NADP⁺-dependent D-xylose dehydrogenase from pig liver

Purification and properties*

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An NADP⁺-dependent D-xylose dehydrogenase from pig liver cytosol was purified about 2000-fold to apparent homogeneity with a yield of 15 % and specific activity of 6 units/mg of protein. An M_r value of 62000 was obtained by gel filtration. PAGE in the presence of SDS gave an M_r value of 32000, suggesting that the native enzyme is a dimer of similar or identical subunits. D-Xylose, D-ribose, L-arabinose, 2-deoxy-D-glucose, D-glucose and D-mannose were substrates in the presence of NADP⁺ but the specificity constant (ratio $k_{cat}/K_m(app.)$) is, by far, much higher for D-xylose than for the other sugars. The enzyme is specific for NADP⁺; NAD⁺ is not reduced in the presence of D-xylose or other sugars. Initial-velocity studies for the forward direction with xylose or NADP⁺ concentrations varied at fixed concentrations of the nucleotide or the sugar respectively revealed a pattern of parallel lines in double-reciprocal plots. K_m values for D-xylose and NADP⁺ were 8.8 mM and 0.09 mM respectively. Dead-end inhibition studies to confirm a ping-pong mechanism showed that NAD⁺ acted as an uncompetitive inhibitor versus NADP⁺ (K_i 5.8 mM) and as a competitive inhibitor versus xylose. D-Lyxose was a competitive inhibitor versus xylose and uncompetitive versus NADP⁺. These results fit better to a sequential compulsory ordered mechanism with NADP⁺ as the first substrate, but a ping-pong mechanism with xylose as the first substrate has not been ruled out. The presence of D-xylose dehydrogenase suggests that in mammalian liver D-xylose is utilized by a pathway other than the pentose phosphate pathway.

INTRODUCTION

The degradation of pentoses in mammalian liver is generally thought to occur exclusively via the pentose phosphate pathway, i.e. through the phosphorylation of free pentose and later conversion into ribulose 5-phosphate (Segal & Foley, 1959). Alternative pathways involving non-phosphorylated sugars are considered to be non-existent, or dismissed as 'vestiges of primitive pathways' (Horecker, 1968). However, non-phosphorylating alternative pathways for pentose utilization may occur, as attested by the finding in mammalian liver (Metzger & Wick, 1967; Schachter *et al.*, 1969; Mobley *et al.*, 1970; Ureta & Radojković, 1970; Yuen & Schachter, 1972; Majub *et al.*, 1973) of several NAD⁺- or NADP⁺-dependent dehydrogenases acting on free pentoses and catalysing the general reaction:

D-Xylose, a pentose present in most organisms, is the main component of hemicellulose, a structural material of all plants, and of heteropolysaccharides of plants, fungi, algae and animal tissues. As part of glycosylaminoglycans bound to proteins, xylose participates as an important structural component of membranes and

connective tissue, especially in mammals (Wissler & Logemann, 1984).

The oxidation of xylose to xylonic acid is known to occur in micro-organisms, and the enzyme involved, D-xylose dehydrogenase [D-xylose:NAD(P)⁺ 1-oxido-reductase, EC 1.1.1.175] has been identified in several prokaryotes (Buchert *et al.*, 1986), but, as far as we know, it has been studied in some detail in a few species only, as in the case of the enzyme from *Arthrobacter* sp. (Yamanaka *et al.*, 1977).

It has been shown in mammals that parenteral administration of [1-¹⁴C]xylose results in ¹⁴CO₂ production. Further, the rate of ¹⁴CO₂ evolution is higher if [1-¹⁴C]xylonic acid is injected instead. Tissue slices are able to oxidize D-xylose through the intermediate production of D-xylonic acid (Weser & Laster, 1968).

