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Kinetics of nucleotide binding to the β-subunit (AKR6A2) of the voltage-gated potassium (Kv) channel

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ABSTRACT

The β-subunits of the voltage-gated potassium (Kv) channels modulate the kinetics and the gating of Kv channels and assists in channel trafficking and membrane localization. These proteins are members of the AKR6 family. They share a common $(\alpha/\beta)_8$ barrel structural fold and avidly bind pyridine nucleotides. Low catalytic activity has been reported for these proteins. Kinetic studies with rat Kvβ2 revealed that the chemical step is largely responsible for the rate-limitation but nucleotide exchange could also contribute to the overall rate. Herein we report our investigations on the kinetics of cofactor exchange using nucleotide-free preparations of Kvβ2. Kinetic traces measuring quenching of Kvβ2 fluorescence by $NADP^+$ were consistent with a two-step binding mechanism which includes rapid formation of a loose enzyme:cofactor complex followed by a slow conformational rearrangement to form a tight final complex. Closing of the nucleotide enfolding loop, which in the crystal structure folds over the bound cofactor, provides the structural basis for this rearrangement. The rate of the loop opening required to release the cofactor is similar for NADPH and $NADP^+$ ($0.9\,min^{-1}$) and is of the same order of magnitude as the rate of the chemical step estimated previously from kinetic studies with 4-nitrobenzaldehyde ($0.3–0.8\,min^{-1}$, [S.M. Tipparaju, O.A. Barski, S. Srivastava, A. Bhatnagar, Catalytic mechanism and substrate specificity of the beta-subunit of the voltage-gated potassium channel, Biochemistry 47 (2008) 8840–8854]). Binding of NADPH is accompanied by a second conformational change that might be responsible for a 4-fold higher affinity observed with the reduced cofactor and the resulting difficulty in removing bound NADPH from the protein. These data provide evidence that nucleotide exchange occurs on a seconds-to-minutes time scale and set the upper limit for the maximal possible rate of catalysis by Kvβ2. Slow cofactor exchange is consistent with the role of the β-subunit as a metabolic sensor implicated in tonic regulation of potassium currents.

1. Introduction

The β-subunits of the voltage-gated potassium channels (Kv) are the members of the aldo-keto reductase (AKR) superfamily [1,2]. Three distinct Kvβ genes (*KCNAB1-3*) that belong to the AKR6A subfamily are conserved in rodents and humans [3]. These genes encode proteins with a highly conserved C-terminus and a variable N-terminal domain. The conserved C-terminus of Kvβ proteins displays 15–30% amino acid identity with sequences of other AKRs and folds into an $(\alpha/\beta)_8$ or triosephosphate isomerase (TIM) barrel motif, which is the structural motif common to all AKRs. The proteins of the AKR6 family form tetramers in solution and crystallize with a 4-fold symmetry [4,5]. The variable N-terminus does not share homology with AKRs and imparts inactivation to the Kvα subunits of Kv1 and Kv4 channels [6].

The Kvβ-subunits associate with the N-terminus of membrane-spanning Kvα-subunits and cause a hyperpolarizing shift in the half activation potential of Kv currents [7,8]. Functionally, they are also implicated in localization and trafficking of Kv channels from endoplasmic reticulum to the plasma membrane. Like other AKRs, the Kvβ proteins bind pyridine nucleotides with high affinity [4]. Our previous studies show that pyridine nucleotides binding to Kvβ differentially regulates Kv currents. We have found that binding of reduced nucleotides supports N-terminus mediated inactivation of Kvα currents by Kvβ1.3, whereas oxidized nucleotides remove inactivation of K^+ currents generated by Kv α-β subunits [8–10].

Recent studies show that Kvβ2 and Kvβ1 possess low catalytic activity with k_{cat} between 0.06 and $0.4\,min^{-1}$ [11–13]. These proteins catalyze the reduction of a broad range of carbonyls including aromatic carbonyls, electrophilic aldehydes and prostaglandins,

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phospholipid and sugar aldehydes. Potential physiological substrates include products of lipid peroxidation POVPC (1-palmitoyl, 2-oxovaleroyl-sn-glycero-3-phosphatidylcholine) and ONE (4-oxo-*trans*-2-nonenal), AGE (advanced glycation end products) precursors such as methylglyoxal and phenylglyoxal, and arachidonic acid metabolites such as oxoeicosatetraenoic acids and prostaglandin J_2 [12]. The catalytic sequence follows ordered bi–bi reaction mechanism with nucleotide cofactor binding first and leaving last [12]. In our previous publication, we showed that in the reduction of 4-nitrobenzaldehyde the catalytic step contributed significantly to the rate-limitation but was not a sole rate-limiting step and hypothesized that the cofactor exchange may be responsible for the remaining portion of rate-limitation [12]. A slow release of $NADP^+$ has been identified to be the rate-limiting step in the AKR1B1-catalyzed aldehyde reduction [14] and is believed to be due to the slow movement of the NADPH binding loop [15,16]. This possibility is consistent with the crystal structure of Kvβ2, which shows that the cofactor is held tightly at the active site [5]. Thus $NADP^+$ release, which requires extensive conformational rearrangement, could contribute to the overall rate of the catalytic cycle.

