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Spin-echo EPR of Na,K-ATPase unfolding by urea

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ABSTRACT

Denaturant-perturbation and pulsed EPR spectroscopy are combined to probe the folding of the membrane-bound Na,K-ATPase active transport system. The Na,K-ATPase enzymes from shark salt gland and pig kidney are covalently spin labelled on cysteine residues that either do not perturb or are essential to hydrolytic activity (Class I and Class II –SH groups, respectively). Urea increases the accessibility of water to the spin-labelled groups and increases their mutual separations, as recorded by D_2O interactions from ESEEM spectroscopy and instantaneous spin diffusion from echo-detected EPR spectra, respectively. The greater effects of urea are experienced by Class I groups, which indicates preferential unfolding of the extramembrane domains. Conformational heterogeneity induced by urea causes dispersion in spin-echo phase-memory times to persist to higher temperatures. Analysis of lineshapes from partially relaxed echo-detected EPR spectra indicates that perturbation by urea enhances the amplitude and rate of fluctuations between conformational substates, in the higher temperature regime, and also depresses the glasslike transition in the protein. These non-native substates that are promoted by urea lie off the enzymatic pathway and contribute to the loss of function.

1. Introduction

The Na,K-ATPase active transport enzyme is an integral protein of the plasma membrane that is responsible for maintaining ionic homeostasis in eukaryotes. The α-subunit has a multiple-spanning transmembrane sector, with associated single-spanning accessory and regulatory β- and γ-subunits, and a large, globular cytoplasmic sector that contains the nucleotide-binding and hydrolytic sites [1–3]. The analogous P-type Ca-ATPase of the sarcoplasmic or endoplasmic reticulum (SERCA) is similar in structure to the α-subunit of the Na,K-ATPase [1,2].

Perturbation by aqueous denaturants affords a valuable means to probe the folding of globular and membrane proteins [4–10]. For instance, both infrared spectroscopy and activity measurements have been used to study denaturant-mediated unfolding of the SERCA Ca-ATPase [11,12]. Fig. 1 illustrates the urea-induced unfolding of Na,K-ATPase from two different species, as monitored by conventional continuous-wave electron paramagnetic resonance (CW-EPR)[1] spectroscopy of a maleimide spin label covalently attached to Class II [13] cysteine residues [14]. Unfolding of those domains of the membranous protein that are accessible to urea results in conversion to a weakly immobilized (*W*) state of spin-labelled residues, which, in the native protein, are strongly immobilized (*S*) on the conventional EPR timescale. The sigmoidal dependence on denaturant concentration reflects the cooperative nature of the unfolding transitions. The effects of urea on the CW-EPR spectra are completely reversible [14], reflecting reversible structural changes associated with the folding/unfolding process.

Recently, we have shown that the combination of conventional CW-EPR with spin-echo EPR spectroscopy [15] can be used to characterize the conformational heterogeneity of the Na,K-ATPase [16]. Conformational substates [17,18], which are frozen in at low temperatures, are detected by inhomogeneous broadening of the CW-EPR spectra and dispersion in phase-memory relaxation times of the spin-labelled protein. Motional averaging of these conformational substates is driven by librational fluctuations that are observed by echo-detected EPR spectra. In addition, modulation of the spin-echo decays by hyperfine interaction with D_2O (^{2}H-ESEEM) was shown to reveal the differential accessibility to water of different classes of spin-labelled –SH groups in the Na,K-ATPase.

In the present work, we use this combination of CW-EPR and pulsed EPR to investigate the urea-induced unfolding of membranous Na,K-ATPase from both shark salt gland and pig kidney. D_2O-ESEEM quantifies the increase in exposure to water of those parts of the protein that are unfolded in urea. ED-EPR spectra detect the onset of librational motions that drive transitions between conformational substates. Echo decay rates probe the increase in conformational heterogeneity that is induced by urea. Deconvolution of the CW-EPR lineshapes shows the effect of urea on motional averaging of the on-pathway substates. In addition, ED-EPR spectra at low temperature,

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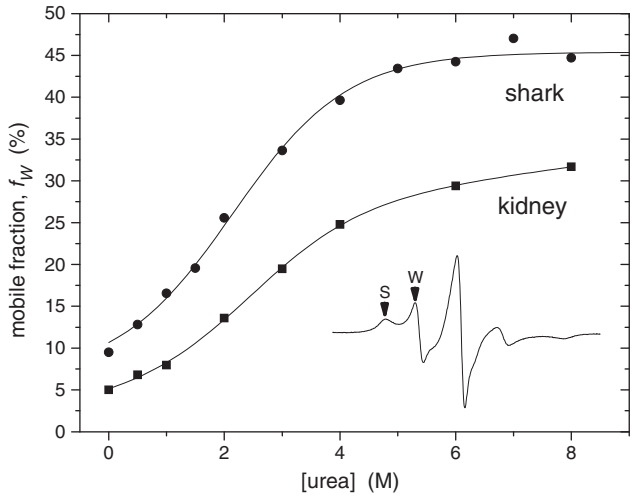

Fig. 1. Fraction, f_W, of weakly immobilized component in the CW-EPR spectra of shark salt gland (circles) and pig kidney (squares) Na,K-ATPase spin labelled with 5-MSL (pH 7.4), as a function of urea concentration, at 4 °C. 5-MSL is covalently bound to Class II sulphydryl groups. Inset: CW-EPR spectrum of shark Na,K-ATPase in 1 M urea; W and S indicate the weakly and strongly immobilized components, respectively. Data are from Ref. [14].

which are sensitive to instantaneous diffusion that arises from mutual spin–spin interactions, reveal the extension of the polypeptide structure that results from urea-induced unfolding.

