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PROPERTIES OF ESTROGEN AND HYDROXYSTEROID SULPHOTRANSFERASES IN HUMAN MAMMARY CANCER

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Summary—Partial purification (~x 140-fold) of estrogen sulphotransferase (EC 2.8.2.4) in human mammary estrogen receptor positive cancer tissue was achieved by affinity chromatography on adenosine-3′,5′-diphosphate-agarose. It had a M_r of approximately 70,000 by gel filtration and upon electrophoresis on concave gradient polyacrylamide gels, showed a major (M_r 70,000) and a minor (M_r 200,000) peak of activity. Kinetics of this preparation (estradiol-17β and estrone as substrates), and also that of hydroxysteroid sulphotransferase (EC 2.8.2.2) contained in the cytosol of human mammary cancer MCF-7 cells (5-androstene-3β,17β-diol and dehydroepiandrosterone as substrates), were compared. The enzymes showed very similar behaviour, characterized by high affinity for their steroid substrates (low nM range) and co-operativity in their binding. For hydroxysteroid sulphotransferase, the adrenal-derived estrogen 5-androstene-3β,17β-diol was the preferred substrate compared to dehydroepiandrosterone in the 0–40 nM concentration range. Such properties of the enzymes might be designed to limit the exposure of nuclear receptor to free ligand. Alternatively, a defined subcellular location would perhaps involve the enzymes in the elimination of estrogen after processing of the ligand-bound receptor.

INTRODUCTION

The ability of human mammary tumours to sulphurylate steroids *in vitro* has been shown to be associated with both prognosis and response to hormone ablation [1]. Patients whose tumours lacked sulphurylating ability uniformly failed to respond to adrenalectomy and this also reflected a very poor prognosis. Hormone sensitivity of the tumour was also indicated by the relative sulphurylation rates of two substrates, viz. estradiol-17β (E_2) and dehydroepiandrosterone [1]. These steroids are substrates for estrogen sulphotransferase (EST) (EC 2.8.2.4) and hydroxysteroid sulphotransferase (HST) (EC 2.8.2.2), respectively. The expression of EST in human mammary tumours and cell lines is positively correlated with the presence of the estrogen receptor [2–6]. More recently, a similar relationship for the expression of HST in human mammary tumours and cell lines, has been established [6].

When the metabolism of 1 nM concentrations of E_2 and the adrenal-derived estrogen 5-androstene-3β,17β-diol (Adiol) [7] (a substrate for HST but not EST) were studied in four estrogen receptor positive and four estrogen receptor negative cell lines, then sulphate ester formation was generally the major route of metabolism in estrogen receptor positive cells. Oxidation of E_2 to estrone, or Adiol to dehydroepiandrosterone, was the major metabolic route in the estrogen receptor negative cell lines [5, 6]. Adiol is capable of acting as an estrogen in human mammary cancer cell lines at concentrations found in the blood of Western women (~2 nM). For example, in MCF-7 cells it induces synthesis of the estrogen-dependent 52 K glycoprotein [8], and in ZR-75-1 cells it stimulates cell growth [9]. At these concentrations it is also estrogenic in the rat [10].

We now report that EST and HST of human mammary cancer origin, share common properties highlighted by very high affinity and cooperativity in binding of their respective steroid substrates.

EXPERIMENTAL

Materials

Sephacryl S-200 and adenosine-3′,5′-diphosphate-agarose were obtained from Pharmacia, Uppsala, Sweden. Adenosine-3′,5′-diphosphate, adenosine-3′-phosphate-5′-phosphosulphate, bovine serum albumin and other protein molecular weight markers, and unlabelled steroids were obtained from Sigma Chemical Co., (St Louis, Mo.). [^{35}S]Sulphate (carrier-free), [2,4,6,7-^{3}H]estrone (80 Ci/mmol), [2,4,6,7-^{3}H]-E_2 (96 Ci/mmol), [1,2,6,7-^{3}H]Adiol and [1,2,6,7-^{3}H]dehydroepiandrosterone (89 Ci/mmol), were obtained from the Radiochemical Centre, Amersham, England. Adenosine-3′-phosphate-5′-phospho-[^{35}S]sulphate was prepared as previously described [11]. Bovine EST was purified from placenta cotyledons [12]. Human mammary tumours

were received on dry ice and stored at −80°C. Assay for estrogen receptor (dextran charcoal method) in the cytosol was carried out as described previously [2] and was performed on these samples within a period of 1–2 weeks. For enzyme purification, estrogen receptor positive tumour tissue was removed after storage at −80°C for periods up to 4 months.