In the present study we measured the kinetics of nucleotide binding and determined the microscopic rate constants for the steps involved in formation of NADPH and $NADP^+$ complexes with Kvβ2. We found that binding and release of the reduced and the oxidized cofactor are accompanied by conformational changes that occur on the same time scale as the chemical step involving hydride transfer. These studies provide a better understanding of the catalytic mechanism of Kvβ2. Our results are also relevant to the electrophysiological behavior of Kvβ2 as nucleotide binding is required for Kvβ-dependent modulation of Kv currents.

2. Materials and methods

2.1. Kvβ2 expression and purification

The Kvβ2 protein was expressed and purified from bacterial culture carrying the plasmid coding for an AKR domain (amino acids 39–360) of rat Kvβ2 (AKR6A2) with a His tag at its N-terminus, as described earlier [4]. The His-tagged protein was purified over a Ni-affinity column (Qiagen) according to manufacturer' instructions. The purified protein was dialyzed against 0.2 M potassium phosphate buffer, pH 7.4 for 16–20 h at 4 °C. The concentration of the protein was measured using Bradford's assay [17].

2.2. Preparation of nucleotide-free protein

As shown previously Kvβ2 protein purified from bacteria remains tightly bound to NADPH, which is not removed by overnight dialysis [4,18]. Hence, to prepare a nucleotide-free apoenzyme, NADPH bound to Kvβ was removed by oxidizing it to $NADP^+$, which dissociates from the protein more readily than NADPH. For this, Kvβ2 purified from bacteria was incubated with 0.6 mM 4-nitrobenzaldehyde and the disappearance of NADPH was monitored at 360 nm [12]. After driving the reaction to completion, the reaction mixture was transferred to a dialysis cassette (10,000 Da cutoff; Pierce) and dialyzed against 0.2 M potassium phosphate (pH 7.4) at 4 °C for 16–20 h.

2.3. Equilibrium nucleotide binding studies

The nucleotide-free Kvβ2 protein prepared as described above was equilibrated at a concentration of 0.21 μM in 2 ml of 0.2 M potassium phosphate buffer at room temperature for 10 min. Aliquots of NADPH or $NADP^+$ were added and fluorescence was recorded using an excitation wavelength of 290 nm and emission at 335 nm. The protein was allowed to bind to the nucleotide for 4 min before addition of the next aliquot. No inner filter effect correction was performed because the maximal nucleotide concentration used did not exceed 1.5 μM.

Two step

$$E + N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EN \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E^*N$$

Three step

$$E + N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EN \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E^*N \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E^{**}N$$

Scheme 1. Two and three step models of nucleotide binding to Kvβ2. In a two-step model, formation of a weak initial *EN* complex is followed by a conformational change to a tightly bound *E*N* complex. In a three step model, two kinetically distinct conformational changes follow the initial nucleotide binding step.

2.4. Binding kinetics

The kinetics of NADPH and $NADP^+$ binding to Kvβ2 was measured by monitoring decrease in protein fluorescence (ex. 290 nm; em. 335 nm) that accompanies cofactor binding to Kvβ2. The nucleotide-free Kvβ2 protein (0.21 μM) was pre-equilibrated at room temperature in 0.2 M phosphate buffer, pH 7.4 and the reaction was initiated by adding the nucleotide (0.1–3.2 μM). The reaction was monitored continuously by measuring changes in fluorescence for 250 s in a Shimadzu RF-1530 instrument.

2.5. Data analysis

The equilibrium nucleotide dissociation constants were calculated from the data on protein fluorescence quenching by using a variation of the Scatchard equation as described previously [19,20].

Nucleotide binding transients were analyzed using either a single or a dual-exponential decay equation:

$$y = ae^{-kt} + y_0 \quad (1)$$

$$y = ae^{-k_{fast}t} + be^{-k_{slow}t} + y_0 \quad (2)$$

The observed exponential rate constants k_{fast} and k_{slow} as a function of total nucleotide concentration were fitted to equations describing two and three step binding models (Scheme 1):

$$k_{fast} = k_4 + k_3\frac{E_{bound}}{E_0}, \text{and } k_{slow} = k_6 + k_5\frac{E_{bound}}{E_0} \quad (3)$$

where E_{bound}/E_0 is the fraction of enzyme bound with the cofactor at the preceding step and is given by:

$$\frac{E_{bound}}{E_0} = \left(\frac{0.5}{E_0}\right)[(E_0 + N + K_{loose}) - ((E_0 + N + K_{loose})^2 - 4E_0N)^{1/2}] \quad (4)$$

where E_0 is the total enzyme concentration, N is the total nucleotide concentration, and K_{loose} is the K_d of the initial *EN* complex (k_2/k_1 in Scheme 1) [14,21,22].

2.6. Statistical analysis

All regression analyses were performed using Sigmaplot 10. Statistical parameters R^2, *F*-values, and the standard errors of the estimates calculated by Sigmaplot were used to estimate goodness of fit of single or dual exponential equations to the data. Calculated values of the parameters are presented as mean ± SEM.