Aldehyde oxidation in human placenta

Purification and properties of 1-pyrroline-5-carboxylate dehydrogenase

Jaume FARRÉS, Pere JULIÀ and Xavier PARÉS

Departament de Bioquímica i Biologia Molecular, Facultat de Ciències, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

The human placenta contains a considerable amount of 1-pyrroline-5-carboxylate dehydrogenase ($23 \pm 6 \mu\text{g/g}$; $n = 12$), about 25 % of the concentration present in liver. The enzyme is the only form in placenta that oxidizes short- and medium-chain aldehydes, which facilitates its purification from this organ. It can be purified to homogeneity by successive chromatographies on DEAE-cellulose, 5'-AMP-Sepharose and Sephacryl S-300. From 500 g of tissue, about 2.1 units of enzyme can be obtained with a 12 % yield. Placental 1-pyrroline-5-carboxylate dehydrogenase is a dimer of M_r -63000 subunits. It exhibits a pI of 6.80–6.65, and is specific for 1-pyrroline-5-carboxylate, the cyclic form of glutamate γ -semialdehyde ($K_m = 0.17 \text{ mM}$, $k_{\text{cat}} = 870 \text{ min}^{-1}$), although it also oxidizes short-chain aliphatic aldehydes such as propionaldehyde ($K_m = 24 \text{ mM}$, $k_{\text{cat}} = 500 \text{ min}^{-1}$). These properties are very close to those of the liver enzyme, indicating a strong similarity between the enzyme forms from both organs. The enzyme is highly sensitive to temperature, showing 50 % inhibition after incubation for 0.8 min at 45 °C or after 23 min at 25 °C. It is irreversibly inhibited by disulfiram, and a molar ratio inhibitor:enzyme of 60:1 produced 50 % inhibition after incubation for 10 min. A subcellular-distribution study indicates that the enzyme is located in two compartments: the mitochondria, with 60 % of the total activity, and the cytosol, with 40 % activity. The physiological role of the enzyme in placental amino acid metabolism is discussed.

INTRODUCTION

We have recently become interested in the enzymes of alcohol and aldehyde metabolism in human placenta [1]. We have demonstrated that the placenta exhibits a single alcohol dehydrogenase isoenzyme, χ -ADH, specific for long-chain alcohols and aldehydes. A possibility existed that placenta could also contain an aldehyde dehydrogenase with a related specificity, that could be involved in the same metabolic pathway as χ -ADH. In the present work we show that the major enzyme in human placenta catalysing the oxidation of propionaldehyde and aldehydes of different structures can be identified as 1-pyrroline-5-carboxylate (P5C) dehydrogenase (EC 1.5.1.12). The enzyme catalyses the oxidation of glutamic γ -semialdehyde to glutamic acid, although the true substrate is the cyclic structure P5C [2]. The reaction represents a central step in the metabolism of arginine, ornithine, proline and glutamic acid. The enzyme has been mostly studied in non-mammalian sources [3], although several reports existed with partially purified enzyme from bovine liver [2,4] and kidney [5], and from rat liver [6]. P5C dehydrogenase has been purified and partially characterized from human liver, demonstrating that this enzyme accounts for the high- K_m aldehyde dehydrogenase activity previously known as E4 [7].

Here we show that the P5C dehydrogenase purified from placenta exhibits physical and kinetic properties very similar to those of the hepatic form. The characterization of the enzyme has been completed with studies on electrophoretic properties, disulfiram inhibition, heat-stability and subcellular localization. In this respect we demonstrate that this enzyme, which had been considered

exclusively mitochondrial [2,3,5], is to a significant extent also localized in the cytosol.

EXPERIMENTAL

Materials

DEAE-cellulose was obtained from Whatman. 5'-AMP-Sepharose 4B, NAD⁺ (grade III), NADP⁺ (grade III), DTT, disulfiram, succinic semialdehyde, 4-nitrophenyl acetate and starch for gel electrophoresis were from Sigma. Glutamic γ -semialdehyde was obtained by hydrolysis of DL-P5C 2,4-dinitrophenylhydrazone hydrochloride (Sigma) by the method of Mezl & Knox [8]. EDTA (Titriplex III), acetaldehyde and octanal were from Merck. Propionaldehyde was from Aldrich, 3-nitrobenzaldehyde was from Fluka, and Sephadex G-200 and Sephacryl S-300 were from Pharmacia Fine Chemicals. All other chemicals were reagent grade. Volatile aldehydes were redistilled shortly before use.