NADP⁺-dependent D-xylose dehydrogenases have been isolated from the crystalline lens of calf and pig (van Heyningen, 1958; Wissler, 1977; Wissler & Logemann, 1984) and from pig liver (Ureta & Radojković, 1970), but none of those enzymes has been adequately purified and characterized. In view of the possible functional relevance of these dehydrogenases to the catabolism of free xylose, we have focused our attention on an NADP⁺-linked dehydrogenase acting on D-xylose and other related pentoses, previously identified in pig liver by Schiwar *et al.* (1968) and by Ureta & Radojković (1970). The enzyme has now been purified to homogeneity and some of its physical and kinetic features

* This work is respectfully dedicated to Professor Dr. Hermann Niemeyer (Departamento de Biología, Facultad de Ciencias, Universidad de Chile) on his 71st birthday.

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were studied. Portions of this work were previously reported and published in abstract form (Zepeda & Ureta, 1986).

EXPERIMENTAL

Materials

Nicotinamide nucleotides, sugars and sugar derivatives, reagents for electrophoresis, phenylmethanesulphonyl fluoride, Reactive Blue 2-agarose and protein markers of known molecular size were products of Sigma Chemical Co. DEAE-cellulose (DE-52) was from Whatman. Hydroxyapatite (Bio-Gel HTP) was from Bio-Rad Laboratories. Other reagents were of the highest purity commercially available. Fresh pig liver was obtained in a local abattoir.

Measurement of xylose dehydrogenase activity

Xylose dehydrogenase activity was assayed at 30 °C by measuring the rate of reduction of NADP⁺ at 340 nm in a medium containing in a final volume of 0.5 ml the following (final concentrations): 10 mM-KCl, 85 mM-Tris/HCl buffer, pH 7.5, 12.6 mM-MgCl₂, 1.6 mM-EDTA, 0.5 mM-NADP⁺, 100 mM-D-xylose and enzyme (Ureta & Radojković, 1970). A system without sugar was used as a blank. Modifications of the standard assay system are described in the legends to Figures. One unit of enzyme activity is that amount catalysing the reduction of 1 μmol of NADP⁺ in 1 min under the described conditions. Specific activity is defined as units/mg of protein.

A Zeiss PMQ II spectrophotometer, thermostatically maintained at 30 °C and equipped with an automatic device for sequential monitoring of six simultaneous reactions, was used for the kinetic studies. Initial velocities were determined from the beginning of the progress curves. Substrate depletion was limited to less than 20%. Reactions were initiated by the addition of enzyme. For each experimental point triplicate determinations were performed.

Statistical analysis of the kinetic data

The initial-velocity and inhibition studies were analysed by using the program NATO, adapted to an IBM personal computer (Cornish-Bowden, 1985), which assesses the required weighting from the data set itself, avoiding the need for the experimenter to make prior assumptions about weights (Cornish-Bowden & Endrenyi, 1986). The non-linear least-squares regression procedure of the NATO program yields the kinetic parameters, the associated standard errors and goodness-of-fit characteristics.

Purification of D-xylose dehydrogenase

All operations were performed at 4 °C. Enzyme from each step of the purification procedure was suspended in buffer A (5 mM-Tris/HCl buffer, pH 8.5, containing 1 mM-EDTA, 10 mM-monothioglycerol and 1 mM-phenylmethanesulphonyl fluoride). Enzyme preparations in buffer A maintained constant activity during 6 months (longer times were not studied). Dialysis and concentration were performed in an Amicon ultrafiltration apparatus.

Preparation of crude extracts. About 300 g of pig liver was homogenized in a Waring blender with 1 vol. of buffer A. The homogenate was centrifuged at 105000 g

for 60 min in a Beckman L5-50B ultracentrifuge. The supernatant liquid was used as starting material.

Step 1: negative adsorption on CM-Sephadex. A 300 ml portion of the supernatant liquid was applied to a column (4.5 cm × 38 cm) of CM-Sephadex equilibrated with buffer A. Non-adsorbed fractions containing the enzyme activity were pooled (about 400 ml).

