

Binding of Pyridine Nucleotide Coenzymes to the β -Subunit of the Voltage-sensitive K^+ Channel*

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The β -subunit of the voltage-sensitive K^+ (K_v) channels belongs to the aldoketo reductase superfamily, and the crystal structure of $K_v\beta 2$ shows NADP bound in its active site. Here we report that $K_v\beta 2$ displays a high affinity for NADPH ($K_d = 0.1 \mu M$) and $NADP^+$ ($K_d = 0.3 \mu M$), as determined by fluorometric titrations of the recombinant protein. The $K_v\beta 2$ also bound NAD(H) but with 10-fold lower affinity. The site-directed mutants R264E and N333W did not bind NADPH, whereas, the K_d^{NADPH} of Q214R was 10-fold greater than the wild-type protein. The K_d^{NADPH} was unaffected by the R189M, W243Y, W243A, or Y255F mutation. The tetrameric structure of the wild-type protein was retained by the R264E mutant, indicating that NADPH binding is not a prerequisite for multimer formation. A C248S mutation caused a 5-fold decrease in K_d^{NADPH} , shifted the pK_a of K_d^{NADPH} from 6.9 to 7.4, and decreased the ionic strength dependence of NADPH binding. These results indicate that Arg-264 and Asn-333 are critical for coenzyme binding, which is regulated in part by Cys-248. The binding of both NADP(H) and NAD(H) to the protein suggests that several types of $K_v\beta 2$ -nucleotide complexes may be formed *in vivo*.

The voltage-sensitive K^+ (K_v) channels participate in several cellular processes. In excitable tissues, these channels play an essential role in establishing the resting membrane potential and in modulating the frequency and the duration of the action potential (1). In nonexcitable cells, they are involved in cell volume regulation, hormone secretion, oxygen sensing, and cell proliferation (2). The functional diversity of these channels is partly due to variations in their structure. The ion-conducting pore of these channels is formed by heterotetramers of different, but structurally related, α subunits (2, 3). Moreover, the cytoplasmic face of the $K_v\alpha$ proteins associates with auxiliary β -subunits ($K_v\beta$), which do not participate in ion conductance but can regulate the activity of the channel (4, 5).

Several homologous genes encoding the $K_v\beta$ proteins have been described. A comparison of the amino acid sequences of the β -subunit proteins shows that these proteins have a variable N terminus and a highly conserved C-terminal domain. The β -subunits have been assigned to three classes: $K_v\beta 1$ to 3. In addition, several splice variants of $K_v\beta 1$, that is, $K_v\beta 1.1$, 1.2,

and 1.3, have been reported (for review, see Refs. 4 and 5). Although some of the β -subunits enhance the inactivation of the $K_v\alpha$ currents (4, 5), the physiological role of these proteins remains unclear. In heterologous systems, coexpression of $K_v\beta$ increases the surface expression of $K_v\alpha$, indicating that the β subunits regulate the expression and/or the localization of the $K_v\alpha$ proteins. Moreover, $K_v\beta 2$, which is the most widely distributed of the β -subunits, does not affect inactivation even though it associates with $K_v\alpha$, suggesting that the β -subunits may have other undetermined physiological functions.

Structural analyses support the view that $K_v\beta$ proteins may have unique regulatory properties not displayed by accessory proteins of other ion channels. The primary amino acid sequence of the $K_v\beta$ proteins is not related to the auxiliary proteins of other voltage-sensitive channels but, unexpectedly, to the proteins of the aldoketo reductase (AKR)¹ superfamily (6, 7). Within this superfamily, the amino acid sequences of the $K_v\beta$ proteins are most closely related to alfatoxin reductase (AKR7) and morphine dehydrogenase and 2,5-diketogluconate reductase (AKR5). On the basis of this homology, the $K_v\beta$ proteins have been assigned to the AKR6 family (8). The AKR proteins catalyze the reduction or the oxidation of a broad range of carbonyl substrates, including aldoses, steroids, prostaglandins, and aldehydes derived from lipid peroxidation (8–11). The sequence homology between the β -subunits and the AKR proteins suggests that the $K_v\beta$ proteins are catalytically competent oxidoreductases that couple metabolic changes to membrane excitability.

The crystal structure of $K_v\beta 2$ shows that the protein folds into $\beta 8/\alpha 8$ or the triosephosphate isomerase barrel motif similar to other AKR proteins (12). A single molecule of $NADP^+$ was found to co-crystallize with each monomer of the protein (12). The cofactor was bound to the C terminus of $K_v\beta 2$ by active site residues, some of which are conserved within the AKR superfamily. Nonetheless, no functional data are available on pyridine nucleotide binding to $K_v\beta$. In the present study, we examined the coenzyme specificity and selectivity of the purified $K_v\beta 2$ and investigated the role of individual active site residues involved in binding pyridine nucleotides.

EXPERIMENTAL PROCEDURES

Construction of the Expression Vector for $K_v\beta 2$ —The cDNA containing the coding sequence for $K_v\beta 2$ was a gift from Dr. Min Li. To generate the $K_v\beta 2$ cDNA fragment with a *NdeI* site at the 5' end and a *XhoI* site at the 3' end, standard polymerase chain reaction procedures were used. The primers for the full-length β -subunit were 5'-CATATGTATCCGGAATCAACC-3' (forward) and 5'-GGATCTGACTTAGGATCTAT-

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¹ The abbreviations used are: AKR, aldoketo reductase; HPLC, high pressure liquid chromatography; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; WT, wild type; AR, aldose reductase; HSD, hydroxysteroid dehydrogenase; 2,5-DKGR, 2,5-diketogluconic acid reductase.

AGTCC-3' (reverse) and for the N-terminal deleted β -subunit were 5'-AGACAGCTCCATATGTACAGGAAC-3' (forward) and 5'-GGATCCTGACTTAGGATCTATAGTCC-3'. The polymerase chain reaction products were inserted into pCR-TOPO (Invitrogen), and the amplified vector was further digested by *NdeI* and *XhoI* to isolate the β -subunit fragments, which were ligated to a linearized pET28a vector cleaved by *NdeI* and *XhoI*.

Expression and Purification of $K_v\beta_2$ —The expression vectors pET28-F β (full-length $K_v\beta_2$) and pET28-C β ($\Delta NK_v\beta_2$, encoding amino acid residues 39–367) were transformed into strain BL21 of *Escherichia coli*. The transformed bacteria were cultured at 37 °C in LB medium containing 50 μ g/ml kanamycin. When the absorbance of the culture medium at 600 nm reached ~ 0.8 , the expression of the $K_v\beta_2$ protein was induced by the addition of 1 mM isopropyl- β -D-thiogalactoside. Induction was continued for another 4 h at 25 °C with constant shaking at 280 rpm. The bacteria were lysed by sonication in a buffer consisting of 20 mM Tris-HCl, pH 7.9, 200 mM NaCl, and 5 mM imidazole. The cell debris was pelleted by centrifugation, and the supernatant was applied to a nickel nitrilotriacetic acid Superflow (Qiagen) column that was pre-equilibrated with the binding buffer. The protein bound to the column was eluted by a step change in the imidazole concentration from 50 to 300 mM. The $K_v\beta_2$ protein was identified by its mobility on 12% SDS-polyacrylamide gel electrophoresis. Fractions containing $K_v\beta_2$ were collected, pooled, and dialyzed against 0.15 M potassium phosphate, pH 7.4. The molecular weight of the purified protein was determined by size-exclusion chromatography using a TSK-GEL G3000SW_{XL} (TosoHass, Montgomeryville, PA) column and a Waters Alliance HPLC. The column was equilibrated with 0.4 M potassium phosphate, pH 7.4, and calibrated using thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (43 kDa), and myoglobin (17.6 kDa).

