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**The catalytic mechanism of NADH-dependent reduction of 9,10-phenanthrenequinone by *Candida tenuis* xylose reductase reveals plasticity in an aldo-keto reductase active site**

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**Running Title:** Mechanism of quinone reduction by AKR2B5.

**Keywords:** aldo-keto reductase; AKR2B5; catalytic mechanism; active-site plasticity; kinetic isotope effect, transient state kinetics

**Abbreviations:** AKR, aldo-keto reductase; AKR1C9, rat liver  $\alpha$ -hydroxysteroid dehydrogenase; AKR2B5, xylose reductase from *Candida tenuis*; Y51A, mutated AKR2B5 in which Tyr<sup>51</sup> was substituted by Ala; K80A, mutated AKR2B5 in which Lys<sup>80</sup> was substituted by Ala; H113A; mutated AKR2B5 in which His<sup>113</sup> was substituted by Ala; PQ, 9,10-phenanthrenequinone; KIE, kinetic isotope effect; s-KIE, solvent KIE; NADD, 4R-[H<sup>2</sup>]-NADH

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## SYNOPSIS

Despite their widely varying physiological functions in carbonyl metabolism, *Candida tenuis* xylose reductase (AKR2B5) and many related enzymes of the aldo-keto reductase protein superfamily utilise 9,10 phenanthrenequinone (PQ) as a common *in vitro* substrate for NAD(P)H-dependent reduction. The catalytic roles of the conserved active-site residues (Tyr<sup>51</sup>, Lys<sup>80</sup>, His<sup>113</sup>) of AKR2B5 in the conversion of the reactive  $\alpha$ -dicarbonyl moiety of PQ are not well understood. Using wild-type and mutated (Tyr<sup>51</sup>, Lys<sup>80</sup> and His<sup>113</sup> individually replaced by Ala) forms of AKR2B5, we have conducted steady-state and transient kinetic studies of the effects of varied pH and deuterium isotopic substitutions in coenzyme and solvent on the enzymatic rates of PQ reduction. Each mutation caused a  $10^3 - 10^4$ -fold decrease in the rate constant for hydride transfer from NADH to PQ whose value in the wild-type enzyme was determined as  $\sim 8 \times 10^2 \text{ s}^{-1}$ . The presented data support an enzymic mechanism in which a catalytic proton bridge from the protonated side chain of Lys<sup>80</sup> ( $pK = 8.6 \pm 0.1$ ) to the carbonyl group adjacent to the hydride acceptor carbonyl facilitates the chemical reaction step. His<sup>113</sup> contributes to positioning of the PQ substrate for catalysis. Contrasting its role as catalytic general acid for conversion of the physiological substrate xylose, Tyr<sup>51</sup> controls release of the hydroquinone product. The proposed chemistry of AKR2B5 action involves delivery of both hydrogens required for reduction of the  $\alpha$ -dicarbonyl substrate to the carbonyl group undergoing (stereoselective) transformation. Hydride transfer from NADH probably precedes the transfer of a proton from Tyr<sup>51</sup> whose  $pK$  of  $7.3 \pm 0.3$  in the NAD<sup>+</sup>-bound enzyme appears suitable for protonation of a hydroquinone anion ( $pK$  8.8). These results show that the mechanism of AKR2B5 is unusually plastic in the exploitation of the active-site residues for catalysis to carbonyl group reduction in  $\alpha$ -dicarbonyls differs from that utilised in the conversion of xylose.

## INTRODUCTION

Aldo-keto reductases (AKR) are NAD(P)H-dependent oxidoreductases that catalyse the conversion of carbonyl groups to alcohols on a great variety of substrates including sugars, steroids, prostaglandins as well as compounds of biogenic and xenobiotic origin [1-4]. In addition to their function in energy-related and biosynthetic metabolic pathways, transformations promoted by AKRs are central to steroid hormone regulation and other forms of signal transduction, and they also contribute to detoxification [4-7]. In spite of their different roles in physiology, many AKRs share the ability to utilise  $\alpha$ -dicarbonyl compounds, especially o-quinones, as highly active substrates for NAD(P)H-dependent reduction *in vitro* [2, 8-11]. The catalytic efficiency for conversion of the  $\alpha$ -dicarbonyl compound often exceeds that for the reaction with the physiological substrate. It was shown that  $\alpha$ -dicarbonyl reduction by rat liver  $\alpha$ -hydroxysteroid dehydrogenase (AKR1C9) relies on a catalytic mechanism different from that used by this enzyme in the conversion of single-carbonyl compounds [8]. The results for AKR1C9 raise the interesting, yet unanswered question of whether it is common among the AKRs that the substrate structure (single carbonyl vs.  $\alpha$ -dicarbonyl) determines the participation of active-site residues in catalysis.