2. Materials and methods

2.1. Enzyme preparation

The Na,K-ATPase membranes from pig kidney and shark salt gland were prepared as described earlier [19,20]. Specific activities were 28 and 32 μmol P_i generated per milligram protein per minute at 37 °C, for kidney and shark preparations, respectively. The shark enzyme was stored at a protein concentration of approx. 5 mg/ml in 20 mM histidine and 25% glycerol (pH 7.0) and the kidney enzyme at a protein concentration of approx. 4 mg/ml in 20 mM histidine, 250 mM sucrose, and 1 mM EDTA (pH 7.0). Protein concentrations were determined by using the Lowry method [21], and enzymatic assays were performed as described previously [22].

2.2. Spin labelling of Na,K-ATPase membranes

Prelabelling of shark Na,K-ATPase with NEM to block Class I –SH groups, and sulphydryl groups of non-Na,K-ATPase proteins in the membrane preparations, was performed as described previously (see Refs. [16] and [23] for details). The prelabelled shark enzyme had more than 95% of the initial specific activity and was stored in 20 mM histidine and 25% (v/v) glycerol at −20 °C. Prelabelling of kidney Na,K-ATPase followed the same route (see Ref. [16] for details). The prelabelled kidney enzyme had more than 90% of the initial specific activity and was stored at −20°C in 20 mM histidine, 250 mM sucrose, and 1 mM EDTA (pH 7.0).

Class I –SH groups in the Na,K-ATPase membranes were spin labelled with 5-MSL (3-maleimido-1-oxyl-2,2,5,5-tetramethylpyrrolidine; Sigma, St. Louis, MO) by using the same protocol as that for prelabelling with NEM. For shark enzyme, the native membranes were treated with 0.2 mM 5-MSL, and the spin-labelled enzyme had more than 95% of the initial specific activity. For kidney enzyme, a spin label concentration of 1.25 mM was used, and the spin-labelled enzyme had more than 90% of the initial specific activity.

Selective spin labelling of the Class II –SH groups, which are essential to the overall Na,K-ATPase activity, was performed with NEM-prelabelled enzyme in the presence of 3 mM ATP as described previously [13,16]. The concentration of 5-MSL used was 0.125 mM for shark enzyme and 1.0 mM for kidney, resulting in residual activities of 8% and 20%, respectively.

2.3. Deuterium exchange of spin-labelled membranes

A buffer was prepared by using D_2O containing the following salts: 11 mM Tris, 11 mM CDTA, and 22 mM NaCl (pH 7.0 at 20 °C). 5-MSL spin-labelled Na,K-ATPase (see above) was pelleted by centrifugation, and approximately 15 mg protein was homogenized in 10 ml of the D_2O buffer. This membrane suspension was incubated at 14 °C for 60 min and pelleted by centrifugation at 100,000×g for 2 h at 14 °C. The pellet was homogenized in 10 ml of the D_2O buffer and incubated at 14 °C for 12 h, after which it was pelleted by centrifugation as above. Samples in H_2O were subjected to the same manipulations as the D_2O samples.

2.4. Treatment of spin-labelled membranes with urea

The pellets after D_2O or H_2O treatment (see preceding paragraph) were weighed, and solid urea was homogenized with the pellet to give final concentrations of 2.5 or 5 M (it was assumed that the pellet as well as the final sample had a density of 1 g/ml). For samples in H_2O buffer, normal urea was used and, for samples in D_2O buffer, fully deuterated urea was used ($[^2H]_4$ urea, Cambridge Isotope Laboratories, MA). Samples without urea were treated as those with urea. The samples were incubated overnight at 14 °C and then transferred to quartz EPR tubes (inner diameter 3 mm) with a sample volume of ca. 100 μl and stored at −20 °C.

2.5. EPR spectroscopy

Pulsed EPR data were collected on an ELEXSYS E580 9-GHz Fourier Transform FT-EPR spectrometer (Bruker, Karlsruhe, Germany) equipped with a MD5 dielectric resonator and a CF 935P cryostat (Oxford Instruments, UK).

To obtain ESEEM spectra, three-pulse, stimulated echo ($\pi/2$–τ–$\pi/2$–T–$\pi/2$–τ–echo) decays were collected by using microwave pulse widths of 12 ns, with the microwave power adjusted to give $\pi/2$ pulses. The time delay T between the second and the third pulses was incremented from 20 ns by 700 steps of $\Delta T = 12$ ns, while maintaining the separation τ between the first and the second pulses constant at 168 ns. A four-step phase-cycling program, $+(x,x,x)$, $-(x,-x,x)$, $-(-x,x,x)$, $+(-x,-x,x)$, where the initial sign indicates the phase of the detection ($\pm y$), was used to eliminate unwanted echoes. The magnetic field was set to the maximum of the EPR absorption. The time-dependent echo amplitudes, $V(\tau,T)$, were processed to yield standardized ESEEM intensities, according to the protocol developed previously [24,25]. The average experimental echo decay, $\langle V(\tau,T)\rangle$, was fitted with a biexponential function. The normalized ESE modulation signal was then obtained as:

$$V_{norm}(\tau,T) = V(\tau,T) / \langle V(\tau,T)\rangle - 1 \quad (1)$$

Three levels of zero filling were added to increase the total number of points to 4 K. The absolute-value ESEEM spectrum was then calculated, with specific inclusion of the dwell time ($\Delta T = 12$ ns) between points, yielding standardized intensities with dimensions of time [25].

By application of the mass-action law, the product of the equilibrium constant, K, for H-bonding and the effective concentration, $[W]$, of free water is related to the normalized intensity, I_{broad}, of the broad D_2O-ESEEM component by [24]:

$$K[W] = \frac{I_{broad} / I_o}{2 - I_{broad} / I_o} \quad (2)$$