Biophysical Chemistry 172 (2013) 61–67

Contents lists available at SciVerse ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

Thermodynamic parameters of anion binding to halorhodopsin from *Natronomonas pharaonis* by isothermal titration calorimetry

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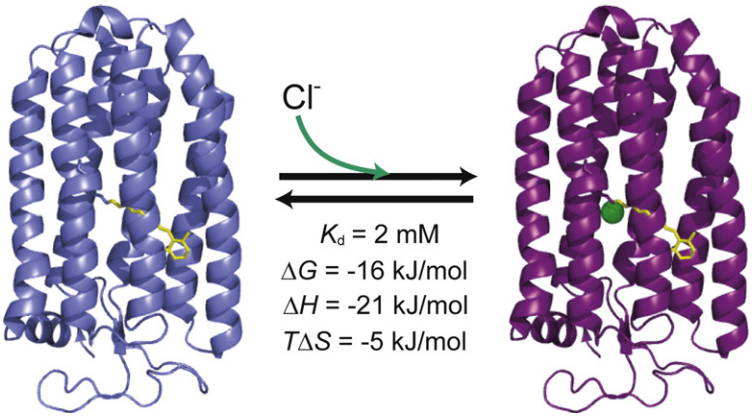
HIGHLIGHTS

- The thermodynamic parameters of anion binding to halorhodopsin were determined.
- The dilution heat was minimalized by considering water activities.
- K_d values were almost equal to those determined by the conventional spectroscopy.
- The protonated Schiff base was essential for the anion binding.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 8 November 2012
Received in revised form 10 January 2013
Accepted 14 January 2013
Available online 20 January 2013

Keywords:
Microbial rhodopsin
ITC
Anion binding
Thermodynamic parameters
Opsin

ABSTRACT

Halorhodopsin (HR), an inwardly directed, light-driven anion pump, is a membrane protein in halobacterial cells that contains the chromophore retinal, which binds to a specific lysine residue forming the Schiff base. An anion binds to the extracellular binding site near the Schiff base, and illumination makes this anion go to the intracellular channel, followed by its release from the protein and re-uptake from the opposite side. The thermodynamic properties of the anion binding in the dark, which have not been previously estimated, are determined using isothermal titration calorimetry (ITC). For Cl^- as a typical substrate of HR from *Natronomonas pharaonis*, $\Delta G = -RT \ln(1/K_d) = -15.9$ kJ/mol, $\Delta H = -21.3$ kJ/mol and $T\Delta S = -5.4$ kJ/mol at 35 °C, where K_d represents the dissociation constant. In the dark, K_d values have been determined by the usual spectroscopic methods and are in agreement with the values estimated by ITC here. Opsin showed no Cl^- binding ability, and the deprotonated Schiff base showed weak binding affinity, suggesting the importance of the positively charged protonated Schiff base for the anion binding.

Abbreviations: HR, halorhodopsin; BR, bacteriorhodopsin; NpHR, halorhodopsin from *Natronomonas pharaonis*; HsHR, halorhodopsin from *Halobacterium salinarum*; λ_{max}, absorption maximum; ITC, isothermal titration calorimeter; K_d, dissociation constant; K_b, binding constant ($=1/K_d$); DDM, *n*-dodecyl-β-D-maltoside; PC, L-α-phosphatidylcholine; MES, 2-(*N*-morpholino)ethanesulfonic acid; EC, extracellular channel; CP, cytoplasmic channel.

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http://dx.doi.org/10.1016/j.bpc.2013.01.001

1. Introduction

Halorhodopsin (HR) is an inwardly directed light-driven Cl^--pump in halobacterial cell membranes [1–5]. The transfer of negative charges across the membrane by HR induces the inside-negative membrane potential that increases the proton-motive force created by bacteriorhodopsin (BR), an outwardly directed light-driven proton-pump in the same halobacterial membranes [6–8]. Like the well-known BR, HR is a seven-helix membrane protein, and retinal binds to a specific lysine residue in the 7th (G) helix via the protonated Schiff base [9]. Since retinal works as a chromophore, this binding affords the visible light absorption whose absorption maximum (λ_{max}) is ~580 nm. Absorption of a photon initiates the photocycle in which the excited HR relaxes to the original ground state via a series of various photo-intermediates referred to as K, L, N and O [4,10–13]. During the transition from N to O intermediate, one Cl^- is released to the intracellular space, and during the transition from O to HR, another Cl^- enters from the opposite side, i.e., the extracellular space [14–17]. Thus, by the completion of the photocycle, one Cl^- transfers from the outside to the inside of cells.

In the unphotolyzed state, Cl^- binds to the binding site located near the positively charged protonated Schiff base in the extracellular channel [18,19]. This binding affinity has been estimated from the spectrum shift of Cl^- titration. For instance, the Cl^- binding to the Cl^--free form of HR from *Natronomonas pharaonis* gives rise to the blue shift of λ_{max} from ~600 (anion-free form) to 580 nm (the bound form) [12,20]. This shift is interpreted to be the stabilization of the ground state energy level due to the electrostatic interaction between the positively charged protonated Schiff base and the negatively charged anion. Analysis of this spectrum shift gives a value of several mM for the dissociation constant, K_d, of HR.