Human tissues

Full-term placentas were frozen at -30°C within 2 h after normal delivery and used within 2 months, except for subcellular-fractionation experiments, in which fresh tissue was employed. Human liver and stomach samples were obtained within 36 h *post mortem* from adults who had died suddenly. Specimens were stored at -30°C . Before analysis samples were thawed, cut into small pieces and homogenized in 10 mM-Tris/HCl (pH 8.2)/1 % (w/v) Triton X-100. The homogenates were centrifuged at 11 000 g for 1 h, and the supernatants were used for activity assays and electrophoretic analysis.

Subcellular fractionation

All steps were carried out at 4 °C. Fresh placenta (20 g) was cleaned of fetal membranes and large blood vessels, washed thoroughly with cold buffer (0.25 M-sucrose/5 mM-Tris/HCl/1 mM-EDTA/1 mM-DTT, pH 7.4) to remove blood, and cut into small pieces. Placental homogenates were prepared by grinding the tissue in 1 vol. of the buffer in a Potter-Elvehjem glass homogenizer with a Teflon pestle. The homogenate was then filtered through four layers of surgical gauze and centrifuged at 754 *g* for 10 min. The sediment was resuspended in the buffer and designated the nuclear fraction (N). The supernatant, named cytoplasmatic extract (E), was centrifuged at 20400 *g* for 10 min. The corresponding sediment was resuspended in buffer and centrifuged at 20400 *g* for 10 min. The final pellet was resuspended in buffer and designated the mitochondrial fraction (M). The combined supernatants were centrifuged at 106000 *g* for 60 min. The resulting pellet was resuspended in buffer and designated the microsomal fraction (P), and the supernatant constituted the cytosolic fraction (S).

Before analysis of marker-enzyme activity, samples were treated with 1% Triton X-100. Lactate dehydrogenase (EC 1.1.1.27) was determined by monitoring the disappearance of NADH during the reduction of pyruvate to lactate. The reaction mixture contained 0.2 mM-NADH and 0.76 mM-sodium pyruvate. Glutamate dehydrogenase (EC 1.4.1.3), monoamine oxidase (EC 1.4.3.4) and NADPH-cytochrome *c* reductase (EC 1.6.2.4) were assayed as described elsewhere [9].

Activity assay

The P5C dehydrogenase activity was determined spectrophotometrically at 340 nm and 25 °C in a Cary 219 instrument. Standard assay of a partially purified enzyme was performed in 0.1 M-sodium phosphate, pH 7.5, 0.5 mM-NAD⁺ and 0.2 mM-glutamic γ -semi-aldehyde, or in 33 mM-sodium pyrophosphate, pH 8.5, 0.5 mM-NAD⁺ and 13.3 mM-propionaldehyde. The latter conditions were also used for measuring total aldehyde dehydrogenase activity of tissue homogenates, whereas 68 μ M-propionaldehyde was employed for determining the contribution of low-*K_m* enzymic forms of aldehyde dehydrogenase. The activity of high-*K_m* enzymes was then calculated by subtracting low-*K_m* activity from total aldehyde dehydrogenase activity. Esterase activity was measured by monitoring the increase in *A*₄₀₀ caused by the release of 4-nitrophenol from 4-nitrophenyl acetate. Activities were expressed as units (1 μ mol of substrate transformed/min).

Kinetic results were analysed by using the Lineweaver-Burk plot and linear regression by least-squares fitting, unless otherwise stated.

Purification of human placenta P5C dehydrogenase

All processes were performed at 4 °C. The buffers contained 1 mM-EDTA and 1 mM-DTT, and were evacuated and saturated with N₂ before use. In a typical experiment, 500 g of placenta was homogenized in 500 ml of 10 mM-Tris/HCl buffer, pH 8.2. The homogenate was treated with 1% Triton X-100 for 15 min, and centrifuged at 11000 *g* for 1 h. The supernatant was then filtered through a DEAE-cellulose bed (2 cm $\times$ 10 cm) and dialysed against 10 mM-Tris/HCl, pH 8.2. The

