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Substrate profiling and aldehyde dismutase activity of the Kvβ_2 subunit of the mammalian Kv1 potassium channel

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Abstract

Voltage-dependent potassium channels (Kv) are involved in various cellular signalling processes by governing the membrane potential of excitable cells. The cytosolic face of these α subunit-containing channels is associated with *β* subunits that can modulate channel responses. Surprisingly, the *β* subunit of the mammalian Kv1 channels, Kv*β*2, has a high level of sequence homology with the aldo-keto reductase (AKR) superfamily of proteins. Recent studies have shown that Kv*β*2 can catalyze the reduction of aldehydes and, most significantly, that channel function is modulated when Kv*β*2-bound NADPH is concomitantly oxidized. As a result, the redox chemistry of this subunit is crucial to understanding its role in K^+ channel modulation. The present study has extended knowledge of the substrate profile of this subunit using a single turnover fluorimetric assay. Kv*β*2 was found to catalyse the reduction of aromatic aldehyde substrates such as 2, 3 and 4-nitrobenzaldehydes, 4-hydroxybenzaldehyde, pyridine 2-aldehyde and benzaldehyde. The presence of an electron withdrawing group at the position para to the aldehyde in aromatic compounds facilitated reduction. Aliphatic aldehydes proved to be poor substrates. We devised a simple HPLC-based assay to identify Kv*β*2 reaction products. Using this assay we showed, for the first time, that Kv*β*2 can catalyze a slow aldehyde dismutation reaction using 4-nitrobenzaldehyde as substrate and have identified the products of this reaction. The ability of Kv*β*2 to carry out both an aldehyde reduction and a dismutation reaction is discussed in the light of current thinking on the role of redox chemistry in channel modulation.

Key words: *β* subunit, K^+ channels, dismutation, substrate specificity and aldo-keto reductase

1. Introduction

Voltage-dependent potassium channels (Kv superfamily) play a key role in the mammalian nervous system by governing the resting membrane potential of excitable cells thereby modulating the frequency of action potentials and neurotransmitter release (Hille, 1991). Four voltage sensing, integral membrane pore-forming Kvα subunits assemble to form the Shaker-related voltage-gated K^+ channels (Kv1 subfamily) (Kolb, 1990; Orlova et al., 2003). The auxillary *β*-subunit, Kv*β*2, associated with the cytoplasmic face of Kvα proteins (Dolly et al., 1994; Parcej et al., 1992; Scott et al., 1994), has a conserved catalytic core that shows a high level of sequence homology with aldo-keto reductase (AKR) enzymes (Gulbis et al., 1999; Long et al., 2005). The catalytic C-terminal of Kv*β*2 has a tightly, but non-covalently, bound nicotinamide (NADPH) cofactor and an aldehyde binding site. Of all the variants of Kv*β* genes (Kv*β*1-3), Kv*β*2 is the most widely distributed (Pongs et al., 1999; Xu and Li, 1998) and it strongly associates with several Kvα proteins.

Weng et al. (2006) showed that Kv*β*2 was catalytically active as an aldehyde reductase, identified substrates for this enzyme activity, and demonstrated modulation of channel function when Kv*β*2-bound cofactor (NADPH) became oxidized (Weng et al., 2006). This important finding focused attention on the redox properties of this channel subunit. It was further speculated that oxidation of the bound NADPH induces a conformational change in Kv*β*, which propagates to Kv1.1 to effect channel inactivation (Pan et al., 2008a; Weng et al., 2006). Initially, two Kv*β* substrates were identified (4-cyanobenzaldehyde and 4-carboxybenzaldehyde) as being able to reversibly oxidize Kv*β*2-bound NADPH (Weng et al., 2006). Tipparaju et al. (2008) carried out a more

detailed study of the Kvβ2 substrate specificity using a multiple turnover reaction and showed that Kvβ2 could catalyse the reduction of aldehydes and ketones with equal affinity.

Understanding the details of the redox chemistry of Kvβ2 is essential for elucidating its mode of action. It has been suggested that it may respond to changes in the concentration of intracellular carbonyl substrates. Thus, an oxygen or metabolism-dependent change in carbonyl substrate concentration could rapidly alter the catalytic rate of Kvβ2, leading to cofactor oxidation and to changes in Kv function (Tipparaju et al., 2008). The physiological substrates responsible for the reduction/oxidation of the enzyme-bound cofactor are not known.

In addition to the well-characterized reversible oxidation of alcohols, a number of pyridine linked dehydrogenases/reductases are known to catalyze the dismutation of aldehydes (Scheme 1) to equimolar concentrations of the corresponding alcohol and carboxylic acid (Daussmann et al., 1997; Henehan et al., 1995; Mee et al., 2005; Oppenheimer et al., 1995; Steinbuchel et al., 1984; Winberg et al., 1998). Kvβ2 bears some similarity to the microbial nicotinoprotein oxidoreductases in having a tightly-bound catalytically-active NADPH cofactor (Schenkels and Duine, 2000). Since nicotinoprotein oxidoreductases function as aldehyde dismutases, it seemed possible that Kvβ2 might also catalyse aldehyde dismutation.

Herein, we extend the information on the substrate profile for this subunit in an attempt to identify compounds capable of interacting with Kvβ2. We identify the reduced reaction products for its optimum substrate, 4-nitrobenzaldehyde. In addition, the ability of Kvβ2

to catalyze an aldehyde dismutation reaction is established for 4-nitrobenzaldehyde and reduced and oxidized reaction products identified.

2. Material and Methods

2.1. Purification of Kvβ2

Kvβ2 gene from rat brain, cloned with an N-terminus His-tag as described in Dolly et al., (1994), was provided by Dr. Oleg Shamotienko. *E. coli* BL21 (DE3, plysS) transformed with pET15b-Kvβ2 construct were cultured at 37°C in LB medium containing 50µg/ml ampicillin. When the $A_{600\,nm}$ of the culture reached ~ 0.8, the expression of Kvβ2 protein was induced for 14hr by the addition of IPTG to a final concentration of 1mM at 25°C with constant shaking at 280rpm (Stuart S1500 incubator/orbital shaker). Cells were resuspended in lysis buffer (20mM Tris-HCl, pH 7.9, 5mM imidazole, 200mM NaCl) and lysed by sonication for 130 seconds on ice (VC-750 ultrasound generator, Sonics and Materials Inc.). Cell debris was collected by centrifugation and the supernatant applied to a nickel-charged iminodiacetic acid (Novagen) column pre-equilibrated with lysis buffer. The Ni^{+} column was washed with 20 column volumes of the equilibration buffer before elution with 20mM Tris-HCl, pH 7.9, 200mM NaCl, and 300mM imidazole. Fractions containing Kvβ2 were identified by SDS-polyacrylamide gel electrophoresis, pooled and dialyzed against 2L of prechilled 0.2M potassium phosphate, pH 7.5 for 36hr with three changes.