Site-directed Mutagenesis—Site-directed mutants of $\Delta NK_v\beta_2$ were prepared using QuikChange mutagenesis kit (Stratagene). Mutation sites were introduced by single-mutant primers in polymerase chain reaction amplification using the *Pfu* Turbo DNA polymerase (Stratagene). The following primers were used: CTGGGGCACATCAATGTGAGCTCCATGGAG (R189M), GGTCGCATGACCGCGTCCCCTCTGCGGTGC (W243A), GGTGCCATGACCTACTCCCCTCTGGCGTGC (W243Y), CTGGTCCCCTCTGGCGTCCGGCATCGTC (C248S), GTCTCAGGGAAGTTTGACAGCGGGATCCAC (Y255F), CCCATCTGCGAGCGAGCGGAATATCAC (Q214R), CACCCTACTCCGAAGCCTCCCTGAAG (R264E), CAACTTATGGAGTGGATTGGAGCAATACAG (N333W). The sequence of the site-directed mutants was confirmed by DNA sequencing. The mutants were expressed and purified as described above.

Fluorescence Titrations—Fluorescence spectra were recorded on a Shimadzu RF-5301 PC fluorescence spectrophotometer. Unless indicated otherwise, an excitation wavelength of 290 nm and an emission wavelength of 335 or 345 nm were used for the fluorometric titrations. Aliquots of the protein were equilibrated with 2.0 ml of 0.15 M potassium phosphate, pH 7.4. The fluorescence of the protein was measured before and after the addition of 2–20 μ l of the pyridine nucleotides. To minimize nucleotide absorbance, a 5 \times 10-mm cuvette was used for titrations with NAD(H). For measuring the pH dependence of coenzyme binding, a three-buffer system was used that consisted of MES, MOPS, and Tris. The pK values of the individual components of the buffer at an ionic strength of 0.2 M and the amount of salt needed to keep the ionic strength constant throughout the experimental pH range were calculated using a computer program (13). The protein concentration was measured by the Bradford dye binding method (14).

Data Analysis—Fluorescence titration data were fitted to a binding equation that takes into account the corrections for scatter, dilution, and cofactor absorbance (15). In this equation, the fluorescence intensity I is a function of the cofactor concentration X , the protein concentration P , and the dissociation constant K_d , as shown below.

$$I(P, X, K_d) = e^{-\rho X} \left(\gamma \left(Y_{\min} - Y_{\max} \right) \frac{[PX]_{P, X, K_d}}{[P]} + Y_{\max} \right) + Y_{\text{bgnd}} \quad (\text{Eq. 1})$$

In this relationship, Y_{\min} and Y_{\max} are the minimum and maximum fluorescence intensities, respectively, Y_{bgnd} is the intensity of the background scatter, γ is $V_{\text{initial}} / (V_{\text{initial}} + V_X)$ (the dilution factor), and ρ is the absorbance coefficient of the cofactor. The fraction of the protein bound to the cofactor is related to these parameters as follows.

$$\frac{[PX]_{P, X, K_d}}{[P]} = \frac{\gamma P + X + K_d}{2\gamma P} - \frac{1}{2} \sqrt{\left(\frac{\gamma P + X + K_d}{\gamma P} \right)^2 - \frac{4X}{\gamma P}} \quad (\text{Eq. 2})$$

Using 0.1–2 μ M protein, we first determined the approximate K_d of the

individual nucleotides. Then, for final measurement of the K_d , the data were acquired at a protein concentration less than the expected value of K_d . The concentration of the active protein $[P]$ was determined by the curve-fitting procedure under the condition when the total concentration of the protein was more than K_d . Typically, $\sim 70\%$ of the protein was found to be active by this method. The absorbance correction used in the curve-fitting procedure was verified by titrating solutions of tryptophan (of equal absorbance as the protein) with NADPH.

The pH dependence of coenzyme binding was analyzed using Equation 3, in which $\log Y (= 1/K_d)$ decreases at both high and low pH (16) as follows.

$$\log Y = \log \left[\frac{c}{1 + \frac{[H^+]}{K_a} + \frac{K_b}{[H^+]}} \right] \quad (\text{Eq. 3})$$

where K_a and K_b are the dissociation constants of the enzyme, and c is the pH-independent value of Y . Additionally, Equation 4 was used to analyze data in which the value of Y decreases at low pH but levels out to a new value.

$$\log Y = \log \left[\frac{a + b \left(\frac{K_a}{[H^+]} \right)}{1 + \frac{K_a}{[H^+]}} \right] \quad (\text{Eq. 4})$$

The ionic strength dependence of pyridine nucleotide binding was analyzed using the Boltzmann relationship as follows.

$$Y = \frac{Y_{\max}}{1 + e^{-\left(\frac{K_{1/2} - X}{C} \right)}} \quad (\text{Eq. 5})$$

where Y is the dissociation constant, X is the ionic strength, Y_{\max} is the maximal value of the parameter, $K_{1/2}$ is the value of X at which Y is half-maximal, and C is the slope factor. In all cases, the best fit to the data was chosen on the basis of the standard error of the fitted parameter and the lowest value of σ , which is the residual sum of squares divided by the degrees of freedom.

RESULTS

As shown in Fig. 1, the purified wild-type (WT) $K_v\beta_2$, its N terminus deleted form ($\Delta NK_v\beta_2$), and the indicated site-directed mutants migrated as single bands on SDS-polyacrylamide electrophoresis gels. The molecular masses of these proteins were between 38 and 40 kDa. When examined by size exclusion chromatography, the $\Delta NK_v\beta_2$ eluted with a retention time of 9.8 min, which corresponds to a Stokes radius of a protein with a molecular mass of 153 kDa, indicating that under these conditions, the protein exists primarily as a homotetramer. No monomeric or dimeric forms of the protein were observed (Fig. 1B). The freshly purified $\Delta NK_v\beta_2$ showed a high absorbance at 260 nm and an additional absorbance band centered near 360 nm (Fig. 2A, inset), indicating that the purified protein remains bound to NAD(P)H. From the absorbance at 363 nm, a stoichiometry of ~ 0.9 mol of NADPH bound/mol of the protein was calculated. To confirm that the purified preparation was indeed a binary complex, the fluorescence spectrum of the nucleotide-bound protein was recorded. When excited at 290 nm, the freshly isolated protein showed two prominent emission bands with peaks at 335 and 450 nm (Fig. 2B). Upon extensive dialysis against 0.15 M potassium phosphate, pH 7.4, the intensity of the 335-nm band increased with a corresponding decrease in the emission band at 450 nm. When 1 μ M NADPH was added to the dialyzed protein, the 450-nm band reappeared, whereas the emission at 335 nm was quenched (data not shown). The emission band at 450 nm was not restored by the addition of NADP⁺, although this did quench the emission at 335 nm. We conclude, based on these observations, that NADPH remains bound to the freshly purified $\Delta NK_v\beta_2$ and that it is lost from the protein upon dialysis. These data also show that the formation of a binary complex between NADPH and $\Delta NK_v\beta_2$ quenches the intrinsic trypto-