sample was applied to a DEAE-cellulose column (5 cm $\times$ 50 cm) equilibrated with 10 mM-Tris/HCl, pH 8.2. The column was washed with 1 litre of the same buffer and the enzyme was eluted with a 3 litre linear gradient of 0–150 mM-NaCl in the buffer. Fractions containing P5C dehydrogenase activity were pooled, dialysed against 10 mM-sodium phosphate, pH 6.0, and applied to a 5'-AMP-Sepharose column (1 cm $\times$ 22 cm) equilibrated with 10 mM-sodium phosphate, pH 6.0. The column was washed successively with this buffer (300 ml) and 50 mM-sodium phosphate, pH 6.0 (300 ml). P5C dehydrogenase activity was eluted with 500 ml of a linear gradient of 0–60 μ M-NADH in 50 mM-sodium phosphate, pH 6.0. Fractions containing P5C dehydrogenase activity were pooled, concentrated and chromatographed on a Sephacryl S-300 column (2.3 cm $\times$ 90 cm), equilibrated with 30 mM-sodium phosphate, pH 7.3. Active fractions were pooled, concentrated and stored at 4 °C in tubes flushed with N₂ and tightly sealed.

Protein concentrations were determined by the method of Bradford [10], with bovine serum albumin (Sigma) as the standard.

Electrophoretic analysis

Horizontal starch-gel electrophoresis was carried out at 4 °C, pH 7.6, for 8 h at 720 V in a Multiphor instrument (LKB). Each electrode reservoir contained 1 litre of 0.3 M-Tris/H₃PO₄, pH 7.6, and 10 mM-EDTA; 200 mg of NAD⁺ was added to the cathodic reservoir. After electrophoresis, the gel was sliced into two layers and stained for aldehyde dehydrogenase activity with 250 ml of 50 mM-Tris/HCl buffer, pH 8.6, containing 200 mg of NAD⁺ (Sigma, grade AA-1), 100 mg of Nitro Blue Tetrazolium (Sigma), 7 mg of phenazine methosulphate (Sigma), 300 mg of sodium pyruvate (Sigma), and 2 ml of freshly distilled propionaldehyde. The procedure was performed at two different temperatures: 20 °C and 45 °C.

Electrophoresis on SDS/polyacrylamide gels and isoelectric focusing were performed as previously reported [11].

M_r determination

The apparent *M_r* of placental P5C dehydrogenase was determined by using columns (2.3 cm $\times$ 90 cm) of Sephadex G-200 and Sephacryl S-300 (both Superfine grade), by using the same conditions as previously described for the analysis of alcohol dehydrogenase [11].

Inhibition with disulfiram

Before the inhibition experiments, DTT was removed from the enzyme by exhaustive dialysis against 50 mM-Hepes, pH 7.0, saturated with N₂. Disulfiram was dissolved in methanol, and added to the 3 ml assay mixture to give final concentrations of 8.4 nM–40 μ M. The highest amount of methanol added to the assay was 10 μ l, which did not affect the enzymic activity.

RESULTS

Aldehyde oxidation in human placenta homogenates

P5C dehydrogenase activity with 0.2 mM-glutamic γ -semialdehyde was measured in homogenates of 12 full-

term placentas. The activity per g of fresh tissue was 95 ± 25 munits (mean $\pm$ s.d.). This enzyme was also responsible for the high- K_m aldehyde dehydrogenase activity with 13.3 mM-propionaldehyde (33 ± 9 munits/g). The contribution of low- K_m aldehyde dehydrogenase isoenzymes (activity with $68 \mu\text{M}$ -propionaldehyde) was very low: 2.2 ± 1.2 munits/g.

Purification of P5C dehydrogenase from human placenta

Table 1 summarizes the purification of P5C dehydrogenase from human placenta. On the DEAE-cellulose column, all propionaldehyde dehydrogenase and P5C dehydrogenase activities co-eluted in a single peak, before haemoglobin, during the NaCl gradient. At this stage the enzyme preparation was contaminated with a high amount of lactate dehydrogenase activity. Subsequently, the two enzymes were separated by affinity chromatography on a 5'-AMP-Sepharose column: P5C dehydrogenase activity was eluted by the NADH gradient, whereas lactate dehydrogenase remained bound to the column under the conditions used.

The last purification step was gel-filtration chromatography on Sephacryl S-300, which eliminated residual contaminating proteins. The purified enzyme was stable for several weeks when stored at 4 °C in the presence of 1 mM-DTT under a N_2 atmosphere. SDS/polyacrylamide-gel electrophoresis of the purified enzyme exhibited only one band when the gel was stained for protein.

