Characterization of xylose reductase from the yeast *Pichia stipitis*: evidence for functional thiol and histidyl groups

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Xylose reductase (EC 1.1.1.21) from the yeast *Pichia stipitis* NRC 2548 was purified to homogeneity via a two-step protocol using anion-exchange and gel-filtration chromatography. The pH-activity profile revealed the presence of two ionizable groups with pK_{app} values of 5.8 and 8.1, suggesting the catalytic involvement of histidyl and thiol groups, respectively. Additional evidence supporting the involvement of these residues was provided by the use of group-specific inhibitors. The enzyme was rapidly inactivated in a pseudo-first order manner by the thiol-specific modifier *p*-chloromercuriphenylsulphonate (PMBS) and analysis of the order-of-reaction suggested that one essential cysteine residue was modified to effect inactivation. Treatment of the enzyme with another thiol-specific modifier, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), showed that modification of one cysteine per monomer led to 90% loss of activity, further supporting the existence of one essential cysteine residue. Inactivation by PMBS was reversed by adding 1 mM- β -mercaptoethanol. Inactivation of xylose reductase by the histidine-specific modifier diethylpyrocarbonate (DEP) followed a pseudo-first order process, and analysis of the order-of-reaction suggested that one essential histidine residue was modified to effect inactivation. Treatment of DEP-inactivated enzyme with 0.2 M-neutral hydroxylamine resulted in the recovery of 45% of enzyme activity. Protection of xylose reductase from PMBS- and DEP-inactivation was provided by NADPH and NADH but not by $NADP^+$, D-xylose or DL-glyceraldehyde. This suggests that the essential histidine and cysteine residues may be involved with binding of cofactor by the *P. stipitis* xylose reductase.

Introduction

D-Xylose, the predominant pentose sugar in plant hemicelluloses, is second only to D-glucose in natural abundance. These sugars represent the majority of all carbohydrates obtained from the hydrolysis of renewable plant biomass, and their efficient utilization is essential for the development of viable biomass to energy conversion processes (Schneider, 1989).

D-Xylose metabolism in pentose-fermenting yeasts proceeds initially through a D-xylose-inducible enzyme pathway (Webb & Lee, 1990). D-Xylose undergoes a two-step isomerization where it is first reduced by NAD(P)H-dependent xylose reductase to xylitol, which is oxidized by NAD^+ -dependent xylitol dehydrogenase to D-xylulose. D-Xylulose is then phosphorylated by xylulose kinase to D-xylulose-5-phosphate which enters

the pentose phosphate, Embden–Meyerhof or phosphoketolase pathways (Evans & Ratledge, 1984; Skoog & Hahn-Hagerdal, 1988). Xylose reductase, the first enzyme in the pathway, has been shown to control the rate of D-xylose utilization (Hagedorn & Ciriacy, 1989; Schneider *et al.*, 1989). In addition, the ability of some pentose-fermenting yeasts such as *Pichia stipitis* to ferment D-xylose anaerobically is linked to the NADPH or NADH cofactor requirement of their xylose reductases (Bruinenberg *et al.*, 1984).

Although xylose reductases have been purified from several yeasts (Bolen *et al.*, 1985, 1986; Ditzelmüller *et al.*, 1984; Ho *et al.*, 1990; Rizzi *et al.*, 1988; Scher & Horecker, 1966; Suzuki & Onishi, 1975; Verduyn *et al.*, 1985; Watson *et al.*, 1969) and the amino acid sequence of the *P. stipitis* xylose reductase has recently been published (Amore *et al.*, 1991; Hallborn *et al.*, 1991; Takuma *et al.*, 1991), little is known about the enzyme's catalytic mechanism or the nature of its catalytic sites. Yeast xylose reductases have been classified as members of the aldose reductase enzyme family (alditol : NAD(P)⁺ 1-oxidoreductase, EC 1.1.1.21) based on similar sub-

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Abbreviations: DEP, diethylpyrocarbonate; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); PMBS, *p*-chloromercuriphenylsulphonate.

strate specificities (Bolen *et al.*, 1986; Verduyn *et al.*, 1985) and response to some chemical inhibitors (Webb & Lee, 1991). Aldose reductases are extensively distributed in various mammalian tissues, where they reduce D-glucose to sorbitol. Under non-physiological conditions this activity has been implicated in the manifestation of serious diabetic complications (Kador *et al.*, 1985). Therefore, there is considerable interest in the properties of mammalian aldose reductases, with the objective of developing more potent and specific inhibitors for the pharmacological management of these complications (Das & Srivastava, 1985; Kador *et al.*, 1985).

