Fine tuning of coenzyme specificity in family 2 aldo-keto reductases revealed by crystal structures of the Lys-274 → Arg mutant of *Candida tenuis* xylose reductase (AKR2B5) bound to NAD^+ and $NADP^+$

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Abstract **Aldo-keto reductases of family 2 employ single site replacement Lys → Arg to switch their cosubstrate preference from NADPH to NADH. X-ray crystal structures of Lys-274 → Arg mutant of *Candida tenuis* xylose reductase (AKR2B5) bound to NAD^+ and $NADP^+$ were determined at a resolution of 2.4 and 2.3 Å, respectively. Due to steric conflicts in the $NADP^+$-bound form, the arginine side chain must rotate away from the position of the original lysine side chain, thereby disrupting a network of direct and water-mediated interactions between Glu-227, Lys-274 and the cofactor 2′-phosphate and 3′-hydroxy groups. Because anchoring contacts of its Glu-227 are lost, the coenzyme-enfolding loop that becomes ordered upon binding of $NAD(P)^+$ in the wild-type remains partly disordered in the $NADP^+$-bound mutant. The results delineate a catalytic reaction profile for the mutant in comparison to wild-type.**

Keywords: Dual coenzyme specificity; Selectivity; Loop ordering; Xylose fermentation

1. Introduction

There is a fundamental distinction between NADH and NADPH in metabolic pathways. NADH is the reductant in catabolic reactions, whereas NADPH generally provides reducing power for biosynthesis. Therefore, most nicotinamide cofactor-dependent enzymes are specific for either NADH or NADPH. Xylose reductase (XR) from the yeast *Candida tenuis* is a dual specific NADPH- and NADH-dependent enzyme that converts D-xylose into xylitol [1]. The xylitol is oxidized to yield D-xylulose which in turn is phosphorylated and enters the pentose phosphate pathway (PPP) as D-xylulose 5-phosphate. The PPP is a main source of NADPH and supplies intermediates for energy production and biosynthesis [2]. The co-substrate selectivity of the XR probably reflects the capacity of the PPP to regenerate the NADPH consumed by XR and the extent to which carbon from xylose partitions between catabolism and anabolism in *C. tenuis* physiology.

Based on sequence homology, *C. tenuis* XR (CtXR) has been classified into family 2 of the aldo-keto reductase (AKR) superfamily of proteins and enzymes and is systematically identified as AKR2B5 [3]. Family 2 is unusual among the 14 AKR families currently recognized because it includes dual specific NADPH/NADH-dependent reductases as well as NADPH-specific enzymes. Crystal structures of wild-type CtXR bound to $NADP^+$ and NAD^+ show that a conformational change of the side chain of Glu-227, to make strong interactions with the 2′-OH and 3′-OH groups when the cofactor 2′-phosphate group is lacking, is most critical for utilization of NADH [4,5]. Glu-227 of CtXR is widely conserved among the dual specific AKRs of family 2.

The side chain of Lys-274 forms a strong hydrogen bond with the 2′-phosphate group and participates in a network of water-mediated contacts with Glu-227 and the cofactor 3′-hydroxyl group. In the NAD^+-bound conformation, the side chain of Lys-274 rotates away slightly and is re-directed to hydrogen bond with solvent molecules. Recent results of mutational studies [6] corroborate the structure-derived notion that ≈20-fold tighter binding of NADPH than NADH results mainly from the interactions of Lys-274, which are specific to the NADP complex. Lys-274 is conserved in all members of family 2 except for XR from *Candida parapsilosis* which shows a Lys → Arg replacement and is the only natural XR known to prefer NADH [7]. A K274R mutant of CtXR utilizes NADH and NADPH with the same specificity, whereas the wild-type prefers NADPH about 33-fold [6]. To provide a molecular basis of how coenzyme selectivity in family 2 AKRs is adjusted by the substitution of Lys-274 with Arg, we determined the X-ray crystal structures of the K274R mutant of CtXR bound to $NADP^+$ and NAD^+.

2. Materials and methods

2.1. Protein preparation and crystallization

All chemicals used were reported previously [6]. The purified K274R mutant [6] was crystallized by using the hanging drop vapor diffusion method at 25 °C and a drop consisting of 1 μl well solution and 1 μl protein solution (15 mg/ml) with 2.5 mM cofactor added. Crystals of the NAD^+-bound holoenzyme were obtained using a well solution of 2.0 M ammonium sulfate and 100 mM sodium citrate, pH 6.2, in the presence of $NADP^+$ the well solution contained additional 160 mM

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Abbreviations: AKR, aldo-keto reductase; XR, xylose reductase; CpXR; *Candida parapsilosis* XR; CtXR, *Candida tenuis* XR; PPP, pentose phosphate pathway; r.m.s., root mean square

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sodium acetate. Crystals of the NAD^+- and $NADP^+$-bound enzymes took 2 and 4 months to grow, respectively, and had final dimensions of approximately $0.5 \times 0.1 \times 0.1$ mm.

