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The pH-sensitive structure of the C-terminal domain of voltage-gated proton channel and the thermodynamic characteristics of Zn^{2+} binding to this domain

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ABSTRACT

The voltage-gated proton channel Hv1 is strongly sensitive to Zn^{2+}. The H^+ conduction is decreased at a high concentration of Zn^{2+} and Hv1 channel closing is slowed by the internal application of Zn^{2+}. Although the recent studies demonstrated that Zn^{2+} interacts with the intracellular C-terminal domain, the binding sites and details of the interaction remain unknown. Here, we studied the pH-dependent structural stability of the intracellular C-terminal domain of human Hv1 and showed that Zn^{2+} binds to His^{244} and His^{266} residues. The thermodynamics signature of Zn^{2+} binding to the two sites was investigated by isothermal titration calorimetry. The binding of Zn^{2+} to His^{244} (mutant H266A) and His^{266} (mutant H244A) were an endothermic heat reaction and an exothermic heat reaction, respectively.

1. Introduction

Voltage-gated proton channel Hv1 is perfectively selective for protons and has no detectable permeability to other cations [1–5]. Hv1 is activated by depolarization and intracellular acidification [1,6], and the gating strongly depends on both the intracellular pH (pH_i) and extracellular pH (pH_o) [1,7]. Hv1 functions as a dimer and each subunit contains its own pore [8–10]. There is strong cooperativity between subunits in the dimeric Hv1. The activation of one monomeric channel subunit affects the gating of the other subunit within the dimeric architecture [11,12]. The intracellular C-terminal domain was found to be responsible for the dimeric architecture, cooperative gating, and structural stability of Hv1 [13,14].

Hv1 is strongly sensitive to polyvalent metal cations, particularly Zn^{2+} [1,2,4,5]. Extracellular Zn^{2+} blocks the channel at a low concentration and the inhibition is dependent on pH_o. The effect of external Zn^{2+} on the channel gating is profoundly enhanced at a high pH_o [15,16]. The pH-dependent inhibition suggests that Zn^{2+} blocks Hv1 by competing with H^+ for a site in the external surface of the channel, and two His residues, His^{140} and His^{193}, were found to be the sites contributing to the inhibition of extracellular Zn^{2+} [1,4,15,16]. In addition, it was previously shown that high concentrations of intracellular Zn^{2+} reduce Hv1 proton conduction and slow down channel closing [15]. In contrast with extracellular Zn^{2+}, intracellular Zn^{2+} has relatively weak and obviously different effects, indicating that extracellular and intracellular Zn^{2+} binds to different sites within Hv1 [15]. Although recent studies have shown that divalent metal ions interact with the C-terminal domain of Hv1 [17], the sites and thermodynamic signature of Zn^{2+} binding to the C-terminal domain remain unknown.

At some times, protein precipitation is induced by multivalent metal binding. In this process, multivalent metals serve as cross-linking agents between protein molecules, and the affinity of the protein for the metal, available metal coordination sites, protein and metal concentrations, and solution pH play important roles [18–20]. In the present work, we studied the pH-dependent structural stability of the intracellular C-terminal domain of human Hv1 and the binding of Zn^{2+} to the coiled-coil domain by Zn^{2+}-induced precipitation and isothermal titration calorimetry (ITC). We found that two His residues His^{244} and His^{266} are indispensable for Zn^{2+} binding. The binding of Zn^{2+} to His^{244} and His^{266} were an endothermic heat reaction and an exothermic heat reaction, respectively. Our results suggest that the two His residues within this domain may be the protonation sites that help regulate channel gating.

2. Materials and methods

2.1. Cloning, mutation, expression and purification

Gene cloning, expression and purification of the wild type C-terminal domain of Hv1 (residues 221–273) were the same as

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described previously [21]. The point mutations in the protein (H244A, H266A and the H244A/H266A double mutant) were created by site-directed mutagenesis. Gene cloning, expression and purification of the mutants were the same as the wild type. Briefly, DNA fragments for the wild type and the mutants were inserted into a pGEX-6p-1 vector, respectively. Escherichia coli strain BL21(DE3) harboring the recombinant plasmids were grown in LB medium at 30 °C and induced with 0.5 mM IPTG at 25 °C for 20 h. The protein was purified with a Glutathione-Sepharose 4B affinity column (GE Healthcare), and GST-tag was removed by pre-Scission protease. The C-terminal domain without the GST-tag was then purified by a High S Cartridge column (GE Healthcare), and a Superdex 75 10/300 gel filtration column (GE Healthcare). The concentrations of the proteins were determined by the BCA method using BSA as a standard.

2.2. Circular dichroism spectroscopy

CD measurements were carried out on an Applied Photophysics Chirascan CD spectrometer (Leatherhead, UK). CD data for far-UV (200–250 nm) were collected at 25 °C with a bandwidth of 0.5 nm using a quartz cuvette with a light path-length of 1 mm. The protein samples were diluted to 0.1 mg/ml with 50 mM sodium phosphate buffers containing 150 mM NaCl and 1 mM dithioerythritol (DTT) with a range of pH values of 5–8. The obtained values were subtracted by the corresponding baseline records for the buffers having the same concentration of salts under similar conditions.