The relationship between yeast xylose reductase and mammalian aldose reductase is of interest from an evolutionary viewpoint. In the present paper, we use group-specific modifiers to provide evidence of the possible involvement of histidyl and thiol residues in the catalytic mechanism of xylose reductase from *P. stipitis*. The involvement of these residues in the catalytic mechanism of aldose reductases from mammalian sources has also been suggested (Bhatnagar *et al.*, 1989; Cromlish & Flynn, 1983; Doughty & Conrad, 1982; Doughty *et al.*, 1982; Flynn *et al.*, 1981; Jedziniak & Kinoshita, 1971; Liu *et al.*, 1989; Wermuth *et al.*, 1982).

Methods

Micro-organism. *Pichia stipitis* NRC 2548 (NRRL-Y7124) was kindly supplied by R. K. Latta, National Research Council Culture Collection, Ottawa, Ontario, Canada. The yeast was maintained at 4 °C on potato dextrose agar (PDA) slants (Difco) and subcultured prior to growth in a liquid medium (Webb & Lee, 1991).

Preparation of inocula. A single colony from a 24 h PDA plate was transferred to 20 ml medium containing 0.67% yeast nitrogen base (YNB, Difco) without amino acids and 2% (w/v) glycerol (Bicho *et al.*, 1988). The culture was incubated for 48 h at 30 °C in a 125 ml Erlenmeyer flask, shaken at 200 r.p.m. in a gyratory shaker (Webb & Lee, 1991).

Culture conditions. Two millilitres of inoculum were transferred to 100 ml medium containing 0.67% YNB without amino acids and 4% (w/v) D-xylose. The culture was kept in a 250 ml Erlenmeyer flask and incubated as above. The cells were harvested during late exponential phase (18–20 h) by centrifugation and stored as a frozen pellet at –15 °C until use (Webb & Lee, 1991). Cell pellets were never stored for more than one week.

Purification of xylose reductase. Unless otherwise stated, all steps were carried out at 2–4 °C. (a) **Preparation of cell extract.** The cell pellet from about 20 g wet cells was thawed and resuspended in sufficient ice-cold 20 mM-Bis-Tris buffer (pH 6.5) containing 0.5 mM-EDTA and 1 mM- β -mercaptoethanol (Buffer A) to form a thin paste. About 1.5 g alumina (Sigma) was added, and cell extracts were prepared by breaking the cells by sonication (Webb & Lee, 1991), followed by centrifugation (Lee *et al.*, 1986). (b) **Anion-exchange chromatography.** The cell extract was applied to a DEAE-Sephacel column (2.6 × 20 cm, Pharmacia) which had been previously equilibrated with Buffer A at 30 ml h⁻¹. The absorbed xylose reductase was eluted with 400 ml of a 0 to 0.5 M linear NaCl gradient in Buffer A. Fractions (each 5 ml) were

collected and their A_{280} measured. Fractions containing xylose reductase activity were pooled and desalted through an Econo-Pac 10DG column (Bio-Rad) into Buffer B, which contained 50 mM-sodium sulphate, 20 mM-sodium dihydrogen phosphate, pH 6.8, and 1 mM- β -mercaptoethanol. (c) **Gel permeation chromatography.** Xylose reductase was further purified using a Bio-Sil SEC 250 column (Bio-Rad) and was eluted with Buffer B at 0.5 ml min⁻¹ at 22 °C. Fractions (1 ml) were collected and their A_{280} was measured. Fractions containing enzyme activity were pooled and stored at 4 °C.