Electrophoretic analysis

Starch-gel electrophoresis of placental homogenate did not demonstrate any activity band when the gel was stained with propionaldehyde or glutamic γ -semi-aldehyde (0.1 M) as a substrate at 45 °C (Fig. 1). However, a very faint band on the anodic side of the gel appeared when the staining procedure was performed at 20 °C. At both temperatures the purified placental P5C dehydrogenase exhibited several anodic bands, although they were much more intense at 20 °C than at 45 °C. Placental P5C dehydrogenase had the same mobility as the liver P5C dehydrogenase, previously known as the E4 iso-

Table 1. Purification of P5C dehydrogenase from human placenta

Starting material was 500 g of full-term human placenta. Activity was measured with 13.3 mM-propionaldehyde and 0.5 mM- NAD^+ in 33 mM-sodium pyrophosphate, pH 8.5.

Step	Vol. (ml)	Concn. (unit/ml)	Total activity (units)	Protein (mg/ml)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Supernatant	750	0.023	17.6	90	0.00026	100	1
DEAE-cellulose	300	0.051	15.2	2.3	0.022	86	85
5'-AMP-Sepharose	50	0.072	3.6	0.078	0.92	20	3500
Sephacryl S-300	15	0.14	2.1	0.10	1.43	12	5500

Fig. 1. Starch-gel electrophoresis of human tissue homogenates and purified P5C dehydrogenase

Electrophoresis was performed at pH 7.6 and 720 V for 8 h. After electrophoresis the gel was divided into two slices, (a) and (b), which were stained for activity with propionaldehyde. Temperature of the staining incubation was 20 °C for slice (a), and 45 °C for slice (b). Samples: 1, liver homogenate; 2, stomach homogenate; 3, purified placental P5C dehydrogenase (P5CD); 4, placental homogenate; 5, same as sample 3; 6, partially purified placental P5C dehydrogenase with similar activity to sample 5, but containing lactate dehydrogenase activity. O, origin. E1, E2 and E3 are isoenzymes of aldehyde dehydrogenase [7,13,17].

enzyme of aldehyde dehydrogenase [7], which, in addition, showed similar sensitivity to the staining temperature. In contrast, all other forms with propionaldehyde dehydrogenase activity of the human liver and stomach stained much better at 45 °C than at 20 °C.

An additional observation was that those partially purified preparations which also contained lactate dehydrogenase activity did not stain as well as the homogeneous placental enzyme (Fig. 1). As indicated in the Experimental section, pyruvate was routinely added to the staining buffer for the purpose of inhibiting the oxidation of endogenous lactate by lactate dehydrogenase. Therefore, a plausible explanation is that NADH produced during aldehyde oxidation is used by lactate dehydrogenase for the reduction of pyruvate, thus interfering with the staining procedure (pyruvate also apparently inhibited the spectrophotometric activity assay of P5C dehydrogenase preparations contaminated with lactate dehydrogenase, but did not inhibit the purified enzyme). In this manner, detection of P5C dehydrogenase from placenta and, possibly, from liver homogenates, is hampered owing to co-migration with a lactate dehydrogenase isoenzyme (probably LDH4, according to its electrophoretic mobility [12]). This phenomenon, and the thermolability at the usual staining temperature (35–45 °C), would explain why previous analysis of propionaldehyde dehydrogenase activity in the human placenta did not detect this enzyme [13,14], and why liver P5C dehydrogenase is poorly stained in the electrophoresis of liver homogenates [7]. Addition to the staining solution of 20 mM-oxalate (an inhibitor of lactate dehydrogenase), instead of pyruvate, greatly increased the sensitivity of the staining procedure for both placental and liver enzymes.

Isoelectric focusing of purified placental P5C dehydrogenase revealed a major band at pI 6.80 and a minor band at pI 6.65.

M_r

Gel filtration of placental P5C dehydrogenase on Sephacryl S-300 and Sephadex G-200 resulted in a single symmetrical peak of activity in both columns, corresponding to an apparent M_r of 100 000 and 114 000 respectively. SDS/polyacrylamide-gel electrophoresis of the purified enzyme resolved a single band from which a subunit M_r of 63 000 was calculated. Therefore the enzyme appears to be a dimer with subunits of identical M_r .