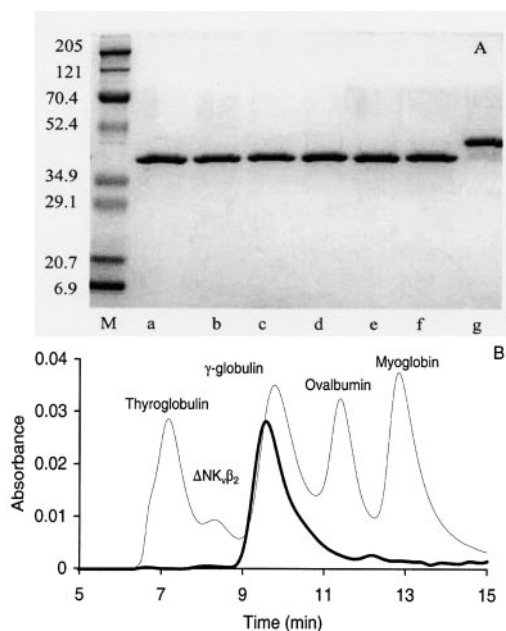


FIG. 1. SDS-polyacrylamide gel electrophoresis and size exclusion chromatography of $K_v\beta 2$. A, wild-type $NK_v\beta 2$ and $\Delta NK_v\beta 2$ and its site-directed mutants were purified from *E. coli* by a nickel affinity column and separated on SDS-polyacrylamide electrophoresis gels. Approximately 2.0 μg of protein was loaded on the gel and visualized by Coomassie Blue. Lane M, molecular weight markers; lanes a–g, $\Delta NK_v\beta 2$, R189M, W243A, W243Y, C248S, Y255F, and full-length $K_v\beta 2$, respectively. B, a 20- μl aliquot of 1 mg/ml $\Delta NK_v\beta 2$ was injected into a TSK-GEL G3000SW_{XL} column equilibrated with 0.4 M potassium phosphate, pH 7.4. The protein eluted with a retention time of 9.8 min, corresponding to a Stokes radius of a protein with a molecular mass of ~ 153 kDa. The broken trace shows the elution pattern of a different run containing the molecular mass standards; thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (43 kDa), and myoglobin (17.6 kDa).

phan fluorescence of the protein and leads to the appearance of a new emission band at 450 nm. However, for all subsequent experiments, we monitored the emission at 335 nm because, in contrast to the alterations at 450 nm, changes at 335 nm were independent of the redox state of the nucleotide.

The titration of the extensively dialyzed $K_v\beta 2$ with NADPH led to a progressive loss of fluorescence at 335 nm (Fig. 3). The change in fluorescence was saturated at high nucleotide concentration, and the addition of more than 0.6 μM NADPH caused no further decrease in fluorescence. Typically, NADPH quenched a maximum of ~ 30 –40% of the total fluorescence. Because the protein concentration was an independent variable in the fitting routine, at protein concentration $>K_d$, we estimate that 60–70% of the total protein was bound to NADPH. The K_d^{NADPH} of the full-length $K_v\beta 2$ was 0.08 ± 0.004 μM and that of $\Delta NK_v\beta 2$ was 0.10 ± 0.006 μM . These results suggest that $K_v\beta 2$ has a high affinity for NADPH that is not affected by the deletion of the N-terminal domain. Thus for all subsequent experiments, the $\Delta NK_v\beta 2$ protein was used.

In addition to NADPH, $\Delta NK_v\beta 2$ also displayed a high affinity for NADP^+ , although $K_d^{\text{NADP}^+}$ was 3-fold higher than K_d^{NADPH} (Table I). The nucleotides, NADH, and NAD^+ were also bound to the protein. However, the large intrinsic absorbance of these nucleotides at the high concentrations required for the assay precluded the accurate determination of the $K_d^{\text{NAD(H)}}$ under conditions identical to those used for measuring K_d^{NADPH} . Hence, to optimize emission and to minimize inner filter effects, a 5×10 -mm cuvette was used for the assay, and instead of 335 nm, the emission of the protein was measured at 345 nm. Under these conditions the absorbance of 0.1

mM NAD(H) was less than 0.05 (see “Experimental Procedures”). The $K_d^{\text{NAD(H)}}$ values thus determined were in the low micromolar range (Table I).

We next determined the interaction of $K_v\beta 2$ with different nucleotide analogs. The K_d of the protein for 3'-acetylpyridine NADP^+ was 10-fold greater as compared with NADP^+ , indicating that the amide side chain of the nicotine ring participates in high affinity binding of NADP^+ to the protein. The removal of the 3'-carbonyl from the nicotine ring also led to a decrease in affinity (compare the K_d values for 3-aminopyridine NADP^+ and NADP^+), suggesting that there are energetically significant interactions between the 3' side chain of the pyridine ring and the binding site residues. Other fragments of the pyridine coenzymes such as ADP-ribose, NMN, and nicotinamide displayed poor affinity for $\Delta NK_v\beta 2$. Moreover, the flavin coenzyme, FAD, bound weakly to the protein, indicating that it is unlikely to be an *in vivo* ligand of $K_v\beta 2$ or to compete with pyridine coenzymes for binding to the active site of the protein.

The crystal structure of the $\Delta NK_v\beta 2$ -NADP⁺ binary complex shows that the coenzyme binds into a deep cleft in the triose-phosphate isomerase scaffolding of the protein (12). When bound, the cofactor displays an extended conformation and makes several contacts with the binding site residues. A schematic representation of these interactions is shown in Fig. 4. The sequence alignment of the $K_v\beta$ proteins, using the program CLUSTLW (17), revealed that most of the residues interacting with the cofactor in $K_v\beta 2$ are conserved in other $K_v\beta$ proteins (Fig. 5). The orientation of the nicotinamide ring in $K_v\beta 2$ is constrained by H bonding with a basic residue (Arg-189) and π -stacking against an aromatic residue (Trp-243). To examine the significance of these interactions, site-directed mutants of $\Delta NK_v\beta 2$ were prepared in which Arg-189 was replaced by methionine, and Trp-243 was replaced by phenylalanine. As shown in Table II, no significant changes in the K_d^{NADPH} were observed with these mutations as compared with the WT protein. To confirm that the lack of change in the K_d^{NADPH} was not due to the retention of hydrophobicity in the tryptophan to phenylalanine substitution, Trp-243 was replaced with alanine. However, the K_d^{NADPH} of W243A was comparable with that of W243Y or the WT protein, indicating that ring stacking or the hydrophobicity of the residue at position 243 does not contribute to NADPH binding. In contrast, the disruption of the hydrogen bond between Asn-333 and the adenine ring in the N333W mutant completely prevented NADPH binding to the protein. Similarly, the replacement of an arginine replacement of glutamine 214, which interacts with the hydrogens attached to N₇N of the nicotinamide ring, led to a 20-fold increase in K_d^{NADPH} . This observation indicates that the amide side chain of the pyridine ring plays a significant role in nucleotide recognition at the $K_v\beta 2$ binding site.

In the $\Delta NK_v\beta 2$ -NADP binary complex the oxygen attached to the ribose phosphate ($\text{OP}_{1\text{R}}$) interacts with Tyr-255 via a water molecule (12), suggesting that this residue may be involved in coenzyme binding. The replacement of Tyr-255 with phenylalanine, however, did affect K_d^{NADPH} (Table II), indicating that this residue does not contribute to pyridine nucleotide binding. In addition to Tyr-255, the water molecule associated with $\text{OP}_{1\text{R}}$ forms a hydrogen bond with Cys-248 (12). This cysteine residue also interacts with the pyrophosphate oxygen ($\text{OP}_{2\text{A}1}$) in a mode reminiscent of the lysine residue (Lys-262) that is responsible for the tight binding of NADPH to aldose reductase (AR; Ref. 18). The replacement of Cys-248 by serine, however, increased the affinity of $\Delta NK_v\beta 2$ for NADPH, as evinced by a decrease in K_d^{NADPH} from 100 to 20 nM (Table II).