Xylose reductase from the yeast *Candida tenuis* (AKR2B5) is a well characterised member of the AKR protein superfamily [12-16]. Like other AKRs for which the crystal structure is known, AKR2B5 adopts the canonical  $(\beta/\alpha)_8$  barrel fold and displays a highly conserved active-site architecture, where a tetrad of residues (Tyr<sup>51</sup>, Lys<sup>80</sup>, His<sup>113</sup>, and Asp<sup>46</sup>) are situated at the base of the substrate binding pocket [17]. Mutational analysis of the catalytic residues of AKR2B5 suggested a mechanism for reduction of xylose by NAD(P)H (Supplementary Scheme 1) [18-21]. In this mechanism, Tyr<sup>51</sup> functions as the catalytic proton donor-acceptor for interconversion of xylose and xylitol [21]. The  $pK$  of its phenolic side chain changes during the reaction from a value of  $9.2 \pm 0.1$  in enzyme-NADH complex to a lower value of  $7.3 \pm 0.3$  in the enzyme-NAD<sup>+</sup> complex. Lys<sup>80</sup> and His<sup>113</sup> facilitate in different

ways the hydride transfer from and to the 4-pro-*R* position on the nicotinamide ring. The protonated side chain of Lys<sup>80</sup> (held in place through the salt-link interaction with Asp<sup>46</sup>) was proposed to provide auxiliary electrostatic stabilisation to the catalytic step [20]. This stabilisation results because approximation of the uncharged reactive groups on substrate, coenzyme, and Tyr<sup>51</sup> are favoured in the preorganised polar environment of the AKR2B5 active site. His<sup>113</sup>, by contrast, is thought to interact directly with the carbonyl group undergoing reduction [19]. A hydrogen bond from Nε2 of the singly protonated His side chain to xylose could contribute to catalysis by optimising C=O bond charge separation and positioning effects.

Penning and coworkers used mutational analysis of AKR1C9 to examine the catalytic mechanism of reduction of 9,10-phenanthrenequinone [8], a highly reactive and common substrate for AKRs [2, 9, 11, 22, 23]. They found that Tyr<sup>55</sup>, the conserved catalytic Brønsted acid of AKR1C9 which was essentially required in the reduction of physiological steroid substrates [24], could be replaced by Phe or Ser without affecting the enzyme activity on PQ dramatically (< 10-fold) [8]. A mechanism was proposed (Supplementary Scheme 2A) in which hydride transfer from NADPH does not rely on catalytic assistance from a general acid and occurs as result of an entropic effect, facilitating the approximation of reactants at the active site of the enzyme. Adjacent carbonyls of PQ serve as hydride ion and proton acceptors during chemical reduction, and bulk water provides the proton. It was also postulated that Lys<sup>84</sup> (the positional homologue of Lys<sup>80</sup> in AKR2B5) has a critical role in positioning the PQ substrate for catalytic reduction by NADPH [8]. Tyr<sup>55</sup> and His<sup>117</sup> from the AKR1C9 active site were thought to function as relevant structural components of the binding pocket.

We have examined here the catalytic mechanism of PQ reduction by AKR2B5. Considering the prior work on AKR1C9 [8], we were particularly interested in delineating roles of the active-site residues of AKR2B5 during conversion of the reactive  $\alpha$ -dicarbonyl group of the substrate. The results reveal that the catalytic strategies utilised by AKR2B5 to

achieve rate enhancement to NADH-dependent reduction of xylose and PQ are different. They also show that there is a marked distinction in how AKR2B5 and AKR1C9 [8] exploit their respective Tyr/Lys/His triad in the conversion of PQ.

## EXPERIMENTAL

A detailed description of the applied methods is available under Supplementary Information.

### Materials and enzymes

Unless indicated otherwise, all materials used were described in previous papers [21, 25]. Wild-type and mutated forms of AKR2B5 were obtained as N-terminally His-tagged proteins using reported methods [21].

### Steady-state kinetic analysis

Previously reported procedures were used to determine steady-state kinetic parameters ( $k_{\text{cat}}$ ,  $k_{\text{cat}}/K_m$ ) for NADH-dependent reduction of single and  $\alpha$ -dicarbonyl substrates [21, 25]. Primary and solvent deuterium kinetic isotope effects on  $k_{\text{cat}}$  and  $k_{\text{cat}}/K_m$  were obtained using published protocols [15] (see Supplementary Methods). The pH dependencies of kinetic parameters for PQ reduction were determined in the pH range 7.0 – 9.5 using reported methods [21]. Substrate solutions were prepared in potassium phosphate (pH 7.0 – 8.0) or Tris-HCl buffer (pH 8.0 – 9.5) and adjusted to a constant ionic strength of  $I = 0.02$  M [26]. Functional complementation of K80A by external primary amines was assayed essentially as described elsewhere [20] using PQ at a constant and saturating concentration of 250  $\mu\text{M}$ .

### Transient kinetic analysis

Rapid-mixing stopped-flow experiments were performed at  $25 \pm 0.1$  °C as described elsewhere [14]. All reactions were started by mixing a buffered PQ solution with an equal volume of a buffered enzyme solution, containing NADH or 4R-[<sup>2</sup>H]-NADH (NADD; fraction of deuterium  $\geq 99\%$ ). Unless stated otherwise, a 50 mM potassium phosphate buffer

(pH 7.0) was used. Oxidation of the coenzyme was measured from the decrease in absorbance at 340 nm. Absorbance traces were recorded in triplicate and averaged.

Kinetic measurements of proton uptake by the enzyme (wild-type AKR2B5, Y51A) during NADH-dependent reduction of PQ were carried out using the pH indicator Phenol Red whose absorbance at 556 was recorded. The response of the pH indicator was calibrated by titration with NaOH. Enzymes were gel-filtered twice to a reaction buffer containing 1.00 mM potassium phosphate (pH 7.0) and 8.0  $\mu$ M Phenol Red. The ionic strength of the buffer was adjusted with NaCl to that of a 50 mM potassium phosphate buffer.

