Exploring the active site of yeast xylose reductase by site-directed mutagenesis of sequence motifs characteristic of two dehydrogenase/reductase family types

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Received 17 April 2001; revised 6 June 2001; accepted 11 June 2001

First published online 21 June 2001

Edited by Pierre Jolles

Abstract Starting from a common tyrosine, yeast xylose reductases (XRs) contain two conserved sequence motifs corresponding to the catalytic signatures of single-domain reductases/epimerases/dehydrogenases (Tyr^{n}-$(X)_3$-Lys^{n+4}) and aldo/keto reductases (AKRs) (Tyr^{n}-$(X)_{28}$-Lys^{n+29}). Tyr^{51}, Lys^{55} and Lys^{80} of XR from *Candida tenuis* were replaced by site-directed mutagenesis. The purified $Tyr^{51} \rightarrow$ Phe and $Lys^{80} \rightarrow$ Ala mutants showed turnover numbers and catalytic efficiencies for NADH-dependent reduction of D-xylose between 2500- and 5000-fold below wild-type levels, suggesting a catalytic role of both residues. Replacing Lys^{55} by Asn, a substitution found in other AKRs, did not detectably affect binding of coenzymes, and enzymatic catalysis to carbonyl/alcohol interconversion. The contribution of Tyr^{51} to rate enhancement of aldehyde reduction conforms with expectations for the general acid catalyst of the enzymatic reaction.

Key words: Aldo/keto reductase; Single-domain reductase/epimerase/dehydrogenase; Catalytic tyrosine

1. Introduction

Xylose reductase (XR) catalyzes the first step of the catabolic pathway for D-xylose in yeasts and fungi. It converts D-xylose into xylitol using NAD(P)H as the reductant. In the systematic sequence-based classification of aldo/keto reductases (AKRs) [1], yeast XRs have been clustered into subfamily 2B (see the AKR Webpage at the URL address, http://www.med.upenn.edu/akr/). A-side stereochemistry of hydride transfer from NADH [2] and positional conservation of the catalytic tetrad residues of AKRs [3] (Tyr^{51}, Lys^{80}, His^{113}, and Asp^{46} in XR from *Candida tenuis* CBS 4435 [2]) are in good agreement with the classification of XR into the AKR superfamily.

Members of the AKR superfamily and single-domain reductase/epimerase/dehydrogenase (RED) superfamily do not display detectable similarities at the levels of primary structure [4,5] and three-dimensional scaffold [6,7]. The RED superfamily is also widely known as the family of short-chain dehydrogenases/reductases. It is intriguing, therefore, that the proposed chemical mechanism of reaction is closely similar for AKRs [8,9] and REDs [10,11]. In both superfamilies, an invariant tyrosine is suggested to act as general acid–base catalyst and a nearby lysine facilitates the reaction by tuning the pK_a of the tyrosine for optimal catalytic effectiveness [8–11]. The catalytic consensus segments which are positionally conserved across virtually all members of each superfamily are: Tyr-$(X)_3$-Lys for REDs [5] and a Tyr/Lys couple separated by usually 28 or 29 amino acids for AKRs [4]. A Ser residue, though not universally conserved throughout the superfamily, completes the catalytic triad of REDs (Ser/Tyr/Lys) [5,10,11]. Point mutation of the respective Tyr and Lys in REDs [10–12] and AKRs [8,9,13] using replacement by Ala and other structurally conservative replacements caused a decrease in enzyme activity by a factor of between 10^4- and 10^6-fold below wild-type level.

An interesting feature of XRs among AKRs and REDs is that these reductases contain the consensus sequences of both superfamilies starting from a common tyrosine [2,3]. Considering the known structure/function relationships for REDs [5] and AKRs [4], each of the conserved lysines is a probable candidate to take part in the reaction catalyzed by XR and in addition to the tyrosine, a clear target for site-directed mutagenesis. The present paper reports experiments designed to study the involvement of the Tyr-$(X)_n$-Lys motifs in the NAD(H)-dependent interconversion of D-xylose and xylitol catalyzed by XR from *C. tenuis* (CtXR). The mutational strategy involved the following replacements: Y51F, K55N, and K80A. Mutation of Lys^{55} into Asn was chosen because Asn^{52} of human aldose reductase (hAR; AKR 1B1) [4] is the positional substitution of Lys^{55} in CtXR [2]. We rationalized, therefore, that the active site of CtXR should be able to accommodate an Asn residue at position 55. At the same time, the effect of removal of the ε-NH_3^+ group could be studied in the K55N mutant. To remove essentially all polar side chain interactions present at position Lys^{80}, the putative catalytic lysine in CtXR, mutation into alanine was chosen.