Step 2: (NH₄)₂SO₄ fractionation. Solid (NH₄)₂SO₄ to 45% saturation was added to the preparation from step 1 and the precipitate was removed by centrifugation. To the supernatant liquid further (NH₄)₂SO₄ was added to reach 70% saturation. The pellet was dissolved in buffer A (usually about 35 ml) and dialysed against the same buffer.

Step 3: chromatography on DEAE-cellulose. The preparation from step 2 was applied to a column (4.5 cm × 38 cm) of DEAE-cellulose equilibrated in buffer A. A linear KCl gradient (0 to 200 mM, total volume 2 litres) was then applied to elute xylose dehydrogenase at about 20 mM-KCl. Fractions with high enzyme activities were pooled, dialysed against buffer A and concentrated to approx. 5 ml.

Step 4: exclusion chromatography on Sephacryl S-300. The enzyme fraction from step 3 was layered on top of a Sephacryl S-300 column (2.5 cm × 75 cm) equilibrated and eluted with buffer A containing 100 mM-KCl. Enzyme fractions were pooled, dialysed against buffer A and concentrated to about 5 ml. Only a small increase of the specific activity is accomplished at this step, but it is essential for the increase in the next step to occur.

Step 5: Chromatography on hydroxyapatite. The enzyme fraction from step 4 was adsorbed on a column (1.3 cm × 17.5 cm) of hydroxyapatite mixed with an equal weight of cellulose and equilibrated with buffer A. Elution was accomplished with a linear gradient of KH₂PO₄ from 1 to 50 mM (pH 8). Fractions with high enzyme activities were pooled, dialysed against buffer A and concentrated.

Step 6: chromatography on Reactive Blue 2-agarose. The preparation from step 5 was adsorbed on a column (0.7 cm × 12 cm) packed with Reactive Blue 2-agarose equilibrated in buffer A and washed with a solution of the same buffer containing 150 mM-KCl. Xylose dehydrogenase was eluted with the same solution containing 1 mM-NADP⁺.

Protein determination

Protein concentrations were determined by the method of Bradford (1976).

PAGE

SDS/6–20%-PAGE was performed at 18 °C according to the procedure of Laemmli (1970). PAGE under non-denaturing conditions was performed in slab gels with the solutions described by Davis (1964).

Determination of M_r

A Sephacryl S-300 column (75 cm × 2.5 cm), equilibrated with buffer A containing 100 mM-KCl, was calibrated with rabbit muscle fructose-bisphosphate

Table 1. Purification of D-xylose dehydrogenase from pig liver

For experimental details see the text.

Step	Activity (units)	Protein (mg)	Sp. activity (units/mg)	Yield (%)	Purification (fold)
Starting supernatant	47	15347	0.0031	100	1
1. CM-Sephadex chromatography	62	5416	0.012	132	4
2. (NH ₄) ₂ SO ₄ fractionation	70	2010	0.035	149	11
3. DEAE-cellulose chromatography	43	276	0.15	91	48
4. Sephacryl S-300 chromatography	24	71	0.34	51	110
5. Hydroxyapatite chromatography	14.6	9.5	1.54	31	497
6. Reactive Blue 2-agarose chromatography	9.9	1.4	7.1	21	2290

aldolase, ovalbumin and cytochrome *c*. Void volume and internal volume were measured with Blue Dextran and [¹⁴C]lysine respectively. M_r values under denaturing conditions were estimated by SDS/PAGE calibrated with a commercial mixture of proteins of known molecular size.

RESULTS

Purification of D-xylose dehydrogenase

A typical protocol of the purification procedure (Table 1) resulted in a preparation with a specific activity of

6.1 ± 0.7 units/mg (mean $\pm$ S.D.; $n = 6$) and showing only one band of protein after SDS/PAGE (Fig. 1). The enzyme was purified over 2000-fold with a yield of about 20%. The protocol can be completed in 5 days and is very reproducible. The key step is the pseudo-affinity chromatography on Reactive Blue 2-agarose.