In this paper, we intended to obtain K_d values of the anion binding with another method such as isothermal titration calorimeter (ITC). However, two problems arise. The first problem is that for the precise determination of binding constants ($K_b = 1/K_d$) with the manufacturer's analysis software, the c value, which is defined as $c = K_b \cdot M_{tot} \cdot n$, where M_{tot} and n represent the sample concentration and the stoichiometry number, respectively, should be in the range of 1–1000. Because, as described above, $K_b \sim 1/5\ mM^{-1}$, M_{tot} should be in the range of 5 mM–5 M (assuming $n = 1$). The preparation of such a high concentration of protein solutions, however, is impossible. Obviously, this problem is due to the fundamentally weak interactions, as manifested in the large values of K_d. The other problem is the production of the dilution heat. Due to the large K_d values, a high concentration of Cl^- is required for the injection solution, which yields appreciable dilution heat at the titration. We found a method to avoid these problems and succeeded in the estimation of K_d values and thermodynamic parameters. Values of K_d estimated by this method were similar to those determined from the λ_{max}-shift. Suggestions on the existence of the other binding sites have been reported [21,22] which may not affect the absorption of HR, and then analysis in this paper was carried out on assumption that there are strong and weak binding sites. The thermodynamic parameters associated with anion binding to HR (strong binding) were first reported in this paper. It is noteworthy that anions do not bind to opsin, a protein part of rhodopsin only without retinal, showing the important role of the positively charged protonated Schiff base in anion binding. This finding was not obtained by the spectroscopic method and was first obtained here by the ITC method.

2. Materials and methods

2.1. Preparation of NpHR

Previous investigation of HR has been performed using two sources. One source is *Halobacterium salinarum*, and the other is *Natronomonas pharaonis* (abbreviated as NpHR). NpHR was used in this study because the expression system using *Escherichia coli* has been established [23,24]. Expression of the (6×) histidine-tagged protein and purification procedures using *E. coli* BL21(DE3) cells harboring the appropriate plasmid were performed as described elsewhere [24,25]. The binding was measured not only for the wild-type but also for various mutants. Given that charged or hydrophilic amino acid residues may play an important role, we made mutants of Arg123, Thr126 and Ser130, which are all located near the binding site in the extracellular channel. In addition, mutants of Lys215 and Thr218 in the cytoplasmic channel were prepared. The construction of these mutants was described in Sato et al. [26–28] and Kubo et al. [29]. The opsin (with a 6× his-tag) was prepared without an addition of retinal at the induction and purified as usual. The protein was solubilized with 0.1% *n*-dodecyl-β-D-maltoside (DDM, from Dojindo, Kumamoto, Japan). The protein concentration was determined using the extinction coefficient of 54,000 at 578 nm in the presence of Cl^- and 50,000 in the absence of Cl^-[20]. For opsin, a large enough amount of retinal was added after the experiment to estimate the concentration by the absorption.

2.2. Reconstitution of NpHR with phospholipids

The sample was reconstituted with phospholipids to increase the stability of NpHR and to make buffer exchange easier. The protein solubilized with 0.1% DDM and L-α-phosphatidylcholine (PC) from egg york (Funakoshi, Tokyo, Japan) was mixed at a molar ratio of 1:50. DDM was removed with biobeads SM2 (1 g for 1 ml of 1% DDM, BIO-RAD, Tokyo) under a nitrogen atmosphere at 4 °C. Details are described elsewhere [30,31].

2.3. ITC measurements

A VP-ITC (MicroCalorimeter, MicroCal, Northampton, MA) was used. PC-reconstituted NpHR solutions of 30–50 μM were added into the lower cell chamber (1.45 mL), and the injection syringe contained a concentrated salt solution whose compositions are shown below. The NpHR sample in the lower cell was suspended in 500 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) adjusted to a pH of 6.0. The dissociation of the protonated Schiff base of the Cl^--free NpHR was observed in alkali solutions, and experiments were carried out at pH 6. The solution compositions of the injection syringe were dependent on the salt used. These compositions were determined by a trial-and-error method so that the dilution heat became minimal. Compositions used were as follows:

NaCl, 150 mM NaCl, 475 mM MES, pH 6.0

NaBr, 150 mM NaBr, 450 mM MES, pH 6.0

NaI, 150 mM NaI, 490 mM MES, pH 6.0

$NaNO_3$ 150 mM $NaNO_3$, 420 mM MES, pH 6.0

NaSCN 150 mM NaSCN, 475 mM MES, pH 6.0

The reason of variations of MES concentrations on anion species is not clear. As described above, these titration solutions having these MES concentrations yielded the smallest dilution heat. Since as described below, the volumes added from the syringe are at most 250 μL (5 μL×50 times) and the lower cell contained 1.45 mL of 500 mM of MES, the ionic strength variations in the cell depending on anion species used were small.

Prior to ITC measurements, both the sample protein and injection solutions were de-gassed with ThermoVac Sample Degassing and Thermostat (MicroCal, Northampton, MA). The temperature was set to 35 °C. The protein solution in the cell was stirred at 300 rpm. The duration and spacing of injection were 10 s and 240 s, respectively. The numbers of injections were either 30 or 50. The first injection volume was 2.5 μL, and the volume increased to 5.0 μL due to the second injection.