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Catalytic mechanism and substrate specificity of the β -subunit of the voltage-gated potassium (Kv) channel

Srinivas M. Tipparaju[‡], Oleg A. Barski[‡], Sanjay Srivastava[‡], and Aruni Bhatnagar^{‡,*}

[‡]*Division of Cardiology, Department of Medicine, Institute of Molecular Cardiology, University of Louisville, Louisville, KY 40202*

Abstract

The β -subunits of voltage-gated potassium (Kv) channels are members of aldo-keto reductase (AKR) superfamily. These proteins regulate inactivation and membrane localization of Kv1 and Kv4 channels. The Kv β proteins bind to pyridine nucleotides with high affinity; however, their catalytic properties remain unclear. Here we report that recombinant rat Kv β 2 catalyzes the reduction of a wide range of aldehydes and ketones. The rate of catalysis was slower (0.06 to 0.2 min⁻¹) than that of other AKRs, but displayed the expected hyperbolic dependence on substrate concentration, with no evidence of allosteric cooperativity. Catalysis was prevented by site-directed substitution of Tyr-90 with phenylalanine, indicating that the acid-base catalytic residue, identified in other AKRs, has a conserved function in Kv β 2. The protein catalyzed the reduction of a broad range of carbonyls including aromatic carbonyls, electrophilic aldehydes and prostaglandins, phospholipid and sugar aldehydes. Little or no activity was detected with carbonyl steroids. Initial velocity profiles were consistent with an ordered bi-bi rapid-equilibrium mechanism in which NADPH binding precedes carbonyl binding. Significant primary kinetic isotope effects (2.0 – 3.1) were observed under single and multiple turnover conditions, indicating that the bond-breaking chemical step is rate-limiting. Structure-activity relationships with a series of para-substituted benzaldehydes indicated that the electronic interactions predominate during substrate binding and that no significant charge develops during the transition state. These data strengthen the view that Kv β proteins are catalytically-active AKRs that impart redox-sensitivity to Kv channels.

Most eukaryotic cells express a diverse range of voltage-gated potassium (Kv) channels (1). In excitable cells, such as nerves and muscles, Kv channels regulate the generation and the duration of action potential, firing patterns, pacemaking and neurotransmitter release (2-4). In non-excitable cells, these channels participate in volume regulation, hormonal secretion as well as proliferation and apoptosis (5-8). Native mammalian Kv channels are large macromolecular complexes composed of 4 pore-forming transmembrane proteins (Kv α subunits) that regulate K⁺ efflux. The cytosolic domains of the Kv 1 and 4 α subunits associate with auxiliary β -subunits that regulate channel assembly and function. In mammals, 3 distinct Kv β genes have been identified. These genes encode proteins with a highly conserved C-terminus and a variable N-terminal domain. The conserved C-terminus of Kv β proteins folds into an (α/β)₈ or triosephosphate isomerase (TIM) barrel motif, which is the most common structural scaffolding among enzymes involved in metabolism and biosynthesis (9). The TIM barrel also forms an efficient fold for high affinity binding of flavin and pyridine nucleotides that

*Corresponding Author: Aruni Bhatnagar, Ph.D., Division of Cardiology, Department of Medicine, University of Louisville, Louisville, KY 40202. Tel. 502-852-5966; Fax. 502-852-3663; Email: aruni@louisville.edu

Supporting Information Available

Table S1 with parameters obtained from the multivariate regression analysis of Hammett plots as described in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

participate in catalyzing oxidation-reduction reactions (9). The TIM barrel structure of Kv β proteins bears a strong structural resemblance and sequence similarity to proteins of the aldo-keto reductase (AKR) superfamily and the catalytic features of the AKR active site are conserved in the C-terminal domain of Kv β proteins (10-12). On the basis of this homology, the Kv β proteins have been classified as a distinct family (AKR6) within the AKR superfamily of proteins (13).

Our previous studies show that like other AKRs, the Kv β proteins bind to pyridine nucleotides with high affinity (14;15). We have found that binding of reduced nucleotides supports N-terminus mediated inactivation of Kv α currents by Kv β , whereas oxidized nucleotides remove inactivation of K⁺ currents generated by Kv α - β subunits (16). These observations support the notion that the β -subunits impart metabolic sensitivity to Kv currents. Such coupling might allow the Kv channel to sense changes in cell metabolism and oxygenation (17). Nevertheless, it remains unclear whether binding of pyridine nucleotide to Kv β proteins by itself is required for the regulation of Kv activity or if catalysis is necessary to change the redox state of the nucleotide bound to Kv β . Clearly, further studies on the catalytic activity and substrate-specificity of Kv β are required to understand how it regulates Kv function.

Several lines of evidence suggest that Kv β proteins are catalytically active and display AKR properties. The AKR active site is conserved in Kv β proteins and the catalytic residues are positioned for efficient carbonyl binding. Moreover, substitution of the active site residues of the protein markedly affects the inactivating properties of Kv β (15;18;19). Indeed, weak catalytic activity of Kv β 2 with aromatic aldehydes has been reported before (18). Nevertheless, the catalytic and kinetic mechanisms of the protein remains poorly understood and its substrate specificity is unknown. Specifically, it is not clear whether the protein displays cooperativity; which steps limit the overall catalytic rate; and which structural features of the substrate facilitate binding to the active site. The current study was therefore designed to identify the reaction sequence, determine the kinetic mechanism of Kv β catalysis and to examine the substrate-specificity of the protein. Our results show that the Kv β follows a rapid equilibrium bi-bi ordered reaction mechanism. These properties may be important aspects of Kv β function in the regulation of Kv channel.

Experimental Procedures

Mutagenesis and expression of Kv β 2

The cDNA encoding rat Kv β 2 (rKv β 2) was provided by Dr. Min Li (Johns Hopkins University). The construct for bacterial expression of rKv β 2 [NP_059000] with His-tag at its N-terminus in pET28a vector was generated as described previously (14). Site-directed mutagenesis for Y90F substitution was performed directly in pET28a vector using QuikChange XL site-directed mutagenesis kit (Stratagene). The sequence of the PCR primers was: forward 5'-CGATACGGCGGAGGTCTTCGAGCTGGCAAGGCTG-3', and reverse 5'-CAGCCTTGCCAGCTGCGAAGACCTCCGCCGTATCG-3'. The complete sequence of the mutated Kv β 2 insert was confirmed by DNA sequencing. Wild-type and mutant Kv β 2 were expressed in the BL-21(DE3) strain of *E.coli* and purified to homogeneity as described before.

Protein purification

The AKR C-terminal domain of Kv β 2 protein (amino acids 39-367) was expressed with a His tag at its N-terminus in the BL-21 strain of *E.coli*. The bacterial pellet was lysed in ice-cold binding buffer containing (in mM): NaCl, 150; Tris, 20; imidazole, 5; pH 7.9. The supernatant was incubated with Ni-affinity agarose beads (Qiagen) for 2-3 h at 4°C. The Ni-column was washed with binding buffer to 10 times the bed volume and the protein was eluted by imidazole in binding buffer. A small volume from each eluted fraction (2 μ l) was separated by SDS-

PAGE. The purified proteins were stored at 4°C in 0.2 M potassium phosphate buffer, pH 7.4 and used within 4 - 6 weeks. The concentration of the protein was measured using Bradford's assay (20)