## RESULTS

### Kinetic parameters for enzymatic reduction of PQ

Table 1 summarises kinetic parameters ( $k_{\text{cat}}$ ,  $k_{\text{cat}}/K_m$ ) for NADH-dependent PQ reduction catalysed by wild-type and mutated forms of AKR2B5. It also shows kinetic parameters for xylose reduction by these enzymes. Results obtained for His-tagged variants of AKR2B5 (Table 1) agree well with literature where xylose reduction was measured using untagged enzymes [12, 19, 20]. While in terms of  $k_{\text{cat}}/K_m$  individual site-directed substitutions of Lys<sup>80</sup> and His<sup>113</sup> by Ala affected reduction of PQ and xylose to a similar extent, kinetic consequences in Y51A were strongly dependent on the substrate used. The  $k_{\text{cat}}/K_m$  for xylose reduction by Y51A was decreased about 400,000-fold as compared to the corresponding efficiency of the wild-type enzyme. Using PQ, by contrast, the relevant decrease in  $k_{\text{cat}}/K_m$  for Y51A was only 70-fold.

A series of other  $\alpha$ -dicarbonyl compounds ( $\alpha$ -keto aldehydes,  $\alpha$ -keto esters) known to be converted by the wild-type enzyme were therefore examined as substrates of Y51A. Kinetic parameters for their reduction are shown in Supplementary Table 1. Compared to native AKR2B5, the loss in efficiency resulting from the substitution of Tyr<sup>51</sup> was relatively moderate ( $\leq$  800-fold) across the chosen compound series, clearly suggesting that reduction of

$\alpha$ -dicarbonyls by AKR2B5 depends much less on catalytic participation from the tyrosine than does the reduction of xylose.

We also determined that, as in the reference reaction catalysed by the wild-type enzyme [27], NADH-dependent conversion of ethyl-benzoyl formate (5 mM; see Supplementary Methods) by Y51A gave the *R*-configured  $\alpha$ -hydroxy ester, the enantiomeric excess in product being about 98 % (ratio *R*:*S* = 99.1:0.9). Note that alcohol enantiomeric purity serves to distinguish between two possible routes of proton transfer during reduction of the  $\alpha$ -dicarbonyl substrate. A mechanism in which adjacent carbonyls act as hydride ion and proton acceptors (see Supplementary Scheme 2B) will yield an endiol product. As the endiol has no center of chirality at the “ $\alpha$ -carbon”, tautomerisation at the enzyme active site or in solution will eventually lead to a racemic  $\alpha$ -hydroxy carbonyl. Therefore, the data support a scenario of ethyl-benzoyl formate reduction by wild-type and Tyr<sup>51</sup>→Ala mutated forms of AKR2B5 where proton transfer occurs directly to the hydride acceptor  $\alpha$ -carbonyl.

### pH-rate profiles for PQ reduction

Figure 1 shows pH profiles of  $\log(k_{\text{cat}}/K_m)$  (panel A) and  $\log k_{\text{cat}}$  (panel B) for PQ reduction by wild-type and mutated AKR2B5 enzymes. All pH profiles except those for K80A and the  $\log k_{\text{cat}}$  profile for the wild-type enzyme decreased with a slope of  $-1$  at high pH. Equation (1) was used to fit these pH dependencies where  $Y$  is  $k_{\text{cat}}$  or  $k_{\text{cat}}/K_{\text{PQ}}$ ,  $C$  is the pH-independent value of  $Y$ , and  $K_a$  is the macroscopic dissociation constant for the ionisable group being titrated.  $[\text{H}^+]$  is the proton concentration.

$$\log Y = \log[C/(1+(K_a/[\text{H}^+]))] \quad (\text{Eqn.1})$$

The pH profile for  $\log k_{\text{cat}}$  decreased from a constant value at low pH to a lower constant value at high pH (Figure 1B), and the data were fitted with Eqn. (2) where  $C_H$  and  $C_L$  is the constant value of  $Y$  at high and low pH, respectively.

$$\log Y = \log[(C_L+C_H K_a/[\text{H}^+])/(1+K_a/[\text{H}^+])] \quad (\text{Eqn.2})$$

Calculated  $pK_a$  values are summarised in Table 2.  $\log k_{\text{cat}}$  and  $\log(k_{\text{cat}}/K_m)$  for K80A did not show a pH dependence.

### Kinetic isotope effects

Primary deuterium KIEs on kinetic parameters for PQ reduction were determined in  $^1\text{H}_2\text{O}$  and  $^2\text{H}_2\text{O}$  solvent at pL 7.0 where L is  $^1\text{H}$  or  $^2\text{H}$ . KIEs resulting from deuteration of the solvent (s-KIEs) were obtained from a separate set of experiments at pL 7.0, comparing initial rates of NADH-dependent reduction of PQ in  $^1\text{H}_2\text{O}$  and  $^2\text{H}_2\text{O}$ . Results are summarised in Table 3. KIEs and s-KIEs for xylose reduction were also determined (Supplementary Table 2). There was no significant KIE on the catalytic efficiency for NADH ( $^Dk_{\text{cat}}/K_{\text{NADH}} = 1.0 \pm 0.1$ ) under conditions of a saturating concentration of PQ ( $50 \mu\text{M}$ ).

