The β-subunit of the voltage-sensitive K⁺ (Kv) channels belongs to the aldo-keto reductase superfam-ily, and the crystal structure of Kvβ2 shows NADP bound in its active site. Here we report that Kvβ2 displays a high affinity for NADPH (Kd = 0.1 μM) and NADP⁺ (Kd = 0.3 μM), as determined by fluorometric titrations of the recombiant protein. The Kβ2 also bound NAD(H) but with 10-fold lower affinity. The site-directed mutants R264E and N333W did not bind NADPH, whereas, the Kd NADP of Q214R was 10-fold greater than the wild-type protein. The Kd 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 Kd NADPH, shifted the pKd of Kd 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 Kβ2-nucleotide complexes may be formed in vivo.

The voltage-sensitive K⁺ (Kv) 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 Kvα proteins associates with auxiliary β-subunits (Kvβ), which do not participate in ion conductance but can regulate the activity of the channel (4, 5).

Several homologous genes encoding the Kvβ 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: Kvβ1 to 3. In addition, several splice variants of Kvβ1, that is, Kvβ1.1, 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 Kvα currents (4, 5), the physiological role of these proteins remains unclear. In heterologous systems, coexpression of Kvβ increases the surface expression of Kvα, indicating that the β subunits regulate the expression and/or the localization of the Kvα proteins. Moreover, Kvβ2, which is the most widely distributed of the β-subunits, does not affect inactivation even though it associates with Kvα, suggesting that the β-subunits may have other undetermined physiological functions.

Structural analyses support the view that Kvβ proteins may have unique regulatory properties not displayed by accessory proteins of other ion channels. The primary amino acid sequence of the Kvβ proteins is not related to the auxiliary proteins of other voltage-sensitive channels but, unexpectedly, to the proteins of the aldo-keto reductase (AKR) superfamily (6, 7). Within this superfamily, the amino acid sequences of the Kvβ proteins are most closely related to alfaftoxin reductase (AKR7) and morphine dehydrogenase and 2,5-diketogluconate reductase (AKR5). On the basis of this homology, the Kvβ 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 Kvβ proteins are catalytically competent oxidoreductases that couple metabolic changes to membrane excitability.

The crystal structure of Kvβ2 shows that the protein folds into ββαβαβ 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 Kvβ2 by active site residues, some of which are conserved within the AKR superfamily. Nonetheless, no functional data are available on pyridine nucleotide binding to Kvβ. In the present study, we examined the coenzyme specificity and selectivity of the purified Kvβ2 and investigated the role of individual active site residues involved in binding pyridine nucleotides.

EXPERIMENTAL PROCEDURES

Construction of the Expression Vector for Kvβ2—The cDNA containing the coding sequence for Kvβ2 was a gift from Dr. Min Li. To generate the Kvβ2 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’-CATATGTATCCGGAATCACAAC-3’ (forward) and 5’-GGATCTTGACTTTAGGATCTAT-
AGTCC-3' (reverse) and for the N-terminal deleted β-subunit were 5'-AGACAGCTTCAATGTGACGAGA-3' (forward) and 5'-GATC-CTGACTTAGGATATCAGTC-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 $K_vβ2$—The expression vectors pET28-F9 (full-length $K_vβ2$) and pET28-C$β2$ (ΔNKβ2, 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 μg/ml kanamycin. The absorbance of the culture medium at 600 nm reached ~0.8, the expression of the $K_vβ2$ 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 7.4, 200 mM NaCl, and 5 mM imidazole. The cell debris was pelleted by centrifugation, and the supernatant was applied to a nickel-nitrioltriacetic 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 $K_vβ2$ protein was identified by its mobility on 12% SDS-polyacrylamide gel electrophoresis. Fractions containing $K_vβ2$ 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-GEI GL3000SW (TosoHass, Montomeryville, 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 ΔNKβ2 were prepared using QuikChange mutagenesis kit (Stratagene). Site-directed mutagenesis sites were introduced by single-mutant primers in polymerase chain reaction amplification using the Pfu Turbo DNA polymerase (Stratagene). The following primers were used: CTGGGGCACATCA (W243Y), CTGGTCCCCTCTGGCGTGC (W243A), GGTGCCATGACC (R189M), GGTGCCATGACC (Q214R), CACCCTACTCCGCAATACAG (T262A), and CGAATTGACATACAG (T262A). The transformed bacteria were cultured at 37 °C in LB medium at 600 nm reached 0.8, the expression of the $K_vβ2$ protein was indicated otherwise, an excitation wavelength of 290 nm and an emission wavelength of 335 or 345 nm were used for the fluorometric titrations. Data Analysis—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. Allosteric and the lowest value of $\gamma$ is a function of the cofactor concentration