Affinity chromatography

Estrogen receptor positive (>6 fmol/mg cytosol protein) human mammary cancer tissue was pooled, cleaned of connective tissue and fat, and a sample (5 g) homogenised in 4 vol of 0.05 M Tris–HCl buffer, pH 7.4, containing 1 mM EDTA, 0.1 mM dithiothreitol and aprotinin (Sigma), 200 KIU/ml (Ultraturrax instrument, Janke and Kunkel, K. G. Ikawerk, Stansen I., Breisgaw, West Germany). Cytosol was prepared by centrifugation at 100,000 *g* for 1 h. The procedure for absorption of EST onto adenosine-3′,5′-diphosphate-agarose and elution with adenosine-3′,5′-diphosphate, was exactly as described previously [5].

Gradient polyacrylamide gel electrophoresis

Concave (2.5–27%) gradient gels (8 cm × 8 cm × 3 mm) were purchased from Gradipore Ltd, Sydney, Australia. These gels were prepared in 2.7 mM Na_2EDTA, 16 mM boric acid and 19 mM $(NH_4)_2SO_4$. A continuous buffer system (0.08 M Tris/boric acid, pH 8.3 containing 3 mM EDTA) was employed, and this was cooled continuously by pumping through coils immersed in ice-water. Before applying to the gradipore gel, EST, eluted from the affinity gel by adenosine-3′,5′-diphosphate, was freed of nucleotide by passage through a small column of Sephadex G-100 equilibrated with homogenization buffer (50 mM Tris–HCl, pH 7.4, containing 1 mM EDTA and 0.1 mM dithiothreitol). Samples were then concentrated in a collodion sac to a vol of 0.1 ml under vacuum at ice bath temperature in the apparatus (SM 16304) supplied by Sartorius Gmbh., Göttingen, Germany. Glycerol (10 μl) and tracking dye (5 μl of 0.01% bromophenol blue in water containing 10% glycerol) were mixed with the sample and 50 μl applied to a gel slot. A sample of bovine serum albumin (60 μg), prepared in the same way, was run as a reference protein. Electrophoresis was carried out (300 V, ~150 mA) until the marker dye was 1 cm from the end of the gel (40 min). Enzyme was assayed in 0.4 mm segments of the gel (see legend to Fig. 2). Protein in companion tracks was stained with colloidal Coomassie Blue G-250 (Gradipore Ltd, Sydney, Australia).

EST assays

Determination of enzyme units (1 pmol estrogen sulphate produced/h) in cytosol, affinity gel eluates and washes, was carried out with [^{3}H]E_2 (10 μM) and adenosine-3′-phosphate-5′-phosphosulphate (0.1 mM) and isolation of the estrogen sulphate by thin-layer chromatography, as previously described [5]. Assay on gradient gels after electrophoresis, or on Sephacryl column fractions, was carried out with [^{35}S]nucleotide as described in figure legends.

Kinetic data

EST assays were carried out in duplicate or triplicate for each concentration of [^{3}H]E_2 or [^{3}H]estrone, exactly as described previously [5]. The amounts of protein used were 0.2–0.3 mg (0.1 ml) for cytosol or 1 μg of affinity gel eluate. For EST derived from bovine placenta, 9 μg of protein was employed. After elution of EST from affinity gels by adenosine-3′,5′-diphosphate, the latter was removed by Sephadex G-100 chromatography and the enzyme used immediately.

Cytosol was prepared from MCF-7 cells harvested at confluence, as previously described [5]. HST in the cytosol was assayed with [^{3}H]Adiol or [^{3}H]dehydroepiandrosterone (in the concentration ranges given in the legend to Fig. 5) in an analogous manner to EST. The volume of cytosol used was 0.1 ml and incubation carried out for 45 min at 37°C. After addition of carriers, labelled conjugates were isolated and counted as for EST, viz. by thin-layer chromatography, employing the same solvent systems [5]. Under these conditions, the maximum conversion of Adiol or dehydroepiandrosterone to sulphate ester, was 1.8%.

Protein

This was determined by the method of Bradford [13].