NADPH binding studies

Binding of NADPH to WT or Y90F:Kvβ2 was measured by fluorescence titration as described earlier (14). Briefly, the Kvβ2 protein (15 μg) was equilibrated in 2 ml of 0.2 M potassium phosphate buffer at room temperature for 10 min and changes in fluorescence were measured using an excitation wavelength of 280 nm and emission at 340 nm. Aliquots of NADPH were added and changes in emission were recorded. Fluorescence changes obtained by titrating tryptophan with NADPH were used to correct for inner-filter effect (14). Dissociation constants were calculated from the following equation:

$$\Delta F = \Delta F_{\max} \text{NADPH} / (K_d + \text{NADPH}) \quad (1)$$

Activity measurements in multiple and single turnover mode

Activity assays were performed in multiple or single turnover mode (18;21). Multiple turnover were measured in quartz microcuvettes with final reaction volume of 250 μl containing Kvβ2 (15-20 μM) and NADPH (150 μM) incubated in 0.2 M phosphate, pH 7.4 at 37°C. The reaction was started by adding the substrate. The loss of NADPH was monitored by following the decrease in absorbance at 340 nm for 15 min using a spectrophotometer (Cary Varian 50). Single turnover activity was measured by monitoring changes in A₃₆₀ nm to follow oxidation of NADPH bound to Kvβ2. The Kvβ2 protein was pre-equilibrated at 37°C and the enzymatic reaction was initiated by adding the substrate (0.2 – 2 mM). The reaction was monitored continuously by measuring changes in A₃₆₀ for 30-60 min at 37°C to follow the oxidation of NADPH bound to Kvβ2.

Synthesis of deuterated NADPH

NADPH and NADPD were synthesized by using alcohol dehydrogenase from *Thermoanaerobium Brockii* (Sigma Chemical Co) as described earlier (22). For synthesis, alcohol dehydrogenase 2U (1.21 mg) was incubated with 2 mM NADP⁺ in 50 mM Tris-HCl (pH7.8) and 300 mM 2-propanol or 2-propanol-*d*₈ (Sigma Chemical Co) for 1 h at 40°C. The progress of the reaction was monitored by measuring changes in absorbance at 340 nm. After completion, the reaction mixture was loaded on an anion exchange column pre-equilibrated with 20 mM Tris-HCl pH 9.0. The column was washed with 25-30 ml of 20 mM Tris-HCl, pH 9.0 and the reduced cofactor NADPH/D was eluted by using 250 mM NaCl, 50 mM Tris, pH 7.8. The absorbance of the eluted fractions at 260 and 340 nm was recorded. Fractions with A₂₆₀/A₃₄₀ ratio of > 2.4 were pooled and the pH of the mixture was adjusted to 9.0 with NaOH. Synthesized pyridine nucleotides were stored at -80°C until further use.

Exhaustion of Kvβ2 bound NADPH and addition of synthesized NADPH or NADPD

The Kvβ2 protein purified from bacteria remains tightly bound to NADPH, which is not removed by dialysis. Hence, for kinetic isotope effect studies, NADPH bound to Kvβ was removed by oxidizing it to NADP⁺, which dissociates from the protein more readily than NADPH. For this, Kvβ2 purified from bacteria was incubated with 4-nitrobenzaldehyde (600 μM) and the disappearance of NADPH was monitored at 360 nm. After driving the reaction to completion, the reaction mixture was transferred to a dialysis cassette (10,000 Da cutoff; Pierce) and dialyzed against 0.2 M potassium phosphate (pH7.4) at 4°C for 16-20 h. Synthesized NADPH or NADPD was added to Kvβ2 and the nucleotide was allowed to bind to the protein for 30 min at 4°C. Excess NADPH/D was removed by equilibrium dialysis. The progress of the reaction was monitored at each step by measuring changes in absorbance at 360 nm.

Kinetic isotope effects

To study kinetic isotope effects, 150 μM synthesized NADPH or NADPD was mixed with 20 μM of Kv β 2 in 0.2 M potassium phosphate, pH7.4 at 37°C and enzyme reaction was started by adding varying concentrations (0.1 – 2 mM) of the substrate. Oxidation of the NADPH/D was monitored at 340 nm for 15 min at 37°C. For calculating initial velocity, only the linear part of the reaction was used. Kinetic isotope effects on single turnover reactions were calculated from the rate of oxidation of NADPH or NADPD bound to Kv β 2 measured from changes in absorbance at 360 nm at 37°C for 30 min after the addition of the substrate.

Product identification

The reduction of phospholipid aldehyde 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphatidylcholine (POVPC) to 1-palmitoyl-2-(5)-hydroxyvaleroyl-sn-glycero-3-phosphorylcholine (PHVPC) by Kv β 2 was followed by electrospray mass spectrometry (ESI⁺/MS) analysis as described earlier (23). The reaction containing 35 μM Kv β 2, 150 μM NADPH and 80 μM POVPC was incubated at 37°C for 3 h. Additional NADPH was added to the reaction mixture at 45 min intervals. The reaction was stopped after 3 h and phospholipids were extracted by using the Bligh and Dryer (24) procedure. For ESI⁺/MS analysis, a MicroMass ZMD 2000 mass spectrophotometer (Waters-Micromass, Milford, MA) was used. The injection solvent was 2:1 methanol/chloroform (v/v) containing 1.0% acetic acid in positive ionization mode and 2:1 methanol/chloroform (v/v) containing 10 mM ammonium hydroxide in the negative ionization mode. Samples were injected into the spectrometer using a Harvard syringe pump at a flow rate of 25 $\mu\text{l/min}$. The ESI⁺/MS operating parameters were: capillary voltage 3.38 kV, cone voltage 25 V, extractor voltage 9V, RF lens voltage 0.9 V, source block, and desolvation temperatures 100 and 200 °C, respectively. Nitrogen was used as the nebulizer gas at a flow rate of 3.4 liters/h. Spectra were acquired at a rate of 275 atomic mass units per s over the mass range of 2–1000 atomic mass units and were averaged over a period of 5 min or 100 scans (23).

HPLC Analysis

Reagent 4-hydroxy-*trans*-2-nonenal (4-HNE), 1,4-dihydroxynonenal (DHN) were synthesized as described before (25). The 4-oxo-2-nonenol was synthesized by the reduction of 4-oxo-2-nonenal (4-ONE) by aldose reductase (25). Reagent 4-HNE, 4-ONE, DHN and 4-oxo-2-nonenol were separated by HPLC using a Varian reverse phase ODS C₁₈ column pre-equilibrated with 0.1% aqueous trifluoroacetic acid. The compounds were eluted using a gradient consisting of solvent A (0.1% aqueous trifluoroacetic acid) and solvent B (100% acetonitrile) at a flow rate of 1 ml/min. The gradient was established such that solvent B reached 26% in 30 min, and was held at this value for 10 min. In the next 10 min, solvent B reached 60%. In an additional 5 min, it reached 100% and was held at this value for 10 min. Under these conditions, DHN, 4-oxo-2-nonenol, 4-HNE, and 4-ONE eluted with a retention time of 31, 37, 43, and 53 min, respectively.

Analysis of reduced product by Gas Chromatography/Mass Spectrometry

HPLC purified *peak II* was extracted in dichloromethane and dried under nitrogen. The samples were derivatized with 50 μl of *BSTFA-TMS* (N,O-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane) for 5 min at 80 °C. An aliquot (1 μl) of the mixture was utilized for gas chromatography/electron impact ionization - mass spectrometry (GC/EI-MS) analysis. GC/EI-MS analysis was performed using a HP-6890/HP5973 GS/MS system (Hewlett Packard; Palo Alto, CA, USA). Compounds were separated on a bonded phase capillary column (DB-5MS, 30 m $\times$ 0.25 mm $\times$ 0.25- μm film thickness from J&W Scientific, Folson, CA, USA). The GC injection port and interface temperature were set at 280 °C. Injections were made in the splitless mode with the inlet port purged for 1 min following injection. The GC oven temperature was

held initially at 100 °C for 1 min, and then increased at a rate of 10 °C/min to 280 °C, and then held there for 5 min. Under these conditions, the retention time of 4-oxo-2-nonenol was 8.06 min.