### Transient kinetic analysis

Multiple-turnover stopped-flow progress curves for PQ conversion by wild-type AKR2B5 displayed a pre-steady state burst of NADH consumption that accounted for  $\sim 60\%$  of the molar concentration of the catalyst present ( $17.3 \mu\text{M}$ ) and was followed by a linear steady-state phase of reaction (Supplementary Figure 1A). The kinetic transient for the reaction of Y51A likewise showed a burst of utilisation of NADH whose magnitude corresponded to the full molar enzyme equivalent (Supplementary Figure 1B). The time courses of PQ reduction by K80A and H113A were characterised by a linear disappearance of NADH absorbance (Supplementary Figure 1B). These results immediately suggest that the steady-state rate of reactions catalysed by wild-type enzyme and Y51 was limited by a slow step after the hydride transfer, likely dissociation of  $\text{NAD}^+$  (wild-type enzyme) and hydroquinone product (Y51A), as discussed later. For K80A and H113A, by contrast, rate limitation is located in the overall hydride transfer step. Supplementary Table 3 summarises transient ( $k_{\text{obs}}$ ) and steady-state ( $k_{\text{ss}}$ ) rate constants obtained from the stopped-flow data.

The pH dependencies of  $\log k_{\text{obs}}$  for wild-type AKR2B5 and Y51A are shown in Supplementary Figure 1C along with the pH profiles of  $\log k_{\text{ss}}$  for K80A. The observed

decrease of  $\log k_{\text{obs}}$  at high pH was fitted with Eqn. (1), and the results are presented in Table 2. The KIE on  $k_{\text{obs}}$  for the wild-type enzyme resulting from deuteration of coenzyme or solvent was  $\sim 1.8$  and  $1.4 \pm 0.2$ , respectively.

Figure 2 compares time courses of oxidation of NADH and uptake of protons during conversion of PQ by wild-type AKR2B5 and Y51A. Unfortunately, K80A precipitated under the low-buffer conditions required for proton uptake measurements, precluding kinetic analysis of proton transfer steps for this mutant. The results confirm that enzymatic conversion of PQ involved the uptake of 1 mol proton per mol of  $\text{NAD}^+$  formed in the steady state. In the transient kinetic phase, however, *only* 0.3 mol proton was taken up per each mol NADH oxidised by the wild-type enzyme. For Y51A, by contrast, oxidation of NADH in the pre-steady state was accompanied by the uptake of an *equal amount* of protons (see Supplementary Table 3). Transient rate constants for proton uptake ( $k_{\text{p,obs}}$ ; see Supplementary Methods) were  $44 (\pm 17) \text{ s}^{-1}$  and  $0.35 (\pm 0.03) \text{ s}^{-1}$  for the wild-type enzyme and Y51A, respectively. The corresponding values of  $k_{\text{obs}}$  measuring the overall hydride transfer from NADH were  $102 (\pm 14) \text{ s}^{-1}$  and  $0.57 (\pm 0.04) \text{ s}^{-1}$ . Observation that  $k_{\text{p,obs}}$  was smaller than  $k_{\text{obs}}$  implies that proton transfer had to take place after the hydride transfer.

### Chemical rescue of K80A by primary amines

When offered in six concentrations between 20 and 500 mM, primary amines (methyl, ethyl, propargyl) could not enhance PQ reductase activity of K80A as compared to the relevant control. However, chemical rescue of xylose reductase activity in K80A occurred as expected from literature [20] (data not shown), indicating that the presence of the His-tag did not interfere with functional complementation of the mutated enzyme. These results suggest that Lys<sup>80</sup> accomplishes a different task in the catalytic reaction with PQ as compared to conversion xylose.

## DISCUSSION

### Kinetic analysis of PQ reduction by AKR2B5

Results of transient kinetic experiments and the pattern of steady-state KIEs suggest an ordered kinetic mechanism of PQ reduction by wild-type AKR2B5 where, as in the reaction with xylose [14], NADH binds to the enzyme before the carbonyl substrate binds and dissociation of the second product, likely  $\text{NAD}^+$ , is rate-limiting for  $k_{\text{cat}}$  (see Supplementary Discussion, Part 1). Assuming an intrinsic KIE of  $\sim 6.5$  on the hydride transfer step ( $^{\text{D}}k$ ) [28], we can use data for the wild-type enzyme ( $k_{\text{obs}} = 1.1 \times 10^2 \text{ s}^{-1}$ ;  $^{\text{D}}k_{\text{obs}} = 1.8$ ) and the relationship  $k_{\text{hydride}} = k_{\text{obs}} (^{\text{D}}k - 1) / (^{\text{D}}k_{\text{obs}} - 1)$  to obtain an estimate of  $7.6 \times 10^2 \text{ s}^{-1}$  for the rate constant of hydride transfer from NADH to PQ ( $k_{\text{hydride}}$ ) catalysed by native AKR2B5. Interestingly,  $k_{\text{hydride}}$  for reduction of PQ was up to 10 times larger than the corresponding  $k_{\text{hydride}}$  for reduction of single-carbonyl aldehyde substrates [14, 15], perhaps reflecting the relatively high chemical reactivity of the quinone substrate towards free [29] and enzyme-bound hydride donor molecules [8]. The rate constant ( $k_{\text{uncat}}$ ) for uncatalysed reduction of PQ (50  $\mu\text{M}$ ) by NADH (280  $\mu\text{M}$ ) at 25 °C and pH 7.0 was determined as  $5 \times 10^{-4} \text{ s}^{-1}$  (data not shown). The enzymatic rate enhancement ( $k_{\text{hydride}}/k_{\text{uncat}}$ ) to PQ reduction by wild-type AKR2B5 was therefore estimated as  $1.5 \times 10^6$ -fold ( $= 763 \times 10^4 / 5$ ). Individual site-directed replacements of Tyr<sup>51</sup>, Lys<sup>80</sup> and His<sup>113</sup> by Ala caused a marked decrease in  $k_{\text{hydride}}$  (K80A:  $1.1 \times 10^{-1} \text{ s}^{-1}$ ; H113A:  $1.3 \times 10^{-1} \text{ s}^{-1}$ ; Y51A:  $7.9 \times 10^{-1} \text{ s}^{-1}$ ; see Supplementary Table 3) and a corresponding loss in rate enhancement. Therefore, the active site residues of AKR2B5 are clearly important for efficient hydride transfer reduction of PQ, Lys<sup>80</sup> and His<sup>113</sup> more so than Tyr<sup>51</sup>.