Determination of M_r

The M_r of the native enzyme was found to be 62000 ± 1300 ($n = 4$) by gel filtration (results not shown). The M_r of the denatured enzyme as determined by SDS/PAGE (results not shown) was 32100 ± 900 ($n = 3$). Thus D-xylose dehydrogenase is a dimer of subunits of equal or similar molecular mass.

Effect of pH

Maximal rates of D-xylose dehydrogenase were found at pH 8 (results not shown).

Substrate-specificity

Several sugars or sugar derivatives (D-xylose, L-xylose, D-ribose, D-arabinose, L-arabinose, D-lyxose, D-glucose, 6-phosphogluconate, D-glucose 6-phosphate, 2-deoxy-D-glucose, D-glucosamine, D-fructose, D-fucose, L-rhamnose and D-mannose) were tested as substrates at concentrations of 100 mM in the presence of either NADP⁺ or NAD⁺. The latter was inactive as hydrogen acceptor in all cases. Sugars found not to be substrates for xylose dehydrogenase include L-xylose (relative rate with respect to D-xylose: 7%), D-fucose and D-lyxose (1%). With D-mannose, D-fructose, L-rhamnose, D-glucosamine and the phosphate esters D-glucose 6-phosphate, 6-phosphogluconate and D-ribose 5-phosphate relative rates were almost undetectable.

Sugars able to reduce NADP⁺ with a relative velocity of at least 10% with respect to D-xylose were studied with greater detail (Table 2). Apparent K_m and V_{max} values were obtained according to the method of Eisenthal & Cornish-Bowden (1974). For the calculation of k_{cat} values ($K_{cat} = V_{max}/[E]_0$), the molar enzyme concentration, $[E]_0$, was calculated by using the M_r value of 62200 (see above). The specificity constant K_{sp} is expressed as the ratio $k_{cat}/K_m(app.)$ (Brot & Bender, 1969).

The enzyme oxidizes several pentoses and hexoses, but D-xylose showed the highest value of K_{sp} . D-Ribose and L-arabinose showed K_{sp} values 6-fold and 11-fold lower respectively than that of D-xylose, and K_{sp} values with hexoses were even lower.

Fig. 1. PAGE of the purified D-xylose dehydrogenase from pig liver

Lane A: PAGE under native conditions. A 5 μ g portion of the enzyme preparation after step 6 was electrophoresed as described in the Experimental section. After the run, the gel was immersed in a reaction mixture similar to the one used for the spectrophotometric assay but containing 0.4 mg of Nitro Blue Tetrazolium/ml and 0.04 mg of phenazine methosulphate/ml. Colour development occurred in the first 2 min. Lane B: PAGE in the presence of SDS. A 5 μ g portion of the same preparation was subjected to SDS/PAGE as described in the Experimental section and the gel was stained with Coomassie Blue. Lane C: a mixture of proteins of known molecular size was run in parallel with lane B (1, phosphorylase *b*; 2, BSA; 3, ovalbumin; 4, carbonic anhydrase; 5, trypsin inhibitor; 6, lysozyme).

Table 2. Substrate-specificity of D-xylose dehydrogenase

$V_{\max}$ and $K_{m(\text{app.})}$ values were measured according to the method of Eisenthal & Cornish-Bowden (1974). None of the sugars studied was able to reduce NAD^+ in the presence of the enzyme.