The coenzyme selectivity of the AKR proteins is in part due to the presence of basic amino acids in their binding pockets

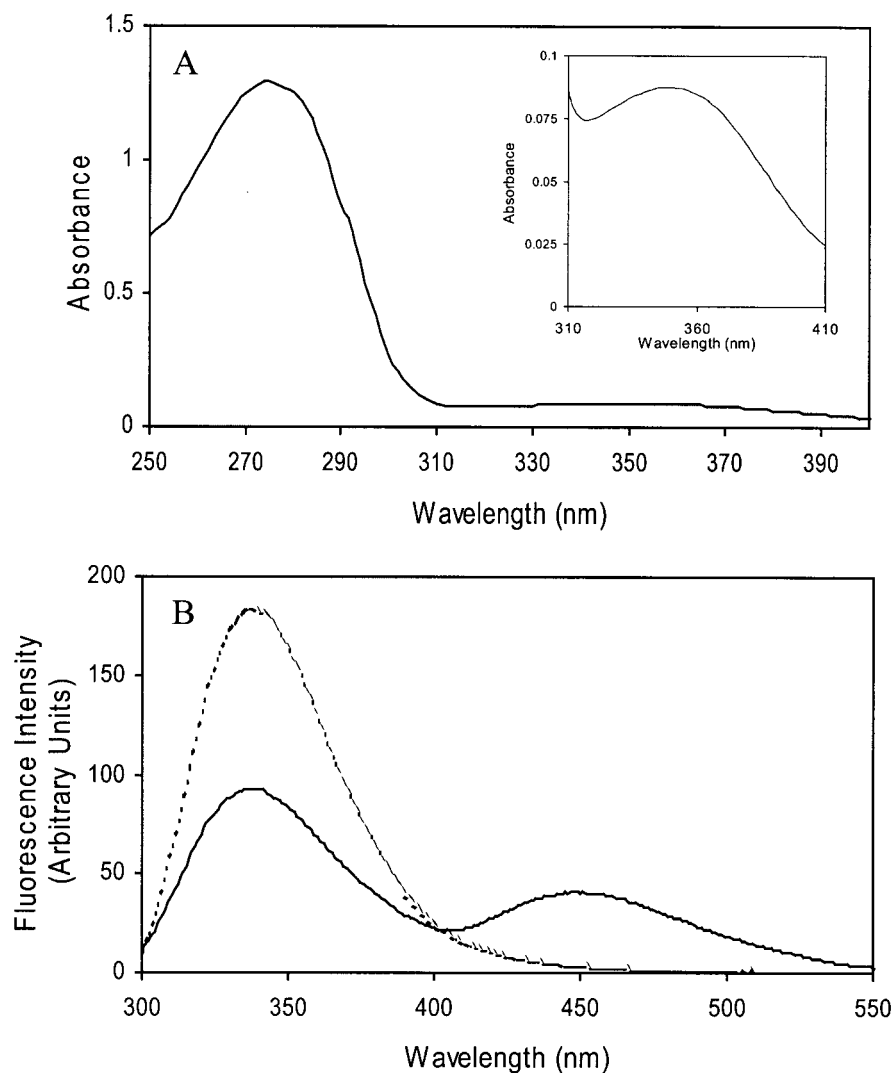


FIG. 2. Retention of NADPH in purified $K_v\beta 2$. Freshly prepared $\Delta NK_v\beta 2$ (~ 1 mg/ml) was suspended in 0.15 M potassium phosphate, pH 7.4, and scanned for absorbance and fluorescence. *A*, absorbance scan of $\Delta NK_v\beta 2$. The *inset* shows the absorbance of the protein between 310 and 400 nm. *B*, emission scan of $\Delta NK_v\beta 2$ in phosphate buffer before (—) and after (---) dialysis against phosphate buffer for 2 weeks. Identical protein concentrations were used for the two emission scans.

that accommodates the 2'-phosphate of NADPH (8, 19, 20). The 2'-phosphate binding pocket of $K_v\beta 2$ contains only one basic residue, that is, Arg-264. This residue forms a hydrogen bond with the free hydroxyl group of the adenine ribose and interacts with OP_{4R} of the 2'-phosphate (Fig. 4). In our experiments, the replacement of Arg-264 with glutamic acid led to a complete loss of NADPH binding. The fluorescence of R264E was not quenched even by the addition of 1 mM of NADPH. These observations suggest that Arg-264 is essential for NADPH binding to $K_v\beta 2$.

To confirm the results obtained from fluorometric titrations, the complete fluorescence spectra of the site-directed mutants were recorded. As expected, the freshly purified N333G and R264E proteins displayed a much stronger emission band at 335 nm than did equimolar concentrations of the WT or the C248S protein. Both the WT and C248S proteins displayed an additional band at 450 nm, which was absent in the emission spectra of the N333W and the R264E proteins (Fig. 6), indicating that the N333W and R264E proteins do not bind NADPH. When excited at 340 nm (to elicit NADPH fluorescence), both the WT and the C248S proteins displayed strong emission near 450 nm, whereas the N333W and the R264E proteins did not, confirming that the N333W and R264E proteins do not contain NADPH bound to their active sites. To examine whether the lack of NADPH binding affects the quaternary structure of the protein, we determined the Stokes radius of R264E using size

exclusion chromatography. The R264E protein eluted from the HPLC column with a retention time of 9.7 min (data not shown), which was similar to the retention time of the WT protein, indicating that binding of NADPH is not essential for the formation of the $\Delta NK_v\beta 2$ homotetramers.

To further characterize coenzyme binding to $K_v\beta 2$, we examined the effects of ionic strength and pH. As shown in Fig. 7A, an increase in the ionic strength of the buffer led to a decrease in K_d^{NADPH} . This dependence was best described by a Boltzmann function (Equation 5), in which the maximal value of the K_d^{NADPH} (Y_{max}) was calculated to be $2.9 \pm 0.3 \mu M$, with a $K_{1/2}$ of 0.59 ± 0.04 M and a slope factor C of $0.15 \pm 0.03 \times 10^{-6}$. The effect of ionic strength on the K_d^{NADPH} of R189M was similar to that observed with the WT protein. However, the ionic strength dependence was significantly altered by the C248S mutation. Compared with the WT protein, C248S was less sensitive to changes in ionic strength. The best fit of Equation 5 to the data provided the following estimates of the parameters: $Y_{max} = 1.6 \pm 0.4 \mu M$, $K_{1/2} = 0.79 \pm 0.15$ M, and $C = 0.22 \pm 0.05 \times 10^{-6}$. These results support the idea that NADPH binding to $K_v\beta 2$ is sensitive to changes in ionic strength within the physiological range and that this sensitivity is in part due to Cys-248.

