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Binding of pyridine coenzymes to the β -subunit of the voltage sensitive potassium channels

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Abstract

The β -subunit of the voltage-sensitive K^+ channels shares 15–30% amino acid identity with the sequences of aldo–keto reductases (AKR) genes. However, the AKR properties of the protein remain unknown. To begin to understand its oxidoreductase properties, we examine the pyridine coenzyme binding activity of the protein *in vitro*. The cDNA of $K_v\beta 2.1$ from rat brain was subcloned into a prokaryotic expression vector and overexpressed in *Escherichia coli*. The purified protein was tetrameric in solution as determined by size exclusion chromatography. The protein displayed high affinity binding to NADPH as determined by fluorometric titration. The K_D values for NADPH of the full-length wild-type protein and the N-terminus deleted protein were 0.1 ± 0.007 and 0.05 ± 0.006 M, respectively — indicating that the cofactor binding domain is restricted to the C-terminus, and is not drastically affected by the absence of the N-terminus amino acids, which form the ball and chain regulating voltage-dependent inactivation of the α -subunit. The protein displayed poor affinity for other coenzymes and the corresponding values of the K_D for NADH and NAD were between 1–3 μ M whereas the K_D for FAD was > 10 μ M. However, relatively high affinity binding was observed with 3-acetyl pyridine NADP, indicating selective recognition of the 2' phosphate at the binding site. The selectivity of $K_v\beta 2.1$ for NADPH over NADP may be significant in regulating the K^+ channels as a function of the cellular redox state. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Voltage sensitive; K^+ channel; β -subunit; Pyridine coenzyme nucleotides; Rat brain

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1. Introduction

The K^+ channels perform a variety of critical functions in both excitable and non-excitable cells [1]. These channels consist of homo- or hetero-tetramers (α -subunit) of membrane spanning proteins that comprise the voltage-sensitive, K^+ -selective pore. In addition, several K^+ channels are associated with cytoplasmic β -subunits. These proteins were first identified as 40 KDa components that copurified with α dendrotoxin acceptor ($K_v\alpha$ 1.2). To date, nine homologous genes encoding β -subunits have been identified (for review, see Ref. [2]; six from mammals, 1 from *Drosophila* and 2 from plants. These sequences have a distinct and variable N-terminus and a highly conserved C-terminal core region. In addition, several splice variants of $K_v\beta$ 1 i.e. $K_v\beta$ 1.1, 1.2, and 1.3 have been reported. These variants have identical core region but variable N-terminus. The amino acid sequence of the β -subunits bears no obvious homology to known auxiliary subunits of other ion channels. However, they display 15–30% sequence homology to aldo-keto reductase (AKR) genes. In the nomenclature proposed by Jez et al. [3], the rat and bovine shaker β -subunits are AKR6A1 and AKR6A2, respectively, related most closely to the member of the AKR7 family i.e. aflatoxin B1 reductase and AKR5 family members such as *Pseudomonas* morphine dehydrogenase (AKR5B) and *Corynebacterium* 2,5-diketo-gluconate reductase (AKR5C).

The three-dimensional structure of the voltage-sensitive K^+ channel β -subunit ($K_v\beta$ 2) closely resembles that of other AKRs ([4,5]). The eight β strands and the α helices correspond to the framework of the AKR barrel. However, the β F- α D loop in 3- α -hydroxysteroid dehydrogenase is shortened in the $K_v\beta$ 2-subunit, creating a wider and more solvent accessible active site. Nonetheless, the active site and the cofactor binding are similar to other AKRs, and the catalytic triad of AKRs consisting of Try-Lys-Asp is conserved, although the active site histidine (His-110 in aldose reductase) is replaced by an asparagine residue. The $K_v\beta$ 2 binds one NADP(H) per active site. Similar to its configuration when bound to other AKRs, NADPH bound to the β -subunit forms multiple contacts with the protein side chains and backbone [4]. However, no functional data on pyridine coenzyme binding to this protein are available. Therefore, we examined the specificity and selectivity of the protein for binding pyridine coenzyme in vitro.

2. Preparation and characterization of the $K_v\beta$ -subunit

The cDNA containing the coding sequence for $K_v\beta$ 2.1 was a generous gift from Dr. Min Li (Johns Hopkins University, Baltimore). To generate the $K_v\beta$ 2.1 cDNA fragment with Nde I at 5' end and Xho I at 3' end, a standard PCR procedure was used for amplifying the gene with two pairs of primers. For the full-length β -subunit, 5'-CATATGTATCCGGAATCAACC-3' (forward) and 5'-GGATCCTGACTTAGGATCTATAGTCC-3'(reverse), and for the N-terminal deleted β -subunit, 5'-AGACAGCTCCATATGTACAGGAAC-3' (forward) and 5'-GGATCCTGACTTAGGATCTATAGTCC-3'. The PCR products were inserted

into pCR-TOPO (Invitrogen), and the amplified vector was further digested by Nde I and Xho I to isolate β -subunit fragments, which were ligated with a linearized pET28a vector cleaved by Nde I and Xho I.