2.2. Fluorescence assay of the single step hydride transfer rate constant for Kvβ2 substrates

The rates of single turnover hydride transfer reaction were monitored using a Perkin Elmer fluorescence spectrometer LS50B at 22°C. Assays were carried out in a quartz cuvette containing 2.0 μM Kv*β*2 and varying substrate concentrations, in a final volume of 2.0 ml. All substrates were prepared in ethanol and then diluted into 0.2M potassium phosphate buffer, pH 7.5 to the required concentration, keeping the content of ethanol in the reaction mixture equal to or below 1 % (v/v). Ethanol at this concentration had no effect on enzyme-bound NADPH. The excitation wavelength used was 360 nm with a slit size of 15nm and the emission spectra were acquired from 300 to 600nm at different times after initiating the reaction by addition of substrate.

The decrease in emission at 454 nm due to enzyme-bound NADPH followed a single exponential function over time, which is consistent with the single-step hydride transfer reaction. Rate constants were estimated from the slope of a plot of ln $[NADPH]_t$ versus time (t) and expressed as the inverse of the exponential time constant (see Figure3). The equation used was:

$$\ln F_t = -kt + \ln F_0$$

Where, F_t the fluorescence intensity at time‘t’, F_0 is the initial fluorescent intensity and ‘k’ is the rate constant. All assays were carried out in duplicate.

2.3. HPLC assay to monitor Kvβ2 catalysed dismutation of 4-nitrobenzaldehyde

Aldehyde dismutation was assayed in duplicate in 0.2M potassium phosphate buffer, pH 7.5 containing 0.2mM $NADP^+$ at 37°C in the presence of ~0.5mg of Kvβ2 and 500μM of 4-nitrobenzaldehyde (final vol of 2.0ml). The reaction was quenched by mixture with HPLC mobile phase (methanol/trifluroacetic acid/water, 60: 0.1: 39.9) in the ratio 1:2. Aliquots, (10μl) of the resultant mixture were analysed on a Nucleosil C18 (3.9 x 150 mm) HPLC column, using a method adapted from Shearer et al. (1993) with monitoring by a Millipore Waters (Mississauga, Canada) liquid chromatography UV detector at 274 nm. Standard curves, constructed for 4-nitrobenzaldehyde, 4-nitrobenzyl alcohol and 4-nitrobenzoic acid were used to determine product concentrations in the reaction mixtures.

3. Results

3.1. Substrate screening studies by fluorimetric assay

Kvβ2, when purified, contains bound NADPH. During purification care must be taken to isolate the protein with the dissociable NADPH still bound. The presence of bound cofactor in recombinant Kvβ2-NADPH was observed by UV and fluorimetric analysis (Figure 1). When excited at 360nm Kvβ2 shows a prominent fluorescence peak at 454nm corresponding to bound NADPH (Figure 1 inset). After addition of substrate to freshly purified Kvβ2 in 0.2M phosphate buffer, pH 7.5, the transfer of a hydride from the Kvβ2-bound NADPH to a substrate was observed as reduction in the 454nm fluorescence peak (see Figure 2 A, B). This single turnover reaction allowed an investigation of the ability of Kvβ2 to catalyze the reduction of various aldehydes. This assay has previously been described for Kvβ1 (Pan et al., 2008a) and is more sensitive than the spectrophotometric assay first described for monitoring the reduction of aldehydes by Kvβ2 (Weng et al., 2006).

A continuous spectrophotometric assay has also been described for the aldehyde reducing activity of Kvβ2 (Tipparaju et al., 2008). Such a multiple turnover assay is possible since the oxidized cofactor resulting from aldehyde reduction will exchange with externally added NADPH to recommence the catalytic cycle. In the present study, the multiple turnover assay was only used to identify the reduced product, 4-nitrobenzylalcohol derived from the reduction of 4-nitrobenzaldehyde. In our hands, multiple turnover initial rates measurements were complicated by slow blanching of enzyme bound NADPH. Thus, multiple turnover initial rates were not used for kinetic constant estimations. The

use of a single turnover reaction allows for the screening of a range of substrates. More detailed studies of kinetic constants were not pursued due to the low activity of Kvβ2 with the carbonyls tested as previously noted (Tipparaju et al., 2008).

When the initial rate of aldehyde reduction was measured by taking the slope of the linear part of the plot of fluorescence intensity at 454nm versus time; the initial rate observed was linearly dependent on Kvβ2 concentration (Figure 3).

3.2. Substrate profiling

Pseudo-first order rate constants were estimated for a range of aromatic and aliphatic aldehydes, ketones and dicarbonyls. All substrates were tested at a concentration of 0.5mM except for certain aliphatic substrates that required a higher concentration to yield observable activity. A range of aldehydes of various structures was reduced by Kvβ2, with aromatic substrates bearing a strongly electron withdrawing substituent giving the largest rate constants (Table 1). Of these, 4-nitrobenzaldehyde gave the highest rate constant. All reactions were carried out at 22ºC due to the aggregation of Kvβ2 at higher temperatures. This assay is similar to that previously used to identify substrates for Kvβ1 (Pan et al., 2008a).