2.2. Data collection, structure determination and refinement

The crystals were flash-cooled in a 100 K nitrogen stream in a buffer containing 80% (v/v) of the well solution, 5 mM coenzyme and 20% (v/v) glycerol as cryo-protectant. Data were collected at the Stanford Synchrotron Radiation Laboratory beamline 9-1 on a MAR Research 345 detector and were integrated and reduced using the programs DENZO and SCALEPACK [8]. The data collection statistics along with the respective space group and lattice constants are presented in Table 1. Since the mutant enzyme unit cells were isomorphous with the wild-type, rigid body refinement using the program CNS [9] commenced with the respective native structures stripped of $NADP^+$ or NAD^+ and water molecules as starting models (PDB-codes 1K8C and 1MI3). Before any refinement was performed, 5% of the observed reflections were flagged for calculation of the free *R*-factor. The structures were refined with CNS [9] and manually refitted into the electron density with the program O [10]. Many iterations of this cycle resulted in the final structures. Waters were automatically picked by CNS [9] and manually checked for electron density and appropriate hydrogen bonding. The statistics associated with the final round of refinement are shown in Table 1.

Table 1
Statistics for the crystal structures of CtXR K274R mutant bound to NAD^+ and $NADP^+$

	K274R-NAD^+	K274R-$NADP^+$
Data collection		
Space group	*C*2	*C*2
Unit cell dimensions		
a (Å)	182.83	180.01
b (Å)	128.67	127.99
c (Å)	80.10	80.07
β (°)	90.11	90.22
Overall temperature factors (Å^2)		
Monomer A	31.4	27.0
Monomer B	25.7	25.3
Monomer C	29.9	25.1
Monomer D	21.6	21.6
Ramachandran plot [20]		
Most favoured regions	90.3%	90.8%
Additional allowed regions	9.5%	9.1%
Generously allowed regions	0.2%	
Disallowed regions		0.1%
Resolution range (Å)	30–2.4	30–2.3
R_{merge} (overall/high resolution shell[a])	0.080/0.257	0.094/0.347
Completeness (overall/high resolution shell [a]) (%)	96.2/95.4	99.7/98.8
Mosaicity (°)	0.96	0.62
$I/\sigma(I)$ (overall/high resolution shell[a])	10.61/3.2	13.4/4.5
Refinement		
R_{cryst}	0.206	0.192
R_{free}	0.256	0.229
r.m.s. deviation from ideal bond length (Å)	0.007	0.006
r.m.s. deviation from ideal bond angle (°)	1.3	1.3

[a]The high resolution shell is 2.49–2.40 Å for the NAD^+-bound and 2.38–2.30 Å for the $NADP^+$-bound structure.

2.3. Steady-state kinetic measurements

Initial rates of $NADP^+$- and NAD^+-dependent oxidations of xylitol catalyzed by K274R were recorded spectrophotometrically (340 nm) at 25 °C in 50 mM potassium phosphate buffer, pH 7.0. Xylitol was varied in seven concentrations between 48 and 2400 mM, and NAD^+ or $NADP^+$ was present at several constant concentrations between 1 and 300 μM. The concentration of K274R was approximately 3 μM in all assays. $NADP^+$-dependent wild-type rates were obtained under identical conditions using similar reactant concentrations. Data fitting was performed as described elsewhere [6].

3. Results and discussion

3.1. Kinetic consequences of replacing Lys-274 by arginine

Kinetic parameters of K274R-catalyzed oxidation of xylitol by NAD^+ and $NADP^+$ are summarized in Table 2 along with previously determined kinetic parameters of K274R for the reduction of xylose by NADH and NADPH [6]. Table 2 also shows the relevant wild-type parameters. For a comparison of the wild-type and the mutant, the ratio of K_i values which is a measure of the selectivity of binding is most informative. In the wild-type, apparent binding of NADPH and $NADP^+$ was about 19- and 39-fold tighter than binding of NADH and NAD^+, respectively. Ratios of K_i values for the K274R mutant were 1.33 ($=K_{iNADH}/K_{iNADPH}$) and 7.3 ($=K_{iNAD+}/K_{iNADP+}$). Therefore, replacement of Lys-274 by an arginine caused a change in NADP versus NAD binding selectivity for reduced and oxidized coenzymes by factors of 14 (=19/1.33) and 5.3 (=39/7.3), respectively. Comparison of catalytic efficiencies for reactions with NADP(H) and NAD(H), indicated in Table 2, shows that the wild-type preferred NADPH and $NADP^+$ in the directions of xylose reduction (33-fold) and xylitol oxidation (23-fold). The mutant displayed a slight preference for reactions with NADH (1.5-fold) and NAD^+ (2-fold). With the exception of k_{cat} for NADPH-dependent reduction of xylose (discussed later), turnover numbers of K274R and wild-type were identical within experimental error. In the absence of a catalytic reaction profile for xylose reduction by CpXR, comparison of the literature data [7] and our results must be qualitative. It indicates however that the Lys-274 → Arg replacement induced kinetic properties in the mutant that most clearly distinguish the NADH-preferring CpXR from CtXR wild-type. In CpXR and K274R, apparent binding affinity for NADH seems to be gained partly at the expense of affinity for NADPH; and the K_m for xylose in the NADPH-linked reaction is strongly increased, compared to CtXR.

3.2. Overall structures of K274R mutant

The K274R mutant bound to $NADP^+$ or NAD^+ folds into the $(\beta/\alpha)_8$ barrel previously described for wild-type CtXR [4,5]. There were four K274R molecules, two enzyme dimers, within the respective crystallographical asymmetric units. Numbering starts with 1 for the initiator methionine. Results are summarized in Table 1.

3.3. Structure of K274R mutant bound to NAD^+

The NAD^+-bound structure of the mutant showed well-defined electron density around residues 4–322 predicted from the protein and the NAD^+ coenzyme molecules. A total of 681 ordered waters were observed in the final structure of K274R but none was placed at a position possibly important for the enzyme function. The root mean square (r.m.s.) devia-