2. Materials and methods

2.1. Materials

T4 polynucleotide kinase, *Pfu* DNA polymerase, T4 DNA ligase

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Abbreviations: XR, xylose reductase; AKR, aldo/keto reductase; RED, single-domain reductase/epimerase/dehydrogenase; hAR, human aldose reductase; PCR, polymerase chain reaction; 3α-HSD, 3α-hydroxysteroid dehydrogenase; CtXR, xylose reductase from *Candida tenuis*

0014-5793/01/$20.00
PII: S0014-5793(01)02609-6

and dNTPs were obtained from Promega. Synergy DNA polymerase was from Genecraft. Nucleotide primers for polymerase chain reaction (PCR) were from BRL-Life Technologies. D-Xylose, xylitol, NAD^+, NADPH and NADH were from Sigma.

2.2. Site-directed mutagenesis

The pET11-CtXR expression vector (pBEAct.1i) used here has been described recently [14]. Site-directed mutagenesis to produce K55N was done by using the PCR-based overlap extension method in which three rounds of amplification were performed [15]. Synergy DNA polymerase was used. Briefly, two fragments of the target gene sequence were amplified in separate PCRs. Each reaction used one flanking primer that hybridized at one end of the target sequences 1: 5′-GGATCCATATGAGCGCAAGTATCC-3′; 2: 5′-GTACCCGGGATCCGTCTT-3′ and one internal primer that hybridized at the site of the mutation and contained the mismatched bases (underlined) 1: 5′-AACGAAAACGAAGTCGG-3′; 2: 5′-CCGACTTCGTTTTCGTT-3′.

After extension and amplification of the overlapping fragments, the mutant construct was digested with *Nde*I and *Bam*HI, and the desired band was gel-purified and subcloned into the pET11-CtXR expression vector from which the respective fragments had been excised.

The Y51F and K80A mutants were made by using inverse PCR and following a published protocol with slight modification [16]. The mutant constructs were obtained by directly amplifying the plasmid vector using *Pfu* DNA polymerase and two oligonucleotide primer pairs (listed below with mismatched bases underlined), which were phosphorylated at the 5′ end using T4 polynucleotide kinase prior to PCR amplification.

Y51F (forward) 5′-GGTGCCGAGGACTTCGGTAACG-3′; Y51F (reverse) 5′-GTCGAACAATCTGTAACCGGCC-3′; K80A (forward) 5′-CTCACCTCCGCGTTGTGGAAC-3′; K80A (reverse) 5′-GAAGATCTCTTCTCTCTTGAC-3′.

The amplification products generated from the PCR reaction were then treated as described above for the K55N mutant. Plasmid miniprep DNA was subjected to dideoxy sequencing to verify that the desired mutations had been introduced and that no misincorporation of nucleotides had occurred as a result of the DNA polymerase.

2.3. Gene expression and purification of wild-type and mutant XR

The expression vectors for wild-type and mutant CtXR were used to transform competent *Escherichia coli* BL21(DE3) cells. Production of recombinant proteins used induction with isopropyl-1-thio-β-D-galactopyranoside (0.5 mM) and followed the procedure described recently [17].

Wild-type and mutated CtXR were purified by using a published two-step protocol [17]. The polishing step employed high-resolution anion exchange chromatography on a MonoQ column (Amersham Pharmacia) which removed minor contaminations present after biomimetic dye ligand chromatography of wild-type [17] and mutants. Protein was determined using the Bio-Rad dye binding assay standardized by using bovine serum albumin (fraction V, USB). The molarity of CtXR solutions was established from measurements of $A_{280\,nm}$ and using a molar extinction coefficient of 54 000 M^{-1} cm^{-1}.