2.3. Temperature-induced denaturation

The thermal melting experiments were carried out by increasing the temperature of the sample in the quartz cell with a path-length of 1 mm using a programmable water circulating bath. The temperature was increased from 25 to 90 °C at a rate of 1 °C/min. The measurements were performed with a step size of 5 °C and paused for 2 min at each temperature before recording. The sample was cooled to 25 °C for 5 min and taken a final measurement to determine the extent of refolding.

Denaturation curves were fitted to the two-state model [22]. The fraction unfolded, F_U, was calculated using Eq. (1):

$$F_U = [\theta_t - \theta_F]/[\theta_U - \theta_F] \quad (1)$$

where θ_t is the observed CD signals at any temperature; θ_F and θ_U are the signals for folded and unfolded state, respectively. The constant of folding for dimmer, K_F, is:

$$K_F = [F_2]/[U]^2 \quad (2)$$

where F_2 is the native dimer, U is the unfolded monomer. The thermal melting values, T_m (the midpoint of the thermal denaturation curve), can be derived using Eq. (3):

$$\Delta G = \Delta H(1 - T/T_m) - R \times T \times \ln P_t - \Delta C_p \times ((T_m - T) + T \times \ln(T/T_m)) \quad (3)$$

where ΔH is the enthalpy change; T is the absolute temperature; R is the ideal gas constant; ΔC_p is the change in heat capacity and P_t is the total protein concentration. The multi-wavelength temperature-dependent analysis was carried out using the Global 3™ global analysis software.

2.4. Zn^{2+} binding sites detected by SDS–PAGE

To determine qualitatively whether Zn^{2+} binds to His residues in the C-terminal domain of Hv1, the interactions of Zn^{2+} with the wild type, H244A, H266A and H244A/H266A, were estimated by SDS–PAGE. For pH 5, 10 mM sodium acetate buffer; for pH 6, 10 mM MES buffer; for pH 7–8, 10 mM HEPES buffer, containing 150 mM NaCl and 0.5 mM dithioerythritol (DTT), were used. The mixtures containing 0.5 mg/ml protein and various concentrations of $ZnCl_2$ were centrifuged at 12,000 rpm for 20 min. The proteins in supernatants and pellets were detected by SDS–PAGE.

2.5. Isothermal titration calorimetry

ITC measurements were carried out on a TAM III microcalorimetric system (TA instruments-Waters LLC, USA). The wild type C-terminal domain of Hv1 and the mutants (H244A, H266A, the H244A/H266A double mutant) were dissolved in buffer (20 mM HEPES buffer containing 100 mM NaCl and 0.5 mM DTT, pH 6.5), and dialyzed against the same solution. The titrant, chloride salt of Zn^{2+}, was dissolved in the buffer as mentioned above, and the concentration was adjusted to be 10–50-fold higher than the protein concentration. The protein samples and titrant were degassed at room temperature for 10 min before each titration. 800 μl of the protein samples at 50–100 μM was placed in the reaction cell. The titration was undertaken by injecting 8 μl × 25 injection of the titrant at 300-S intervals with stirring at 60 rpm to ensure a complete equilibration. Control experiments, that were used to determine the dilution heats, were carried out by making identical injections in the absence of protein. All titrations were recorded at 25 °C. The dilution heats were subtracted from the corresponding total heats of reaction to obtain the reaction heats. The titration data were analyzed by the NanoAnalyza software. The data of Zn^{2+} binding to the two single mutants (H244A and H266A) and the wild type C-terminal domain were fit to the independent and multiple site model, respectively.

3. Results

3.1. pH-dependent secondary structure and thermostability of the C-terminal domain

As shown in Fig. 1, the far-UV CD spectra of the protein showed two pronounced double minima at 222 and 208 nm with a range of pH 5–8, characteristic of α-helical secondary structure [23]. Although the characteristic double minima of α-helix remained, the α-helical content was decreased with a pH increase (Supplementary Fig. S1 and Table S1). The ratios of $\theta_{222}/\theta_{208}$ that provide the information on the likelihood of the coiled-coil dissociation were more than 1.0 (Supplementary Table S1), indicating that the protein remains a dimeric structure between pH 5 and 8 [24,25], which is consistent with the result from analytical ultracentrifugation [26].

The thermal-melt curves of the C-terminal domain in response to pH values were shown in Fig. 1A and C. At pH 5 and 6, the fractions unfolded were sharply increased from 45 and 37 to 65 and 58 °C, and reached a plateau at 75 and 62 °C (Fig. 1A), respectively. However, at pH 7 and 8, the fraction unfolded was increased from 37 and 25 to 58 and 65 °C, and reached a plateau at 68 and 75 °C (Fig. 1C), respectively. The mid-point temperatures (T_m) of the protein in response to pH values were calculated and listed in Table 2.

Van't Hoff plots calculated from the data of Fig. 1A and C were shown in Fig. 1B and D, respectively. The enthalpy change (ΔH) of transition at any particular temperature will be equal to the slope of these curves times the gas constant (R = 8.314 J/mol K) [27,28]. At T_m, the enthalpy changes (ΔH_m) of unfolding of the protein were 57.9, 56.2, 53.8 and 28.6 kJ mol^{-1} at pH 5, 6, 7 and 8, respectively. The thermodynamic parameters obtained from the thermal stability curves of the protein at different pH values at T_m were summarized in Table 1.

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