Thermal stability

The electrophoretic bands of placental and hepatic P5C dehydrogenase on starch gel stained much better at 20 °C than at 45 °C, suggesting that the enzyme is especially sensitive to high temperatures. Therefore, we studied the thermolability of the propionaldehyde dehydrogenase activity in liver and placental homogenates, and in a purified preparation of placental P5C dehydrogenase.

Heat-denaturation curves at 45 °C for the enzyme-forms with propionaldehyde dehydrogenase activity revealed significant differences in thermal stability between low- K_m and high- K_m enzymes (Fig. 2). After 10 min incubation at 45 °C, low- K_m isoenzymes of liver homogenates retained more than 90 % of their initial activity. In contrast, high- K_m forms present in hepatic (E3 and P5C dehydrogenase) and placental (P5C dehydrogenase)

Fig. 2. Thermal stability of human propionaldehyde dehydrogenase activity at 45 °C

■, Low- K_m activity of liver homogenate with 68 μ M-propionaldehyde as substrate; ●, high- K_m activity of liver homogenate, calculated as the difference between total (13.3 mM-propionaldehyde) and low- K_m (68 μ M-propionaldehyde) activities; ○, placental homogenate activity with 13.3 mM-propionaldehyde.

Fig. 3. Thermal stability of purified placental P5C dehydrogenase

Activities at 25 °C (○), at 45 °C without any protective agent (●), at 45 °C with 1 mM-DTT (▲) and at 45 °C with 0.5 mM-NAD⁺ (■) are shown.

homogenates, and the purified enzyme from placenta (Fig. 3), were much more unstable, retaining less than 10 % of their original activity under the same conditions. When 0.5 mM-NAD⁺ or 1 mM-DTT was added to incubation buffer, this provided some protection for the purified P5C dehydrogenase, but only during the first few minutes (Fig. 3). The enzyme, in the absence of any protective agent, was much more stable at 25 °C, although a 25 % inactivation was observed after 10 min incubation at this temperature.

For the purified P5C dehydrogenase, a plot of log activity versus incubation time (not shown) results in thermal inactivation constants [15] of 84 min⁻¹ at 45 °C and 0.03 min⁻¹ at 25 °C. The time required for 50 % inactivation ($t_{1/2}$) was 0.8 min at 45 °C and 23 min at 25 °C.

Kinetic properties

Apparent K_m values for NAD⁺ and NADP⁺ were determined with 13.3 mM-propionaldehyde as the substrate in 33 mM-sodium pyrophosphate, pH 8.5. The

Table 2. Kinetic constants of human placenta P5C dehydrogenase

Activity was measured in 33 mM-sodium pyrophosphate, pH 8.5, with 0.5 mM-NAD⁺, except where otherwise indicated.

Substrate	K_m (mM)	$k_{cat.}$ (min ⁻¹)	$k_{cat.}/K_m$ (mM ⁻¹ ·min ⁻¹)
Acetaldehyde	22	150	6.8
Propionaldehyde	24	500	21
Octanal	4	130	33
Succinic semialdehyde	0.03*	9.6	320
Glutamic γ -semialdehyde†	0.17	870	5200
3-Nitrobenzaldehyde‡	1.3	110	86
4-Nitrophenyl acetate‡§	0.4	50	150

* The value represents the $s_{0.5}$ (semi-saturation constant) calculated from the Hill plot.

† Activity measured in 0.1 M-sodium phosphate, pH 7.0.

‡ Activity measured in 33 mM-sodium phosphate, pH 7.5.

§ Esterase activity was measured as indicated in the Experimental section.

enzyme preferentially used NAD⁺ as the coenzyme, but it was also active with NADP⁺. Thus the K_m for NADP⁺ (0.62 mM) was 35 times that for NAD⁺ (0.017 mM), whereas the maximum velocity was twice as great for NAD⁺.

Aldehydes having a wide variety of molecular structures were assayed as substrates and the kinetic constants for them calculated (Table 2). Propionaldehyde was one of the best substrates in terms of $k_{cat.}$, and, because of its availability, it was used as substrate for the determination of standard activity. Glutamic γ -semialdehyde, or more correctly its cyclic form P5C, was the most outstanding substrate, with the highest $k_{cat.}$ value. Although its K_m is not as low as that for succinic semialdehyde, glutamic γ -semialdehyde showed the best specificity constant, $k_{cat.}/K_m$, indicating that the enzyme is relatively specific for this substrate.