2.4. Initial velocity measurements

They were carried out in the directions of D-xylose reduction and xylitol oxidation at 25°C in 50 mM potassium phosphate buffer, pH 7.0, as described recently and measuring spectrophotometrically the consumption and formation of NADH or NADPH at 340 nm, respectively. The assay time varied between 5 and 30 min, depending on the specific activity of the enzyme used. Initial velocity data for the wild-type and the K55N mutant were obtained under conditions in which one substrate was varied at several constant concentrations of the second substrate. The data were fitted to Eq. 1 using the least square method. Apparent kinetic parameters for NADH-dependent reduction of D-xylose catalyzed by wild-type CtXR and the Y51F and K80A mutants were determined from non-linear fits of Eq. 2 to initial velocities recorded at varied [D-xylose] and a constant saturating concentration of NADH (220 μM).

$$V = k_{cat}[E][S][Nu]/(K_{i\,Nu}K_S + K_{Nu}[S] + K_S[Nu] + [Nu][S]) \quad (1)$$

$$V = k_{cat}[E][S]/(K_S + [S]) \quad (2)$$

where V is the reaction rate as Δ[NADH]/Δtime; [E], [S], and [Nu] are enzyme, substrate and coenzyme concentration; $K_{i\,Nu}$ is the dissociation constant of the enzyme/coenzyme complex; K_S and K_{Nu} are Michaelis constants for S and Nu; k_{cat} is the turnover number based on a molecular mass of 36 kDa for CtXR.

3. Results

3.1. Expression and purification of wild-type and mutated CtXR

Using the reported procedure for expression of the CtXR gene in *E. coli* [14,17], cell extracts contained approximately 10% recombinant wild-type or mutant protein. Formation of inclusion bodies was not observed under these conditions. The recombinant proteins were purified to homogeneity as determined by SDS–PAGE and isoelectric focusing (not shown). Wild-type and mutated CtXR showed closely similar elution profiles in biomimetic dye ligand chromatography, high-resolution anion exchange chromatography and gel filtration chromatography, indicating that no major conformational changes occur in CtXR as result of the mutations. All proteins migrated to exactly the same position in SDS–PAGE, corresponding to a molecular mass of 36 kDa which is in good agreement with the size of the protein subunit calculated from the sequence [2]. The K55N mutant showed significantly greater mobility in non-denaturing anionic PAGE than the wild-type and the other mutants (Fig. 1). Therefore, this implies an altered mass/charge ratio for the K55N mutant, compared to the other proteins, and reflects an increase in negative net charge brought about by removal of the cationic side chain of Lys^{55}.

3.2. Characterization of wild-type CtXR and the K55N mutant

Purified K55N mutant displayed specific enzyme activities for NAD(P)H-dependent reduction of D-xylose which were identical within experimental error to those of the wild-type enzyme, namely 13 U/mg with NADH and 20 U/mg with NADPH as the coenzyme. Results of a preliminary steady-state kinetic analysis, in which [D-xylose] was varied while [NADH] was constant and saturating, and vice versa, revealed almost identical values of k_{cat}, K_{NADH} and K_{xylose} for wild-type and K55N. Therefore, a full kinetic study was carried out for the forward and reverse reaction catalyzed by K55N and

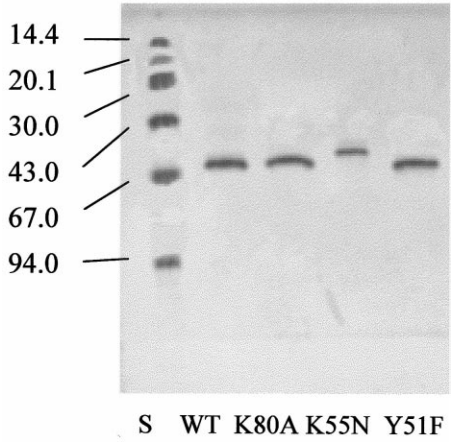

Fig. 1. Non-denaturing anionic PAGE of wild-type (WT) and mutant CtXR. Molecular mass standards (S) are in kDa, and staining with Coomassie was used to visualize protein bands.

Table 1
Kinetic parameters for wild-type CtXR and K55N

	Wild-type	K55N
$k_{cat,red}$ (s^{-1})[a]	13.0 ± 0.4	12.3 ± 0.4
$K_{i\ NADH}$ (μM)	28 ± 7	55 ± 13
K_{NADH} (μM)	30 ± 3	37 ± 4
K_{xylose} (mM)	99 ± 10	84 ± 10
$k_{cat,ox}$ (s^{-1})[a]	0.93 ± 0.05	0.96 ± 0.06
$K_{i\ NAD^+}$ (μM)	127 ± 12	135 ± 15
K_{NAD^+} (μM)	24 ± 6	34 ± 8
$K_{xylitol}$ (mM)	303 ± 22	309 ± 26
K_{eq}[b]	194	116