\[
\log_2 I = \log_2 \frac{I_{max}}{I_{bgnd}}
\]

where $Y$ is the dissociation constant, $X$ is the intrinsic strength, $Y_{max}$ is the maximal value of the parameter, $K_{\beta2}$ 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 $\gamma$, which is the residual sum of squares divided by the degrees of freedom.

**RESULTS**

As shown in Fig. 1, the purified wild-type (WT) $K_vβ2$, its N-terminus deleted form (ΔNKβ2), 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 ΔNKβ2 eluted with a retention time of 9.8 min, which corresponds to a Stokes radius of a monomeric protein. The absorbance correction used 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 ΔNKβ2 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 NADP+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 μM 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 ΔNKβ2 and that it is lost from the protein upon dialysis. These data also show that the formation of a binary complex between NADPH and ΔNKβ2 quenches the intrinsic trypto-
Binding of Pyridine Nucleotides to K\textsubscript{v,2}

A, wild-type NK\textsubscript{v,2} and ΔNK\textsubscript{v,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, ΔNK\textsubscript{v,2}, B183M, W243A, W243Y, C248S, Y255F, and full-length Kv\textsubscript{v,2}, respectively. B, a 20-μl aliquot of 1 mg/ml ΔNK\textsubscript{v,2} was injected into a TSK-GEL G3000SWXL column equilibrated with 0.4 M potassium phosphate, pH 7.4. The protein eluted with a retention time of 9.8 min, respectively.

The titration of the extensively dialyzed K\textsubscript{v,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\textsubscript{d}, we estimate that 60–70% of the total protein was bound to NADPH. The K\textsubscript{d,NADPH} of the full-length K\textsubscript{v,2} was 0.08 ± 0.004 μM and that of ΔNK\textsubscript{v,2} was 0.10 ± 0.006 μM. These results suggest that K\textsubscript{v,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 ΔNK\textsubscript{v,2} protein was used.

In addition to NADPH, ΔNK\textsubscript{v,2} also displayed a high affinity for NADP\textsuperscript{+}, although K\textsubscript{d,NADP\textsuperscript{+}} was 3-fold higher than K\textsubscript{d,NADPH} (Table I). The nucleotides, NADH, and NAD\textsuperscript{+} 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\textsubscript{d,NADH} under conditions identical to those used for measuring K\textsubscript{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\textsubscript{d,NADH} values thus determined were in the low micromolar range (Table I).

We next determined the interaction of K\textsubscript{v,2} with different nucleotide analogs. The K\textsubscript{d} of the protein for 3′-acetylpyridine NADP\textsuperscript{+} was 10-fold greater as compared with NADP\textsuperscript{+}, indicating that the amide side chain of the nicotine ring participates in high affinity binding of NADP\textsuperscript{+} to the protein. The removal of the 3′-carbonyl from the nicotine ring also led to a decrease in affinity (compare the K\textsubscript{d} values for 3-aminopyridine NADP\textsuperscript{+} and NADP\textsuperscript{+}), 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 ΔNK\textsubscript{v,2}. Moreover, the flavin coenzyme, FAD, bound weakly to the protein, indicating that it is unlikely to be an in vivo ligand of K\textsubscript{v,2} or to compete with pyridine coenzymes for binding to the active site of the protein.

The crystal structure of the ΔNK\textsubscript{v,2}-NADP\textsuperscript{+} binary complex shows that the coenzyme binds into a deep cleft in the triosephosphate 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\textsubscript{v,2} proteins, using the program CLUSTLW (17), revealed that most of the residues interacting with the cofactor in K\textsubscript{v,2} are conserved in other K\textsubscript{v,2} proteins (Fig. 5). The orientation of the nicotinamide ring in K\textsubscript{v,2} is constrained by H bond 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 ΔNK\textsubscript{v,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\textsubscript{d,NADPH} were observed with these mutations as compared with the WT protein. To confirm that the lack of change in the K\textsubscript{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\textsubscript{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 the pyridine ring plays a significant role in nucleotide recognition at the K\textsubscript{v,2} binding site.