The expression vectors, pET28-F β (full length β -subunit) and pET28-C β (C-terminal β -subunit), were transformed into *Escherichia coli* strain BL21. The transformed bacteria were cultured at 37°C in LB containing 50 μ g/ml kanamycin. The expression of K ν β 2.1 protein was induced by addition of 1 mM IPTG, when the absorbance at 600 nm in the culture medium reached approximately 0.8. The induction was continued for another 4 h at 37°C with a constant shaking at 280 rpm. The bacterial cells were lysed by sonication in binding buffer containing 20 mM Tris–HCl, 200 mM NaCl, and 5 mM imidazole, pH 7.9. The lysate was loaded onto the column packed with Ni-NTA superflow (Qiagen), pre-equilibrated with binding buffer. The protein bound to the column was eluted using a step gradient generated by increasing the imidazole concentration from 50 to 300 mM. The expressed β -subunit was identified by 12% SDS–PAGE, and the fractions with β -subunit were pool and dialyzed against 150 mM potassium phosphate buffer.

The full length wild type (WT) and the N-terminus deleted (Δ N) K ν β 2.1, expressed in *E. coli*, migrated a single band on SDS–PAGE, corresponding to molecular weights of 40 and 38 KDa, respectively (Fig. 1). The quaternary structure of the β -subunit in solution was analyzed by size exclusion chromatography using

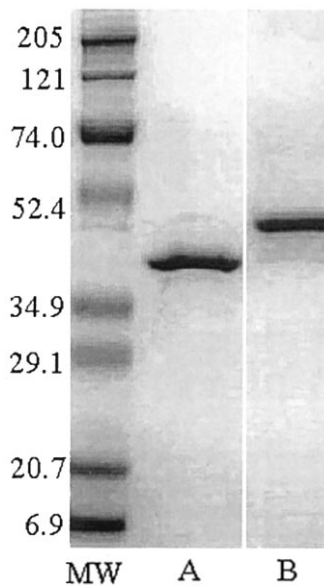


Fig. 1. SDS–PAGE of recombinant K ν β 2.1. cDNA encoding full length WT and Δ N K ν β 2.1 containing a His-Tag leader sequence were expressed in *E. coli* and purified on a Ni-affinity column, \sim 5.0 μ g of each protein was loaded and visualized by the Coomassie Blue stain. Molecular weight markers in Lane A are indicated in the figure. Lane B; full length and Lane C; Δ N K ν β 2.1.

TSK-GEL G3000SW_{XL} (TosoHass, Montgomeryville, PA) column and Waters Alliance HPLC. The column was equilibrated with 0.4 M K-phosphate, pH 7.0. The column was calibrated using globulin (158 000), bovine serum albumin (67 000) and ovalbumin (43 000). Under the conditions used, the retention time of the homogenous ΔN K_v β 2.1 corresponded to molecular weight of ~ 156 000 Da, indicating that the protein exists as a tetramer in solution. No monomeric or dimeric forms of the protein were observed. These results demonstrate for the first time that the β -subunit exists as a homotetramer in dilute solution, and are in agreement with previous co-immunoprecipitation [6] and yeast-two hybrid experiments [7], which also suggest a β_4 stoichiometry of the protein, and are consistent with a tetrameric form of the protein in crystals [4].

To characterize the protein further, absorbance and fluorescence spectra of the proteins were determined. Both the full length, and ΔN K_v β 2.1, showed a prominent absorption peak at 363 nm, indicating the presence of NAD(P)H bound to the protein. The presence of the NAD(P)H bound to the purified protein was also suggested by a broad emission peak at 340 nm upon excitation at 280 nm. These observations indicate that the purified K_v β 2.1 retains NAD(P)H at the active site.

3. Nucleotide binding to K_v β 2.1

The affinity of K_v β 2.1 for nucleotides was determined by fluorometric titrations. The fluorescence spectra were recorded on a Shimadzu RF-5301 PC fluorescence spectrophotometer. Changes in fluorescence were measured using an excitation wavelength of 280 nm and an emission wavelength of 335 nm. Five to ten microliters of the protein (0.1 mg/ml in 0.15 M K-phosphate, pH 7.4) were added to the 1.8 ml of the assay buffer with 2–10 μ l addition of the pyridine coenzymes. Concentrations of the coenzymes were corrected for dilution. A control titration curve without the enzyme in the same volume of the buffer was carried out to correct for non-specific changes in the fluorescence of the nucleotides.