Sugar	$V_{\max}$ (units/ mg of protein)	$K_{m(\text{app.})}$ (M)	$k_{\text{cat.}}$ (s^{-1})	$K_{\text{sp.}}$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)
D-Xylose	7.6	0.0065	58	8923
D-Ribose	5.2	0.027	40	1481
L-Arabinose	5.9	0.057	45	789
2-Deoxy- D-glucose	2.9	0.054	22	407
D-Glucose	1.1	0.049	16	326
D-Galactose	1.5	0.14	12	86
D-Arabinose	1.2	0.13	9	69

Initial-velocity studies

Double-reciprocal primary plots of velocity against D-xylose and NADP^+ show basically parallel lines (Figs. 2 and 3). The lines were obtained by fitting the experimental data to eqn. (1) for a ping-pong mechanism (Cleland, 1963):

$$v = \frac{V_{\max} [A][B]}{K_m^A [B] + K_m^B [A] + [A][B]} \quad (1)$$

where [A] and [B] are the concentrations of xylose and NADP^+ respectively and K_m^A and K_m^B are the Michaelis constants for xylose and NADP^+ respectively.

The K_m value for NADP^+ of $93 \mu\text{M}$ is two orders of magnitude lower than the value for xylose (8.8 mM). The NADP^+ double-reciprocal primary plots exhibit strong upward curvatures at high NADP^+ concentrations (Fig. 3a), indicating substrate inhibition by NADP^+ . The inhibition tends to disappear at saturating xylose concentrations (Fig. 3a). The value of K_{si} (substrate

Fig. 2. Effect of D-xylose on the initial velocity of xylose dehydrogenase at various NADP^+ concentrations

Assays were made at 30°C in a total volume of 0.5 ml under the conditions described in the Experimental section, with NADP^+ and 0.04 mM ($\circ$), 0.05 mM ($\bullet$), 0.06 mM ($\square$), 0.08 mM ($\blacksquare$) and 0.1 mM ($\triangle$) and xylose concentrations varied from 1.5 mM to 30 mM. The lines for the reciprocal plot were drawn by using the parameters determined by fitting the data to a ping-pong reaction model by non-linear least-squares regression. The initial velocity is in μmol of NADP formed per min.

inhibition constant) for NADP^+ was dependent on the concentration of xylose. Thus at 50 mM- and 1.5 mM-xylose the values for K_{si} were 48.6 mM and 2.1 mM respectively. Secondary plots of the intercepts and slopes of Fig. 3(b) versus the reciprocal value of xylose showed straight lines. Similar results were obtained with L-arabinose as the sugar substrate (results not shown). With this pentose the K_m value for NADP^+ , calculated assuming a ping-pong mechanism, was $95 \mu\text{M}$. If the data were fitted to a sequential mechanism the corresponding value was $70 \mu\text{M}$. K_m values for L-arabinose were 88 mM and 71 mM respectively. Similarly to D-xylose, inhibition by high concentrations of NADP^+ was observed. Detailed studies of the kind summarized above were not performed

Fig. 3. Effect of NADP^+ on the initial velocity of xylose dehydrogenase at various D-xylose concentrations

(a) High (0.1–2 mM) NADP^+ concentrations. Xylose concentrations were 1.5 mM ($\circ$), 2 mM ($\bullet$), 3 mM ($\square$), 10 mM ($\blacksquare$) and 30 mM ($\triangle$). Each line was drawn by using the calculated values for an excess-of-substrate-inhibition model by non-linear least-squares regression. (b) Low (0.04–0.1 mM) NADP^+ concentrations. Xylose concentrations were 1.5 mM ($\circ$), 2 mM ($\bullet$), 3 mM ($\square$), 6 mM ($\blacksquare$), 10 mM ($\triangle$) and 30 mM ($\blacktriangle$). The lines for the reciprocal plot were drawn by using the parameters determined by fitting the data to a ping-pong reaction model by non-linear least-squares regression. Other details are as in the legend to Fig. 2.

with other sugar substrates (i.e. those listed in Table 2), mainly because their $V_{\max}$ values were very low and apparent K_m values were not very different to that of L-arabinose, which is about 10 times higher than the value for D-xylose (Table 2).

Tsopanakis & Herries (1975) have proposed a kinetic method to discriminate between ping-pong and sequential mechanisms. In this method velocities are measured at substrate concentrations such that the second substrate concentration occurs in a number of fixed ratios to the first. Straight lines are obtained in double-reciprocal plots with ping-pong mechanisms and parabolas with sequential mechanisms. In the case of xylose dehydrogenase double-reciprocal plots of velocity versus D-xylose or L-arabinose and NADP^+ concentrations at a constant ratio showed straight lines (results not shown).