The binding of NADPH to $K_v\beta 2$ was also found to be sensitive to pH. A systematic evaluation of the effects of pH revealed that the values of K_d^{NADPH} were enhanced at low pH but decreased at high pH. A plot of $\log(1/K_d)$ reached a plateau at

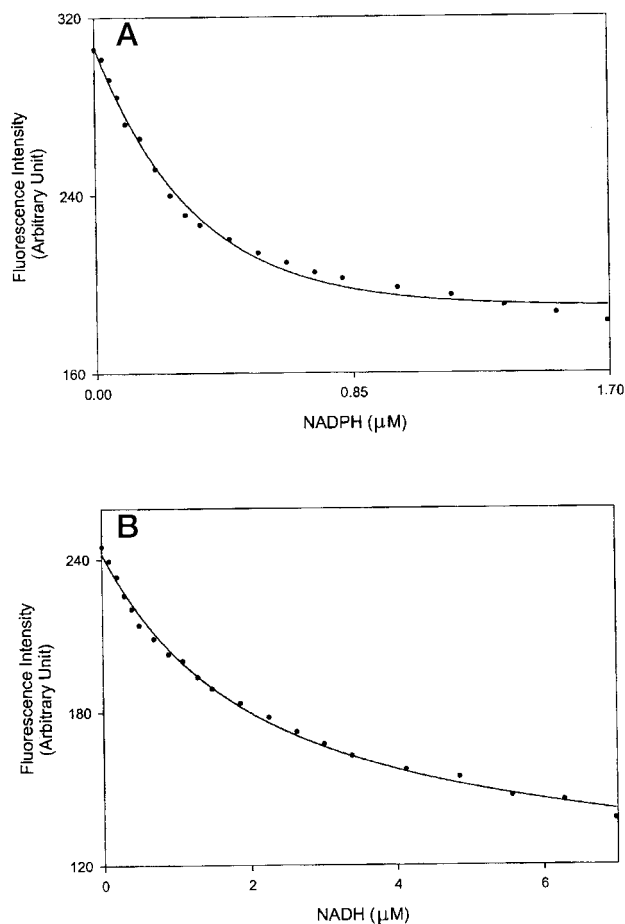


FIG. 3. **Binding of pyridine coenzymes to $K_v\beta 2$.** Dialyzed $\Delta N K_v\beta 2$ was suspended in 0.15 M potassium phosphate, pH 7.4, and changes in emission were monitored either at 335 nm (for NADPH) or 345 nm (for NADH) using 290 nm as the excitation wavelength. The protein was titrated with the indicated concentrations of NADPH (A) or NADH (B). The concentration of the protein was adjusted to be below the K_d . Data are shown as discrete points, and the curves are the best fits to the data estimated as described in the text.

TABLE I

The binding of pyridine nucleotide coenzymes and analogs to $K_v\beta 2$

Recombinant $\Delta N K_v\beta 2$ protein was suspended in 2 ml of 0.15 M potassium phosphate, pH 7.4, and changes in emission at 335 or 345 nm were monitored using an excitation wavelength of 290 nm. The concentration of the protein used was less than the K_d . Aliquots of the indicated ligands were added to the cuvette, and steady-state fluorescence was recorded. The K_d values were determined as described under "Experimental Procedures." Data are shown as the mean \pm S.D. ($n = 3-7$). FAD, flavin adenine dinucleotide; NMN, nicotinamide mononucleotide; N.D., no detectable change in fluorescence observed after the addition of 100 μ M ligand.

Ligand	K_d
	μ M
NADPH	0.12 ± 0.004
NADP ⁺	0.36 ± 0.014
NADH	1.23 ± 0.16
NAD ⁺	3.61 ± 0.4
3'-Acetylpyridine NADP ⁺	4.24 ± 1.15
3'-Aminopyridine NADP ⁺	14.35 ± 1.34
ADP-ribose	412.01 ± 23.5
FAD	10.2 ± 5.99
NMN	144 ± 15.3
Nicotinamide	N.D.

low pH, giving rise to a wave-like pH dependence (Fig. 7B). Using Equation 4, a pK_a of 6.9 ± 0.4 was calculated. At high pH, a slight decrease in K_d was observed, but even at pH 10, $\log 1/K_d$ did not decrease to half its maximal value, thereby pre-

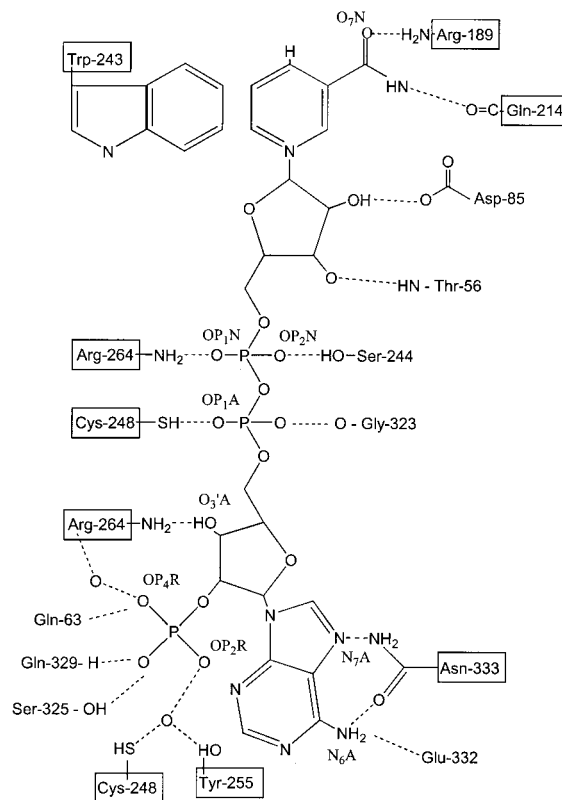


FIG. 4. **Schematic diagram of the interactions between NADPH and $K_v\beta 2$.** The schematic is based on the crystal structure of the $K_v\beta 2$:NADP binary complex solved by Gulbis *et al.* (12). The amino acid residues examined in the present study are shown in boxes. For clarity, multiple contacts with individual residues are shown separately. The atoms of NADPH interacting with the indicated residues are numbered.

cluding accurate estimates of pK_b . An approximate calculation using Equation 3 indicated that the pK_b is near 9.6. This ionization may be due to Arg-264, but the role of this residue could not be tested further because the R264E mutant did not bind NADPH. However, our data show that the pK_a value depends in part on Cys-248 because the C248S mutation shifted the pK_a value from 6.9 to 7.4 ± 0.2 (Fig. 7B).

DISCUSSION

The results of this study show that the β -subunit of the K_v channel preferentially binds NADPH, suggesting that NADPH may be the most probable ligand bound to $K_v\beta$ *in vivo*. Our data further show that NADPH binds to the C terminus or the conserved AKR core of the protein and that this binding is not affected by the variable N terminus of the protein. The high affinity with which $K_v\beta 2$ binds NADPH is comparable with the affinity of other AKR proteins for this cofactor (21, 22). Upon binding NADPH, the intrinsic fluorescence of $K_v\beta 2$ was quenched, and an additional emission band appeared that centered around 450 nm. These changes are similar to those observed upon NADPH binding to other AKR proteins, such as 3α -hydroxysteroid dehydrogenase (3α -HSD; Ref. 21) and AR (22). The 450-nm emission of the 3α -HSD-NADPH complex has been suggested to be due to the formation of a charge-transfer complex between the reduced nicotinamide ring and Trp-87 located within 10 Å of the ring (21). The Trp-87 of 3α -HSD is located in the $\beta 3$ sheet of the triosephosphate isomerase barrel and is conserved in AR (Trp-79), $K_v\beta$ (Trp-121), and other AKR proteins (8), indicating that similar interactions are likely in most AKRs. Hence, the formation of a low energy charge-

FIG. 5. The alignment of the amino acids sequences of the conserved C terminus core of $K_v\beta$ proteins. The sequences were aligned using the program CLUSTALW (17). The filled circles indicate the residues forming contacts with NADP(H). The residues mutated in this study are boxed. The sequences were obtained from the NCBI protein data bank: $K_v\beta 1$ (human, S66503), $K_v\beta 2$ (rat, X76724), $K_v\beta 3$ (rat, S7562), and $K_v\beta 4$ (mouse, U65593).