Fluorescence titration curves were obtained by measuring the change in fluorescence intensity at 335 nm with excitation at 280 nm. The data were fitted to a quadratic binding equation using standard non-linear regression techniques. The binding equation included corrections for scatter, dilution, and cofactor absorbance. The fluorescence intensity I as a function of the cofactor concentration X is given by

$$I = e^{-\Delta X}(G((F_{\min} - F_{\max})Q(P, X, k_d) + F_{\max}) + F_{\text{bgnd}}),$$

Where F_{\min} and F_{\max} are the minimum and maximum fluorescence intensities above the background F_{bgnd} due to scatter. $Q(E, X, k_d)$ is the quadratic solution to the equation: $P + L \rightarrow PL$, where P is the protein and L is the ligand.

$$Q(P, X, k_d) = -(b + \sqrt{(b^2 - 4a \cdot c)})/2a,$$

$$a = 1,$$

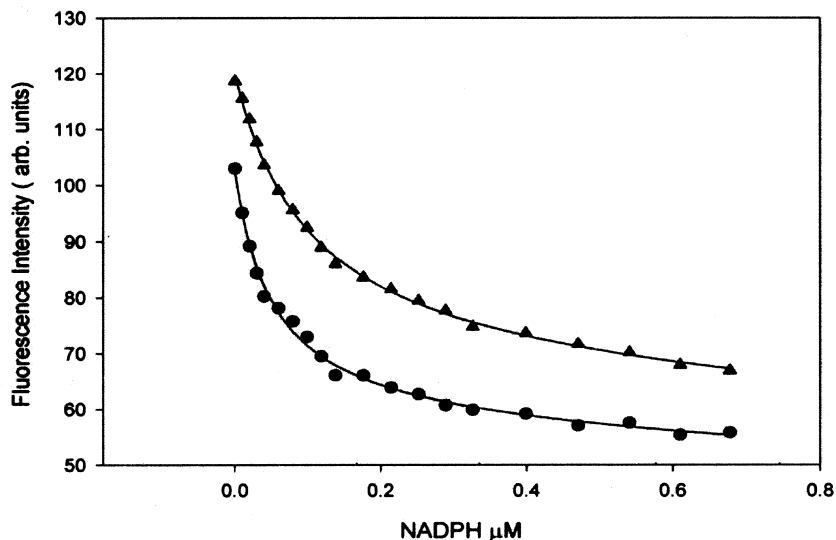


Fig. 2. Measurement of NADPH binding to the β -subunit. The full length (filled circle) or ΔN (filled triangles) were suspended in 10 mM K-phosphate, pH 7.0 and the indicated concentrations of NADPH were added to the cuvette. Data are shown as discrete points, while the continuous curve represents the best fit of the data to the binding isotherm (see text for details).

$$b = -(GP + X + k_d)/(PE),$$

$$c = X/(PE),$$

Δ is the absorbance of the cofactor X , G is the dilution correction ($V/(V + V_x)$ V is the initial volume and V_x is the volume of cofactor titrated).

Data for determining the binding constant K_D were acquired with $[E] < K_D$. The active protein concentration $[E]$ was determined $[E] > K_D$. Typically, when the total protein concentration was assayed by the Bradford test, 70% of the protein was found to be active by this method.

Titration of the protein with increasing concentrations of NADPH in 10 mM K-phosphate buffer, pH 7.0 led to progressive loss of fluorescence (Fig. 2). After the addition of 0.6 μM of NADPH, no further decrease in fluorescence was observed. Typically, NADPH quenched 35–50% of the total fluorescence. In three separate experiments, the average K_D NADPH determined under these conditions was $0.1 \pm 0.007 \mu\text{M}$ for the full-length WT protein, and $0.05 \pm 0.005 \mu\text{M}$ for the ΔN - $K_v\beta 2.1$. These data show that $K_v\beta 2.1$ displays unusually high affinity for NADPH and that deletion of the N-terminal domain does not dramatically alter the affinity of the protein for the coenzyme-indicating selective interaction of NADPH for the C-terminal or the AKR core of the protein. Thus, for all subsequent experiments, the ΔN - $K_v\beta 2$ protein was used.