The data summarized so far suggest a ping-pong mechanism for the order of addition of substrates and release of products from the enzyme. However, in an ordered sequential mechanism, if NADP^+ were the first

substrate with a lower value for K_{ia} than for the other kinetic constants, the equation for the initial velocity becomes similar to eqn. (1). To rule out this possibility, the experimental values were fitted to an equation for a sequential mechanism. The values obtained for K_m^A and K_m^B were similar to those shown above but that for K_{ia} was negative, and the effective sums of squares were rather similar for a ping-pong mechanism and for a sequential mechanism. With L-arabinose as substrate the K_{ia} value was $9 \mu\text{M}$ (with a rather large experimental error of $\pm 5 \mu\text{M}$) and the effective sums of squares were also similar for both mechanisms. These findings suggest that the best fitting was to eqn. (1).

Dead-end-inhibitor studies

In order to resolve the ambiguity between the mechanisms proposed above, a search for dead-end inhibitors was undertaken (Fromm, 1983). D-Lyxose was found to be a linear competitive inhibitor with respect to xylose at a NADP^+ concentration equivalent to $2.15 \times K_m$, with a value for the inhibition constant of $1.53 \pm 0.29 \text{ mM}$ (Figure not shown). Lyxose with respect to NADP^+ showed an uncompetitive type of inhibition at a xylose concentration equivalent to $4 \times K_m$, $0.8 \times K_m$ and $0.4 \times K_m$. The reciprocal plots of the data (Fig. 4) showed in all cases inhibition by NADP^+ at concentrations over approx. $5 \times K_m$.

Fig. 5 shows double-reciprocal plots for the inhibition produced by NAD^+ with NADP^+ as the substrate whose concentration was varied at xylose concentrations equivalent to $4 \times K_m$ (Fig. 5a) and $6.6 \times K_m$ (Fig. 5b). At both sugar concentrations the lines were essentially parallel at low NADP^+ concentrations. This behaviour

Fig. 4. Effect of D-xylose on the initial velocity of xylose dehydrogenase at various NADP^+ concentrations

Conditions were the same as for Fig. 2 with xylose at 30 mM (a), 6 mM (b) and 3 mM (c) and D-xylose absent ($\circ$) and at 100 mM ($\bullet$). NADP^+ concentrations varied from 0.05 to 2 mM. Each line was drawn by using the calculated values for an excess-of-substrate-inhibition model by non-linear least-squares regression. Other details are as in the legend to Fig. 2.

Fig. 5. Effect of NAD^+ on the initial velocity of xylose dehydrogenase at various NADP^+ concentrations

Conditions were the same as for Fig. 2 with xylose at 50 mM (a) and 30 mM (b) and NAD^+ absent ($\circ$) and at 5 mM ($\bullet$). NADP^+ concentrations varied from 0.05 to 2 mM. Each line was drawn by using the calculated values for an excess-of-substrate-inhibition model by non-linear least-squares regression. Other details are as in the legend to Fig. 2.

Table 3. NAD⁺-dependence of the value for the inhibition constant (K_{si}) by excess of NADP⁺ in the xylose dehydrogenase reaction

$K_{si(app.)}$ and $V_{max(app.)}$ were determined from each curve of the double-reciprocal plots shown in Fig. 5, by using the NATO Program (see the Experimental section).