### Lys<sup>80</sup> determines the pH dependence of PQ reduction by AKR2B5

An apparent single group in the enzyme-NADH complex ( $\text{p}K = 8.8 \pm 0.1$ ;  $\log(k_{\text{cat}}/K_{\text{m}})$  profile) and in the enzyme-NADH-PQ complex ( $\text{p}K = 8.6 \pm 0.1$ ;  $\log k_{\text{obs}}$  profile), respectively, had to be protonated for PQ reduction by wild-type AKR2B5. The pH profile of  $\log k_{\text{cat}}$  was affected by a different ionisation ( $\text{p}K = 7.7 \pm 0.2$ ) which was not further pursued here, considering that

the  $k_{\text{cat}}$  of the wild-type enzyme is controlled by the overall process of dissociation of  $\text{NAD}^+$ . Relevant pH-rate profiles for Y51A and H113A showed retention of the titratable group with  $\text{pK}$  8.2 – 8.5. The absence of a pH dependence for K80A therefore suggests that  $\text{Lys}^{80}$  is the protonated group required for the enzymatic reaction with PQ. Note that the pH dependence of xylose reduction by AKR2B5 is controlled by the ionisation of  $\text{Tyr}^{51}$  [21].

### **Lys<sup>80</sup> participates in a catalytic proton bridge that facilitates hydride transfer to PQ**

Sizeable s-KIEs on  $k_{\text{cat}}/K_{\text{m}}$  and  $k_{\text{obs}}$  for PQ reduction by wild-type AKR2B5 are interpreted in terms of a catalytic proton bridge that is formed at the ternary complex and becomes tightened in the transition state to facilitate the hydride transfer step (see Supplementary Discussion, Part 2). The inverse s-KIEs on  $k_{\text{cat}}/K_{\text{mPQ}}$  for the mutated enzymes (Supplementary Table 2) are consistent with a substantial weakening of this proton bridge as result of each site-directed substitution and most probably reflect an equilibrium solvent effect on the binding of NADH (see Supplementary Discussion, Part 2).  $^{D_2O}k_{\text{cat}}/K_{\text{m}}$  for xylose reduction is also inverse (Supplementary Table 2), clearly indicating that one or more steps in the sequence of steps from PQ binding up to product release are considerably more sensitive to solvent deuteration than are analogous reaction steps during the AKR2B5-catalysed conversion of xylose.

Running the reaction in  $^2\text{H}_2\text{O}$  as compared to  $\text{H}_2\text{O}$  (pL 7.0) caused enhancement of  $^Dk_{\text{cat}}/K_{\text{mPQ}}$  for wild-type AKR2B5, Y51A and H113A, implying that the isotope-sensitive step of hydride transfer was affected by solvent deuteration in these enzymes. Attenuation of  $^Dk_{\text{cat}}/K_{\text{mPQ}}$  for K80A resulting from the exchange  $\text{H}_2\text{O} \rightarrow ^2\text{H}_2\text{O}$  reveals that, as in the reaction of the wild-type enzyme with xylose ([14]; Supplementary Table 2), the solvent-sensitive step in the catalytic mechanism of the mutant was not coincident with hydride transfer. With the assumption that the s-KIE on  $k_{\text{cat}}/K_{\text{mPQ}}$  does not report on the chemical proton-transfer step as discussed later, selective removal of the solvent dependence of the hydride transfer step in K80A provides good evidence in favour of the suggestion that  $\text{Lys}^{80}$  is the group responsible for formation of the proposed catalytic proton bridge.

### Proton transfer steps during reduction of PQ

Measurement of the relative amount of protons taken up in the kinetic transient of PQ conversion by wild-type AKR2B5 and Y51A reveals that the pH-dependent ionisation of the group responsible for proton transfer is sensitive to substitution of Tyr<sup>51</sup> by Ala. Because the  $pK_a$  of Lys<sup>80</sup> in the enzyme-NADH- and in the enzyme-NAD-PQ-complex was not perturbed in Y51A from where it was in wild-type AKR2B5, this result strongly suggests that a group different from Lys<sup>80</sup> serves a role in (catalytic) proton transfer.

Considering the  $pK_a$  of 8.8 for phenantrene 9,10 hydroquinone in solution [30], the phenolic OH of Tyr<sup>51</sup> is sufficiently acidic in NAD<sup>+</sup>-bound enzyme ( $pK_a \approx 7.3$  [21]) to function as proton donor for PQ reduction [31]. The overall process of enzymatic proton transfer at pH 7.0 would thus involve *partial* re-protonation of the Tyr<sup>51</sup> before or after dissociation of the hydroquinone, however, prior to the exchange of NAD<sup>+</sup> by NADH. In Y51A, proton uptake may be from bulk solvent and directly by the hydroquinone anion, hence it will be stoichiometric with the NADH consumed in the reaction. This course of proton transfer is consistent with the apparent irreversibility of hydride transfer by Y51A suggested by KIE data (see Supplementary Discussion, Part 3) and likewise concurs with a kinetic behaviour for this mutant where dissociation of the hydroquinone product is completely rate-limiting.