Gel electrophoresis. The purity of xylose reductase preparations was assessed using 15% (w/v) polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (1970). SDS-PAGE was carried out at 150 V for 40–60 min in a Bio-Rad Mini Protean II Gel Electrophoresis apparatus. The proteins were stained with 0.1% Coomassie Brilliant Blue R-250 and destained with 40% (v/v) methanol and 10% (v/v) acetic acid until the background was clear. In some instances, gels were also stained with silver nitrate using the Bio-Rad silver-staining kit.

Analytical methods. Xylose reductase activity was determined at 25 °C as previously described (Webb & Lee, 1991) with some modifications. The standard assay volume of 1 ml contained 50 mM-potassium phosphate buffer, pH 6.5, 0.1 mM-NADPH or NADH, and 100 mM-D-xylose or 50 mM-DL-glyceraldehyde. In most instances, the reaction was started by adding an appropriate amount of enzyme and activity was followed spectrophotometrically by monitoring the initial rate of decrease in A_{340} . One unit (U) of activity was defined as the amount of enzyme which oxidized 1 μ mol NADPH or NADH per min. Specific activity was based on protein determined by the method of Bradford (1976) with bovine serum albumin as the standard.

Chemical modification of xylose reductase. (a) **Effect of pH on xylose reductase-catalysed reaction.** The kinetic properties of xylose reductase were determined over the pH range 4.0–10.6 using the following buffers: 100 mM-citric acid/Na₂HPO₄ (pH 4.0–6.4), 100 mM-KH₂PO₄/NaOH (pH 6.0–8.0), 100 mM-Tris/HCl (pH 7.5–8.9) and 100 mM-glycine/NaOH (pH 8.6–10.6). The effect of pH was determined by adding 37.5 nM-xylose reductase at various substrate (D-xylose or DL-glyceraldehyde) concentrations into buffer at the pH being evaluated, in a total volume of 1 ml. Substrate (D-xylose) concentration was varied in the presence of saturating NADPH (0.2 mM) concentrations. The molarity of xylose reductase was estimated using a molecular mass of 64000 Da as determined by Verduyn *et al.* (1985) and Rizzi *et al.* (1988). Substrate-dependency data were plotted on Eadie-Hofstee plots to calculate $V_{\max \text{ app}}$. (b) **Modification of cysteine residues by p-chloromercuriphenylsulphonate (PMBS).** Solutions (1 ml) containing 0.36 μ M-xylose reductase in 10 mM-Tris/HCl buffer, pH 8.0, were incubated with the indicated concentrations of PMBS for 120 min at 25 °C. The modifications were carried out under pseudo-first order conditions. Samples (10 μ l) were removed from the incubation mixture at specified intervals and added immediately to cuvettes containing the assay mixture for determination of enzyme activity. The reversibility of PMBS-inactivated xylose reductase was measured by adding 1 mM- β -mercaptoethanol to the incubation mixture and then following the recovery of enzyme activity thereafter at specified intervals. Various compounds were tested for ability to protect the enzyme from inactivation by PMBS. These experiments were conducted as above except that the test compound was included in the incubation mixture for 4 min prior to the addition of PMBS. The compounds tested were NADPH, NADH, NADP⁺, D-xylose and DL-glyceraldehyde. In all experiments, controls which did not contain modifying reagents were included, and fractional activity of the modified enzyme at any given time was calculated relative to the appropriate control. (c) **Modification of cysteine residue by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB).** To assess the number of thiol groups modified to effect inactivation, 1 ml solutions containing 1.2 μ M-xylose reductase in 10 mM-Tris/HCl buffer,