Hammett Plot analysis

The kinetic constants, K_m and k_{cat} were obtained with the series of benzaldehyde compounds. For each para-substituted benzaldehyde, values for electronic (σ^+), steric (ν) and hydrophobic (Π) parameters were obtained from published literature (26-28). The $\log k_{cat}$ or the $\log K_m$ or the $\log k_{cat}/K_m$ values were plotted against σ^+ and the correlation coefficients were obtained from multivariate weighted analysis as described before (22;29) for the following equation using SAS 9.1:

$$\log k_{cat}/K_m \quad \text{or} \quad \log k_{cat} \quad \text{or} \quad \log K_m = \rho\sigma^+ + A\nu + B\Pi + C \quad (2)$$

where σ^+ is the Hammett constant for electronic effects, Π is the Hansch constant for the hydrophobic interactions, and ν is the steric constant; ρ , A , B and C are parameter coefficients.

Data Analysis

Steady-state kinetic constants k_{cat} and K_m were calculated by fitting the Michaelis-Menten equation directly in the hyperbolic form to the initial velocities using Marquardt-Levenberg algorithm. For nonlinear regression analysis, Sigmaplot 9.0 software was used. Single turnover traces were analyzed using a single-exponential decay equation:

$$A_{360} = ae^{-k_{trans}t} + b \quad (3)$$

Calculated pseudo-first order rate constants, k_{trans} , were then plotted against substrate concentration and analyzed using nonlinear regression. Kinetic isotope effects were calculated from the initial velocities obtained with NADPH and NADPD at various substrate concentrations using the following equations:

$$v = V_M A / \left[K_m \left(1 + F_i E_{k_{cat}/K_m} \right) + A \left(1 + F_i E_{k_{cat}} \right) \right] \quad (4)$$

$$v = V_M A / [(K_m + A)(1 + F_i E)] \quad (5)$$

where A is the concentration of aldehyde substrate, F_i is the fraction of deuterium in the NADPH/NADPD cofactor, E_{k_{cat}/K_m} and $E_{k_{cat}}$ are the isotope effects minus 1 on k_{cat}/K_m and k_{cat} , respectively. Equation 5 assumes that $E_{k_{cat}/K_m} = E_{k_{cat}}$. Multivariate regression analysis was performed using SigmaPlot 9.0. Data are presented as mean $\pm$ SEM. Intergroup comparisons were made using the unpaired Students t test and $P < 0.05$ were considered to be statistically significant.

Results

Kv β 2-mediated catalysis

To determine whether the purified Kv β 2 protein was enzymatically active, fundamental properties of its catalysis were determined. For this, the pH and ionic dependence of the reaction and the dependence of the reaction velocity on the concentration of the protein and its substrates were studied. In addition, we also established the identity of the acid-base catalyst and authenticated the formation of reduced products generated by the protein. In the first series of experiments, the rate of NADPH reduction was measured using 4-nitrobenzaldehyde (4-NB) as a model AKR substrate. When incubated with 1 mM 4-NB, a time-dependent decrease in A_{340} was observed, indicating oxidation of NADPH to NADP⁺ (Fig. 1A). The loss of absorbance was linear for the first 10 min. The linear portion of the progress curve was used for all initial velocity measurements. The initial rate of NADPH reduction showed an increase

with an increase in protein concentration (Fig. 1B), indicating that the reaction was dependent on Kv β 2 and not a spurious outcome of reaction conditions. No saturation of the reaction rate was observed when the protein concentration was increased from 10 to 50 μ M. Hence, in all future experiments, a concentration of 15-25 μ M protein was used.

To optimize activity assays, the pH-dependence of the reaction was investigated. When the enzyme activity was measured in reaction mixture of pH 6.5 to 7.9, maximal activity was observed between 7.2-7.4. The enzyme activity declined at pH >7.5 or < 6.5 and the pH-dependence conformed to a bell-shaped curve (Fig. 1C). In all subsequent measurements, a pH of 7.4 was used. Next, using a pH of 7.4, the ionic dependence of catalysis was measured. The catalytic activity of the protein was insensitive to changes in ionic strength, when the concentration of phosphate in the reaction mixture was varied between 0.1 and 0.25 M, however, a significant decrease in the enzyme activity was observed at phosphate concentrations < 50 mM (Fig. 1D). At 0.15 M potassium phosphate (pH 7.4), the maximal activity was $\sim$ 2.5 mU/mg protein, and the turnover number was between 0.08 and 0.3 min⁻¹. At conditions optimal for NADPH oxidation, no oxidation of NADH was observed (data not shown). Moreover, inclusion of oxidized cofactor NADP⁺ (40 μ M) or the reduced product p-nitrobenzyl alcohol (40 μ M) did not influence the initial velocity of the reaction with 4-NB, suggesting that neither product inhibition nor reverse reaction occurs to an appreciable extent during initial velocity measurements. Collectively, these data provide optimal values of protein concentration, pH and ionic strength for measuring Kv β catalysis and are consistent with the view that Kv β 2 is an enzymatically active protein that preferentially catalyzes aldehyde reduction. These results also show that the catalytic reduction of aldehydes by Kv β 2 is specific for NADPH and is not limited or affected by product inhibition.

To establish specificity of catalysis, a site-directed Kv β 2 mutant was generated in which Tyr-90 was replaced with phenylalanine. Although previous studies show that D85N and K118M substitutions decrease Kv β 2-mediated reduction of 4-CB, neither of these residues act as acid-base catalyst and could indirectly affect reduction by preventing substrate or NADPH binding (18). The residue corresponding to Tyr-90, on the other hand, is widely accepted to be a direct participant in acid-base catalysis and substitution of this residue with phenylalanine abolishes catalytic activity in other AKRs (21;30;31). The absorbance spectrum of purified Kv β 2:Y90F was similar to that of the WT protein, with absorbance peaks at 275 and 360 nm (Fig. 2 A), indicating that the mutant protein remains tightly-bound to NADPH. To quantitatively determine whether the Y90F mutation affects NADPH binding, K_d^{NADPH} of the WT and mutant proteins was determined by fluorometric titrations. As shown in Fig. 2B, addition of different concentrations of NADPH led to a similar decrease in the fluorescence of WT and Y90F Kv β 2. The K_d^{NADPH} for Kv β 2 WT was 0.2 ± 0.005 μ M, which is similar to that determined for Kv β 2:Y90F (0.13 ± 0.011 μ M), indicating that substitution of Tyr-90 with Phe does not significantly alter NADPH binding (Table 1). Nevertheless, the catalytic activity of the protein was profoundly affected. As shown in Fig. 2 C, significant NADPH oxidation by 4-NB was observed with the WT protein, whereas NADPH oxidation by Kv β 2:Y90F was near background levels. The turnover rate of WT Kv β 2 with 4-NB was reduced from 0.12 to 0.009 min⁻¹ by the Y90F mutation (Table 1). From these results, we conclude that the aldehyde reductase activity of Kv β 2 is inherent to the protein and could not be ascribed to a contaminating protein. Moreover, the catalytic activity of the protein appears to be due to acid-base catalysis provided by Tyr-90, which corresponds to the proton donor identified in other AKRs.