The effect of the *position* of the electron withdrawing group on the pseudo-first order rate constant was explored by comparing 2-nitrobenzaldehyde, 3-nitrobenzaldehyde and 4-nitrobenzaldehyde. It was found that the rate constants for these substrates decreased in the order: 4-nitrobenzaldehyde>3-nitrobenzaldehyde>2-nitrobenzaldehyde. All nitro-

substituted benzaldehydes had higher rate constants than any of the other substrates tested. Thus, it was clear that the position of this group strongly influenced catalysis, with the para position being optimal. The single turnover rate constant for the hydride transfer reaction was estimated to be 0.22 min^{-1} for 0.5 mM 4-nitrobenzaldehyde and assigned as 100%. Activities with all other substrates were expressed as a percentage of 4-nitrobenzaldehyde reductase activity (Table 1). 4-cyanobenzaldehyde, one of the original substrates reported for Kvβ2, had a rate constant (0.014 min^{-1}) more than 10-fold lower than 4-nitrobenzaldehyde. The rate constant with 4-cyanobenzaldehyde was similar to that for benzaldehyde showing that the nitro group significantly accelerated the Kvβ2-mediated aldehyde reduction. 2-cyanobenzaldehyde had a rate constant half that of 4-cyanobenzaldehyde, supporting the observation that the position of the electron withdrawing group para to the aldehyde is important for optimal enzyme activity. This study indicates that a range of known AKR substrates may be reduced by this protein. Surprisingly, Kvβ2 showed no detectable activity with the aromatic aldehydes 4-chlorobenzaldehyde and 4-fluorobenzaldehyde.

Pyridine-2-aldehyde, a well known AKR substrate, gave a rate constant of 0.011 min^{-1}. An aromatic alpha-keto aldehyde, phenylglyoxal, had a rate constant (0.0054 min^{-1}) about half that of the 4-cyanobenzaldehyde while an aliphatic alpha-keto aldehyde, methylglyoxal was a poorer substrate than both phenylglyoxal and 4-cyanobenzaldehyde. These data indicated the importance of activation of the aldehyde group for catalysis, and the importance of the aromatic moiety, presumably for binding.

4-Hydroxybenzaldehyde, with a moderately strong electron donating group in position para to the aldehyde was 5-fold less active than the 4-nitrobenzaldehyde but better than benzaldehyde. Aromatic aldehydes with moderate electron donating groups such as tolualdehyde and anisaldehyde showed no detectable activity. These data seem to indicate a complex picture of substrate preference. Nitrobenzaldehyde derivatives are clearly among the best aromatic substrates but other aromatic substrates, even those with electron withdrawing substituents such as chlorobenzaldehyde, showed no clear pattern of preference.

A para-substituted ketone, 4-nitroacetophenone, had an activity 50-fold less than 4-nitrobenzaldehyde indicating that Kvβ2 had rather poor affinity towards ketones. This finding contrasts with the conclusion of Tipparaju et. al., (2008) who indicated that Kvβ2 could reduce aldehyde and ketone substrates with equal affinity. However, these workers noted that when presented with a choice between aldehyde and keto functional groups on a single substrate, 4-oxo-nonenol, only the aldehyde was reduced.

Aliphatic aldehydes are poor substrates with rate constants <100-fold or lower than aromatic aldehydes even at concentrations up to 10mM. In this study, 2-methylbutyraldehyde was identified as a Kvβ2 substrate with a rate constant of ~10-fold higher than all other tested aliphatic aldehydes (Figure 4). In mammals, isoleucine is one of three branched chain amino acids (leucine, isoleucine and valine) that can cross the blood brain barrier (Oldendorf, 1971a, 1971b) where they serve as substrates for meeting

parenchymal energy demand (Pellerin, 2005; Yudkoff et al., 1996). Formaldehyde, cinnamaldehyde and octanal showed no detectable activity with Kvβ2.

3.3. HPLC-based assay for the identification of reduced product of multiple turnover reaction using 4-nitrobenzaldehyde as substrate

Kv*β*2-mediated formation of reduced product using a phospholipid aldehyde, 1-palmitoyl-2-oxovaleroyl phosphatidylcholine, has been reported (Tipparaju et al., 2008). To date, 4-nitrobenzaldehyde is the most effective substrate for Kv*β*2. Since this aldehyde has a high extinction coefficient, it was used as the basis for the development of a simple HPLC assay for aldehyde reduction/oxidation The HPLC assay using this aldehyde proved to be a convenient and sensitive way to monitor aldehyde reduction and dismutation and will be broadly applicable to the assay of aldehyde dismutating enzymes. Separation of 4-nitrobenzaldehyde, and its corresponding alcohol and acid derivatives by HPLC is shown in Figure 5A.

In the presence of externally added NADPH, Kv*β*2 catalyses a multiple turnover reaction (see above). When the HPLC assay was used to monitor its reduction of 4-nitrobenzaldehyde, in the presence of NADPH, the formation of reduced product *i.e.*, 4-nitrobenzylalcohol (Figure 5B), was clearly observed.

3.4. Dismutation of aldehydes by Kvβ2

A number of pyridine linked alcohol dehydrogenases/aldo-keto reductases have been reported to catalyse the dismutation of aldehydes (Daussmann et al., 1997; Henehan et al., 1995; Mee et al., 2005; Oppenheimer et al., 1995; Steinbuchel et al., 1984; Winberg et al., 1998). This reaction has previously been described for horse liver alcohol

dehydrogenase; hence, this enzyme was chosen to validate the assay conditions in this study (see below) (Oppenheimer et al., 1995).

Purified Kvβ2.NADPH was first treated with 250μM 4-nitrobenzaldehyde to oxidise all enzyme bound cofactor before dialysis for 24 hr against 0.2M potassium phosphate buffer, pH 7.5 to remove remaining substrates. This step ensured that all Kvβ2 bound cofactor was in the oxidised form (Kvβ2.oxd). The reaction mixture containing ~10 μM Kvβ2.oxd, 200μM of NADP, 0.2M potassium phosphate buffer (pH 7.5) and 0.5mM of 4-nitrobenzaldehyde was incubated at 37^{o}C. Aliquots were removed from the reaction mixture every hour and the products analysed by HPLC. This analysis revealed a clear peak for 4-nitrobenzoic acid (oxidized product) along with an equivalent amount of 4-nitrobenzylalcohol (reduced product) (see Figure 6).

The HPLC based dismutation assay method was validated by using horse liver alcohol dehydrogenase (HLADH) and oxidised cofactor (NAD, 200μM) under the same conditions for which dismutation had been reported (Oppenheimer et al., 1995) (see Figure 7A). Dismutation may be conveniently monitored by following the accumulation of 4-nitrobenzoic acid, a reaction product unique to the dismutation reaction. Kvβ2 was shown to catalyze a slow dismutation reaction where the amount of 4-nitrobenzoic acid produced was found to be >1400 times less than HLADH in 2.0 hr (see Figure 7B).