$K_v\beta 1$	LGTFTPQHHSILKSTAKQTGMKYRNLGKSGLRVSCGLGLTWTWTFGGQISDEVAERLMTI	109
$K_v\beta 2$	-QTGSPGMIYSTRYGSFKRQLQFYRNLGKSGLRVSCGLGLTWTWTFGGQITDEMAHMLTL	75
$K_v\beta 3$	VVPRFPAPAGALRESTGRGTGMKYRNLGKSGLRVSCGLGLTWTWTFGGQISDEVAERLMTI	116
$K_v\beta 4$	-----MSRGYGLIFS-----LKVVFPT-----FLSL	20
$K_v\beta 1$	AYESGVNLFDTAEVYAA-GKAEVILGSIKKKGRWRRSSLVITTKLYWGGKAETERGLSRK	168
$K_v\beta 2$	AYDNGINLFDTAEVYAA-GKAEVVLGNIKKKGRWRRSSLVITTKIFWGGKAETERGLSRK	134
$K_v\beta 3$	AYEHGVNLFDTAEVYAA-GKAEVILGSIKKKGRWRRSSLVITTKIFWGGKAETERGLSRK	175
$K_v\beta 4$	PHPP-----	24
$K_v\beta 1$	HIIEGLKGLRQLRQLEYVDVVFANRPPDNTPMEEIVRAMTHVINQGMAMYWGTSRWSAME	228
$K_v\beta 2$	HIIEGLKASLERLRQLEYVDVVFANRPPDNTPMEEIVRAMTHVINQGMAMYWGTSRWSAME	194
$K_v\beta 3$	HIIEGLQGLDLRQLRQLEYVDVVFANRSDPSSPMEEIVRAMTYVINQGLALYWGTSRWSAAB	235
$K_v\beta 4$	----GLQSSDLRQLRQLEYVDVVFANRSDPNSPMEEIVRAMTYVINQGLALYWGTSRWSAAB	80
$K_v\beta 1$	IMEAYSVARQFNMIIPVCEQAIEYHLPQREKVEVQLPELYHKIGVGAMTWSPLACGIISGK	288
$K_v\beta 2$	IMEAYSVARQFNLIIPVCEQAIEYHMFQREKVEVQLPELPHKIGVGAMTWSPLACGIIVSGK	254
$K_v\beta 3$	IMEAYSMARQFNLIIPVCEQAENHFFQREKVEVQLPELYHKIGVGAMTWSPLACSLITSK	295
$K_v\beta 4$	IMEAYSMARQFNLIIPVCEQAENHFFQREKVEVQLPELYHKIGVGAMTWSPLACGLITSK	140
$K_v\beta 1$	VGN-GVPESASLKYQWLKERIVS-----EGRKQNKLDLSPIAER	332
$K_v\beta 2$	VDS-GIPPYASLKYQWLKDKILS-----EGRQRQAKLKLQIAER	298
$K_v\beta 3$	VDG-QVPDCAATVKGQWLKEKQVS-----EDGKKQARVTDLLPIAHQ	339
$K_v\beta 4$	VDG-RVPDCAATVKGQWLKEKQVS-----EGRKQNKLDLSPIAER	184
$K_v\beta 1$	LGCTLPQLAVAWCLRNREGVSSVLLGSSSTPEQLIENLGAIQVLPKMTSHVNEIDNLRNK	392
$K_v\beta 2$	LGCTLPQLAIAWCLRNREGVSSVLLGASNAEQLMBENIGAIQVLPKLSSEIIVHEIDSILGNK	358
$K_v\beta 3$	LGCTVAQLAIAWCLRNREGVSSVLLGVSSAEQLMBHLLGSLQVLGQLTPTQVMEIDALLGNK	399
$K_v\beta 4$	LGCTVGLAIAWCLRNREGVSSVLLGVSSAEQLMBHLLGSLQVLSQLTPTQVMEIDALLGNK	244
$K_v\beta 1$	PYSKKDYRS----	401
$K_v\beta 2$	PYSKKDYRS----	367
$K_v\beta 3$	SHSKK-----	404
$K_v\beta 4$	SHSKK-----	249

TABLE II

The binding of NADPH to $\Delta NK_v\beta 2$ and its site-directed mutants

The K_d values of the protein were determined in 0.15 M potassium phosphate, pH 7.4, as described under "Experimental Procedures." Data are the mean \pm S.D. N.D., no detectable change in fluorescence after the addition of 1 mM NADPH.

Protein	K_d
	μM
$\Delta NK_v\beta 2$	0.11 ± 0.02
R189M	0.093 ± 0.01
W243Y	0.068 ± 0.003
W243A	0.106 ± 0.005
N333W	N.D.
Q214R	2.14 ± 0.4
Y255F	0.096 ± 0.001
C248S	0.017 ± 0.001
R264E	N.D.

transfer complex in the $K_v\beta 2$ -NADPH complex suggests that the functional characteristics of pyridine coenzyme binding as well as the solution conformation of the AKR active site are conserved in $K_v\beta 2$.

The crystal structure the $K_v\beta 2$ -NADP complex shows that the coenzyme forms multiple contacts with the protein (12). The two ends of the coenzyme molecule, the nicotinamide and the adenine rings, interact with residues that are similar to those observed in AR, 3 α -HSD, and 2,5-DKGR (23–25). The binding of the nicotinamide ring by 3 α -HSD is defined by the interactions between Asn-167 and O₇N, Ser-166 and N₇N, and Gln-190 and N₇N of NADP⁺ (24). The corresponding residues in AR are Asn-160, Ser-159, and Gln-183 (23). Although in $K_v\beta 2$ the serine residue (at position 188) and the glutamine residue (at position 214) are conserved, the O₇N of NADP⁺ interacts with Arg-189 (12). However, the interaction between O₇N and Arg-189 in $K_v\beta 2$ does not seem to contribute to the stabilization of the nicotinamide ring at the binding site, because R189M mutation did not affect K_d^{NADPH} . Thus, the recognition of the nicotinamide ring appears primarily to be due to the interactions between the Ser-188 and Gln-214 of $K_v\beta 2$ and the amino group of the nicotinamide ring. This view is supported by the observation

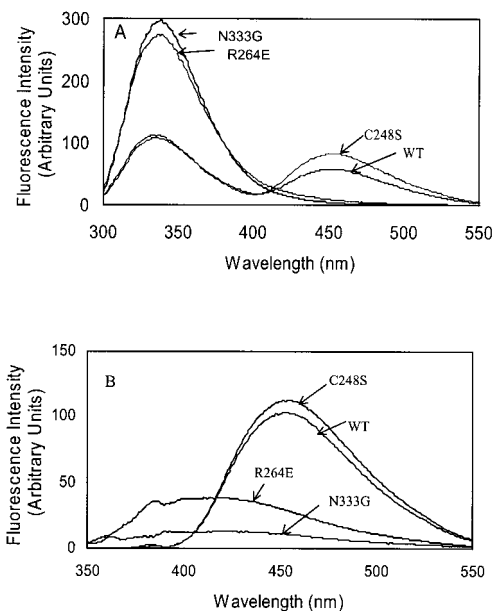


FIG. 6. The fluorescence spectra of the WT, C248S, R264E, and N333G $\Delta NK_v\beta 2$. The indicated proteins were purified and suspended in 0.15 M potassium phosphate, pH 7.4. Aliquots of equal concentrations of the proteins were excited at either 290 (A) or 340 nm (B).

that the K_d values of $K_v\beta 2$ for 3'-acetyl pyridine NADP⁺ and 3'-amino pyridine NADP⁺ were 10–30-fold higher than that for NADP⁺ (Table I). Nicotinamide by itself did not bind to the protein, although ADP-ribose and NMN displayed high, but measurable, K_d values, suggesting that the coupling of the adenine and the nicotinamide rings to ribose enhances the binding of the cofactor to the protein.