Substrate Specificity

To determine whether Kv β reductase activity is limited to aldehydes or whether ketone compounds are also reduced, the enzyme activity with series of acetophenones was measured. Of the several acetophenones tested, only 4-nitroacetophenone (4-NAP) showed appreciable

activity. The catalytic efficiency (k_{cat}/K_m) of the protein with 4-NAP ($0.46 \text{ min}^{-1} \cdot \text{mM}^{-1}$) was similar to that observed with 4-NB ($0.59 \text{ min}^{-1} \cdot \text{mM}^{-1}$), indicating that Kv β 2 catalyzes the reduction of ketones as well as aldehydes. The protein also displayed high catalytic activity with 9,10-phenanthrenequinone ($K_m = 0.24 \pm 0.14 \text{ mM}$; $k_{\text{cat}} = 0.19 \pm 0.07 \text{ min}^{-1}$ and $k_{\text{cat}}/K_m = 0.79$) and the endogenous carbonyl methylglyoxal ($K_m = 12 \pm 1.0 \text{ mM}$; $k_{\text{cat}} = 0.32 \pm 0.04 \text{ min}^{-1}$ and $k_{\text{cat}}/K_m = 0.03$). Measurable catalytic activity was also detected with several other carbonyls (Table 2) such as 4-oxo-*trans*-2-nonenal, phenylglyoxal, succinic semialdehyde, and the phospholipid aldehyde 1-palmitoyl-2-oxovaleroyl phosphatidylcholine (POVPC). Low activity and poor affinity of the protein for these carbonyls, however, precluded accurate estimates of kinetic constants. Several other compounds which are generally good substrates for AKR enzymes were also tested. These include DL-glyceraldehyde, glucose, xylose, D-glucuronic acid, D-fructose, 5 α -androstene 3 α ,17 β diol, and cortisone. We found little or no activity with these compounds at conditions optimal for other substrates. From these data we conclude that Kv β 2 reduces both aldehydes and ketones with equal affinity.

To establish whether Kv β 2 catalysis results in the appearance of a reduced alcohol product, the protein was incubated with POVPC, which is a good substrate of AKR1B1 and other related AKRs (32). ESI⁺/MS analysis of POVPC revealed a predominant ion at m/z 594.6 (Fig. 3A). To examine reduction, the phospholipid aldehyde was incubated with 35 μM Kv β 2 for 180 min, after which the phospholipid was extracted using the Bligh and Dryer (24) procedure and re-analyzed by mass spectrometry. The phospholipid mixture recovered after incubation with Kv β 2 showed the appearance of an additional ion with m/z 596.4, consistent with reduction of POVPC to PHVPC. The total conversion of POVPC to PHVPC was $18 \pm 2\%$ ($n=4$), which corresponds to the amount of NADPH oxidized. These observations confirm the AKR nature of the Kv β 2 active site and substantiate its catalytic activity leading to the generation of a reduced product.

Because Kv β 2 can reduce both aldehydes and ketones, we asked which of these groups is preferentially reduced by Kv β 2 in a keto-aldehyde? To test this, the protein was incubated with 4-ONE, which contains a keto group at C-4 and an aldehyde at C-1. As shown in Scheme 1, reduction of this compound at C-1 will result in the formation of 4-oxo-nonenol, whereas reduction at C-4 will form 4-HNE. Further reduction of 4-HNE and 4-oxo-nonenol would lead to DHN formation. The Kv β -derived product of 4-ONE was first analyzed by HPLC. As shown in Fig. 4, an appropriate separation protocol was developed in which the retention times of 4-HNE, DHN, 4-ONE, and 4-oxo-nonenol were measurably different. Incubation with Kv β 2 shifted the retention time of 4-ONE from 53 min to 37 min, which corresponds to the retention time of 4-oxo-nonenol. No products eluted with a retention time corresponding to 4-HNE or DHN, indicating that Kv β 2 reduces 4-ONE to 4-oxo-nonenol. To further confirm the structural identity of the product, the Kv β 2 derived peak at 37 min was derivatized and analyzed by GC-MS. On GC, the product displayed a retention time of 8.06, which was identical to that of reagent 4-oxo-nonenol. On the mass spectrum of the product (Fig. 4 C), the derivatized molecular ion with 228 m/z (M) was identified, along with major fragments with m/z 213 (M-CH₃), 157 (M-C₅H₁₁), 129 (M-C₆H₁₁O₁). This fragmentation pattern corresponds to 4-oxo-nonenol, but not 4-HNE, 4-ONE or DHN (data not shown), indicating that the keto-group at C-4 position remains unreduced whereas the aldehyde group at C-1 position is reduced; leading to the formation of 4-oxo-2-nonenol. These data indicate that Kv β prefers aldehydes to ketones and that carbonyls with high electron density at oxygen (e.g., C-1 of 4-ONE) are reduced more efficiently than less polarized carbonyls (e.g., C-4 of 4-ONE or C-1 of 4-HNE).

Reaction sequence

To determine the reaction sequence of the catalytic cycle, initial velocity of the reaction was measured at different 4-NB and NADPH concentrations. For these experiments, 4-NB was

chosen as the model substrate because of all the carbonyls examined, highest k_{cat}/K_m values and low K_m values were observed with 4-NB. Double reciprocal plots of initial velocity vs. substrate concentration were linear and showed no signs of product or substrate inhibition. Double reciprocal plots at different NADPH concentration conformed to a pattern of straight lines intersecting at the ordinate (Fig. 5). This pattern is consistent with a rapid-equilibrium ordered kinetic mechanism in which NADPH binds first to the enzyme followed by the carbonyl substrate (Scheme 2). To determine kinetic constants, the following relationship was used:

$$v = \frac{V_M AB}{K_{ia} K_b + K_b A + AB} \quad (6)$$

where A is the concentration of NADPH, and B is the concentration of 4-NB, V_M is V_{max} , K_b is the Michaelis constant for 4-NB; and K_{ia} is the binding constant of NADPH. Best fit of the equation 6 to the data yielded $K_{ia} = 107 \pm 33 \mu\text{M}$, $K_b = 490 \pm 119 \mu\text{M}$, and $k_{\text{cat}} (V_M/E_t) = 0.15 \pm 0.015 \text{ min}^{-1}$. When the equation for a steady-state ordered mechanism containing the term $K_a B$ in the denominator of equation 6 was fitted to the data an undefined K_a was obtained. A replot of slopes (K_m^B/V_m apparent) (determined from Fig. 5) versus $1/\text{NADPH}$ was linear with slope of $K_b K_{ia}/V_m$ and an intercept on the ordinate axis of K_b/V_m . The intercept on the $1/[\text{NADPH}]$ axis was equal to $-1/K_{ia}$. Values obtained from each curve separately (slope replot; Fig. 5; inset) are similar with those determined by global analysis (i.e., by fitting equation 6 to the data). These data confirm that Kv β 2 follows a rapid-equilibrium ordered scheme in which NADPH binding precedes substrate binding (Scheme 2).

Kinetic Isotope Effects

To understand the mechanism of Kv β 2 catalysis in greater detail we investigated the rate-determining step. For this, the primary kinetic isotope effects were determined by using NADPH and NADPD as cofactors. NADPH or NADPD was synthesized as described under *Material and Methods* and initial velocity was measured at different concentrations of the aldehyde substrate. In the first series of experiments, purified Kv β 2 was used for activity measurements directly, using 4-NB or 4-NAP as a variable substrate. Both equations 4 and 5 were used to fit the data obtained (Fig. 6). Equation 5, however, gave a better fit to the data than equation 4 as determined by F values and the standard errors in the values of the kinetic constants. The best-fit values of kinetic constants obtained from equation 5 are shown in Table 3. Similar values of kinetic isotope effects were obtained with either 4-NB or 4-NAP as the varied substrate (Table 3). High values of primary kinetic isotope effect indicate that the chemical step is rate-limiting in Kv β 2-catalyzed reduction. The lack of isotope effect on $K_m B$ suggests that K_m is not a true steady-state kinetic parameter, but a dissociation constant for aldehyde binding to the E:NADPH binary complex. The reverse reaction, i.e., oxidation of 4-nitrobenzyl alcohol by NADP^+ was also investigated. However, with 4-nitrobenzyl alcohol as the substrate, no measurable activity under multiple turnover conditions was detected. This precluded determination of Haldane relationship and calculation of several microscopic constants including k_{-3} which is the rate constant for alcohol oxidation (Scheme 2).