pH 8.0, were incubated with 0.2 mM-DTNB for 80 min at 25 °C. Modifications were carried out under pseudo-first-order conditions. To assay for enzyme activity, 10 µl aliquots were removed from the incubation mixture at various times and added immediately to cuvettes containing the assay mixture as described above. In all experiments, controls which did not contain DTNB were included and fractional activity of the modified enzyme at any given time was calculated relative to the appropriate control. The change in A_{412} was monitored at 25 °C for 80 min, and the number of cysteine groups modified was estimated by formation of thionitrobenzoate anion using a molar extinction coefficient (ϵ) at 412 nm of $1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (Ellman, 1959). (d) *Modification of histidine residues by diethylpyrocarbonate (DEP)*. Modification of histidine residues was carried out at 25 °C by incubating xylose reductase (0.24 µM) with the indicated concentrations of DEP in 50 mM-potassium phosphate buffer, pH 6.0. The DEP was freshly diluted to the desired concentrations in ice-cold absolute ethanol prior to each experiment. Samples (10 µl) were removed from the incubation mixture at various intervals and added to cuvettes containing the assay mixture for determination of enzyme activity. Recovery of enzyme activity from DEP inactivation was assayed with 0.2 M-neutral hydroxylamine. Protection of xylose reductase activity by substrates or substrate analogues from DEP inactivation was done as described above. The final concentration of ethanol in the incubation mixture was 5% (v/v) and this concentration was included in the controls.

Data analysis. All experiments were repeated two to five times. In each instance, similar trends were observed.

Results and Discussion

Xylose reductase purification

Previous studies have shown that *P. stipitis* contains a single xylose reductase enzyme which can utilize either NADPH or NADH as cofactor. The enzyme is a dimer composed of two identical 32 kDa subunits (Verduyn *et al.*, 1985; Rizzi *et al.*, 1988). Recent genetic analysis of *P. stipitis* has confirmed that xylose reductase activity arises from a single gene which encodes a 32 kDa product (Hagedorn & Ciriacy, 1989; Takuma *et al.*, 1991). Although the functional enzyme is a dimer, previous studies have found no evidence of allosteric interaction between the two subunits (Rizzi *et al.*, 1988; Verduyn *et al.*, 1985; Webb & Lee, 1991).

Earlier protocols developed by Verduyn *et al.* (1985) and Rizzi *et al.* (1988) to purify the *P. stipitis* xylose reductase required four and six steps, respectively. In the present study, xylose reductase was purified to homogeneity on SDS-PAGE gels in two steps. The first chromatographic step employed a DEAE-Sephacel anion-exchange column under conditions suitable for binding xylose reductase. Anion-exchange chromatography separated the cell extract into three major protein peaks (Fig. 1). The majority of xylose reductase activity (about 68% of the amount applied) was associated with the upswing arm of the first protein peak. The pooled fractions (nos 16 & 17, Fig. 1) exhibited specific xylose

Fig. 1. Anion-exchange chromatography of *P. stipitis* cell extract using a DEAE-Sephacel column. Symbols: ■, xylose reductase activity; ●, protein; ▲, NaCl gradient. The figure shown is a representative plot chosen from ten independent experiments.

reductase activity of $25.4 \text{ U (mg protein)}^{-1}$, an increase of about 51-fold over that in the cell extract [$0.5 \text{ U (mg protein)}^{-1}$].

Xylose reductase was further purified by gel filtration with a Bio-Sil SEC 125 column. The total enzyme activity recovered after the two chromatographic steps was about 51% of that in the cell extract. The specific enzyme activity after gel filtration [$43.2 \text{ U (mg protein)}^{-1}$] represented an 86-fold purification from that in the cell extract. This compares favourably to the 52- and 59-fold purification of the same enzyme reported earlier by Rizzi *et al.* (1988) and Verduyn *et al.* (1985), respectively. The purified enzyme had an apparent molecular mass of $62000 \pm 1000 \text{ Da}$ as estimated by gel filtration chromatography (data not shown). The molecular mass of the subunits was approximately $38100 \pm 1000 \text{ Da}$ by SDS-PAGE (data not shown). Both are in agreement with previously reported values (Rizzi *et al.*, 1988; Verduyn *et al.*, 1985). Xylose reductase was stable for several weeks at 4 °C in the presence of 1 mM- β -mercaptoethanol, but freezing of the purified or partially purified enzyme resulted in immediate and complete loss of activity.