RESULTS

Isolation of EST from human mammary cancer tissue by affinity chromatography

Conventional methods, such as $(NH_4)_2SO_4$ fractionation and ion-exchange chromatography, which were successfully used in the isolation of EST from bovine sources [12], proved unrewarding in the case of human mammary cancer tissue. This was evidently due to the far greater instability of the human enzyme. A rapid method of isolation, such as affinity chromatography, was then indicated. Previous work, in which E_2 was linked at various positions in the molecule via spacer arms to agarose, yielded uniformly negative results employing bovine placenta as a source of EST (Clarke and Adams, unpublished work). However, adenosine-3′,5′-diphosphate (a potent inhibitor of EST [14]) when linked to agarose, proved to be effective as an affinity chromatography system for the partial purification of EST in small amounts from human mammary carcinoma MCF-7 cells [5]. This system was then employed with cytosolic preparations derived from pooled estrogen receptor positive human mammary cancer tissue. EST was absorbed onto the affinity gel and eluted with an

Table 1. Purification of estrogen sulphotransferase (EST) from pooled human mammary carcinoma tissue

Fraction	Protein (mg)	Units	Specific activity	Recovery (%)
Cytosol	112	2318	20.7	100
Affinity gel supernatant	104	400	3.8	17
Buffer wash 1	3	0	0	0
PAP eluant[a]	0.32	1014	3168	44
Sephacryl gel[b]	0.013	30	2308	1

Cytosol was prepared from pooled estrogen receptor positive human mammary cancer tissue (5 g).

[a]PAP = adenosine-3′,5′-diphosphate.

[b]250 μg of the protein in the PAP eluant was applied to the Sephacryl S-200 column. Values quoted for protein and enzyme Units have been adjusted to total protein in the PAP eluant.

Two additional buffer washes were carried out prior to elution of the affinity gel with PAP. Protein levels in these washes were not determined. Although hydroxysteroid sulphotransferase activity (HST) (dehydroepiandrosterone as substrate) was present in the cytosol (1300 Units), very low activity (35 Units) was found in the PAP eluant and no activity was detected in the pooled fraction after Sephacryl gel filtration.

excess of adenosine-3′,5′-diphosphate. Results are shown in Table 1.

Molecular weight of EST by gel filtration

Although the affinity chromatography system enabled considerable purification of enzyme to be achieved, subsequent steps such as gel filtration led to rapid loss of enzyme activity (Table 1). However, gel filtration on a calibrated Sephacryl S-200 column, revealed a single peak of enzyme activity with a M_r of approximately 70,000 (Fig. 1). The results depicted in this figure was obtained with two separate enzyme preparations, but a third preparation showed the presence of some associated active enzyme in addition to the main M_r 70,000 peak. This latter enzyme profile was almost superimposable on that of a sample of bovine serum albumin when chromatographed on the column under identical conditions and monitored by u.v. absorption (data not shown). The sample of bovine serum albumin was shown to contain monomer, dimer and trimer species by gradient polyacrylamide gel electrophoresis (see below).

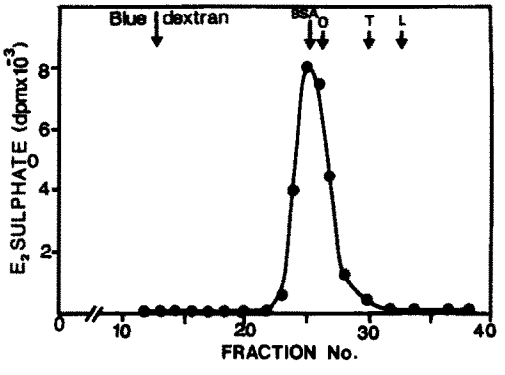

Fig. 1. Gel filtration of EST isolated by affinity chromatography. Enzyme eluted by adenosine-3′,5′-diphosphate (Table 1) was concentrated to 1 ml and applied to a column (69 × 0.8 cm) of Sephacryl S-200 equilibrated with 25 mM Tris/HCl, 0.1 mM dithiothreitol, 0.1 M NaCl, pH 7.4. Fractions (0.6 ml, 3 ml/h) were collected and 0.2 ml aliquots assayed in an incubation which contained: 5 μM adenosine-3′-phosphate-5′-phospho-[^{35}S]sulphate (17 × 10^4 dpm), 10 μM E_2, 10 mM $MgCl_2$, 0.1 mM dithiothreitol and 40 mM Tris/HCl, pH 7.4 in a total vol of 0.4 ml. Incubation was carried out for 2 h at 37°C and E_2-[^{35}S]sulphate assayed by the method of Wengle [26]. The positions of reference proteins (bovine serum albumin monomer (BSA), ovalbumin (O), trypsinogen (T), and lysozyme (L)] are indicated.