Single-turnover Measurements

Transient kinetic measurements, such as single turnover allow determination of some of the microscopic rate constants associated with enzyme catalytic mechanism. The Kv β 2 binds tightly to NADPH and forms a binary complex. Free NADPH in solution absorbs at 340 nm, whereas binding to the β 2 subunit causes a red shift of this peak to 360 nm. Hence for single turnover experiments, we monitored changes in absorbance at 360 nm to measure the activity of the β 2:NADPH binary complex with 4-NB.

To measure isotope effects under single turnover conditions the Kv β 2 protein was preloaded with NADPD. Because Kv β 2 purified from bacteria is tightly bound to NADPH (Fig. 7A), which is not removed by dialysis, NADPH bound to Kv β 2 was first oxidized to NADP⁺ in a single turnover reaction using 4-NB as the substrate. As shown in Fig. 7A, incubation with 4-NB led to a progressive decrease in A₃₆₀, indicating that all of the NADPH bound to the protein was oxidized to NADP⁺. The unutilized 4-NB and the 4-nitrobenzyl alcohol generated from the reaction were removed by equilibrium dialysis, and the protein was reloaded with either NADPH or NADPD by dialysis with these cofactors. This led to an increase in the absorbance at 360 nm, indicating that NADP⁺ bound to the protein was completely replaced by NADPH or NADPD. Analysis of the absorbance at 275 nm and 360 nm using an extinction coefficient of 60,880 for the protein and 6,200 M⁻¹cm⁻¹ for NADPH indicated that approximately 90-100% of the protein was saturated with NADPH/D (Fig. 7A). These preparations were then used for subsequent kinetic isotope studies under single turnover conditions.

Absorbance at 360 nm decreased with time upon addition of 4-NB (0.2 – 2mM) to the NADPH- or NADPD-loaded enzyme (Fig. 7 B & C). Experimental time courses for NADPH/D oxidation were best described by a single exponential function. Values of k_{trans} increased linearly with 4-NB concentration and showed no significant saturation within the concentration range of 4-NB that could be practically achieved (Fig. 7D). From these experiments a kinetic isotope effect of 3.13 ± 0.2 was obtained (Table 3), indicating that the chemical step is rate-limiting in single turnover conditions.

Structure-activity relationship

To understand the catalytic mechanism further, Hammett plots with catalytic efficiencies (k_{cat}/K_m) and turnover numbers (k_{cat}) were generated with a series of para-substituted benzaldehydes (Fig. 8A and B). A plot of $\log k_{cat}/K_m$ versus σ^+ displayed a linear relationship (Fig. 8A), suggesting that the reaction mechanism does not change within this series, and that substituent effects was primarily electronic in nature. A Hammett plot with k_{cat}/K_m showed a positive ρ coefficient, the value (1.95 ± 0.36) of which is similar to that reported for other AKRs (22;29). This fit was only marginally improved by incorporating hydrophobicity or the steric constants (Supplemental Table 1), indicating that these characteristics make only minor contributions to benzaldehyde binding. A plot of k_{cat} versus σ^+ gave a slope of 0.04 ± 0.02 (Fig. 8B). Among the series tested, the only exception was 4-NB. Reasons for the anomalous behavior of 4-NB are unclear but may relate to specific interaction of the nitro group with the active site residues of the protein. $\log K_m$ displayed a linear inverse relationship with σ^+ with a correlation coefficient (ρ) of -1.85 ± 0.52 (Fig. 8C) and no significant contribution of steric or hydrophobic features of the benzaldehydes was evident (Supplemental Table 1).

Discussion

The β -subunits of voltage-gated (Kv) potassium channels belong to the AKR superfamily of enzymes (13). These proteins display 20-26 % sequence identity with other AKRs, and the architecture of their active site resembles that of other AKR oxidoreductases (12). Nevertheless, the catalytic properties of Kv β are largely unknown. We, therefore, studied the substrate-specificity and the kinetic behavior of Kv β 2 in order to identify features of catalysis that are unique to Kv β proteins and those that they share with other AKRs. Our results show that Kv β 2 displays significant enzyme activity and that it utilizes NADPH to catalyze the reduction of both aldehydes and ketones. As expected of an enzyme reaction, the rate of reduction was increased with an increase in protein and substrate concentration; the reaction showed distinct pH and ionic strength dependence; and the initial velocity was saturated at high substrate concentration. Importantly, catalysis, but not NADPH binding, was prevented by substituting tyrosine-90 with phenylalanine, indicating that, as in other AKRs (21;30;31),

this residue in Kv β 2 participates in general acid-base catalysis and that carbonyl reduction is not due to contamination by other proteins in the preparation or a direct covalent reaction with NADPH, but that it is a controlled conversion catalyzed at the Kv β 2 active site.

Substrate-specificity studies show that the protein was active with several aldehydes and ketones. Highest activity was observed with aromatic substrates. Comparable activity was observed with benzaldehydes and acetophenone as well as 9,10 phenanthroquinone. Most AKRs display high catalytic activity with 4-nitrobenzaldehyde (K_m 2 μ M (AKR1B1) (36) – 3mM (AKR7A2) (37)) and methylglyoxal (K_m 8 μ M (AKR1B1) – 1.2 mM (AKR1A1) (38)) with k_{cat} ranging from 24 to 360 min^{-1} (36;39). 4-nitroacetophenone and 9, 10 phenanthrenequinone are typical model substrates for the AKR1C family members, which have substantial catalytic activity with ketone as well as aldehyde compounds. These enzymes possess lower k_{cat} values (2-190 min^{-1}), however K_m values typically lie in the low micromolar range (1-200 μ M) (31;37). Several physiological aldehydes such as lipid peroxidation products 4-HNE and POVPC are specific substrates for the AKR1B enzymes (32;40). Our results show that Kv β catalyzes the reduction of a similar range of aldehydes and ketones with K_m values in the range observed with other AKRs. On the basis of substrate specificity showing that it could catalyze the reduction of both aldehydes and ketones, we propose that Kv β 2 should be considered a general AKR that catalyzes the reduction of both aldehydes and ketones. Nonetheless, when both aldehyde and ketone groups were presented to the enzyme in the same substrate (*e.g.*, in 4-ONE), the enzyme reduced the aldehyde, indicating that it prefers more electronegative carbonyls.

The physiological substrate of Kv β 2 remains unknown. The protein, however, displayed high catalytic activity with several carbonyls that are generated endogenously from enzymatic and non-enzymatic oxidation of lipids. High activity was observed with 2 enzymatically generated carbonyls, 12-oxo-ETE and PGJ₂. 12-oxo-ETE is leukotriene B₄ receptor agonist (42) and is derived from the oxidation of arachidonic acid by 12-lipoxygenase (43), whereas PGJ₂ is a precursor of 15-deoxy Δ 12,14-PGJ₂, which is thought to be a natural ligand of peroxisome proliferator-activated receptor (PPAR- γ) (44). Carbonyl compounds generated from non-enzymatic oxidation of phospholipids such as 4-HNE, 4-ONE, and POVPC were also reduced by Kv β 2. These aldehydes are reactive chemicals and they affect a variety of signaling pathways leading to changes in vasodilation (45), myocyte excitability (46) and cell adhesion (47). Steroid-based natural carbonyl compounds such as 5 α -androstene-3,17-dione, 4-androstene-3,17-dione and cortisone, on the other hand, were minimally reduced under conditions that were optimal for the reduction of other carbonyls. Whether any of these carbonyls are reduced by the protein *in vivo* remains unclear. Nonetheless, the preference of Kv β 2 for hydrophobic aldehydes raises the possibility that the function of this protein is related to the reduction of membrane-derived oxidized lipids.