In the ΔNK\textsubscript{v,2}-NADP\textsuperscript{+} binary complex the oxygen attached to the ribose phosphate (OP\textsubscript{1}) 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\textsubscript{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 OP\textsubscript{1} forms a hydrogen bond with Cys-248 (12). This cysteine residue also interacts with the pyrophosphate oxygen (OP\textsubscript{2a}) 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 ΔNK\textsubscript{v,2} for NADPH, as evinced by a decrease in K\textsubscript{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.
that accommodates the 2'-phosphate of NADPH (8, 19, 20). The 2'-phosphate binding pocket of Kvβ2 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 OP4R 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 Kvβ2.

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 ΔNKβ2 homotetramers.

To further characterize coenzyme binding to Kvβ2, 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 $K_d^{\text{NADPH}}$. This dependence was best described by a Boltzmann function (Equation 5), in which the maximal value of the $K_d^{\text{NADPH}}$ ($Y_{\text{max}}$) was calculated to be $2.9 \pm 0.3 \mu$M, with a $K_{1/2}$ of $0.59 \pm 0.04 \mu$M and a slope factor $C$ of $0.15 \pm 0.03 \times 10^{-6}$. The effect of ionic strength on the $K_d^{\text{NADPH}}$ 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: $Y_{\text{max}} = 1.6 \pm 0.4 \mu$M, $K_{1/2} = 0.79 \pm 0.15 \mu$M, and $C = 0.22 \pm 0.05 \times 10^{-6}$. These results support the idea that NADPH binding to Kvβ2 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 Kvβ2 was also found to be sensitive to pH. A systematic evaluation of the effects of pH revealed that the values of $K_d^{\text{NADPH}}$ were enhanced at low pH but decreased at high pH. A plot of $\log (1/K_d^{\text{NADPH}})$ reached a plateau at
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 precluding 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).

**TABLE I**

| Ligand                   | $K_d$ (μM) |
|--------------------------|------------|
| 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.       |

DISCUSSION

The results of this study show that the $\beta$-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 $K_v\beta$ binds NADPH is comparable with the affinity of other AKR proteins for this cofactor (21, 22). Upon binding NADPH, the intrinsic fluorescence of $K_v\beta$ was quenched, and an additional emission band appeared that centered around 450 nm. These changes are similar to those observed upon NAPDH 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-
transfer complex in the Kβ2-NADP complex suggests that the functional characteristics of pyridine coenzyme binding as well as the solution conformation of the AKR active site are conserved in Kβ2.

The crystal structure the Kβ2-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, 3α-HSD, and 2,5-DKGR (23–25). The binding of the nicotinamide ring by 3α-HSD is defined by the interactions between Asn-167 and O7N, Ser-166 and N7N, and Gln-190 and N7No of NADP (24). The corresponding residues in AR are Asn-160, Ser-159, and Gln-183 (23). Although in Kβ2 the serine residue (at position 188) and the glutamine residue (at position 214) are conserved, the O7N of NADP1 interacts with Arg-189 (12). However, the interaction between O7N and Arg-189 in Kβ2 does not seem to contribute to the stabilization of the nicotinamide ring at the binding site, because R189M mutation did not affect KdNADPH. Thus, the recognition of the nicotinamide ring appears primarily to be due to the interactions between the Ser-188 and Gln-214 of Kβ2 and the amino group of the nicotinamide ring. This view is supported by the observation that the Kd values of Kβ2 for 3'-acetyl pyridine NADP1 and 3'-amino pyridine NADP1 were 10–30-fold higher than that for NADP1 (Table II). Nicotinamide by itself did not bind to the protein, although ADP-ribose and NMN displayed high, but measurable, Kd 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 Arg-269 (23). The binding of NADPH to ΔNKβ2 and its site-directed mutants

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

| Protein            | Kd (μM) |
|--------------------|---------|
| ΔNKβ2              | 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.    |

Fig. 5. The alignment of the amino acids sequences of the conserved C terminus core of Kβ 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: Kβ1 (human, S66503), Kβ2 (rat, X76724), Kβ3 (rat, S7562), and Kβ4 (mouse, U65593).