Chemical modification of xylose reductase

Effect of pH on the xylose-reductase-catalysed reaction. Yeast xylose reductases have previously been shown to exhibit nearly symmetrical pH-activity profiles, with optima near pH 6.0 under standard assay conditions (Scher & Horecker, 1966; Suzuki & Onishi, 1975; Verduyn *et al.*, 1985). These bell-shaped curves suggest that at least two ionizable groups are operative in the catalytic mechanism of yeast xylose reductase. However, if pH activity profiles are to provide useful information

Fig. 2. Effect of pH on the maximum activity ($V_{\max \text{ app}}$) of xylose reductase from *P. stipitis*. Assay buffers used were: ●, 100 mM-citric acid/sodium phosphate, pH 4.0–6.4; ▲, 100 mM-potassium dihydrogen phosphate/NaOH, pH 5.8–8.0; ○, Tris/HCl, pH 7.5–8.9, and ■, 100 mM-glycine/NaOH, pH 8.6–10.6. The figure shown is a representative plot chosen from three independent experiments.

on the nature of essential amino acid residues, kinetic analysis at each pH value is required (Engel, 1977).

Values for $V_{\max \text{ app}}$ (D-xylose) at each pH were estimated at saturating NADPH (0.2 mM) concentrations from Eadie-Hofstee plots, and the change in $V_{\max \text{ app}}$ as a function of pH is plotted in Fig. 2. The pH activity profile suggests that at least two essential ionizing residues with pK_{app} of about 5.8 and 8.1 are required for xylose reductase activity. These estimated pK_{app} values are in agreement with the pK values reported for imidazolium and thiol groups in proteins (Engel, 1977). From this result, we surmised that the essential amino acids might be histidine and cysteine residues. Chemical modification studies on mammalian aldose reductases have also suggested the involvement of histidyl and thiol groups in the enzyme's catalytic mechanisms (Bhatnagar *et al.*, 1989; Cromlish & Flynn, 1983; Doughty & Conrad, 1982; Doughty *et al.*, 1982; Flynn *et al.*, 1981; Jedziniak & Kinoshita, 1971; Liu *et al.*, 1989; Wermuth *et al.*, 1982). Some researchers have suggested that arginyl and lysyl residues may also be involved in aldose reductase activity (Flynn *et al.*, 1989; Doughty & Conrad, 1982; Doughty *et al.*, 1982). However, the low pK_{app} from the basic arm of the pH activity profile in Fig. 2 does not support the involvement of these groups in yeast xylose reductase activity. To confirm the involvement of histidyl and thiol residues in the catalytic mechanism of xylose reductase, enzyme modification with group-specific inhibitors was undertaken.

Inactivation of xylose reductase by PMBS and DTNB

Previous studies on yeast xylose reductase (Scher & Horecker, 1966; Suzuki & Onishi, 1975; Verduyn *et al.*,

Fig. 3. Kinetics of inactivation of *P. stipitis* xylose reductase by PMBS at various concentrations. The incubation mixture contained enzyme (0.36 μM) and PMBS at 5 (○), 7 (▲), 10 (■), 15 (●) or 20 (△) μM . The figure shown is a representative plot chosen from five independent experiments.

1985) and mammalian aldose reductase (Cromlish & Flynn, 1983; Jedziniak & Kinoshita, 1971; Liu *et al.*, 1989; Wermuth *et al.*, 1982) have shown that a variety of thiol-modifying reagents can cause almost complete loss of enzyme activity. In the present study when excess PMBS was incubated with the enzyme, a rapid time-dependent, concentration-dependent decrease in xylose reductase activity was observed. The semi-logarithmic plots of residual activity as a function of time for the various concentrations of PMBS were linear (Fig. 3), indicating that the inactivation process obeys pseudo-first-order kinetics. The linearity of the plot of k_{app} (min^{-1}) against PMBS concentration indicated that a reversible PMBS-xylose reductase complex was not formed before inactivation (data not shown) (Cardemil, 1987). Analysis of the order of reaction by the method of Levy *et al.* (1963) resulted in a slope of 0.96 (data not shown), suggesting that at least one PMBS molecule binds to one xylose reductase monomer to effect inactivation. The amino acid sequence of the *P. stipitis* xylose reductase indicates the presence of three cysteine residues per subunit (Amore *et al.*, 1991; Hallborn *et al.*, 1991; Takuma *et al.*, 1991) and the order of reaction determined from our study suggests that one of these residues was modified to effect inactivation.