Our kinetic analyses suggest that Kv β 2 follows rapid-equilibrium kinetics in which the bond-breaking step is rate-limiting (Scheme 2). This scheme is indicated by the lack of isotope effect of K_m and the convergence of double reciprocal initial velocity plots at different substrate concentrations on the ordinate. This plot also indicates that the enzyme follows an obligate ordered reaction sequence in which coenzyme binding precedes substrate binding. Significant primary kinetic isotope effects were observed on k_{cat} , indicating that chemistry is rate-limiting. Single and multiple turnovers displayed similar isotope effects (Table 3), although the slightly higher values for single (3.13) versus multiple (2.07) turnover may be due to kinetic steps that contribute to multiple, but not single, turnover reactions. These steps include those leading to NADPH binding (k_1 , Scheme 2) or NADP⁺ (k_5) release. They appear to be nearly as slow as the bond-breaking step (k_3) and could thus contribute to the rate of the overall reaction cycle. The value of the microscopic rate constant for the chemical step (k_3) can be estimated from the following relationship:

$$k_3 = k_{\text{cat}} \left({}^Dk_3 - 1 \right) / \left({}^Dk_{\text{cat}} - 1 \right) \quad (7)$$

If we assume that the value of ${}^Dk_3 = 6.5$, as calculated from NADPH-dependent reduction of D-xylose by AKR1B1 (51) and consistent with the kinetics of *Candida tenuis* xylose reductase (29), then calculations using formula (7) show that $k_3 = 0.82 \pm 0.15 \text{ min}^{-1}$ for 4-NB, and $0.73 \pm 0.05 \text{ min}^{-1}$ for 4-NAP. However, if we use a value of 3.1 estimated by Jin *et al.* for the intrinsic isotope effect on AKR1C2-catalyzed reduction of 5 α -DHT by NADPH (33) k_3 for 4-NB is $0.31 \pm 0.06 \text{ min}^{-1}$. Both these values are in agreement with the estimate for k_3 obtained from our single turnover experiments. Fitting of multiple single turnover traces obtained with various 4-NB concentrations (Fig. 6) using Dynafit predicts that k_3 is between 0.3 and 0.8 min^{-1} . This prediction suggests that 20 to 50 % of the overall rate limitation is due to the hydride-transfer step with the remaining contributed by NADP^+ or product release. A slow release of NADP^+ has been identified to be the rate-limiting step in the AKR1B1-catalyzed aldehyde reduction (51), and is believed to be due to the slow movement of the NADPH binding loop (51;52). This possibility is consistent with the crystal structure of Kv β 2, which shows that the cofactor is held tightly at the active site (12). Thus NADP^+ release, which requires extensive conformational rearrangement, could contribute to the inequality of isotope effects between single and multiple turnover reactions, and by implication, to the overall rate of the catalytic cycle.

Our data exclude cooperative or allosteric effects on catalysis and are consistent with our previous work showing a lack of cooperativity in NADPH or NADP^+ binding to Kv β 2 (14). Native Kv β proteins are, however, arranged as a tetramer around the T1 domain of Kv α (53) and Kv β 2 purified from bacteria (used for the kinetic experiments described here) is a tetramer in solution (14), indicating that cooperative behavior is possible. Nevertheless, no cooperatively was observed. Perhaps in solution, as in crystal structure with T1 (53), the active site of Kv β faces the solvent and is minimally affected by other β monomers in the complex. Nonetheless, the possibility of allosteric effects in the regulation of Kv current by the β -subunits *in situ* cannot be ruled out.

The catalytic mechanism for carbonyl reduction by Kv β 2 appears to be similar to that observed for other AKRs. Catalytic inactivation by the Y90F mutation suggests that this residue play a similar role in Kv β 2 as it does in other AKRs (21;30;48). In addition to tyrosine (Tyr- 48 in AKR1B1), an active site histidine residue (His-110 in AKR1B1) has also been suggested to play an important role in AKR catalysis and substrate binding (21;30;49). This residue is located at the center of the hydrogen bonding network that facilitates substrate binding and polarization of the substrate carbonyl (48;50). The imidazole of this histidine in AKR2B5 plays an important role in precise positioning of the carbonyl group for catalysis (50). Significantly, His-110 is absent in Kv β and is replaced by an asparagine residue. This shift in charge is likely to prevent tight binding of the carbonyl at the active site and may be responsible for the low catalytic activity of Kv β 2 (0.06 to 0.2 min^{-1} versus 2 to 600 min^{-1} for other AKRs). Low catalytic rates of Kv β 2 could thus be due to loose positioning and partial charge delocalization at the carbonyl cause by the sole tyrosine phenolate residue rather than a complex between a tyrosine and histidine as in other AKRs. Indeed, replacement of His-113 in AKR2B5 with alanine causes a 600- to 1000-fold decrease in the catalytic activity of the protein (50).

Despite the absence of an active site histidine, the binding of aldehydes to Kv β active site was similar to other AKRs. Our previous experiments with AKR1A1 show a strong dependence of k_{cat}/K_m as well as k_{cat} on variations in substituents on the aromatic ring of benzaldehyde substrate (22). Similar results have been reported for AKR2B5 (29). Indeed, the ρ value obtained from Hammett plot of k_{cat}/K_m for Kv β 2 (1.95) was comparable to the ρ values obtained for AKR1A1 (1.95) and AKR2B5 (1.4). These data suggest that the active sites of AKRs are similar and that electron-withdrawing groups stabilize the formation of the ternary

complex in the catalytic cycle of these proteins. Moreover, a negative correlation of K_m with σ^+ in Kv β 2 indicates that the binding of benzaldehydes is strongly dependent on the electron-withdrawing potential of para substituents. The ρ value for K_m of Kv β 2 (−1.85) is, however, higher than that of AKR1A1 (−0.95) (22), indicating that electronic features play a more important role in substrate binding to Kv β 2 (AKR6) than in AKR1A1. The importance of electronic feature is further underscored by the observation that Kv β 2 was active with PGJ₂, which contains a carbonyl polarized by α,β -unsaturation, but not PGD₂, (Table 2) which is identical to PGJ₂ except that it lacks the conjugated system and therefore has a less polarized carbonyl. Likewise, the more electrophilic C-9 aldehyde 4-ONE was a substrate, but the less polarized 4-HNE, despite a similar structure, was not reduced. Hence, a polarized carbonyl with a partial negative charge on the oxygen appears to favor substrate binding. Furthermore, correlation between substrate binding and σ^+ within the benzaldehyde series suggests that a negative charge develops on the substrate carbonyl during ground state interaction, which may be indicative of the presence of a positively charged group that forms a hydrogen bond with the substrate (Scheme 3; Complex 1). The identity of this group remains to be established, but Tyr-90 is a strong possibility and binding to this residue could increase polarization of the enzyme carbonyl to facilitate acid-base catalysis (Scheme 3).