[a]red, aldehyde reduction; ox, alcohol oxidation.
[b]Calculated by using the Haldane relationship, $K_{eq} = k_{cat,red^-} \times K_{i\ NAD^+} K_{xylitol}/(k_{cat,ox} K_{i\ NADH} K_{xylose})$; the experimental value for the expression [NAD^+][xylitol]/[NADH][D-xylose] at pH 7.0 and 25°C is between 350 and 500.

the wild-type. Initial velocities (v) for NADH-dependent reduction of D-xylose were analyzed in double reciprocal plots of the form $1/v$ against 1/[D-xylose]. The plots were linear and revealed an intersecting line pattern in each case (not shown). Non-linear fits of the data to Eq. 2 were used to obtain kinetic parameters for the wild-type and K55N mutant, which are summarized in Table 1. The internal consistency of the kinetic parameters was verified by using the Haldane relationship for the ordered bi–bi mechanism of CtXR, which relates the kinetic parameters to the thermodynamic equilibrium constant of the reaction at pH 7.0 and 25°C (shown in Table 1). The results in Table 1 indicate that replacement of Lys^{55} by Asn does not have profound effects on coenzyme binding, turnover numbers and Michaelis constants for D-xylose reduction and xylitol oxidation.

3.3. Characterization of Y51F and K80A mutants

The two mutants displayed specific enzyme activities for NAD(P)H-dependent reduction of D-xylose that were below wild-type level by about the same factor of 4000. The activity ratio measured with NADPH and NADH was 1.5 for Y51F and K80A, and thus closely similar to the value observed with wild-type CtXR [18]. Using extended assay times of up to 30 min, accurate initial velocities could be determined with both mutants using D-xylose as the varied substrate. Eq. 2 was used to fit the data, and results are shown in Table 2 and compared to the corresponding kinetic parameters of the wild-type enzyme. The observed large decreases in k_{cat} and k_{cat}/K_{xylose} for the mutants, relative to the wild-type, support the notion of Tyr^{51} and Lys^{80} having important roles in catalysis of CtXR.

4. Discussion

This paper provides clear evidence that the Tyr/Lys couple of the catalytic tetrad of AKRs is important to the enzymatic function of XR. The data reveal several features of XR that are unique among AKRs, and provide an essential step towards establishing the catalytic mechanism of these yeast reductases. Results obtained in initial velocity studies with the recombinant wild-type XR and their comparison with published data for the enzyme isolated from *C. tenuis* [18] show that the native properties of XR were retained fully during heterologous expression.

4.1. Lys^{55}

The results rule out a critical role of the ε-NH_3^+ group of Lys^{55} in the mechanism of D-xylose reduction catalyzed by CtXR. This conclusion pertains to all steps, physical and chemical, of the catalytic pathway of CtXR which was previously shown to involve binding of NADH before D-xylose binds and dissociation of xylitol before NAD^+ is released [18]. Hence, the data confirm the notion that the Tyr^{51}-$(X)_3$-Lys^{55} segment is not a catalytic motif in XR. Discussing a possible relationship of XR to REDs [3], it is interesting to note that Ser of the catalytic triad of REDs [5] in not conserved in XR. The apparent increase in negative net charge of the K55N mutant of CtXR, compared to the wild-type, suggests that the ε-NH_3^+ group of Lys^{55} may be positioned at or close to the protein surface in wild-type CtXR.

The occurrence of a Tyr-$(X)_3$-Lys motif in CtXR is not unprecedented in the AKR superfamily. Like XR, members of subfamilies 1C and 4C display a Tyr^n-$(X)_3$-Lys^{n+3} motif that involves the putative catalytic acid–base tyrosine [19]. Results for the K55N mutant of CtXR are, therefore, of relevance for these two subfamilies. Two members of subfamily 1B, namely aldose reductases from *Mus musculus* (AKR 1B3) and *Rattus norvegicus* (AKR 1B4), show a reverse Asn → Lys replacement at the position corresponding to Lys^{55} in CtXR. The finding supports the conclusion of our mutagenesis study that an Asn/Lys interchange at this position is functionally silent. Rat liver 3α-hydroxysteroid dehydrogenase (3α-HSD; AKR 1C9) contains Tyr^{205} and Lys^{209}. The Y205F mutant of this enzyme was fully active [20] as expected from its position at the periphery of the protein structure [21]. Noteworthy, however, Tyr^{55} of 3α-HSD is the residue corresponding to Tyr^{51} in CtXR (see later).