Concn. of NAD ⁺ (mM)	Concn. of xylose (mM)	$V_{max(app.)}$ (mol/min)	$K_{si(app.)}$ (mM)
0	30	0.33 ± 0.013	28.3 ± 20.4
5	30	0.28 ± 0.06	1.1 ± 0.53
0	50	0.38 ± 0.0017	48.6 ± 7.58
5	50	0.33 ± 0.011	5.1 ± 1.03

was confirmed with four concentrations of NAD⁺ from 0 to 0.5 mM and six concentrations of NADP⁺ from 0.04 to 0.2 mM (results not shown). The effect of high xylose concentrations on the inhibition produced by excess of NADP⁺ was cancelled by NAD⁺ (Fig. 5 and Table 3). With xylose as the substrate whose concentration was varied in the presence of NAD⁺ (results not shown), the lines in a double-reciprocal plot intersected on the ordinate. The experimental data were fitted to a competitive-inhibition model and the calculated inhibition constants were 3.98 ± 0.35 and 4.94 ± 0.49 mM at NADP⁺ concentrations equivalent to $2.2 \times K_m$ and $0.9 \times K_m$ respectively. These values are at least 40-fold higher than the K_m value for NADP⁺ and in the same range of K_{si} . When the data were fitted to a mixed-inhibition model, the competitive component of the inhibition constant was in the same range of the values shown above and the inhibition constant for the uncompetitive component was two orders of magnitude higher. Therefore a mixed type of inhibition by NAD⁺ was rejected because the residual sum of squares resulted in a higher value than that for the competitive-inhibition model. A sequential mechanism would explain the inhibition by NAD⁺ with respect to both xylose and NADP⁺, since NAD⁺ could bind to E-NADP⁺ to form a ternary complex NAD⁺-E-NADP⁺, which increases when the concentration of NADP⁺ is increased (Fig. 5). It must be pointed out, however, that this explanation implies site-site interaction, which is not supported by the kinetics observed.

DISCUSSION

Several enzymes catalysing the nicotinamide nucleotide-linked oxidation of free pentoses have been described. The best known are those acting on D-arabinose, as well as on L-fucose (6-deoxygalactose), in *Pseudomonas* (Palleroni & Doudoroff, 1956; Cline & Hu, 1965a,b,c) and *Neurospora* (Pincheira *et al.*, 1973; Carrasco *et al.*, 1981), and also in mammals (Metzger & Wick, 1967; Schiwarra *et al.*, 1968; Schachter *et al.*, 1969; Ureta & Radojković, 1970; Mobley *et al.*, 1970; Maijub *et al.*, 1973; Carper *et al.*, 1974; Mobley & Metzger, 1978; Endo & Hiyama, 1979).

D-Xylose dehydrogenases have been recognized in micro-organisms (Buchert *et al.*, 1986, and references cited therein), but, as far as we know, only that from *Arthrobacter* (Yamanaka *et al.*, 1977) has been partially purified and characterized. An NADP⁺-dependent xylose

dehydrogenase was briefly described in pig liver (Schiwarra *et al.*, 1968; Ureta & Radojković, 1970). A short account on a similar enzyme from pig lens has been reported (Wissler, 1977; Wissler & Logemann, 1984). Newton *et al.* (1982) have published a detailed genetic study of an NADP⁺-dependent xylose dehydrogenase from mouse liver.

The NADP⁺-dependent D-xylose dehydrogenase from pig liver is a dimeric protein of M_r about 62000. This value is identical with the one obtained by gel filtration of a semipurified NADP⁺-dependent D-xylose dehydrogenase from *Arthrobacter* (Yamanaka *et al.*, 1977). On the other hand, an M_r value of 48000 was reported by Wissler & Logemann (1984) for the NADP⁺-dependent D-xylose dehydrogenase of pig lens.

Most sugar dehydrogenases display wide substrate-specificity, a fact that should advise against undue haste in naming or assigning an EC number to a newly isolated enzyme of this type. In fact, Metzger *et al.* (1970) suggested that the xylose dehydrogenase activity from rat or sheep liver may be merely a marginal reaction of some other enzyme. The specificity studies discussed below suggest that the enzyme now isolated from pig liver may be considered as a xylose dehydrogenase inasmuch as D-xylose showed the highest specificity constant among 38 sugars or sugar derivatives, most of them reasonably potential substrates. The relatively high value of the K_m for D-xylose (8.8 mM) should not be disqualifying, since K_m values in the millimolar range are not uncommon. The xylose dehydrogenase of *Arthrobacter* (specifically induced by D-xylose) has a K_m value for the sugar of about 17 mM, which is even higher than that of the pig liver enzyme.