Retention of substantial level of PQ reductase activity in Y51A suggests that proton transfer from Tyr<sup>51</sup> does not make a large contribution to catalytic rate enhancement. This is in agreement with literature stating that hydride reduction of PQ proceeds in the absence of catalytic assistance from a Brønsted acid (see Supplementary Scheme 2A) [8, 29]. It would follow that during chemical reduction of PQ by AKR2B5 the hydride transfer probably proceeds ahead of the proton transfer, in agreement with measurement that  $k_{p,obs}$  is smaller than  $k_{obs}$ . Now, irrespective of the exact relative timing of the hydrogen transfer steps involved in PQ reduction that is not revealed by the evidence presented herein, protonation by Tyr<sup>51</sup>

should be a highly committed reaction step. Therefore, this implies that despite oxidation of NADH being kinetically faster than proton uptake from solution in the pre-steady state, a deuterium solvent effect on the overall proton transfer could be poorly expressed in the experimentally derived kinetic parameters (for precedent of a similar stepwise mechanism, see [32]).

### **The proposed catalytic mechanism of PQ reduction by AKR2B5 suggests active-site plasticity in this aldo-keto reductase**

NADH-dependent reduction of PQ by AKR2B5 proceeds by a catalytic mechanism different from that utilised for reduction of the physiological substrate xylose [19-21]. Scheme 1 summarises the proposed participation of Tyr<sup>51</sup>, Lys<sup>80</sup>, and His<sup>113</sup> in the catalytic steps of PQ conversion.

The protonated side chain of Lys<sup>80</sup> and the carbonyl group adjacent to the hydride acceptor carbonyl form a catalytic solvation proton bridge at the reactant state, and strengthening of this interaction occurs at the transition state. The observed s-KIEs on  $k_{\text{cat}}/K_{\text{mPQ}}$  and  $k_{\text{obs}}$  for the wild-type enzyme are interpreted in terms of this proton bridge, explaining the solvent sensitivity of the hydride transfer step revealed in multiple deuterium KIE measurements. Partial protonation of substrate by Lys<sup>80</sup> could stabilise a substrate enol (Scheme 1) in which non-favourable interactions of the neighbouring carbonyl dipoles are alleviated and hydride transfer to the reactive carbonyl is therefore facilitated. The proposed role for Lys<sup>80</sup> is probably more demanding with respect to the precise relative orientation of interacting groups than is the electrostatic stabilisation during xylose reduction (Supplementary Scheme 1), providing a tentative explanation for the absence of functional complementation of PQ reductase activity in K80A by external amines.

The catalytic function of His<sup>113</sup> seems similar to that utilised for conversion of xylose [19], except that it relies on a suitable “depolarisation” of the adjacent carbonyl by Lys<sup>80</sup>. In marked contrast to the key general acid catalytic role of Tyr<sup>51</sup> during reduction of xylose,

hydride transfer to PQ requires only auxiliary participation from the Tyr. Proton transfer from Tyr<sup>51</sup> to the (partial) hydroquinone anion completes the reduction.

Summarising, the AKR2B5-catalysed conversion of xylose and PQ represents an uncommon example where the enzymatic mechanism of carbonyl group reduction by NADH is plastic and the exploitation of active-site residues for catalysis is dictated by the structure of the substrate ( $\alpha$ -hydroxy-aldehyde vs.  $\alpha$ -dicarbonyl). Substrate-dependent usage of alternative catalytic mechanisms has to our knowledge limited precedence among dehydrogenases / reductases [9] and has only been described for selected other enzyme systems including hydrolases [33] and transferases [34]. The observed “mechanistic plasticity” for AKR2B5 must be distinguished clearly from “binding-site plasticity” that is common in broadly specific enzymes such as the xylose reductase. Considering the results of previous studies [8, 9], it would seem that the catalytic mechanism of  $\alpha$ -dicarbonyl reduction is not fully conserved among the AKRs. This is interesting and may stimulate further research because for some enzymes of the superfamily  $\alpha$ -dicarbonyl reduction may well be a reaction of physiological relevance [35-37]. The evidence for AKR2B5 is also relevant in light of different biotechnological applications of enzymatic reduction of  $\alpha$ -dicarbonyl substrates [25, 27, 38, 39].

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## FIGURE LEGENDS

**Figure 1.** pH profiles of  $\log(k_{\text{cat}}/K_m)$  (panel A) and  $\log k_{\text{cat}}$  (panel B) for NADH-dependent reduction of PQ by AKR2B5 wild-type (●), Y51A (■), H113A (▲) and K80A (▼). Lines indicate fits of Eqns. (1) or (2) to the data.

**Figure 2.** Stopped flow progress curves of  $\text{NAD}^+$  formation and proton uptake during NADH-dependent reduction of PQ by wild-type AKR2B5 and Y51A. Panel A and B display absorbance traces of proton release (I) and  $\text{NAD}^+$  formation (II) under conditions where the concentrations of wild-type AKR2B5 and Y51A were 26  $\mu\text{M}$  (panel A) and 19.5  $\mu\text{M}$  (panel B), respectively.  $[\text{PQ}]$  (= 150  $\mu\text{M}$ ) and  $[\text{NADH}]$  (= 300  $\mu\text{M}$ ) were constant and saturating. Lines in grey correspond to fits of Eqns (5) and (6) to experimental data. Areas in grey depict the standard deviations, calculated from the average of 9 time courses recorded at 340 nm and 556 nm, respectively.

**Scheme 1.** Proposed mechanism of NADH-dependent reduction of dicarbonyl compounds catalysed by AKR2B5. Charge delocalisation facilitated by Lys<sup>80</sup> is shown for enzymatic reductions of PQ and ethyl benzoylformate.