Gradient polyacrylamide gel electrophoresis

When EST, contained in affinity chromatography eluates (Table 1) was subjected to gradient polyacrylamide gel electrophoresis, a major enzymically active peak, travelling in a position close to bovine serum albumin monomer, was present in 4/4 separate preparations. In 3/4 preparations, a minor enzymically active peak was present in the area corresponding to bovine serum albumin trimer. Protein staining of a companion enzyme track revealed a band corresponding to the main active peak in all cases. Other protein species were also evident (Fig. 2). Electrophoresis on concave gradient acrylamide gels enables an assessment to be made of the molecular weight of a protein by comparison with standards run concurrently. This is so since the mobility to a great extent depends on protein size relative to the pore size of the medium. On this basis, the molecular weight of the main enzymically active fraction would be slightly higher than bovine serum albumin monomer and thus in good agreement with the value of ~70,000 found by gel filtration.

HST in human mammary MCF-7 cells

Rozhin *et al.* [15] have reported that HST present in the cytosol of MCF-7 cells can be partially purified by Sephadex G-200 chromatography. Using the exact procedure described by these workers for the preparation and chromatography of cytosol from MCF-7 cells, we found that the enzyme did not survive gel filtration. The reason for this is unknown, but perhaps may reside in differences in enzyme levels in the cell strains employed.

HST present in human adrenal glands was also found to be unstable and this prevented purification by conventional means. The enzyme from this source

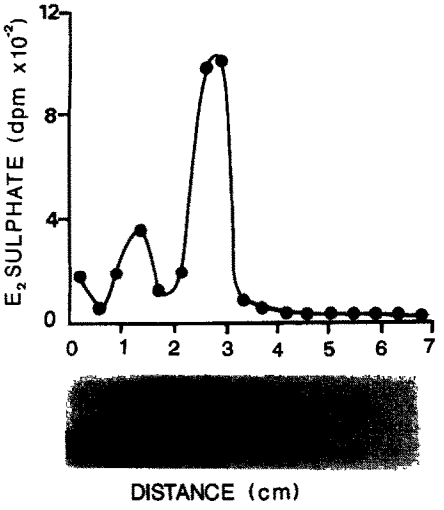

Fig. 2. Electrophoresis of EST on concave (2.5–27%) gradient polyacrylamide gels. Enzyme obtained from pooled human mammary tissue and purified by affinity chromatography was freed of adenosine-3′,-5-diphosphate and concentrated (see Experimental). The concentrate was divided into 2 parts and subjected to electrophoresis: one channel assayed for enzyme activity, and the second stained for protein. Segments (0.4 cm) of gel were placed in tapered glass centrifuge tubes, macerated with a glass rod with a sharpened point, and assayed for enzyme activity as in Fig. 1. The stained gel reveals monomer (M), dimer (D), and trimer (T) species of bovine serum albumin (top channel) and the enzyme (lower channel).

was obtained in pure form by affinity chromatography on gels obtained by coupling dehydroepiandrosterone-17-(O-carboxymethyl)oxime to AH Sepharose 4B [16]. When applied to an $(NH_4)_2SO_4$ cut from MCF-7 cell cytosol preparations, some 43% of enzyme activity was absorbed. However, eluates made with high concentrations of dehydroepiandrosterone, which was the procedure successfully employed in the isolation of HST from human adrenals, did not contain detectable levels of HST. In addition, barely detectable levels of HST were found in the adenosine-3′,5′-diphosphate eluates of adenosine-3′,5′-diphosphate-agarose affinity gel, employed in the isolation of EST from MCF-7 cells [5]. Since attempts to purify the HST were unsuccessful, kinetic data for this enzyme were subsequently obtained with separate MCF-7 cytosolic preparations made from cells grown at different passage numbers.