In contrast to binding, the bond-breaking step ($=k_{cat}$) was relatively insensitive to the polarizing effects of the substituents. In this regard, Kv β 2 is different from AKR1A1 which gives a ρ value of 0.8 for the Hammett plot of k_{cat} (22). The most straightforward explanation for the lack of substituent effects on k_{cat} is that the charge developed on the carbonyl in the transition state is small. Given, however, that acid-base catalysis by other AKRs involves polarization of the substrate carbonyl; we suggest that in Kv β the charge develops in the ground state of the carbonyl group. This polarization leads to the development of a slightly negative carbonyl O₁ and a slightly electron-deficient C₁ (Scheme 3; Complex 1). During transition state these charges are rapidly compensated by a proton transfer to the O₁ and hydride transfer to C₁ (Scheme 3; Complex 2). Because these events occur in concert, no net charge develops during the transition state. This mechanism appears to be similar to that of AKR2B5, which also shows no significant substituent effects (29), but differs from AKR1A1 catalysis. Substituent effects on AKR1A1 are more consistent with a stepwise hydride and proton transfer leading to the formation of a charged transition state. Overall, it appears that in Kv β 2-mediated catalysis, the lack of hydrogen bonding (due to the absence of an active site histidine) prevents stabilization of a transition state in which the carbonyl is hydrogen bonded before the proton transfer occurs. Therefore, the reaction proceeds with a concerted, rather than stepwise hydride and proton transfer (Scheme 3; Complex 2). We speculate that this mechanism may be particularly relevant to the regulation of Kv β catalysis by membrane voltage and for the ability of Kv β to alter Kv inactivation.

It has been suggested that the reason why Kv channels associate with auxiliary subunits possessing oxidoreductase activity relates to two possibilities (54). The first possibility is that the Kv β proteins are redox sensors that couple changes in the intracellular redox state with membrane excitability. This possibility is consistent with Kv β -dependent changes in Kv inactivation in cells subjected to hypoxia (55). Based on the high NADPH binding affinity of Kv β and the observation that Kv β -mediated Kv inactivation is removed by NADP⁺, but not NADPH, we had suggested that changes in intracellular oxygen or intermediary metabolism could affect Kv currents by altering redox state of nucleotides bound to Kv β (16). The redox state of the nucleotide bound to the protein could also be changed by catalysis and, in view of the high catalytic activity of the protein with several endogenous carbonyls, it appears likely that changes in endogenous NADPH-reducible endogenous carbonyls could regulate Kv β activity, and in turn the inactivation or the voltage-dependence of Kv channels. Furthermore, because the dissociation constants of Kv β for NADPH and NADP⁺ (0.1 to 0.6 μ M) are far below the range of physiological changes in pyridine nucleotide concentration, the protein

under most conditions is likely to respond to changes in the concentration of the carbonyl substrate. Thus any oxygen- or metabolism-dependent changes in substrate carbonyl concentration will rapidly alter the catalytic rate of Kv β , leading to cofactor oxidation and changes in Kv function (15;16).

A second scenario is that voltage-dependent changes in Kv channel could regulate Kv β catalysis by affecting the configuration of Kv β active site. We suggest that a mechanism in which catalysis is rate-limiting is ideally suited for such a voltage-sensitive oxidoreductase. Because catalysis or the rate of interconversion of the ternary complexes could be regulated by a single step (independent of cofactor or substrate concentration) voltage changes could efficiently modulate the overall rate of the reaction. Clearly, further experiments are required to fully evaluate and assess the physiological implications of the kinetic behavior of Kv β proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations and Textual Footnotes

4-NB, 4-nitrobenzaldehyde; 4-NAP, 4-nitroacetophenone; 4-HNE, 4-hydroxy-*trans*-2-nonenal; DHN, 1,4-dihydroxy-*trans*-2-nonenal; 4-ONE, 4-oxo-*trans*-2-nonenal; Kv, voltage-gated potassium channel; Kv β , voltage-gated potassium channel β -subunit; AKR, aldo-keto reductase.

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Figure 1. Optimal conditions for catalysis of aldehyde reduction by Kv β 2

Recombinant rat Kv β 2 was purified from *E.coli* and the catalytic activity was measured in reaction mixtures containing 0.15 mM NADPH and 1 mM 4-nitrobenzaldehyde (4-NB). Time course of change in absorbance at 340 nm (A_{340}) was determined at 10 (dashed line) and 50 μM (solid line) concentrations of the protein in 0.2 M potassium phosphate, pH 7.4, at 37 °C (A) and the initial velocity for 10 min was calculated at different protein concentration (B), pH (C) and ionic strength (D). Data are presented as mean $\pm$ SEM ($n = 3$). Line in panel B is the best fit of linear equation to the data ($R^2=0.99$). Lines in panels C and D are the best fits of (C), three parameter normal logarithmic distribution ($R^2=0.97$) and (D), hyperbolic ($R^2=0.96$) function ($y=y_0+a \cdot x/(b+x)$) to the data.

Figure 2. Cofactor binding and catalytic activity of Kv β 2:Y90F

(A) Absorbance spectra of WT (solid line) and Y90F (dashed line) Kv β 2 purified from bacteria. Peak absorbance was observed at 275 nm with a shoulder at 360 nm indicating NADPH bound to the protein. The inset shows SDS-PAGE of purified WT and Y90F Kv β 2 protein; (B) NADPH-dependent changes in the fluorescence of WT (filled circles) and Y90F (open circles) Kv β 2 (15 μ g) measured in 0.2 M potassium phosphate, pH 7.4 using an excitation wavelength of 280 nm and emission wavelength of 340 nm. Data are shown as discrete points and curves are best fits of eq. [1] to the data ($R^2=0.98$ for WT, and 0.95 for Y90F). Changes in fluorescence were normalized to the value of the initial fluorescence of the protein; and (C) time-dependent

changes in absorbance at 340 nm in a reaction mixture containing 0.15 mM NADPH and 1 mM 4-nitrobenzaldehyde and WT (solid line) or Y90F (dashed line) Kv β 2.

Figure 3. Kv β 2-catalyzed reduction of phospholipid aldehyde

Electrospray mass spectra of 1-palmitoyl-2-oxovaleroyl phosphatidyl choline (POVPC) incubated without (A) and with (B) Kv β 2. The formation of the reduced product - 1-palmitoyl-2-oxovaleroyl phosphatidyl choline (PHVPC) is evident by the appearance of a new ion with $m/z = 596.4$. For reduction, POVPC (80 μ M) was incubated without or with Kv β 2 (35 μ M) and NADPH (0.15 mM) for 180 min at 37°C. The 2Da increase in m/z was assigned the reduction of the aldehyde at the *sn*-2 position to an alcohol (as indicated).

Figure 4. Identification of Kvβ2-derived product of 4-oxo-2-nonenal (4-ONE)

Panel A shows the separation of reagent ^3H -1,4-dihydroxynonenol (DHN; 3000 cpm), 4-oxo-2-nonenol, 4-hydroxy-2-nonenal (4-HNE) and 4-ONE, 40 nmoles each on ODS C₁₈ reverse phase HPLC column. Y-axis on the right side shows the radioactivity profile of ^3H -DHN whereas Y-axis on the left side shows OD₂₂₄. Peaks I-IV eluting with retention times of 31, 37, 43 and 53 min are due to reagent DHN, 4-oxo-2-nonenol, 4-HNE and 4-ONE, respectively. *Panel B* shows the HPLC traces of the Kvβ2-catalyzed reduction product of 4-ONE. Reagent 4-ONE (100 nmoles) was incubated with 0.6 mg Kvβ2 and 0.3 mM NADPH in 0.2 M potassium phosphate for 2h at 37 °C and the product was separated on HPLC. *Panel C*: inset shows a representative gas chromatogram of derivatized peak II (from *panel B*), eluting at 8.06 min and *panel C* shows the electron impact ionization-mass spectrum of the silylated metabolite of the GC peak eluting at 8.06 min. Ion with m/z 228 represents molecular ion (M) of 4-oxo-2-

nonenol. Ions with m/z 213, 157 and 129 respectively, correspond to $M-CH_3$, $M-C_5H_{11}$ and $M-C_6H_{11}O$ fragments of 4-oxo-2-nonenol.

