-glycerol-3-phosphocholine (DHPC), and 1,2-dimyristoyl-*sn*-glycero-3-phosphoetholamine-N-[poly(ethylene glycol) 2000] (PEG2000) were purchased from Avanti Polar Lipids Inc. HEPES (N-[2-hydroxyethyl] piperazine-N-2-ethanesulfonic acid), trifluoro acetic acid (TFA), 2, 2, 2-trifluoroethanol (TFE), CLS, 5-DSA, and 16-DSA were purchased from Sigma/Aldrich. 7-DSA was purchased from ICN. 12-DSA and (MTSSL) were obtained from Toronto Research Chemicals Inc. The cholesterol was obtained from Avanti polar lipids, Ltd. All lipids, cholesterol, and spin-labels were dissolved in chloroform and stored at −20 °C prior to use. Ascorbic acid was purchased from Fisher Scientific. Aqueous solutions of ascorbic acid prepared fresh each day. All aqueous solutions were prepared with deuterium-depleted water purchased from Isoec Inc. (Miamisburg, OH). Double distilled water can alternately be used to prepare these solutions.

2.2. Peptide synthesis of M2δ AChR synthesis

Solid-phase peptide synthesis using Fmoc-protection strategy was performed on a 433A peptide synthesizer from Applied Biosystems Inc. (Foster City, CA) to synthesize the model membrane peptides A12C M2δ AChR and TOAC1 M2δ AChR ([1] SL-M2δ AChR). The peptide synthesizer was equipped with a UV detector (wavelength set to 301 nm) to monitor the Fmoc removal from the N-terminus of the growing peptide. The *FastFmoc chemistry*-0.1 mmol protocol provided in the *SynthAssist* 2.0 software (Applied Biosystems Inc.) was modified by our lab to optimize the yield of the synthesis and allow for customized functionality [18]. An appropriate amount of Fmoc-Arginine(pbf)-*NovaSyn TGA* resin (substitution number = 0.22 g resin/mmol peptide) was used to synthesize 0.1 mmol of peptide (based on a theoretical yield of 100%). All amino acids were purchased as Fmoc-protected with chemically sensitive side-chain residues being chemically modified (side-chain protected) to minimize any side reactions. Synthesized peptides were removed from the synthesizer and cleaved from the resin. The spin-labeled [1] SL-M2δ AChR peptides were treated with NH_3 (aq) to reoxidize the spin-label back to the nitroxide form, because TFA from the cleavage mixture reduces nitroxides to the hydroxylamine form. The Fmoc-TOAC synthesis was reformed according to published procedures [19,20]. The α-helical M2δ transmembrane segment of AChR consists of 23 amino acid residues and has the following amino acid sequence:

$$\left(NH_2-E^1K^2M^3S^4T^5A^6I^7S^8V^9L^{10}L^{11}A^{12}Q^{13}A^{14}V^{15}F^{16}L^{17}L^{18}L^{19}T^{20}S^{21}Q^{22}R^{23}-COOH\right).$$

The amino acids underlined represent the spin-labeled sites used in this study.

2.3. Peptide purification

The peptides (A12C M2δ AChR, [1] SL-M2δ AChR, and [12] SL-M2δ AChR) were purified on an Amersham Pharmacia Biotech AKTA Explorer 10S HPLC controlled by Unicorn version 3 system software. A C4 protein column (214TP104, 10 μm, 300 Å pore size, 0.46 × 25 cm, column volume of 4.15 mL) from Grace-Vydac was used to purify the peptides. A 1 mL aliquot of the (5 mg/mL) peptide sample was injected into the column, and the peptide elution was achieved with a linear gradient to a final solvent composition of 95% of solvent B at a flow rate of 8 mL/min. The purified peptide fraction was lyophilized and analyzed by MALDI-TOF mass spectrometry. The lyophilized M2δ AChR peptide was dissolved in TFE and centrifuged to eliminate insoluble particles. The column was equilibrated with 90% solvent A/10% solvent B. Solvent A consisted of H_2O and 0.1% TFA and solvent B was 80% IPA, 20% H_2O, and 0.1% TFA. The lyophilized [1] SL-M2δ AChR peptide was dissolved in 5% HFIP, 50% n-propanol, and 45% H_2O (at a concentration of 5 mg/mL). The column was equilibrated with 90% solvent A/10% solvent B. Solvent A consisted of H_2O and 0.1% TFA and solvent B was 50% n-propanol, 30% ACN, 20% H_2O, and 0.1% TFA. The lyophilized [12] SL-M2δ AChR peptide was dissolved in methanol (at a concentration of 5 mg/mL). The column was equilibrated with 80% solvent A/20% solvent B. Solvent