SDS-PAGE of PMBS-modified xylose reductase showed similar mobility compared to unmodified xylose reductase, indicating that inactivation of the enzyme was not due to cross-linking or aggregation of xylose reductase proteins (data not shown). Inactivation of xylose reductase by PMBS was found to be reversible by treatment with excess β -mercaptoethanol. To demonstrate reversibility, enzyme was first incubated in the presence of PMBS. At 50 to 60 min, enzyme activity was almost completely (98 to 99%) lost (Fig. 4). When β -mercaptoethanol was added, activity of the PMBS-

Fig. 4. Reactivation of PMBS-inactivated *P. stipitis* xylose reductase with β -mercaptoethanol (1 mM). The enzyme (0.36 μ M) was inactivated by PMBS for 60 min, at which time β -mercaptoethanol was added to the modified enzyme as indicated by the arrow. The figure shown is a representative plot chosen from two independent experiments.

inactivated enzyme was rapidly restored such that within 15 min, about 90% of the activity was recovered (Fig. 4).

To further characterize the role of essential thiol residue(s) in xylose reductase activity, the protective effect of cofactors, substrates and various analogues on the rate of PMBS inactivation was studied. Maximum protection was provided by NADPH, followed by NADH, while NADP⁺, D-xylose and DL-glyceraldehyde offered no apparent protection (data not shown). Protection by cofactors was concentration-dependent (Fig. 5). A plot of protection as a function of cofactor concentration according to Scrutton & Utter (1965) could be extrapolated to the origin (Fig. 5, inset), indicating that PMBS inactivation of the enzyme-cofactor complex is not possible.

Protection by cofactors and not by substrates suggests that the critical thiol group may be situated at or near the cofactor-binding site. Similarly, Liu *et al.* (1989) have suggested cysteine residues in human placental aldose reductase are involved in cofactor binding. Alternatively, formation of the enzyme-cofactor complex may result in conformational changes in the enzyme, thereby making the thiol group inaccessible to PMBS inactivation. The inability of DL-glyceraldehyde and D-xylose to protect the enzyme from inactivation is not surprising as Rizzi *et al.* (1988) showed earlier that the xylose-reductase-catalysed reaction proceeds by an ordered-bi-bi mechanism with isomerization of a stable enzyme form in which cofactor binds first to form an enzyme-cofactor complex followed by binding of substrate.

To estimate the number of cysteine groups modified to effect inactivation, the enzyme was treated by DTNB. The enzyme lost about 90% of initial activity when incubated with DTNB for 10 min. During this period, only one cysteine group per subunit had been modified as estimated by the formation of thionitrobenzoate anions

Fig. 5. Protection of *P. stipitis* xylose reductase from PMBS inactivation by NADPH. The incubation mixture contained enzyme (0.24 μ M) and 1 (Δ), 0.8 ($\circ$), 0.5 ($\bullet$), 0.3 ($\blacksquare$) or 0.1 ($\blacktriangle$) mM-NADPH. Inset: the apparent pseudo-first-order rate constants obtained in the presence (K_p) and the absence (K_s) of NADPH plotted according to the method of Scrutton & Utter (1965). The figure shown is a representative plot chosen from two independent experiments.

(data not shown). Prolonged incubation for 70 min led to modification of the other two cysteine groups with a further slight decrease in activity.

Inactivation of xylose reductase by DEP

Xylose reductase was inactivated by DEP in a time-dependent and concentration-dependent manner. The linear nature of the semi-logarithmic plots (Fig. 6) indicated that DEP inactivation of xylose reductase obeyed pseudo-first-order kinetics. Plots of k_{app} versus DEP concentration were linear, indicating that a reversible DEP-enzyme complex was also not formed prior to inactivation (data not shown) (Cardemil, 1987). Analysis of the order of reaction by the method of Levy *et al.* (1963) resulted in a slope of 1.1 (data not shown), indicating that an average of at least one molecule of DEP binds to one monomer of xylose reductase when inactivation occurs. The amino acid sequence of the enzyme indicates there are six histidyl residues per subunit (Amore *et al.*, 1991; Hallborn *et al.*, 1991; Takuma *et al.*, 1991), the inactivation data suggests that DEP may modify one essential histidine per subunit to effect inactivation. No evidence of cross-linking or aggregation of xylose reductase proteins was observed when DEP-inactivated enzyme was analysed on SDS-PAGE (data not shown).