Fig. 6. Ring structures of sugars of which some are substrates for the D-xylose dehydrogenase reaction

Sugars listed in Table 2 were able to reduce NADP^+ in the presence of the dehydrogenase. The β -pyranose structures of some of the substrates and related inactive sugars are shown in Fig. 6. The best substrate is a β -D-pentopyranose with equatorial orientation of hydroxy groups at C-2, C-3 and C-4, i.e. β -D-xylopyranose. An axial hydroxy group at C-2, as in D-lyxose or D-mannose, is incompatible with oxidation, but its absence, as in 2-deoxy-D-glucose, is not. An equatorial hydroxy group at C-3, as in D-ribose, drastically decreases 6-fold the value of K_{sp} , mainly because of an increase in the apparent K_m value. A similar effect is apparent with respect to the hydroxy group at C-4. The primary alcohol group must be on C-5, since with hexoses the values of K_{sp} decrease to about 5% of the value obtained with D-xylose. Phosphorylation of the primary alcohol group, as in ribose 5-phosphate, precludes oxidation.

The same occurs with aldohexose 6-phosphates, although in the case of the pentose phosphate a predominant furanose conformation, as in D-fructose, may account for a compound's inertness as a substrate.

The parallel lines in the double-reciprocal plots of Figs. 2 and 3, the straight lines obtained in double-reciprocal plots of velocity *d*-xylose and NADP^+ concentrations (or L-arabinose and NADP^+ concentrations at a constant ratio, and the inhibition by excess of NADP^+ that is abolished by high xylose concentrations readily suggest a ping-pong mechanism. However, a consideration of all the results, especially the characteristics of the inhibition by NAD^+ and the similarity (within the experimental error) of the K_m values for NADP^+ with D-xylose or L-arabinose as the sugar substrate, leads us to propose a sequential addition of the substrates to the enzyme in a compulsory order with NADP^+ as the first substrate. It is generally accepted that nicotinamide nucleotide-dependent dehydrogenases follow a sequential ordered mechanism with the nucleotide as the first ligand to bind the enzyme (Dalziel, 1975), although in the case of a fish hexose-6-phosphate dehydrogenase the substrate binds before the coenzyme (Ropson & Powers, 1988). The sugar dehydrogenase NADP^+ -dependent glucose dehydrogenase and NAD^+ -dependent arabinose (fucose) dehydrogenase, both from pig liver, show a sequential ordered Bi Bi mechanism (Carper *et al.*, 1976). It is true, however, that under certain conditions some NAD^+ -dependent dehydrogenases display ping-pong kinetics. That is the case for the horse and mouse liver alcohol dehydrogenases (Gershman & Abeles, 1973) and the malate/lactate dehydrogenase (Allen & Patil, 1972). These enzymes bind the nucleotide in such a strong way that the cofactor has even been considered as a prosthetic group rather than a substrate (Spector, 1982). In the case of pig liver D-xylose dehydrogenase further studies will be necessary to distinguish between a sequential or a ping-pong mechanism, since several enzymes have shown parallel lines in double-reciprocal plots in spite of kinetics other than ping pong, as in the case of brain hexokinase (Purich & Fromm, 1971), a phosphofructokinase from *Dictyostelium* (Baumann & Wright, 1968) and a protein kinase from *Neurospora* (Gold & Segel, 1974). The recent description (Aird *et al.*, 1987) of a prokaryotic sulphurtransferase showing parallel or converging lines in double-reciprocal plots according to the acceptor substrate employed is just another example of the difficulties that may arise in the kinetic analysis of some enzymes.

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