With succinic semialdehyde, a metabolite of the γ -aminobutyrate shunt pathway, the enzyme displayed a non-linear Lineweaver-Burk plot. A semi-saturation constant ($s_{0.5}$) of 30 μ M was calculated by fitting the experimental values to the Hill equation for negative cooperativity. The enzyme is also active with aromatic aldehydes such as 3-nitrobenzaldehyde, and has esterase activity with 4-nitrophenyl acetate as a substrate.

Inhibition by disulfiram

Placental P5C dehydrogenase was inhibited by disulfiram, a well-known inhibitor of aldehyde dehydrogenase. When the enzyme (18 nM) was incubated in the presence of 40 μ M-disulfiram for 10 min at 25 °C, activity was fully suppressed. However, when the incubation buffer also contained 0.5 mM-NAD⁺ or 1 mM-DTT, the remaining activity was nearly 100%. When 1 mM-DTT was added after the 10 min incubation with disulfiram, only 10% of the original activity was restored, indicating that inhibition was practically irreversible.

In order to investigate the stoichiometry of the reaction, enzymic activity was measured after 10 min incubation at 25 °C, as a function of the disulfiram/enzyme molar ratio (Fig. 4a). A molar ratio of 60:1

Fig. 4. Inhibition of placental P5C dehydrogenase by disulfiram

(a) Percentage of remaining activity versus disulfiram/enzyme molar ratio. Incubation time was 10 min. (b) Inhibition kinetics at a constant molar ratio of 100 mol of disulfiram/mol of enzyme. In both experiments the 100% activity was that of the enzyme incubated without disulfiram, at 25 °C.

produced 50% inhibition, indicating a much lower susceptibility to disulfiram than for the cytosolic E1 aldehyde dehydrogenase, which is fully inhibited at a molar ratio of 4:1 [16]. The kinetics of the inhibition was studied at a constant disulfiram/enzyme ratio of 100:1 (Fig. 4b). The inhibition is rapid during the first few minutes. Thus, after 4 min incubation, activity is decreased by 50%. The inhibition rate slows down after this initial period, and after 1 h incubation the enzyme still retains 15% of its initial activity.

Subcellular distribution of P5C dehydrogenase

Table 3 shows the activity of P5C dehydrogenase and marker enzymes in different subcellular fractions. About 60% of the activity appeared to be clearly associated with the mitochondria, but a considerable amount of activity (about 40%) was recovered in the cytosolic fraction. This cytosolic activity apparently did not arise from the mitochondrial matrix or the mitochondrial outer membrane, as judged by the low recovery of the marker enzymes glutamate dehydrogenase and monoamine oxidase in the final supernatant. Moreover, little P5C dehydrogenase activity was detected in washings of isolated mitochondria. Starch-gel electrophoresis of the mitochondrial and cytosolic preparations revealed P5C dehydrogenase bands of identical mobility (Fig. 5). In addition, K_m values of both fractions for propionaldehyde were similar.

Table 3. Intracellular distribution of P5C dehydrogenase and marker enzymes in human placenta

Fractions: N, nuclear; M, mitochondrial; P, microsomal; S, final supernatant or cytosol; E, initial cytoplasmic extract. The values are means of two different experiments. Recovery = $100 \times (N + M + P + S)/(N + E)$.

Enzymatic activity	Total activity (N + E) (munits/g)	Distribution (%)				Recovery (%)
		N	M	P	S	
P5C dehydrogenase	8.85	8.3	58.5	5.8	40.1	112.7
Glutamate dehydrogenase	319.8	16.0	52.4	13.4	6.4	88.7
Monoamine oxidase	1.3	0.0	58.3	10.8	5.6	80.4
NADPH-cytochrome <i>c</i> reductase	229.1	13.9	25.8	43.2	13.5	96.4
Lactate dehydrogenase	6520	4.2	0.9	1.8	78.8	85.7
Alcohol dehydrogenase	14.5	1.6	0.0	0.0	93.5	95.1

Fig. 5. Starch-gel electrophoresis of P5C dehydrogenase from mitochondrial and cytosolic preparations of human placenta

Electrophoresis was performed as indicated in Fig. 1, with a staining temperature of 20 °C. Two different placentas were analysed. Samples: M1, mitochondrial fraction of placenta 1; M2, mitochondrial fraction of placenta 2; C1, cytosolic fraction of placenta 1; C2, cytosolic fraction of placenta 2.