Control reactions with no enzyme were incorporated in every experiment. Equimolar concentrations of 4-nitrobenzaldehyde, 4-nitrobenzoic acid, 4-nitrobenzyl alcohol and $NADP^{+}$ were incubated at 37^{o}C as a control to monitor any possible background

chemical reactions. The data clearly showed that the products of the reaction observed were due to Kvβ2 catalyzed dismutation.

3.5. Comparison of Kvβ2-mediated multiple turnover and dismutation reactions

The amount of reduced product (4-nitrobenzylalcohol) formed from the multiple turnover reaction over the course of 30 minutes was ~ 30 times higher than that seen for the dismutation reaction. This was expected since dismutation involves the generation of the Kvβ2- bound NADPH by the formation of 4-nitrobenzoic acid which is presumably slow and expected to be rate limiting (Oppenheimer et al., 1995).

4. Discussion

The substrate screening data in this study help to further define Kvβ2 substrate specificity and establish that Kvβ2 has a preference for aromatic aldehydes with strong electron withdrawing groups at a position para to the aldehyde moiety. It has been suggested that *in vivo* modulation of channel activity may be mediated by an aldehyde substrate. The identity of such a substrate is difficult to define when an enzyme has the broad specificity of Kvβ2. Most of the substrates we and others have examined are unlikely to be physiologically significant and physiological analogs are not easily identified. However, it has been suggested that this subunit is involved in reduction of membrane-derived oxidized lipids (Tipparaju et al., 2008). However, it is difficult to envisage how production of such aldehydes could be controlled sufficiently to regulate channel activity since they arise from a variety of processes (both enzymic and non enzymic) mostly as a result of oxidative stress and would be expected to be competitive substrates.

The suggestion that aldehydes have a direct role in channel modulation (Tipparaju et al., 2008; Weng et al., 2006) presents some challenges. Firstly, most aldehydes are reactive species that readily react with, and modify, cellular proteins. Most cells contain efficient enzyme systems to remove aldehydes in order to avoid such harmful reactions (Kawamura et al., 2002; Svensson et al., 1996). Thus, it would be necessary for such aldehydes to be generated in close proximity to Kvβ2 in order to be effective. However, such a system has not yet been identified. Secondly, there is the issue of competition between aldehydes for Kvβ2 due to its broad specificity. Many aldehydes generated in cells might be expected to compete for the relatively open active site of Kvβ2; this

necessitates very tight control of aldehyde access to Kvβ2 for effective, controlled, channel modulation. The requirement for tight control may indicate a high degree of specificity for the true physiological substrate for this reaction. As a result, the true substrate may prove difficult to identify. A final difficulty lies in the requirement that oxidized Kvβ2 bound cofactor must be reduced in order to reset the channel modulation signal following its oxidation by an aldehyde substrate.

It might be argued that the slow rate of aldehyde reduction by Kvβ2 makes it difficult to envisage that such a reaction could modulate channel activity in real time as has been proposed (Tipparaju et al., 2008; Weng et al., 2006). A possible explanation lies in the requirement that the true substrate, as yet unidentified, has a significantly higher turnover than those identified in this and previous studies. Another possibility is that turnover may be upregulated by association of Kvβ2 with the channel subunits or with other proteins or indeed by other signaling events.

Dismutation of aldehydes has proven difficult to monitor since it is a redox silent reaction (Henehan et al., 1995; Oppenheimer et al., 1995). In this study we have devised a sensitive HPLC assay, based on the absorbance of nitrobenzaldehyde and its derivatives, capable of detecting both net aldehyde reduction and aldehyde dismutation. The assay will be generally useful for screening for dismutation, albeit confined to a single substrate. From these studies it is clear that Kvβ2 can dismutate aldehydes but at a rate far slower than HLADH. This is consistent with the slow turnover of aldehyde substrates

by aldo keto reductases from a number of sources when compared to corresponding alcohol dehydrogenases (Barski et al., 2008). Although dismutation was slow it was clear that acid formation was occurring. Control experiments showed no acid formation under the same conditions thereby discounting the possibility that acid formation was due to non enzymatic aldehyde oxidation. Moreover, acid formation was time dependent and acid was formed in equimolar concentrations to that of the alcohol product.

If it were postulated that channel activity were controlled by aldehyde dismutation it would obviate the necessity to regenerate reduced Kvβ2-bound cofactor since this reaction alternately oxidizes and reduces NADP(H). Notwithstanding this point, it is difficult to argue for a significant physiological role for the slow dismutation observed here with 4-nitrobenzaldehyde but we cannot discount the possibility that dismutation might be faster for other, as yet untested, substrates. It is of interest for inhibitor design to consider that Kvβ2 is capable of generating a carboxylate, presumably from the hydrated form of the aldehyde, at its active site. A similar dismutation reaction has previously been reported for the carbonyl reductase from pig lung (Hara et al., 1991).

Recent findings have shown that cortisone binds to Kvβ2 and promotes its dissociation from the K^+ channel (Pan et al., 2008b). Structural studies identified two types of cortisone binding sites on Kvβ2, one close to the bound NADPH and the other at the interface of two neighbouring Kvβ subunits (Pan et al., 2008b). Only the interface binding site was reported to be required for channel modulation. These findings indicate that cortisone may act to further regulate aldehyde-mediated channel modulation by

promoting Kvβ2 dissociation from α subunits. It is conceivable that fine control of channel activity could be modulated by a specific aldehyde substrate but that cortisone could abolish such control by dissociation of Kvβ2 from the α subunits. The relationship of these two very different modes of K^+ channel modulation remains to be explored. In particular, data on the influence of low concentrations of cortisone on channel activity would be of great interest since reports to date used concentrations of 500μM and above that may be difficult to attain physiologically. Interestingly, cortisone was shown to bind to the active site of Kvβ2 but was not a substrate. Thus, cortisone is expected to act as an inhibitor of aldehyde reduction *in vitro*. Whether aldehyde substrates act in opposition to cortisone or in synergy with cortisone as channel modulators remains to be elucidated.