Kinetic data

Velocity vs estrogen substrate curves for EST obtained from pooled ER positive cancer tissue by affinity chromatography are shown in Fig. 3. For comparison, data obtained with purified EST from bovine placenta cotyledons is also shown in Fig. 3. For EST isolated from human mammary tumour tissue by affinity chromatography, estrone was sulphurylated at a lower rate than E_2 in the 0–20 nM

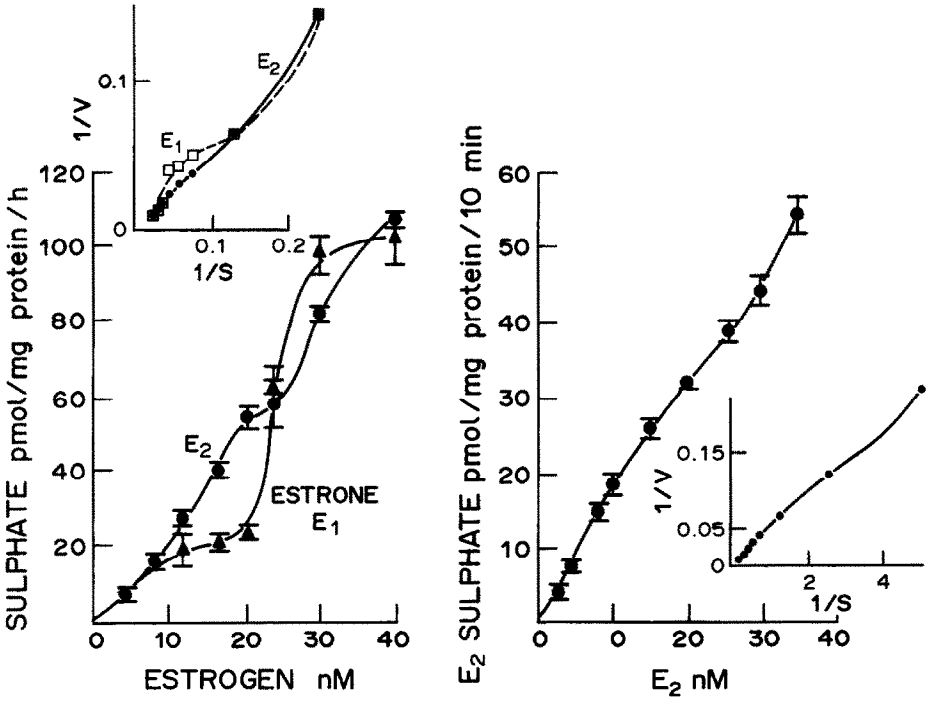

Fig. 3. Velocity–substrate curves for EST preparations. Left: enzyme isolated from human mammary cancer tissue by affinity chromatography was used. Right: for comparative purposes, the enzyme purified from bovine placenta was employed. Bars represent range of values of assays performed in duplicate or triplicate. In each individual assay, product was isolated and counted after thin layer chromatography [5]. Conversion of estrogen to product did not exceed 2.5% in any one instance. Inserts: double reciprocal plots.

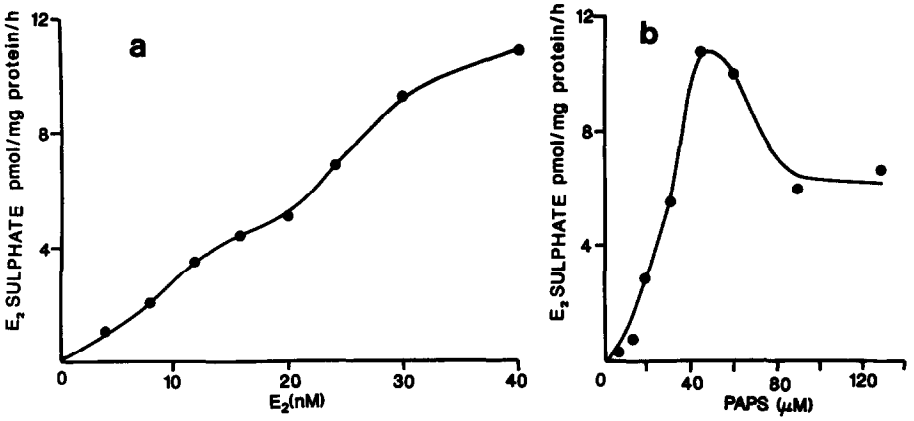

Fig. 4. (a) Influence of electrolyte on kinetics obtained with variable concentrations of E_2. Enzyme (a separate preparation from that used in Fig. 3) was isolated by affinity chromatography, freed of adenosine-3′,5′-diphosphate (see Experimental), NaCl added to a concentration of 0.1 M, and allowed to stand at 0°C for 2 h. Assay was then carried out as in Fig. 3. The final concentration of NaCl was 0.066 M. (b) Kinetic data with variable adenosine-3′-phosphate-5′-phosphosulphate (E_2 concentration; 40 nM). The same enzyme preparation as in (a) was employed, but treatment with NaCl was omitted.