The results shown in Table 3 confirm our previous report on the exclusive localization of human placenta alcohol dehydrogenase (χ -ADH isoenzyme) in the cytosol [1].

DISCUSSION

In the present study we have purified and characterized a P5C dehydrogenase from human placenta. This enzyme exhibits some typical properties of human liver aldehyde dehydrogenases, such as esterase activity, instability in the absence of reducing agents, and disulfiram-sensitivity. It is clear, however, that the enzyme is quite different in molecular properties from the E1 and E2 isoenzymes of aldehyde dehydrogenase. Whereas these low- K_m isoenzymes are tetramers of M_r around 220 000 [17], the placental enzyme appears to be a dimer of approx. M_r 114 000. Moreover, the placental enzyme is the least anodic-migrating form that is stained with propionaldehyde as a substrate, with an electrophoretic mobility similar to that of the liver P5C dehydrogenase. The hepatic enzyme has been recently charac-

terized [7], and its properties are practically identical with those of the placental P5C dehydrogenase purified in the present work. This indicates that the placental and hepatic P5C dehydrogenases are very similar enzyme forms.

The placenta is a suitable source of human P5C dehydrogenase for several reasons. It is the most available human tissue and can be obtained a few minutes after being functional. The amount of enzyme is considerable, $23 \pm 6 \mu\text{g/g}$ of placenta, about one-fourth of the concentration found in liver. Whereas liver contains four or five enzymic forms with aldehyde dehydrogenase activity of broad specificity, P5C dehydrogenase is the only form in placenta that is active with short- and medium-chain aldehydes. This simplifies its identification and purification from this tissue. Thus in three fast purification steps (1 week of work) 2.1 units of homogeneous enzyme can be obtained. A fast procedure such as this is convenient because of the instability of the enzyme during the process.

Starch-gel electrophoresis of the purified preparation resolved three or four activity bands, whereas two bands were detected by isoelectric focusing. However, heterogeneity was observed neither in the M_r determination of the placental enzyme by gel filtration nor in polyacrylamide-gel electrophoresis in the presence of SDS. Furthermore, no evidence for different enzymic forms was detected during the purification procedure or in the kinetic analysis. Therefore, the heterogeneity displayed in electrophoresis is probably a consequence of post-translational modifications or sample manipulation. The fact that the number of bands increases in parallel with the progress of purification (compare Figs. 1 and 5) supports the last hypothesis. On the other hand, an activity band close to the origin was always observed in starch-gel electrophoresis (but not in isoelectric focusing on polyacrylamide gel) of both purified enzyme and crude fractions (Figs. 1 and 5). This band is more intense at the first stages of purification (Fig. 5) and may be due to aggregation of active protein, which prevents its migration through the starch gel. However, the possibility cannot be discarded that this band corresponds to a different P5C dehydrogenase form which is partially lost during the purification process.

Human P5C dehydrogenase is a protein highly sensitive to heat. At 45 °C it exhibits a very short time of inactivation ($t_{1/2} = 0.8 \text{ min}^{-1}$). Even at an assay temperature of 25 °C, $t_{1/2}$ is rather small, 23 min^{-1} . It is clearly

understood from this value that even during the short period of the assay the enzyme is rapidly losing activity, and this should be taken into account in the kinetic analysis. In contrast, the enzymes E1 and E2 of human aldehyde dehydrogenase are very stable at these temperatures (Figs. 2 and 3).

The oxidation of glutamic γ -semialdehyde is probably the main physiological function of P5C dehydrogenase in the placenta, an organ of active amino acid metabolism [18,19]. The enzyme can also oxidize succinic semialdehyde, although with a $k_{\text{cat.}}/K_m$ value 15 times lower than that for glutamic γ -semialdehyde. In any case, the low $s_{0.5}$ for succinic semialdehyde (30 μM) indicates that it can be oxidized under physiological conditions. Succinic semialdehyde is an intermediate in the metabolism of γ -aminobutyrate. A concentration of 50 nmol/g (100 times that in plasma) has been recently demonstrated for this neurotransmitter in human placenta [20]. Thus, a major role of P5C dehydrogenase in placenta may be related to the metabolism of γ -aminobutyrate, acting as a succinic semialdehyde dehydrogenase.