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Legends to Tables:

Table 1. Comparison of the Rate constants (min^{-1}) for the single turnover hydride transfer reaction (first order) for Kvβ2 substrates.

Legends to Figures:

Figure 1: UV/Vis and fluorescence spectra of freshly purified Kvβ2 protein. The UV spectrum of Kvβ2 gave two absorbance peaks at 280nm and 360nm corresponding to the protein and protein-bound NADPH, respectively (Liu et al., 2001; Schenkels et al, 2000). The *inset* is a fluorescence emission scan of purified Kvβ2 showing an emission band at 454nm for bound NADPH. The excitation wavelength was 360nm.

Figure 2: Kvβ2 mediated reduction of 4-nitrobenzaldehyde**. (A)** Fluorescence emission spectra of purified Kvβ2 protein at different time points after mixing with 0.5mM 4-nitrobenzaldehyde. **(B)** Fluorescence intensity at 454nm plotted versus time for Kvβ2 after mixing with 4-nitrobenzaldehyde. This plot was fitted to a first order exponential decay to obtain a pseudo first order rate constant for aldehyde reduction.

Figure 3: Initial velocity of the hydride transfer reaction plotted versus Kvβ2 concentration for the single turnover reaction. The substrate (4-nitrobenzaldehyde) concentration was kept constant at 0.5mM and initial velocities were measured by taking the slope of the linear part of the plot between fluorescence intensity and time. The concentration of enzyme employed was between 0.04-0.14 mg/ml.

Figure 4: Kvβ2 mediated reduction of 2-methylbutyraldehyde. Fluorescence emission spectra of purified Kvβ2 at different time points after mixing with 10mM 2-methylbutyraldehyde which had a rate constant of ~10-fold higher than all other tested aliphatic aldehydes.

Figure 5: (A) Standards HPLC profile of a mixture of equimolar (1mM) concentrations of 4-nitrobenzylalcohol (4-NBalc), 4-nitrobenzaldehyde (4-NBald) and 4-nitrobenzoic acid (4-NBacid) containing 200μM of NADP; the retention times are 3.83, 4.33, 5.13 and 2.18 minutes, respectively. **(B)** Identification of products of Kvβ2 mediated multiple turnover aldehyde reduction reaction. The data show formation of 4-nitrobenzylalcohol (reduced product) with a retention time of 3.83 minutes in a reaction mixture containing ~10 μM Kvβ2, 0.2M potassium phosphate buffer (pH 7.5), 0.2 mM NADPH and 0.5 mM of 4-nitrobenzaldehyde. This mixture was incubated for 15 minutes at 37^{o}C. The peaks at 4.33 and 2.17 minutes represent unreacted 4-nitrobenzaldehyde and NADPH, respectively.

Figure 6: Identification of the products of Kvβ2 mediated dismutation reaction. HPLC profile of the reaction mixture containing ~10 μM Kvβ2.oxd, 0.2M potassium phosphate buffer (pH 7.5), 0.2mM NADP and 0.5mM of 4-nitrobenzaldehyde incubated for 7 hr at 37^{o}C showing the formation of 4-nitrobenzylalcohol (reduced product) along with an equimolar concentration of 4-nitrobenzoic acid (oxidized product, *peak indicated in red*) giving respective retention times of 3.85 and 5.09 minutes. The peaks at 4.35 represent unreacted 4-nitrobenzaldehyde. This experiment was repeated several times with

identical results. Control experiments with no enzyme showed no alcohol or acid formation over the same time period.

Figure 7: (A) Progress curves showing 4-nitrobenzoic acid formation resulting from the dismutation of 4-nitrobenzaldehyde by horse liver alcohol dehydrogenase (HLADH). The data shows the formation of 4-nitrobenzoic acid at different starting concentrations of 4-nitrobenzaldehyde (250μM, 500 μM, 1mM and 5mM). After addition of the HLADH to the assay mixture aliquots were removed at 15, 30, 45, 60, 75, 90, 105 and 120 minutes and the amount of 4-nitrobenzoic acid formed was estimated by HPLC. Results are expressed as the mean of duplicate measurements. The concentration of HLADH and NAD^+ used was 20μg/ml and 200μM, respectively, in a final reaction volume of 2ml. **(B)** Dismutation of 4-nitrobenzaldehyde by Kvβ2. The data show the Kvβ2 mediated dismutation of 4-nitrobenzaldehyde with externally added NADP. After addition of substrate (500μM 4-nitrobenzaldehyde) to the assay mixture, aliquots were removed at 0.5, 1, 2, 3, 4, 5, 6 and 7 hours and the amount of 4-nitrobenzoic acid formed was estimated by HPLC. Results are expressed as the mean of duplicate measurements. Assays were carried out as described in Material and Methods.

Scheme I. Kinetic mechanism of aldehyde dismutation proposed for Kv*β*2 at pH 7.5. RCHO is an aldehyde, $RCH(OH)_2$ is its corresponding hydrate, RCOOH and RCH_2OH are the corresponding alcohol and carboxylic acid respectively. The dismutation of aldehyde substrate consists of two coupled half reactions. In the first half (the upper pathway), hydrated aldehyde is oxidised irreversibly to the corresponding carboxylic acid forming E_{NADPH}. In the second half reaction (lower pathway), another molecule free aldehyde binds to the E_{NADPH} complex and is reduced reversibly to corrosponding alcohol. Hence, aldehyde is dismutated into equimolar concentration of corresponding alcohol and carboxylic acid in a redox silent reaction with no observable change in A_{340}. Ψ denotes the cofactor exchange step. The steps denoted by Ψ are insignificant during dismutation as cofactor remains enzyme bound throughout alternate oxidation and reduction.

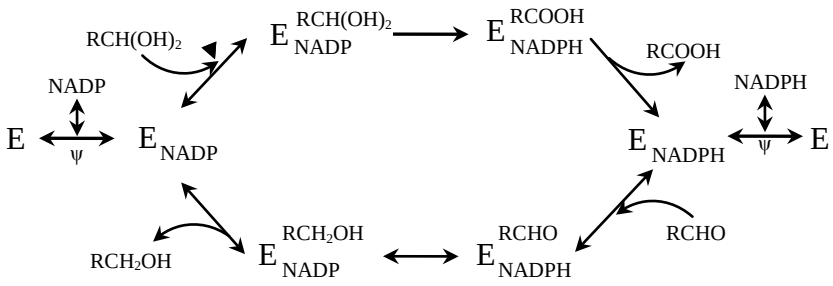


Table 1. Comparison of the Rate constants (min^{-1}) for the single turnover hydride transfer reaction (first order) for Kvβ2 substrates.