Again, as might be expected for an enzyme reaction which operates according to an ordered-bi-bi mechanism with cofactor binding first (Rizzi *et al.*, 1988), NADPH and NADH protected xylose reductase from modification by DEP. As with the PMBS, cofactor protection from DEP inactivation was concentration-dependent

Fig. 6. Kinetics of inactivation of *P. stipitis* xylose reductase by various concentrations of DEP. The incubation mixture contained enzyme ($0.24 \mu\text{M}$) and DEP at 0.5 (Δ), 0.4 ($\bullet$), 0.3 ($\blacksquare$), 0.2 ($\blacktriangle$), 0.15 ($\circ$) or 0.1 ($\square$) mM. The figure shown is a representative plot chosen from four independent experiments.

Fig. 7. Protection of *P. stipitis* xylose reductase from DEP inactivation by NADPH. The incubation mixture contained enzyme ($0.36 \mu\text{M}$) in the presence of NADPH at 2.0 ($\circ$), 1.5 ($\square$), 1.2 (Δ), 1.0 ($\bullet$) or 0.5 ($\square$) mM. Inset: the apparent pseudo-first-order rate constants obtained in the presence (K_p) and in the absence (K_s) of NADPH plotted according to the method of Scrutton & Utter (1965). The figure shown is a representative plot chosen from two independent experiments.

(Fig. 7). When this data was plotted as described by Scrutton & Utter (1965) it also indicated that DEP inactivation of the enzyme-cofactor complex was not possible (Fig. 7, inset). Again, substrates did not offer any protection to DEP-modification (data not shown). These results suggest that DEP-modification of xylose reductase occurs at or near the cofactor-binding site. This finding agrees with a previous study which suggested that histidyl residues may be responsible for binding of NADPH to bovine, rat and human lens aldose reductase (Doughty & Conrad, 1982; Doughty *et al.*, 1982). In a closely related enzyme family, the aldehyde reductases, binding of NADPH can cause conformational changes in the enzyme (Davidson & Flynn, 1979). Likewise, the protection afforded here by the cofactor

may be due to conformational changes to the enzyme that rendered the essential histidyl residue inaccessible to DEP.

Although DEP reacts preferentially with histidyl groups, under certain conditions it may also react with lysine, tyrosine, tryptophan and cysteine (Burstein *et al.*, 1974). These reactions with other amino acid groups occur more frequently at high pH and high DEP concentrations (Miles, 1977); conditions which were not used in this study. Further evidence for the involvement of an essential histidine residue was shown by the ability of neutral hydroxylamine to partially restore the activity of DEP-inactivated xylose reductase. In this experiment, the enzyme was first inactivated almost completely (about 1.5% residual activity) by incubation in the presence of DEP for 30 min. Addition of neutral hydroxylamine to DEP-inactivated enzyme led to a rapid recovery of about 20% of enzyme activity in the first 20 min. Further recovery to about 45% of enzyme activity occurred gradually on incubation for additional 60 min (data not shown). The inability of neutral hydroxylamine to restore enzyme activity fully may be attributed to DEP-modification of other amino acid residues such as lysine which may cause conformational changes in the enzyme (Kerstens-Hilderson *et al.*, 1984) or to the irreversible inactivation of some histidyl residues in the presence of excess DEP (Burstein *et al.*, 1974).

Modification of an essential lysine or cysteine residue by DEP can be ruled out since DEP-modification of these residues is not reversed by neutral hydroxylamine (Lenard *et al.*, 1971). Although DEP-modification of histidine and tyrosine residues is reversed by neutral hydroxylamine, evidence of DEP-modification of tyrosine, a decrease in A_{278} (Miles, 1977), was not observed. However, there was an increase in A_{242} , and this is indicative of carbethoxylation of histidine residues (data not shown).

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