The major difference in NADPH binding by the AKR proteins is their interaction with the pyrophosphate backbone and the 2'-phosphate of the nucleotide. In AR, the pyrophosphate oxygens interact with the basic residues Lys-21 and Lys-262, and the 2'-phosphate forms electrostatic links with Arg-268 and

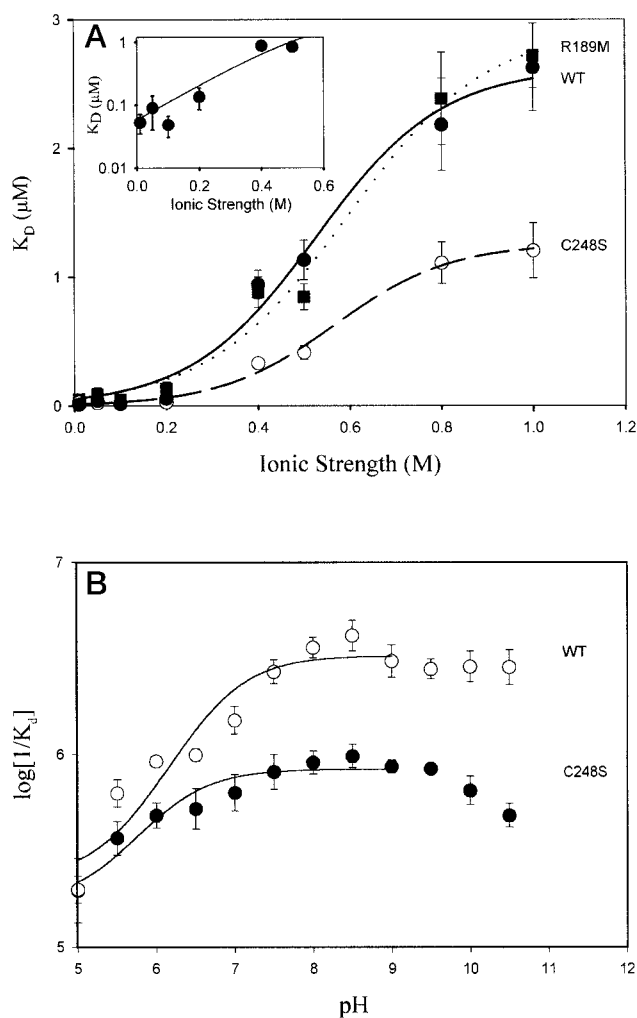


FIG. 7. pH and ionic strength dependence of NADPH binding to $K_v\beta 2$. A, The K_d^{NADPH} of the WT (○), R189M (●), and C248S (□) $\Delta\text{NK}_v\beta 2$ was determined in 10 mM HEPES, pH 7.0 at the indicated ionic strength adjusted with KCl. For clarity, the inset shows a semi-logarithmic plot of the ionic strength dependence of NADPH binding to the WT protein. B, the pH-dependence of K_d^{NADPH} of WT (○) and C248S (●) $K_v\beta 2$. Buffers of different pH were prepared using MES, MOPS, and Tris at a constant ionic strength of 0.2 M. The data are shown as mean \pm S.D. ($n = 3$), and the curves are drawn from the best fits of the data ($R^2 = 0.96\text{--}0.99$) to Equation 4 for pH dependence and Equation 5 for ionic strength dependence of nucleotide binding.

Lys-262 (23). These links form a safety belt, constraining the coenzyme with an unusually high affinity ($K_d^{\text{NADPH}} = 6$ nM) (26). The cofactor binding loop of AR and the residues involved in tight binding (that is, Lys-21, Lys-262, and Arg-268) are absent in other AKR proteins. In 3α -HSD, the 2'-phosphate of the nucleotide forms hydrogen bonds with Arg-276 and Arg-270, and these interactions are responsible for the preference of the protein for NADP^+ over NAD^+ (24). Similarly, a pair of basic residues (Lys-232 and Arg-238) interacts with the 2'-phosphate of NADPH at the active site of 2,5-DKGR (25). However, in $K_v\beta 2$, Arg-264 is the only basic residue interacting with the coenzyme pyrophosphates, the hydroxyl of the adenine ribose, and, via a water molecule, the 2'-phosphate (12). An arginine residue is also located at the same position in $K_v\beta 1$, although $K_v\beta 3$ and -4 show a conserved lysine substitution at this site. The predominant role of Arg-264 in pyridine coenzyme binding to $K_v\beta 2$ is suggested by the observation that the R264E mutation prevented NADPH binding ($K_d > 1$ mM).

Thus, in $K_v\beta 2$, Arg-264 seems to play a more important role in coenzyme binding than do the analogous residues of other AKR proteins.

Site-directed mutations of the phosphate-interacting basic residues of AR (Arg-268 and Lys-262) and 3α -HSD (Arg-276) increase K_d^{NADPH} by 50- to 150-fold (27, 28) but do not completely prevent NADPH binding. In fact, the R276M mutant of 3α -HSD has a 14-fold lower K_d^{NADH} than does the WT enzyme, indicating that residues other than Arg-276 are more important in binding the pyrophosphate backbone (28). In contrast, the lack of coenzyme binding to the R264E mutant of $K_v\beta 2$ demonstrates that the same arginine residue binds to both the 2'-ribose phosphate and the pyrophosphate backbone of NADPH, as is evident from the crystal structure (12). The lack of basic residues other than Arg-264 may also be responsible for the relatively low selectivity of $K_v\beta 2$ for NADPH. The $K_v\beta 2$ binds NADP(H) with a 10-fold higher affinity than NAD(H) (Table I). In comparison, 3α -HSD (28) and AR (27), which have multiple cofactor-interacting basic residues, display a 800- to 1000-fold higher selectivity for NADP(H) as compared with NAD(H).

In contrast to Arg-264, the contribution of other residues interacting with the coenzyme phosphates appears to be minimal. Although the phosphate-binding site of $K_v\beta 2$ contains an aromatic residue (Tyr-255) analogous to Phe-272 in 3α -HSD and Tyr-265 in AR, the role of this residue in coenzyme binding appears to be limited because the K_d^{NADPH} of Y255F was similar to that of the WT $K_v\beta 2$. Interestingly, the OP_{1R} of NADPH, which is bound to Lys-262 in AR (23), interacts with a cysteine residue (Cys-248) in $K_v\beta 2$. In the crystal structure of $K_v\beta 2$:NADP, Cys-248 also interacts with the 2'-phosphate via a water molecule (12). The K262M mutation in AR prevents tight binding of NADPH (K_m^{NADPH} increases > 60 -fold) and leads to an increase in the overall catalytic rate of the enzyme (18). However, the C248S mutation led to a decrease in K_d^{NADPH} , suggesting that the interaction of this residue with NADPH prevents tight binding of the cofactor. Moreover, because the C248S mutation also affected the pH and the ionic strength dependence of K_d^{NADPH} , it appears that the redox state or the ionization of Cys-248 may be an important determinant of NADPH binding to $K_v\beta 2$.