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## Tables

**Table 1. Kinetic parameters for NADH-dependent reduction of xylose and PQ catalysed by wild-type AKR2B5, Y51A, H113A and K80A at 25°C and pH 7.0**

<sup>a</sup> values are from ref [21]. n.a., not applicable; standard errors on all parameters were  $\leq 15\%$ .

Substrate	Wild-type		Y51A		H113A		K80A	
	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$k_{\text{cat}}/K_{\text{m}}$ ( $\text{M}^{-1}\text{s}^{-1}$ )						
xylose	10 <sup>a</sup>	150 <sup>a</sup>	n.a.	4 × 10 <sup>-4 a</sup>	0.02	0.2	0.002	9 × 10 <sup>-4</sup>
PQ	12	2.3 × 10 <sup>6</sup>	0.2	3.3 × 10 <sup>4</sup>	0.043	2 × 10 <sup>4</sup>	0.027	500

**Table 2.  $pK_a$  values from pH profiles for steady state and transient kinetic parameters of PQ reduction catalysed by wild-type and mutated forms of AKR2B5.** <sup>a</sup>pH independent values for the indicated kinetic constants.  $k_{cat}$  and  $k_{obs}$  values are in  $s^{-1}$ ,  $k_{cat}/K_m$  values are in  $M^{-1}s^{-1}$ . <sup>b</sup>pH independent value at high pH; <sup>c</sup>pH independent value at low pH. n.d., not determined

Enzyme	$pK_a$ [ $\log k_{cat}/K_m$ ]	$C^a$	$pK_a$ [ $\log k_{cat}$ ]	$C^a$	$pK_a$ [ $\log k_{obs}$ ]	$C^a$
Wild-type	$8.8 \pm 0.1$	$2 \times 10^6 \pm 2 \times 10^5$	$7.7 \pm 0.2$	$4 \pm 1^b / 10 \pm 1^c$	$8.6 \pm 0.1$	$7.5 \pm 1$
Y51A	$8.2 \pm 0.1$	$14500 \pm 1740$	$8.5 \pm 0.2$	$0.08 \pm 0.01$	$8.5 \pm 0.1$	$0.14 \pm 0.02$
H113A	$8.4 \pm 0.1$	$12600 \pm 1638$	$8.5 \pm 0.1$	$0.13 \pm 0.02$	n.d.	n.d.
K80A	eliminated	-	eliminated	-	eliminated	-

**Table 3. KIEs and s-KIEs on kinetic parameters for NADH-dependent reduction of PQ catalysed by wild-type and mutated forms of AKR2B5.**

<sup>a</sup> superscript DD2O indicates KIEs determined in D<sub>2</sub>O solvent.

Enzyme	<sup>D</sup> $k_{cat}/K_m$	<sup>D</sup> $k_{cat}$	<sup>D2O</sup> $k_{cat}/K_m$	<sup>DD2O</sup> $k_{cat}/K_m$ <sup>a</sup>
wild-type	1.6 ± 0.1	1.0 ± 0.1	2.82 ± 0.04	2.4 ± 0.1
Y51A	2.2 ± 0.2	2.4 ± 0.2	0.88 ± 0.01	2.8 ± 0.1
H113A	3.0 ± 0.2	2.8 ± 0.1	0.9 ± 0.1	5.0 ± 0.2
K80A	2.1 ± 0.2	2.3 ± 0.1	0.85 ± 0.03	1.3 ± 0.1