4.2. Tyr^{51} and Lys^{80}

Upon mutation of Tyr^{51}, k_{cat} for NADH-dependent reduction of D-xylose drops by a factor of approximately 3000. This decrease in rate enhancement for the Y51F mutant, compared to the wild-type, is smaller by at least 10-fold than the decrease in rate enhancement observed for the corresponding Tyr → Phe mutant of 3α-HSD [8]. The Tyr → Phe mutant of hAR is reported to have lost all enzyme activity [9,13]. Considering the k_{cat} value of 0.2 s^{-1} for NADPH-dependent aldehyde reduction catalyzed by wild-type hAR [9], enzyme activities between 10^3- and 10^4-fold below the wild-type level may be very difficult to measure with the conventional spectrophotometric assays used (discussed in [8]). Alternative explanations for the observed differential effect of the Tyr → Phe replacement in AKRs are that hAR shows a significantly greater requirement for general acid catalysis to NADPH-de-

Table 2
Apparent kinetic parameters of wild-type CtXR and mutants Y51F and K80A for NADH-dependent reduction of D-xylose

	k_{cat} (s^{-1})	K_{xylose} (mM)	k_{cat}/K_{xylose} (M^{-1} s^{-1})	(k_{cat}/K_{xylose}) ratio	k_{cat} ratio
Wild-type	12.1 ± 0.1	78 ± 10	154	–	–
Y51F	0.004 ± 0.0006	75 ± 5	0.05	3100	3000
K80A	0.005 ± 0.0006	174 ± 8	0.03	5100	2400

pendent carbonyl-group reduction than CtXR and 3α-HSD; water can partly replace the hydroxy group of tyrosine in the reactions catalyzed by mutants of CtXR and 3α-HSD but not the hAR mutant.

If we consider Tyr^{51} to be the proton donor during D-xylose reduction by CtXR, its contribution to rate enhancement is at the lower end of the 10^3–10^5-fold range commonly quoted for the rate acceleration brought about by general acid catalysis in enzymatic reactions [22]. A loss of rate enhancement by a factor of 2400 was observed for the K80A mutant of CtXR, and this agrees well with data for the corresponding K84M mutant of 3α-HSD [8]. It supports the contention that the conserved lysine plays a crucial role in assisting the catalytic process of CtXR.

Except for CtXR it has not been possible so far to determine k_{cat} and k_{cat}/K for carbonyl-group reduction by an AKR point mutant in which the putative catalytic tyrosine or lysine had been replaced. Y55F and Y55S mutants of 3α-HSD are notable exceptions since they were nearly as active as the wild-type towards reduction of the reactive dicarbonyl substrate, 9,10-phenanthrenequinone [23]. However, reactions of 3α-HSD with quinones and the less reactive, physiological keto-steroid substrates are thought to proceed via two fundamentally different chemical mechanisms [23]. The results for CtXR mutants show that the observed decreases in k_{cat}/K_{xylose} reflect decreases in k_{cat} by about the same factor, which is what one would expect for the removal of an amino acid that participates in the chemical step of an enzymatic reaction rather than substrate binding.

Since replacement of Tyr^{51} or Lys^{80} slows the reaction to give the alcohol product by 2500-fold or greater, binding of D-xylose with the complex of the mutant and NADH will probably come to equilibrium, and the observed K_{xylose} approximates $K_{d\ xylose}$. An estimate of approximately 200 mM was obtained for $K_{d\ xylose}$ of wild-type CtXR (M.K. and B.N., unpublished results), indicating that aldehyde binding is not weakened as a result of the mutations. In marked contrast, equilibrium dialysis studies with wild-type and catalytic tetrad mutants of 3α-HSD have shown that the Y55F and K84M mutant bind the competitive inhibitor testosterone by 10-fold and ≥20-fold less tightly than the wild-type, respectively [9]. The data for CtXR now provide good evidence that although the catalytic tetrad has been conserved across the superfamily, subtle differences prevail among individual AKR members in regard to the contribution of the Tyr/Lys couple to a differential binding mechanism of carbonyl-group reduction. They clearly support a classification of the superfamily into individual subfamilies [1].

Acknowledgements: Financial support from the Austrian Science Funds is gratefully acknowledged (P-12569-MOB to B.N.).

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