The binding of NADPH to $K_v\beta 2$ was also prevented by the N333W mutation. The Asn-333 forms H bonds with N₇A and interacts with the hydrogen attached to N₆A. Together with Glu-332, this residue appears to be important in holding and orienting the adenine ring at the active site (12). This binding motif is conserved in all AKR proteins. The corresponding residues are Glu-279 and Asn-280 in 3α -HSD, Glu-217 and Asn-272 in AR, and Asn-242 and Glu-241 in 2,5-DKGR. The Asn-333 of $K_v\beta 2$ is also conserved in $K_v\beta 1$. However, the corresponding residue in $K_v\beta 3$ and -4 is a histidine (Fig. 5). Although the role of these residues in NAD(P)(H) binding to other AKR proteins has not been examined, our results show that Asn-333 is critical for NADPH binding to $K_v\beta 2$. Moreover, the interaction of Asn-333 with the adenine ring appears to be more significant than the corresponding interaction of the amino groups of the nicotinamide ring with Gln-214 because the Q214R mutation did not completely prevent binding, even though K_d^{NADPH} was increased 10-fold. Surprisingly, we found that π -stacking against tryptophan does not contribute to NADPH binding. It has been suggested that the π -stacking of the nicotinamide ring against an aromatic residue (Tyr-216 in 3α -HSD, Tyr-209 in AR, and Trp-187 in 2,5-DKGR) stabilizes the binding of the cofactor at the active site of AKR proteins (23, 24). However, the K_d^{NADPH} of W243A was similar to that of the WT protein, suggesting that ring stacking does not make a

significant contribution to nucleotide binding. Nonetheless, our results do not rule out the possibility that this stacking may be important for the proper orientation of the nicotinamide ring and for facilitating hydride transfer from the B-face of the cofactor.

Our observation that $K_v\beta$ displays high affinity for NADPH further strengthens the view that the β -subunit may be an enzyme with oxidoreductase properties. Although the catalytic properties of $K_v\beta$ have not been reported, the higher affinity of this protein for NADPH as compared with $NADP^+$ indicates that the β -subunit is more likely to be a reductase rather than an oxidase. Moreover, because the protein did not bind NADPH as tightly as AR, it appears likely that the range of the substrates of $K_v\beta$ may be more restricted than that of AR. The wide substrate specificity of AR is in part due to its tight binding to NADPH, that provides most of the energy required to achieve the transition state. The contribution of the substrate binding step is minimal, thereby enabling AR to catalyze the reduction of a wide range of aldehydes (29). In contrast, AKR proteins such as 3 α -HSD, which do not bind NADPH very tightly, recognize a narrower range of structural motifs (30). Therefore, the $K_v\beta$ is likely to recognize a limited set of substrates, making the empirical identification of this set somewhat difficult.

The binding of pyridine nucleotides to $K_v\beta$ could also serve noncatalytic functions. Nucleotide binding may be required for the structural stability of the protein or for the formation of $K_v\beta$ - $K_v\beta$ or $K_v\beta$ - $K_v\alpha$ multimers. Our results showing that R264E, which does not bind NADPH, is a homotetramer suggest that nucleotide binding is not a prerequisite for maintaining structural integrity or for $K_v\beta$ - $K_v\beta$ interactions. Additionally, the binding of different nucleotides to $K_v\beta$ may be able to differentially regulate K_v channel activity, thereby allowing the channels to "sense" the redox state of the cellular pyridine nucleotides pool. The relatively poor nucleotide discrimination by $K_v\beta 2$ is consistent with this idea and indicates that several forms of protein-nucleotide complexes can exist *in vivo*.

Although our measurements show that the $K_d^{NADP(H)}$ is 10-fold higher than $K_d^{NAD(H)}$, the nature of the cofactor bound to $K_v\beta 2$ *in vivo* will depend upon the relative cellular concentration of pyridine coenzymes that will compete with NADPH for binding. For the NADPH/ $NADP^+$ couple this competition could be described by the following relationship (32).

$$Y_{NADPH} = \left(1 + \frac{K_d^{NADPH}}{[NADPH]} + \frac{K_d^{NADPH}[NADP^+]}{K_d^{NADP^+}[NADPH]} \right)^{-1} \quad (\text{Eq. 6})$$

where Y_{NADPH} is the fraction of the protein bound to NADPH. At $NADPH = 50 \mu\text{M}$ and $NADP^+ = 15 \mu\text{M}$, which is near their cellular concentrations (31), we estimate that more than 90% of the protein will be bound to NADPH (assuming the K_d values listed in Table I). However, when NAD^+ is the competing nucleotide, only 75% of $K_v\beta$ will be bound to NADPH because the cellular concentration of NAD is 10-fold higher than that of NADPH (31). Thus, the β -subunit may be capable of sensing the relative concentrations of pyridine coenzymes such that its conformation and its ability to bind and modulate $K_v\alpha$ may depend upon whether it is bound to $NADP(H)$ or $NAD(H)$. Furthermore, because the cytoplasmic concentrations of $NADP(H)$ and $NAD(H)$ vary with the rate of metabolism and the oxygen concentration (48), differential nucleotide binding may be relevant to the oxygen-sensing ability of $K_v\beta$ that has been demonstrated recently (33).

Despite their low affinity, the binding of nucleotide analogs to $K_v\beta 2$ may also be of physiological significance. Nucleotide

analogues such as NMN and ADP-ribose are generated during cellular metabolism and by DNA degradation (34), for instance, during apoptosis. Because apoptosis in some cases is mediated by the activation of K_v channels (35) and the bacterial apoptotic proteins Reaper and Grim increase the inactivation of the Shaker-type K^+ channels (36), the regulation of $K_v\beta$ by the metabolites of pyridine coenzymes warrants further investigation. Additionally, our observation that the high affinity form of $K_v\beta$ can change to a low affinity form by changing the ionic strength of the medium raises the possibility that *in vivo* the $K_v\beta$ protein may exist in two discrete states. Although demonstrated here in terms of ionic strength, the transition between these two states may also be regulated by other conditions, such as coupling with $K_v\alpha$ or the membrane voltage.

In summary, we report here for the first time that $K_v\beta 2$ displays high affinity and selectivity for NADPH and other related nucleotides. The key residues involved in the recognition and binding of NADPH were identified by site-directed mutagenesis. Specific mutations that can increase (C248S), decrease (Q214E), or prevent (R264E and N333W) NADPH binding to $K_v\beta 2$ were identified. These mutations may be useful for further probing the role of pyridine nucleotides in regulating the function(s) of $K_v\beta$. Because, with the exception of Asp-333, these residues are conserved among the $K_v\beta$ family of proteins, it appears that the residues identified in $K_v\beta 2$ play a similar role in the binding of pyridine nucleotide coenzymes to other $K_v\beta$ proteins as well. Further characterization of NADPH binding to $K_v\beta$ is necessary to understand the mechanisms by which pyridine nucleotides modulate the activity of the voltage-sensitive potassium channels and their role in surface excitability, osmo-regulation, oxygen sensing, and cell survival.

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