Group	Substrate	Concentration of substrate (mM)	Rate constant (min^{-1})	Relative rate constant (%)
Aromatic aldehydes	4-Nitrobenzaldehyde	0.5	0.220	100*
	3-Nitrobenzaldehyde	0.5	0.116	53
	2-Nitrobenzaldehyde	0.5	0.048	22
	Pyridine 2 aldehyde	0.5	0.011	5
	Benzaldehyde	0.5	0.017	8
	4-Hydroxybenzaldehyde	0.5	0.044	20
	2-Cyanobenzaldehyde	0.5	0.007	3
	4-Cyanobenzaldehyde	0.5	0.014	6
	4-Chlorobenzaldehyde	0.5	-	-
	4-Fluorbenzaldehyde	0.5	-	-
	Tolualdehyde	0.5	-	-
	Anisaldehyde	0.5	-	-
Aliphatic aldehyde	Formaldehyde	10	-	-
	2-Methlybutyraldehyde	10	0.035	16
	Butanal	10	0.011	5
	Crotanaldehyde	10	0.004	2
	Valeraldehyde	10	0.002	1
	Hexanal	0.5	0.001	1
	Octanal	0.5	-	-
	Succinic Semialdehyde	20	0.012	5
	Cinnamaldehyde		-	-
Ketones	Isatin	0.5	-	-
	4-Nitroacetophenone	0.5	0.004	2
Dicarbonyls	Methlyglyoxal	10	0.015	7
	Phenylglyoxal	0.5	0.005	3

* The single turnover hydride transfer rate for 4-nitrobenzaldehyde was 0.22 min^{-1}. Enzyme activity was measured as described in Material and Methods. Activities are expressed as a percentage of 4-nitrobenzaldehyde reductase activity. All estimates of rate constants are the mean of triplicate measurements.

Figure 1

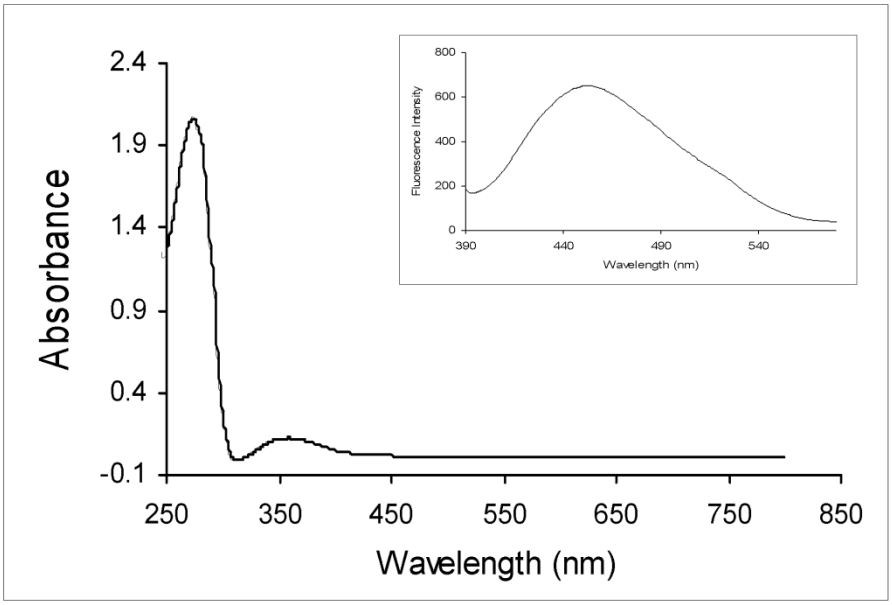
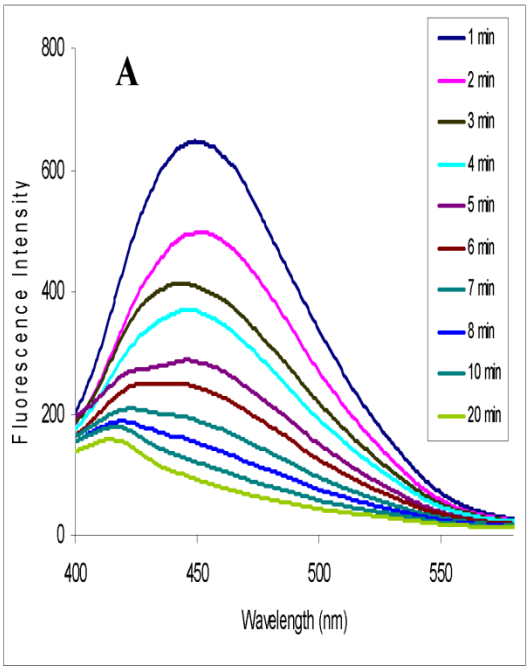