estrogen concentration range. At concentrations beyond 20 nM, then estrone exhibited a stronger positive cooperatively compared to E_2 for the preparation illustrated in Fig. 3. Cooperativity in the binding of both estrogens was observed for all enzyme preparations. In repeated experiments, data similar to that shown in Fig. 3 was obtained with peaks and/or inflections occurring at generally the same estrogen concentrations, but with some variability in their magnitude. Purified estrogen sulphotransferase exhibits non-Michaelis–Menten kinetics when estrogen is varied in the nanomolar range (Fig. 3), or micromolar range [12, 14]. In the present experiments, data has been limited to studies in which the estrogen concentration approached physiological intracellular levels.

Since the cooperativity in binding of estrogen may have been due to an association–dissociation of enzyme protein—and indeed evidence for the presence of an associated form of the enzyme was seen from the gradipore gel electrophoresis experiments—the influence of addition of an electrolyte which might be expected to influence the equilibrium in an associating system was investigated. Enzyme isolated by affinity chromatography was allowed to stand for 2 h in 0.1 M NaCl. However, as shown in Fig. 4(a), no effect of prior treatment with 0.1 M NaCl was evident on the shape of the velocity–substrate curve.

Kinetic data was also obtained by varying adenosine-3′-phosphate-5′-phosphosulphate at constant E_2 concentration (Fig. 4b). In common with other sulphotransferase enzymes, substrate inhibition was apparent. However, this was observed at an unexpectedly low value of $\sim 60\,\mu$M and thus the concentration of 0.1 mM used routinely (Fig. 3) would not be optimum.

Velocity–substrate plots for HST contained in cytosol preparations from MCF-7 cells are shown in Fig. 5. A similar steroid concentration range to that used to EST was employed. Three separate preparations from MCF-7 cells grown at different times yielded very similar results and the collective data are presented as means ± SEM for each concentration point. For Adiol, there is a strong resemblance to the velocity vs E_2 concentration curves obtained with EST (Fig. 3). Although dehydroepiandrosterone appeared to exhibit normal Michaelis–Menten kinetics, the double reciprocal plots were curvi-linear and similar to the data obtained with Adiol (Insert, Fig. 5). This was also the case when dehydroepiandrosterone was varied in a higher concentration range (0–1 μM) (data not shown). Controls were carried out with 2×10^5 dpm (20 nM) [^{3}H]Adiol incubated with MCF-7 cytosol in the absence of adenosine-3′-phosphate-5′-phosphosulphate. After chromatography in the solvent systems used to

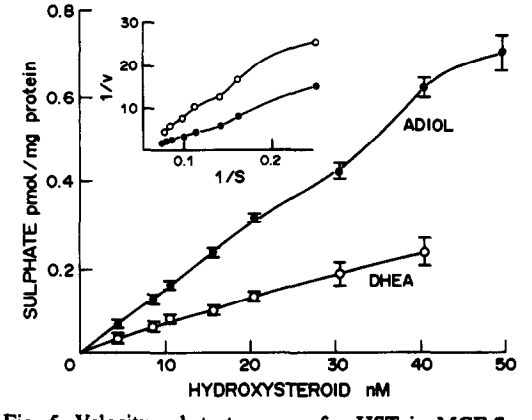

Fig. 5. Velocity–substrate curves for HST in MCF-7 cell cytosols. Data are presented as means ± SEM for three separate cytosol preparations. Assays were carried out in duplicate and the labelled sulphate esters counted after isolation by thin layer chromatography. Insert: Double reciprocal plots. An incubation time of 45 min was employed for each velocity–substrate curve. DHEA = dehydroepiandrosterone.

separate and measure [^{3}H]Adiol sulphate in the kinetic experiments, radioactivity scanning showed that no change had occurred to the [^{3}H]Adiol substrate.

