HUMAN PLACENTAL 17β-OESTRADIOL DEHYDROGENASE. SEQUENCE OF A TRYPTIC PEPTIDE CONTAINING AN ESSENTIAL CYSTEINE

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1. Introduction

Recent studies of the 17β-oestradiol dehydrogenase from human placenta have indicated that the active enzyme is a dimer with a sub-unit molecular weight of 34,000 [1–4]. Inhibition of the enzyme with *N*-ethylmaleimide (NEM) or *p*-chloromercuribenzoate (*p*CMB) has been shown to be due to reaction of these reagents with at least two out of a total of twelve cysteines per mole. These cysteines are not reactive in the presence of coenzyme (NADP) but the substrate (17β-oestradiol) has no such protective effect [4].

In order to obtain more information concerning the subunit structure of the enzyme and the chemical environment of the essential thiol groups a study of the primary structure around these residues has been undertaken. A tryptic peptide containing the chemically modified cysteines essential for the enzymatic activity has been obtained and its amino acid sequence has revealed that the two reactive cysteines occur in a unique sequence in the primary structure of the enzyme.

2. Materials and methods

17β-Oestradiol dehydrogenase was purified as previously described [5]. The enzyme (0.5 μmole) was reacted at ambient temperature with [2–^{14}C] *N*-ethylmaleimide (10 μmoles) in 0.06 M K-phosphate buffer (pH 7.2) containing 20% glycerol. The extent of reaction was determined by measuring the incorporation of ^{14}C into the protein, and the optical density at 310 nm; and by following the loss of enzymatic activity [6]. After 1 hr the activity was reduced to less than 20% and the reaction was then terminated by addition of excess β-mercaptoethanol. After dialysis against 0.01 M potassium phosphate (pH 8.0) solid guanidine-HCl was added to 6 M and residual thiol groups were reacted with non-radioactive iodoacetic acid (10 μmoles) for 2 hr at 20–22°.

In other separate experiments samples of enzyme were totally labelled with [2-^{14}C] NEM, or [2-^{14}C]-iodoacetic acid by reaction in 6 M guanidine as described above. The radioactive protein derivatives were digested with trypsin (1 : 50 by weight) at pH 8.0 and 37° for 4 hr or 18 hr. Tryptic peptides were digested further with thermolysin in 50 mM ammonium acetate/20 mM Ca^{2+} at pH 8.0 for 2, 4 and 18 hr.

The subsequent experimental procedures were similar to those described and referred to by Jörnvall and Harris [7]. Tryptic digests were fractionated by gel-filtration on Sephadex G-50 (superfine grade). Columns were developed with 0.1 M ammonium bicarbonate and effluents were monitored continuously for ^{14}C content and for absorbance at 254 nm using a Uvicord flow-cell and recorder. Column fractions were pooled and freeze-dried, and peptide mixtures were purified further by high voltage electrophoresis on Whatman No. 3 MM paper. Electrophoresis buffers were pyridine–acetic acid at pH 6.5 and 3.5, and 1% ammonium carbonate at pH 8.9. Chromatography of peptides was carried out in *n*-

butanol/acetic acid/water/pyridine (15 : 3 : 12 : 10, by vol). Radioactive peptides were detected by autoradiography and, where necessary, appropriate guide strips were stained with the ninhydrin/cadmium and chlorine/*O*-tolidine reagents. Purified peptides were hydrolysed with 6 N HCl (containing 1% phenol) at 105° for 24 hr and amino acid analyses were determined with a Beckman 120C automatic analyser. End-group and sequence analysis of peptides was carried out by the dansyl- Edman method [8] and amide groups in peptides containing aspartic and/or glutamic acid were assigned from a consideration of their electrophoretic mobilities at pH 6.5.