Figure 5. Initial velocity profile of aldehyde reduction by Kvβ2

Double reciprocal plots of initial velocity were derived from measuring the rates of change of absorbance at 340 nm at different concentrations of 4-nitrobenzaldehyde at 75 (filled circle), 100 (open circle) and 200 (filled inverted triangle) μM NADPH. Lines represent best fit of equation 6 to the data ($R^2=0.97$). Inset shows a replot of individual slopes of the double reciprocal plot versus $1/[\text{NADPH}]$.

Figure 6. Primary deuterium kinetic isotope effects on Kvβ2-catalyzed aldehyde reduction
Initial velocity plots of the reaction at different concentrations of 4-nitrobenzaldehyde (A; 4-NB) and 4-nitroacetophenone (B; 4-NAP) with 0.15 mM NADPH or NADPD as indicated are shown. For each measurement, the Kvβ2 protein was mixed either with NADPH or NADPD (0.15 mM) and the reaction was initiated by the addition of the substrate. Time-dependent changes in total sample absorbance (multiple turnover mode) were measured. The values of initial velocity are normalized to the concentration of the total Kvβ2 protein in the cuvette. Data are shown as discrete points and curves are best fits of eq. [5] to the data ($R^2=0.99$ for 4-NB and 0.97 for 4-NAP).

Figure 7. Kinetic isotope effects on single turnover reactions of Kvβ2

(A) Replacement of intrinsically bound NADPH with NADPD. The NADPH bound to Kvβ2 (black) was oxidized by incubating the protein with 1 mM 4-nitrobenzaldehyde which led to a progressive decline in absorbance of the protein at 360 nm (inset; red line), indicating oxidation of NADPH to NADP⁺. The reaction mixture was then dialyzed for 16-20 h at 4°C against 0.2 M potassium phosphate, pH7.4. The protein was then incubated with synthesized NADPH or NADPD for 30 min at 4°C and dialyzed for 16-20 h. Reloading with NADPH (blue) or NADPD (green) led to an increase in A₃₆₀, indicating re-formation of the protein-nucleotide binary complex; Time-dependent decrease in A₃₆₀ of NADPH (B) and NADPD (C) -bound Kvβ2 incubated with the indicated concentrations of 4-NB; and (D) The values of the pseudo-first order rate constant (k_{trans}), obtained by fitting a single exponential equation to the progress curve data shown in panels B and C, plotted as function of 4-NB concentration. Data are shown as discrete points $\pm$ SEM and the lines are best fit of Eq. (5) to the data ($R^2=0.96$); solid line, NADPH; dotted line, NADPD.

Figure 8. Structure-activity relationships for Kvβ2-mediated reduction of substituted benzaldehydes

Relationship between $\log$ (A) k_{cat}/K_m , (B) k_{cat} and (C) K_m of para-substituted benzaldehydes and the Hammett constant - σ^+ . The enzyme activity was determined under multiple turnover condition with 0.15 mM NADPH. Data are shown as discrete points and the lines are best weighted fits of a linear equation to the data ($R=0.79-0.93$). Calculated slope values are listed on the upper right hand corner of the panel. Results of multivariate regression analysis are shown in supplemental Table 1.

Scheme 1. Potential products of 4-oxo-2-nonenal (ONE) reduction

Reduction of 4-oxo-2-nonenal at C-1 aldehyde would lead to the formation of 4-oxo-2-nonenol, whereas reduction of the C-4 keto group should yield 4-hydroxy-2-nonenal (HNE). Additional reduction of HNE or 4-oxo-2-nonenol would form 1,4-dihydroxy-2-nonenal (DHN). *Note:* Reduction in the presence of Kvβ2 led to the appearance of only 4-oxo-2-nonenol and no HNE or DHN formation was observed (see, Fig. 4). The reduction of the C-1 aldehyde to an alcohol is indicated by a dotted box.

Scheme 2. Catalytic cycle for NADPH-dependent carbonyl reduction by Kvβ2

Sequential binding of the protein to NADPH and the carbonyl substrate results in the formation of a ternary complex in which the carbonyl is reduced to its corresponding alcohol. The release of products is also shown to be ordered. The microscopic rate constants for each reaction step in the forward and reverse direction are indicated as k_n and k_{-n} , respectively.

Scheme 3. Mechanism of NADPH-dependent carbonyl reduction catalyzed by Kvβ2

In complex 1, Tyr-90 is shown poised to form a hydrogen bond with the substrate carbonyl which results in carbonyl polarization, accelerating the hydride transfer of the pro-R hydrogen from the nicotinamide ring of NADPH to the carbonyl carbon of the substrate. Complex 2 shows a non-charged transition state in which the polarization at the carbonyl is quenched by the proton transfer from the protein tyrosine and a concerted hydride transfer to the carbonyl carbon. The reduced carbonyl then dissociates from the acid-base catalyst and a net charge develops on the oxidized cofactor and the tyrosinate anion (Complex 3).

Table 1

Specific activity and NADPH binding of WT and Y90F:Kvβ2.

		K_d^{NADPH} (μM)	Specific activity (min^{-1})	
			4-nitrobenzaldehyde (1 mM)	4-nitroacetophenone (1 mM)
1.	Kvβ2:WT	0.2 ± 0.005	0.118 ± 0.02	0.050 ± 0.008
2.	Kvβ2:Y90F	0.13 ± 0.011	$0.009 \pm 0.002^*$	$0.006 \pm 0.003^*$

Values of K_d^{NADPH} and specific activity represent a mean of 3 independent measurements.

* $P < 0.05$ versus WT.

Table 2

Kvβ2 specific activity with biologic carbonyls.

	Substrate	Structure	Specific activity (min^{-1})
1.	Phenylglyoxal (100 μM)		0.014 ± 0.0006
2.	Methylglyoxal (12 mM)		0.32 ± 0.04
3.	3-deoxyglucosone (5 mM)		0.039 ± 0.01
4.	2-carboxybenzaldehyde (10 mM)		0.010 ± 0.005
5.	Acrolein (100 μM)		0.010 ± 0.003
6.	4-oxo-nonenal (100 μM)		0.028 ± 0.004
7.	4-hydroxy-nonenal (100 μM)		-- ^a
8.	Succinic semialdehyde (25 mM)		0.011 ± 0.004
9.	12-oxoETE (10 μM)		0.044 ± 0.007
10.	PGJ ₂ (100 μM)		0.086 ± 0.019
11.	PGD ₂ (100 μM)		-- ^a
12.	PGF _{2α} (100 μM)		-- ^a
13.	9, 10 Phenanthrenequinone (200 μM)		0.19 ± 0.07

	Substrate	Structure	Specific activity (min^{-1})
14.	POVPC (100 μM)		0.078 ± 0.016
15.	5 α -androstan-17 β -ol-3-one (100 μM)		-- ^a
16.	Cortisone (100 μM)		-- ^a

^a Activity less than two fold over the background.

Table 3

Kinetic constants and primary kinetic isotope effects (KIE) on Kvβ2-mediated carbonyl reduction.

Substrate	K_m (μM)	k_{cat} ($\min^{-1}$)	k_{cat}/K_m ($mM^{-1}\min^{-1}$)	KIE
4-nitroacetophenone	209 ± 49	0.092 ± 0.006	0.46 ± 0.09	1.69 ± 0.1
4-nitrobenzaldehyde	283 ± 31	0.16 ± 0.030	0.59 ± 0.17	2.07 ± 0.4
4-nitrobenzaldehyde ^a	--- ^b	--- ^b	--- ^b	3.13 ± 0.2

The value of each parameter is a representative of 2-5 separate determinations using different preparations of the protein. For each determination, the initial velocity was measured at 6-9 different concentrations of the substrate.

^aData from single turnover experiments.

^bNot determined.