DISCUSSION

Human EST has proven to be much more unstable than the bovine enzyme. Instability was also a major problem in attempting to isolate the enzyme from human placenta [17]. Employment of an affinity gel based on adenosine-3′,5′-diphosphate has enabled a 140-fold purificataion of the enzyme in human mammary tumours to be achieved. Further purification, for example by gel filtration, was not possible due to a marked loss of activity. The M_r of 70,000 obtained by this technique is similar to the value of 74,000 for the purified bovine enzyme [14], and that of 68,000 for the partially purified human placenta enzyme [17]. The affinity gel eluate did survive rapid gradipore gel electrophoresis where the major enzymically active peak ran in a similar position to bovine serum albumin monomer (M_r 66,000). Adenosine-3′,5′-diphosphate-agarose has also been exploited in the partial purification of EST from guinea pig liver and chorion, and the estimated M_r in this instance was ~50,000 [18].

Tseng *et al.*[4] first reported non-Michaelis–Menten kinetics for EST from human breast cancer cytosol preparations when E_2 was varied in the 0–1 μM range. A departure from Michaelis–Menten kinetics, when either E_2 or estrone was varied in the 0–0.5 μM range, has also been described using EST isolated from guinea-pig chorion by chromatofocusing and affinity chromatography [18]. Enzyme present in the cytosol of human endometrial cancer cells showed a very high affinity for E_2 and estrone [19]. Although the kinetics obtained by varying estrone in the 0–30 nM range were stated to be "non-complex" in this particular study, close examination of the data shows that this is not the case, and the double reciprocal plot is curvi-linear. Brooks *et al.*[20] have partially purified EST from porcine endometrium, and although high affinity for E_2 and estrone were reported, it was not stated whether a depature from Michaelis–Menten kinetics occurred.

Adiol was observed to have a high affinity for HST in MCF-7 cytosol and cooperativity was exhibited at Adiol concentrations in the 0–50 nM range. It is important to emphasise that EST is specific for phenolic estrogens [21] and will not sulphurylate other steroids; the sulphurylation of Adiol and dehydroepiandrosterone then being catalysed by HST. Thus, both EST and HST present in human mammary cancer cells share common properties characterized by high affinity for their steroid substrates and cooperativity in the velocity vs steroid concentration curves. Highly purified HST from human adrenal glands also exhibited cooperativity when dehydroepiandrosterone was the variable substrate [22]. In the latter studies, departure from Michaelis–Menten kinetics ([^{3}H]dehydroepiandrosterone as substrate) as seen by double-reciprocal plots, was more pronounced upon addition of unlabelled steroid substrates. Non-Michaelis–Menten kinetics have also been reported for HST purified from female rat liver [23]. Early studies with HST contained in cytosol preparations from human adrenal glands revealed the presence of an association–dissociation system involving monomeric, dimeric and trimeric states. Both dehydroepiandrosterone and adenosine-3′-phosphate-5′-phosphosulphate altered the position of equilibrium as shown by density gradient ultracentrifugation. Cysteine and Mg^{2+}, which individually activated the enzyme, were shown to favour association of the enzyme [24].

The close similarity then in the data obtained with EST and HST (Figs 3 and 5) would indicate a common feature responsible for the non-Michaelis–Menten kinetics. Detection of a trimeric enzymically active species in the human mammary tumour EST parallels the behaviour of bovine adrenal EST [25] and human HST from adrenal glands. Changes in composition of an association–dissociation system by the presence of substrate might be responsible for the unusual kinetics. A more complete discussion of this question is given in Ref. [24]. An alternative explanation for the non-Michaelis–Menten kinetics would be the presence of multiple interacting steroid binding sites. This model allows the generation of theoretical curves closely resembling those seen with the sulphotransferase enzymes [5].

Since expression of EST, HST and the estrogen receptor, are linked in human mammary cancer tissue and cell lines [2–6], this could reflect a functional relationship between estrogen action (E_2 and Adiol) via the receptor and sulphotransferase activity in mammary cancer cells. Whilst the exact nature of this relationship is still unknown, one possibility is that the enzymes may function to eliminate excess steroid from the cell as water-soluble conjugates incapable of combining with the estrogen receptor. The properties of the enzymes, as judged by their relatively high affinities for their steroid substrates coupled with cooperativity of binding, might be designed for limiting the exposure of nuclear receptor to free ligand. As an alternative, a defined subcellular location of EST and HST in human mammary cancer cells could perhaps allow the elimination of estrogen after processing of the ligand-bound receptor in the nucleus. Although both enzymes appear in the cytosol, this could conceivably occur by disruption of a membrane-bound site within the cell.

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