Figure 2



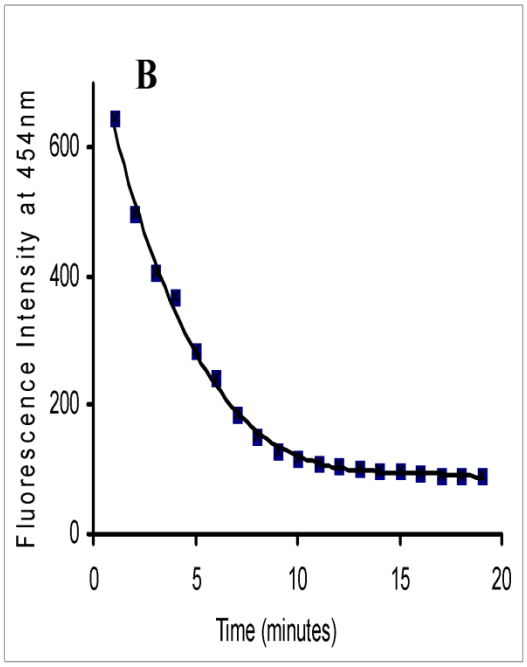


Figure 3

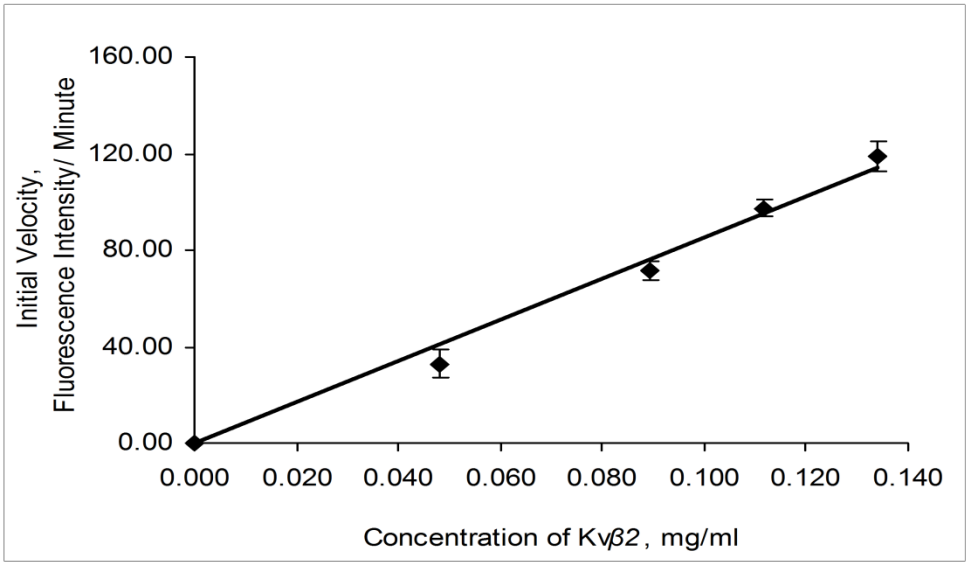


Figure 4

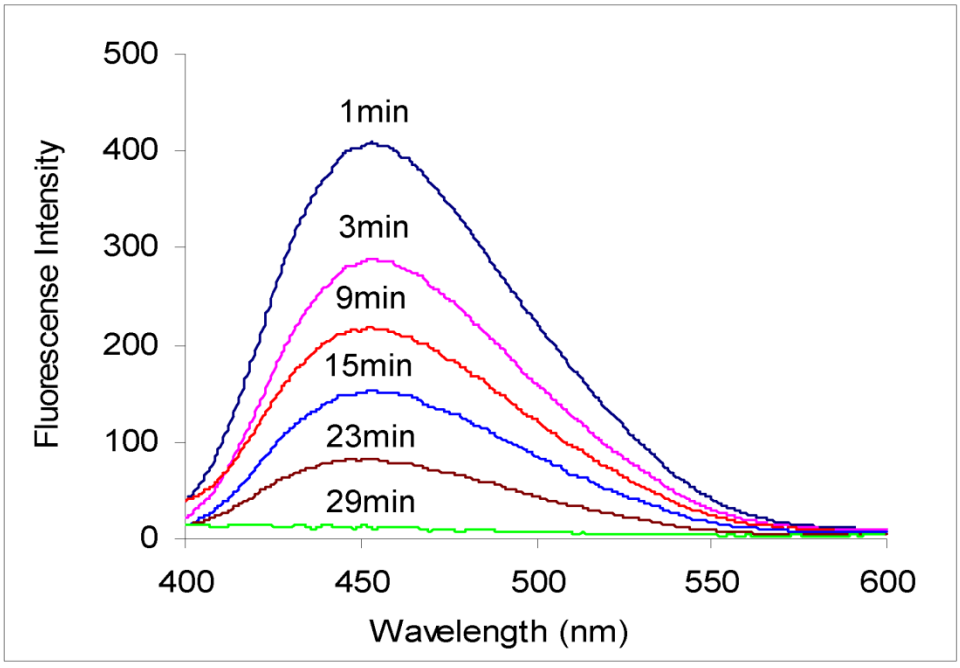


Figure 5

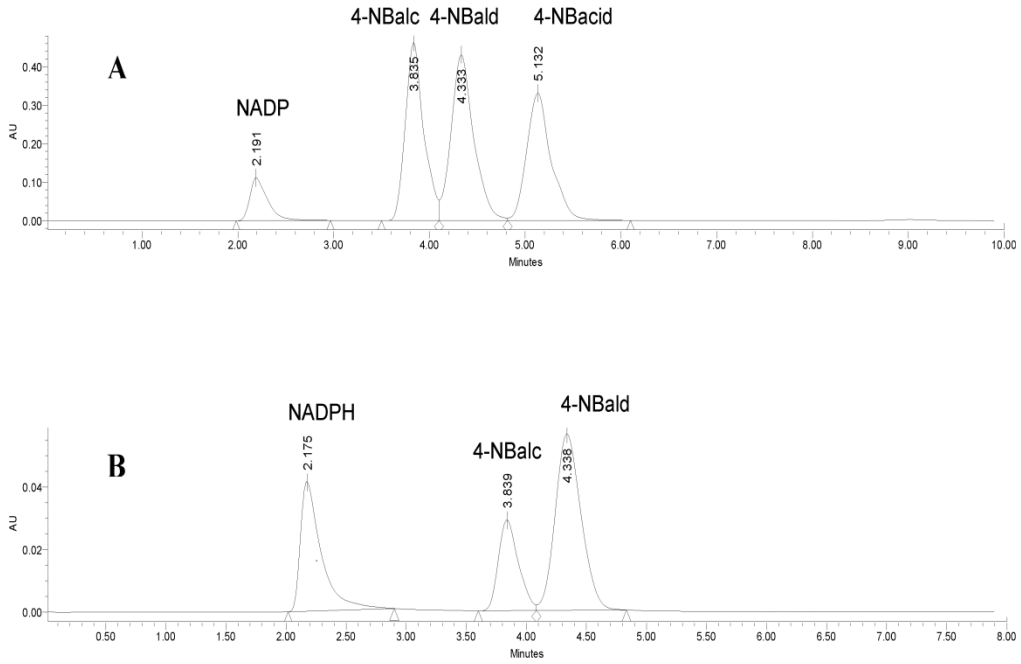


Figure 6

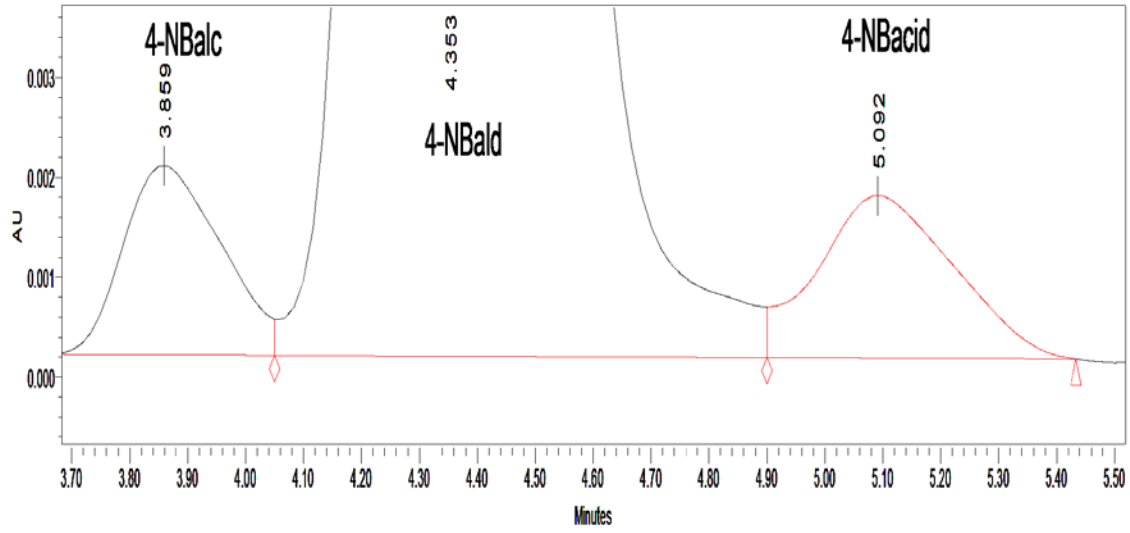
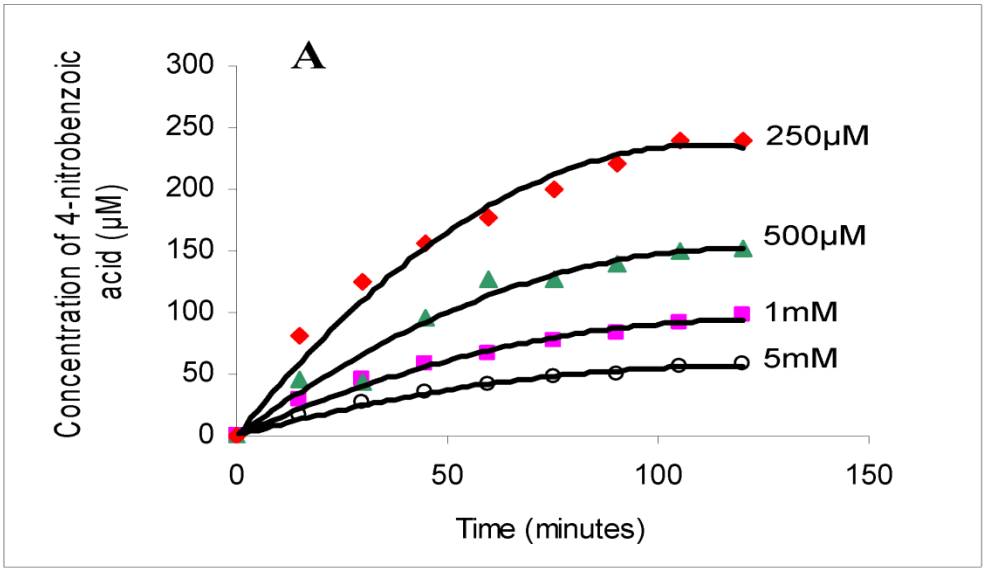