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## Figures and Schemes

Figure 1

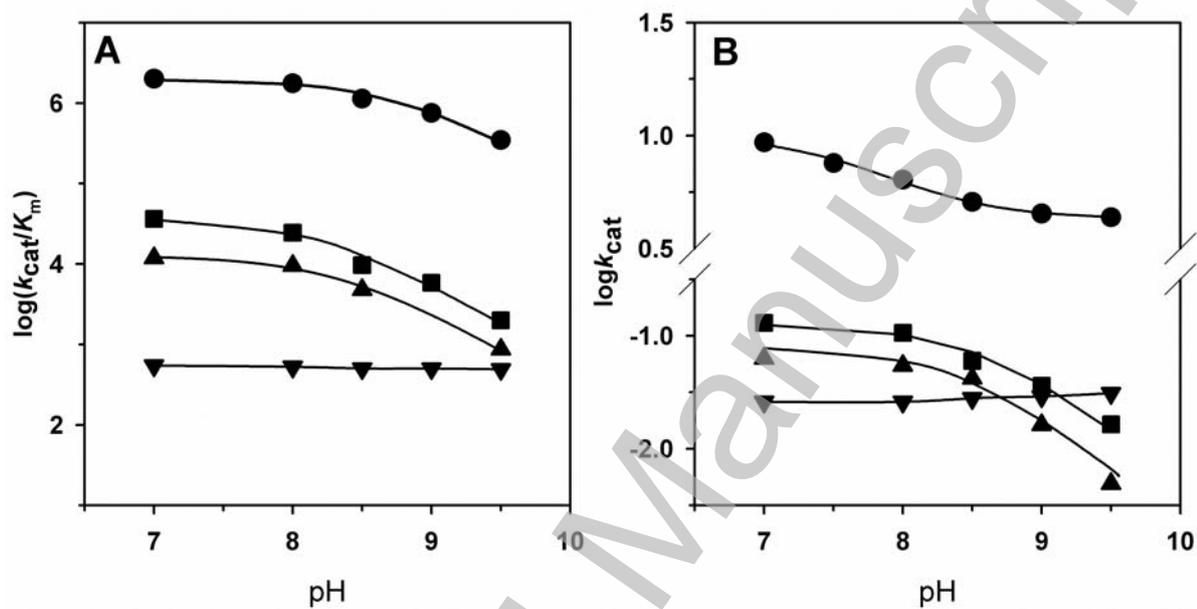
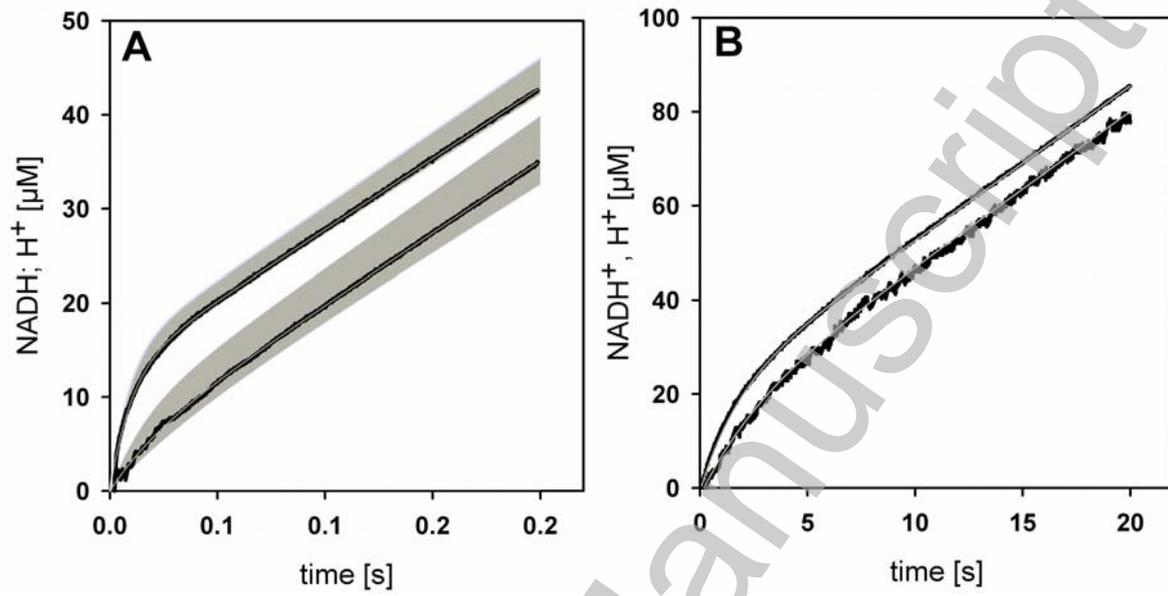
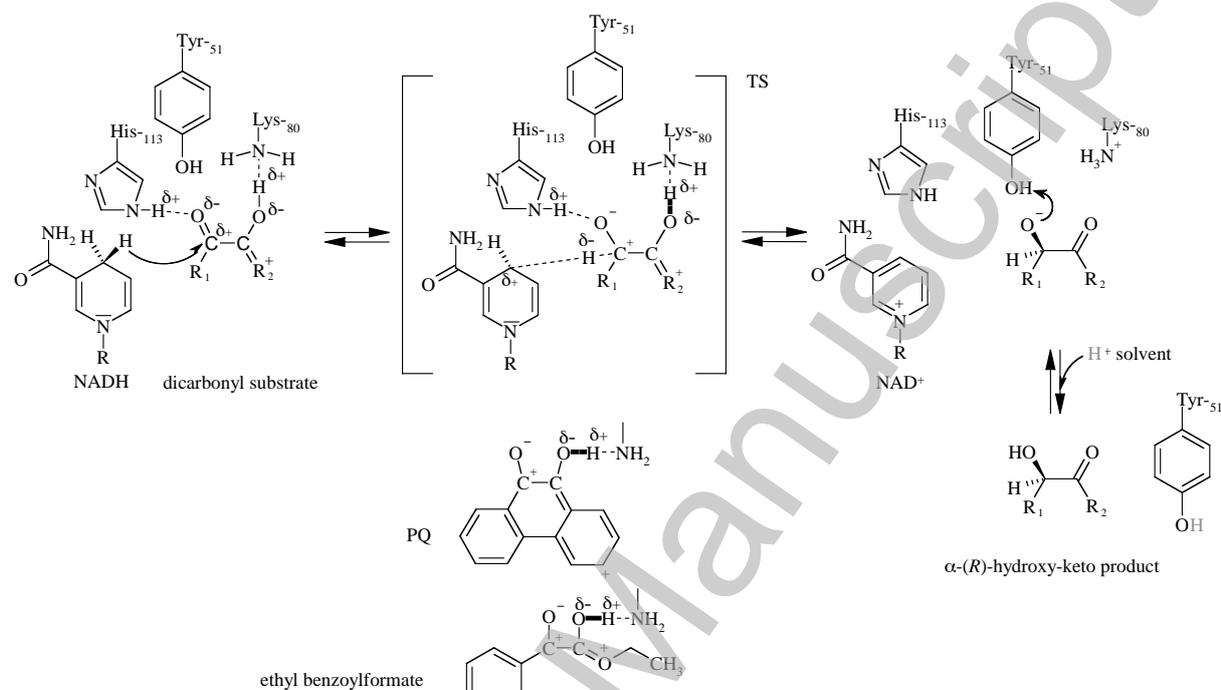


Figure 2



## Scheme 1



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