The availability characteristics of the human placenta have allowed reliable subcellular-localization experiments that would not have been possible with human tissue *post mortem*. About 60% of placental P5C dehydrogenase activity is localized in the mitochondrial fraction and 40% in the cytosol. Electrophoretic and kinetic properties of the enzymes isolated from both subcellular fractions are practically identical. Moreover, our purified enzyme is with all probability a mixture of the cytosolic and mitochondrial forms, and no heterogeneity in M_r or in kinetic properties was found. All these observations indicate that the enzymic forms from both subcellular fractions are very similar, although further studies are required to prove their identity.

Our findings on the P5C dehydrogenase localization are consistent with previous studies. Thus P5C dehydrogenase from rat liver was found to be 50% in the mitochondrial matrix and 50% in the cytosol [6], although this result was not considered in further works and reviews. More recently, a similar distribution was reported for the high- K_m aldehyde dehydrogenase activity in human placenta [21], probably caused by P5C dehydrogenase. Thus an enzyme that was assumed to be exclusively mitochondrial [3] has now been demonstrated to be also present in the cytosol. This may be of importance in the understanding of amino acid metabolism, since glutamic γ -semialdehyde is a central intermediate in the interconversions of proline, arginine and glutamic acid.

We thank Dr. Consuelo Guerri for useful suggestions. This work was supported by grant 1404-82 from Comisión Asesora de Investigación Científica y Técnica, grant 83/0764 from Fondo de Investigaciones Sanitarias de la Seguridad Social, and a grant from CIRIT (Generalitat de Catalunya). J. F. and P. J. were recipients of fellowships from Ministerio de Educación y Ciencia, and Fondo de Investigaciones Sanitarias de la Seguridad Social, respectively.

REFERENCES

1. Parés, X., Farrés, J. & Vallee, B. L. (1984) *Biochem. Biophys. Res. Commun.* **119**, 1047-1055
2. Strecker, H. J. (1960) *J. Biol. Chem.* **235**, 3218-3223
3. Adams, E. & Frank, L. (1980) *Annu. Rev. Biochem.* **49**, 1005-1061
4. Strecker, H. J. (1971) *Methods Enzymol.* **17**, 262-265
5. Lundgren, D. & Ogur, M. (1972) *Biochem. Biophys. Res. Commun.* **49**, 147-149
6. Brunner, G. & Neupert, W. (1969) *FEBS Lett.* **3**, 283-286
7. Forte-McRobbie, C. M. & Pietruszko, R. (1986) *J. Biol. Chem.* **261**, 2154-2163
8. Mezl, V. A. & Knox, W. E. (1976) *Anal. Biochem.* **74**, 430-440
9. Tottmar, S. O. C., Pettersson, H. & Kiessling, K.-H. (1973) *Biochem. J.* **135**, 577-586
10. Bradford, M. (1976) *Anal. Biochem.* **72**, 248-254
11. Julià, P., Farrés, J. & Parés, X. (1987) *Eur. J. Biochem.* **162**, 179-189
12. Harris, H. & Hopkinson, D. A. (1976) *Handbook of Enzyme Electrophoresis in Human Genetics*, North-Holland, Amsterdam
13. Meier-Tackmann, D., Korenke, G. C., Agarwal, D. P. & Goedde, H. W. (1985) *Enzyme* **33**, 153-161
14. Teng, Y.-S. (1981) *Biochem. Genet.* **19**, 107-114
15. Segel, I. H. (1975) *Enzyme Kinetics*, pp. 926-929, John Wiley and Sons, New York
16. Vallari, R. C. & Pietruszko, R. (1982) *Science* **216**, 637-639
17. Greenfield, N. J. & Pietruszko, R. (1977) *Biochim. Biophys. Acta* **483**, 35-45
18. Edwards, E. M., Rattenbury, J. M., Varnam, G. C. E., Dhan, U. K., Jeacock, M. K. & Shepherd, D. A. L. (1977) *Biochim. Biophys. Acta* **497**, 133-143
19. Remesar, X., Arola, L., Palou, A. & Alemany, M. (1980) *Eur. J. Biochem.* **110**, 289-293
20. Erdő, S. L., László, Á., Kiss, B. & Zsolnai, B. (1985) *Gynecol. Obstet. Invest.* **20**, 199-203
21. Kouri, M., Koivula, T. & Koivusalo, M. (1977) *Acta Pharmacol. Toxicol.* **40**, 460-464