Fig. 6. The fluorescence spectra of the WT, C248S, R264E, and N333G ΔNKβ2. 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).
Thus, in K₃β2, 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 $K_{\text{d NADPH}}^*$ 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 $K_{\text{d NADPH}}^*$ 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 K₃β2 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 K₃β2 for NADPH. The K₃β2 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 AR-264, the contribution of other residues interacting with the coenzyme phosphate appears to be minimal. Although the phosphate-binding site of K₃β2 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 $K_{\text{d NADPH}}^*$ of Y255F was similar to that of the WT K₃β2. Interestingly, the $K_{\text{m NADP}}$ of NADPH, which is bound to Lys-262 in AR (23), interacts with a cysteine residue (Cys-248) in K₃β2. In the crystal structure of K₃β2-NADP, Cys-248 also interacts with the 2'-phosphate via a water molecule (12). The K262M mutation in AR prevents tight binding of NADPH ($K_{\text{m NADPH}}$ 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 $K_{\text{d NADPH}}^*$, 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 $K_{\text{d NADPH}}^*$, it appears that the redox state or the ionization of Cys-248 may be an important determinant of NADPH binding to K₃β2.

The binding of NADPH to K₃β2 was also prevented by the N333W mutation. The Asn-333 forms H bonds with N1A and interacts with the hydrogen attached to N3A. 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 Kᵥβ2 is also conserved in Kᵥβ1. However, the corresponding residue in Kᵥβ3 and -4 is a histidine (Fig. 5). Although the role of these residues in NADP(H) binding to other AKR proteins has not been examined, our results show that Asn-333 is critical for NADPH binding to Kᵥβ2. 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 $K_{\text{d NADPH}}^*$ 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 $K_{\text{d NADPH}}^*$ 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 Kβ 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 Kβ 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 Kβ 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 3α-HSD, which do not bind NADPH very tightly, recognize a narrower range of structural motifs (30). Therefore, the Kβ is likely to recognize a limited set of substrates, making the empirical identification of this set somewhat difficult.

The binding of pyridine nucleotides to Kβ could also serve noncatalytic functions. Nucleotide binding may be required for the structural stability of the protein or for the formation of Kβ-Kβ or Kβ-Kα 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 Kβ-Kβ interactions. Additionally, the binding of different nucleotides to Kβ may be able to differentially regulate Kβ channel activity, thereby allowing the channels to “sense” the redox state of the cellular pyridine nucleotides pool. The relatively poor nucleotide discrimination by Kβ2 is consistent with this idea and indicates that several forms of protein-nucleotide complexes can exist in vivo.

Although our measurements show that the Kβ2 NADPH is 10-fold higher than Kβ NADPH, the nature of the cofactor bound to Kβ2 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).

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

where Y_{NADPH} is the fraction of the protein bound to NADPH. At NADPH = 50 μM and NADP+ = 15 μM, which is near their cellular concentrations (31), we estimate that more than 90% of the protein will be bound to NADPH (assuming the Kβ values listed in Table I). However, when NADP+ is the competing nucleotide, only 75% of Kβ 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 Kβ may depend upon whether it is bound to NADP(H) or NAD(H).

Furthermore, because the cytoplasmic concentrations of NADPH 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 Kβ that has been demonstrated recently (33).

Despite their low affinity, the binding of nucleotide analogs 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 Kβ channels (35) and the bacterial apoptotic proteins Reaper and Grim increase the inactivation of the Shaker-type K+ channels (36), the regulation of Kβ by the metabolites of pyridine coenzymes warrants further investigation. Additionally, our observation that the high affinity form of Kβ can change to a low affinity form by changing the ionic strength of the medium raises the possibility that in vivo the Kβ 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 Kα or the membrane voltage.

In summary, we report here for the first time that Kβ2 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 Kβ2 were identified. These mutations may be useful for further probing the role of pyridine nucleotides in regulating the function(s) of Kβ. Because, with the exception of Asp-333, these residues are conserved among the Kβ family of proteins, it appears that the residues identified in Kβ2 play a similar role in the binding of pyridine nucleotide coenzymes to other Kβ proteins as well. Further characterization of NADPH binding to Kβ 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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