To probe the selectivity and specificity of ΔN - $K_v\beta 2$ for NADPH, the K_D of the protein with several pyridine nucleotides and analogs was determined. These values

are listed in Table 1. As compared to NADPH, the K_D NADP was three-fold higher, indicating a significantly higher affinity of the protein for the reduced nucleotide. However, with NAD(H) as the ligand, the protein displayed a 10-fold higher affinity for the oxidized as compared to the reduced coenzyme. Nonetheless, the K_D NAD(H) was substantially higher than that for NADP(H) indicating specific recognition of the ribose 2' phosphate by the protein. Evidence for the functional significance of the amide side chain of the nicotinamide ring in binding to the protein is provided by the observation that the absence of this group in 3'-acetylpyridine NADP led to a 10-fold increase in K_D of this molecule as compared to NADPH. Removal of the 3' carbonyl from the nicotine ring led to additional decrease in affinity (compare 3'-aminopyridineNADP and NADP), suggesting additional stabilization of cofactor binding due to interaction between the 3'-side chain of the ring with the active site residues. Other fragments of the pyridine coenzyme nucleotide such as ADP-ribose, NMN and nicotinamide generally displayed poor affinity for ΔN -K $_{v\beta 2}$. In addition, the flavin coenzyme FAD bound weakly to the protein, suggests that it is unlikely to an *in vivo* ligand of the K $_{v\beta}$.

The high affinity of K $_{v\beta}$ for pyridine nucleotides is similar to that observed for other members of the AKR superfamily. This is not surprising given the close similarity between the configuration of NADP bound to the K $_{v\beta}$ and other members of the AKR superfamily. However, the K_D of K $_{v\beta}$ for NADPH (0.05 μ M) is at the low end of the dissociation constants of other AKRs — (which are usually between 0.1 and 3 μ M; [8–10]). The unusually high affinity for K $_{v\beta}$ for NADPH may be due to more efficient binding of NADPH at the active site of the protein. In contrast to other AKRs, the bound cofactor is completely buried in the deep cleft of K $_{v\beta}$ and covered by the $\beta 1$ - α G loop, which may be responsible for the tight binding of the cofactor. Like aldose and aldehyde reductases, the K $_{v\beta}$ displays 2-fold greater affinity for NADPH over NADP. Interestingly, the AKR dehydroge-

Table 1
Binding of ΔN K $_{v\beta 2}$ to pyridine coenzyme and analogs^a

Nucleotide	K_D (M)
NADPH	0.16 \pm 0.07
NADP ⁺	0.34 \pm 0.08
NADH	1.17 \pm 0.77
NAD ⁺	3.72 \pm 0.38
3-acetyl pyridine NADP ⁺	4.24 \pm 1.15
3-amino pyridine NADP ⁺	14.35 \pm 1.34
ADP-Ribose	412.01 \pm 23.50
FAD	10.2 \pm 5.99
NMN	144.0 \pm 15.3
Nicotinamide	ND

^a Identical ΔN K $_{v\beta 2}$ protein aliquots were titrated by varying concentrations of nucleotide and analogs in 0.15 M, potassium phosphate, pH 7.4 and changes in emission at 340 nm (excitation 280 nm) were monitored. Data are shown as mean \pm SD ($n = 3$). ND: not detected.

nases, e.g. 20 α -hydroxysteroid dehydrogenase prefer NADP ($K_D = 0.36 \mu\text{M}$) over NADPH ($K_D = 0.64 \mu\text{M}$; [10]). Thus, the higher affinity of $K_v\beta$ for the reduced cofactor suggests that the catalytic activity of the protein, if any, is more likely to be carbonyl reduction rather than oxidation.

In addition to NADP(H), the β -subunit also displayed high affinity for NAD(H). Significantly, the preference of the protein for the reduced and the oxidized form of the coenzymes was different. The NAD bound to $K_v\beta$ with a 3-fold higher affinity than NADH, whereas, the binding of NADP was two-fold weaker as compared to NADPH. We speculate that the greater preference of $K_v\beta$ for [NADPH and NAD] over [NADP and NADH] may be of physiological significance. In most tissues [11], the concentration of NAD(H) is at least 3–10 times more than that of NADP(H). Moreover, most of the NAD(H) is in the oxidized form and $\text{NAD}/\text{NADH} = 750\text{--}1000$, whereas most of NADP(H) is reduced ($\text{NADP}/\text{NADPH} = 0.01$). Hence under normal physiological conditions, the entire population of $K_v\beta$ is likely to be bound to NADPH (since $K_D \text{ NADPH}/K_D \text{ NAD} > 20$). However, conditions leading to a decrease in the concentration of NADPH and an increase in NAD (e.g. hypoxia and oxidative stress) may result in significant changes in the nature of the $K_v\beta$ -nucleotide binary complex, leading to an increase in the fraction of the $K_v\beta$ population bound to NAD. Whether changes in the nature of the cofactor bound to $K_v\beta$ alter its function and its regulation of the K^+ channels remains to be determined. However, recent evidence demonstrates that in HEK293 cells, cotransfection of $K_v\beta$ with the α -subunit, confers oxygen sensitivity to K^+ channels [12] suggesting that selective cofactor recognition and changes in cofactor binding to $K_v\beta$ may be a potential mechanism by which $K_v\beta$ sense changes in the cellular redox state and alter the rate of inactivation and the voltage dependence of the potassium currents.

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