Figure 7


A
Concentration of 4-nitrobenzoic acid (μM)
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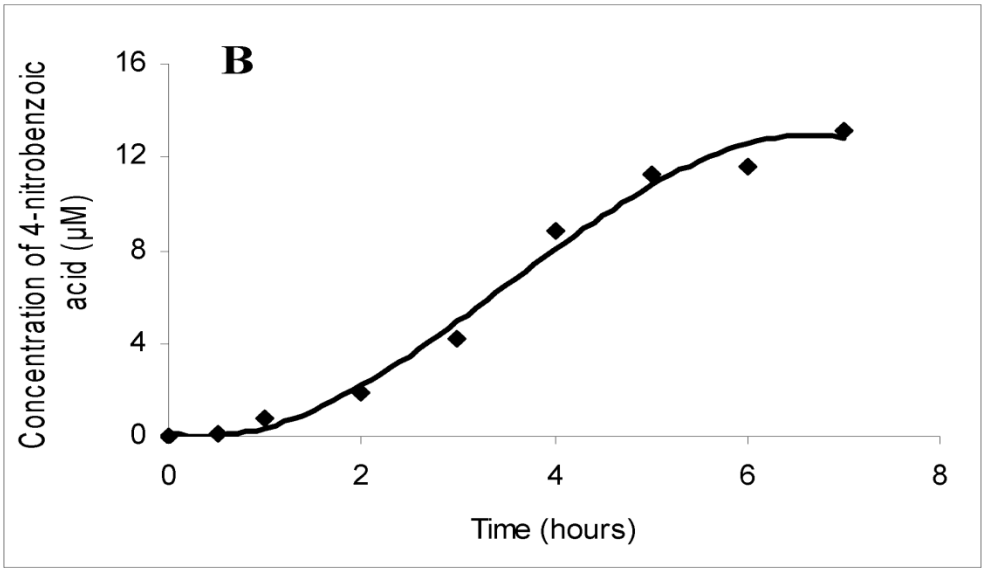
200
150
100
50
0
0
50
100
150
Time (minutes)
250μM
500μM
1mM
5mM


B
Concentration of 4-nitrobenzoic acid (μM)
16
12
8
4
0
0
2
4
6
8
Time (hours)