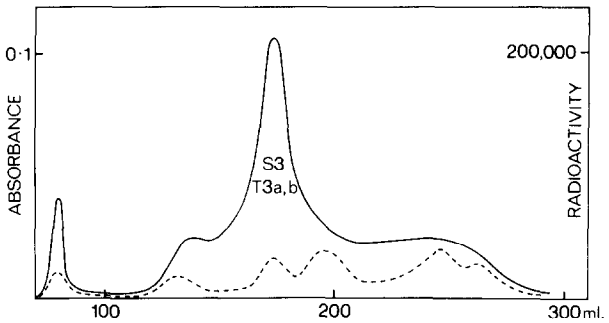

Fig. 1. Gel-filtration of a tryptic digest of the 17β-oestradiol dehydrogenase (0.5 μmole) following its inhibition with [2-^{14}C]NEM (3.2 g atoms of ^{14}C were incorporated per mole (68,000 g)). Sephadex G-50 (120 × 2.5 cm column "superfine" grade) in 0.1 M NH_4HCO_3. (———) Radioactivity; (– – – – –) absorbance at 254 nm.

3. Results

Inactivation of the native enzyme by reaction with [2-^{14}C]NEM is accompanied by the incorporation of approximately 3 g atoms of ^{14}C per mole [6]. Tryptic digestion of this material followed by gel-filtration gave rise to one major radioactive component (fraction S3, fig. 1). Two other minor radioactive fractions (each representing less than 20% of the total radioactivity) were also present and are presumably due to a slower reaction of NEM with other cysteine residues in the protein. Electrophoresis at pH 6.5 of the material in fraction S3 (fig. 1) yielded two acidic radioactive components, T3a and T3b. T3a, however, was completely converted into the more acidic component T3b by an overnight incubation in dilute ammonia (pH ≃ 11). This presumably occurs by hydrolysis of *S*-ethylsuccinimidocysteine [9] to *S*-succinylcysteine, and as such it forms the basis of a "diagonal" electrophoretic procedure for the purification of peptides that are produced following the reaction of protein thiol groups with NEM. Peptide T3b was obtained pure by successive ionophoresis at pH 8.9, 6.5

Table 1

Amino acid composition and electrophoretic mobilities of the tryptic peptide (and its thermolytic fragments) containing an essential cysteine derived from S [2-^{14}C] carboxymethylated 17β-oestradiol dehydrogenase.

	Tryptic peptide T3b	Thermolytic fragments					
		th_1	th_2	th_3	th_4	th_5	th_6
Arginine	1.02 (1)						1
S-carboxymethylcysteine*	1.10 (1)		1	1			
Aspartic acid	1.10 (1)					1	
Threonine	0.90 (1)			1			
Serine	1.05 (1)		1	1			
Glutamic acid	2.01 (2)			1	1		
Proline	1.82 (2)		2	2			
Glycine	1.15 (1)		1	1			
Alanine	1.87 (2)	(1)	2	2			
Valine	0.95 (1)						1
Leucine	3.55 (4)		1	2	1	1	
Total	17	1	8	11	2	2	2
Electrophoretic mobility	− 0.35	0	−0.31	− 0.48	−0.5	−0.65	+0.6

* S-succinylcysteine in NEM-labelled enzyme.

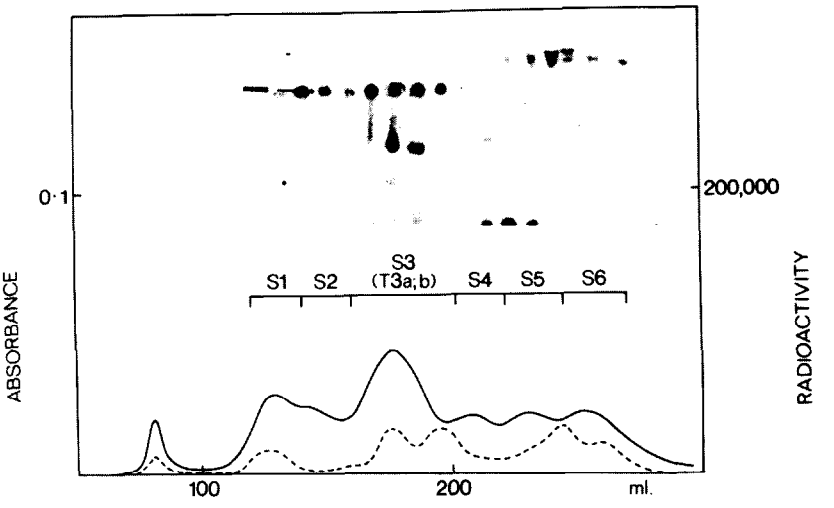

Fig. 2. Gel-filtration of a tryptic digest of the fully S[2-^{14}C]carboxymethylated derivative (0.8 μmole containing 11.8 g atoms of ^{14}C per mole) of 17β-oestradiol dehydrogenase. Sephadex G-50 (120 × 2.5 cm column) in 0.1 M NH_4HCO_3. Samples of effluent fractions were submitted to paper electrophoresis at pH 6.5 and radioactive peptides were revealed by autoradiography (cf. [7]). (———) Radioactivity; (– – – – – –) absorbance at 254 nm.

and 3.5. The N-terminal residue was alanine and amino acid analysis showed it to be a heptadecapeptide with the composition given in table 1.

The same peptide was obtained from a tryptic digest of enzyme that had been totally carboxymethylated with [2-^{14}C] iodoacetic acid in 6 M guanidine-HCl. This material gave rise to a total of 7 major radioactive fractions by gel-filtration (fig. 2) and, of these, fraction S3 yielded the same radioactive heptadecapeptide T3 (except that it contained S [2-^{14}C] carboxymethylcysteine instead of S [2-^{14}C] succinylcysteine) as that obtained from the NEM-labelled enzyme. The animo acid sequence of this heptadecapeptide was established by dansylation following 16 successive cycles of Edman degradation. Samples were counted for ^{14}C after each step and all the radioactivity in the peptide was located in residue 4, later identified as S[2-^{14}C] carboxymethylcysteine. The sequence was confirmed by analysis of thermolytic fragments as shown in table 2.

Table 2

Amino acid sequence of the tryptic peptide (T3) (and its thermolytic fragments (th)) containing an active site cysteine in human placental 17β-oestradiol dehydrogenase.

Peptide	
T36:	Ala–Leu–Ala–Cys*–Pro–Pro–Gly–Ser–Leu–Glu–Thr–Leu–Gln–Leu–Asp–Val–Arg
th_1 →	Ala
th_2 →	Ala–Leu–Ala–Cys*–Pro–Pro–Gly–Ser
th_3 →	Ala–Leu–Ala–Cys*–Pro–Pro–Gly–Ser–Leu–Glu–Thr
th_4 →	Leu–Gln
th_5 →	Leu–Asp
th_6 →	Val–Arg

Residues identified as dansyl amino acids: ——›
Residues identified following digestion with carboxypeptidase A and B: ‹——
Sites of cleavage by thermolysin: ↑ after 1 hr; ⇑ 4 hr; ⇑ 20 hr
*Present as S[2-^{14}C] carboxymethylcysteine.

4. Discussion

Inactivation of 17β-oestradiol dehydrogenase with ^{14}C-labelled NEM followed by tryptic digestion gives rise to one main radioactive peptide. The amount of radioactivity found in this peptide is equivalent to that expected from the reaction of the inhibiter with two cysteines per mole (68,000 g) of enzyme. S [2-^{14}C] carboxymethylcysteine was identified as the only radioactive component following acid hydrolysis and amino acid sequence analysis showed it to be a heptadecapeptide containing one residue of S [2-^{14}C] carboxymethylcysteine in unique sequence. It follows that this heptadecapeptide sequence must occur twice in the molecule of native enzyme, in accord with a structure consisting of two similar and probably identical polypeptide chains (cf. Burns et al. [3]). The sequence in the vicinity of the reactive cysteine is relatively hydrophobic, and does not appear to be related to the sequence around active-site cysteine residues in other NAD-linked dehydrogenases such as, for example, glyceraldehyde 3-phosphate, and alcohol dehydrogenases [10]. Another noteworthy feature is the occurrence of the –Pro–Pro– sequence which might have the effect of locking the adjacent cysteine into a fairly rigid and partly "buried" conformation in the tertiary structure. In this conformation it does not react with iodoacetic acid or iodoacetamide, nor is it particularly reactive towards NEM, a 10-fold excess being required to inactivate the enzyme to the extent of 80% to 90% during 1 hr at 25°.

The precise relationship of the chemical modification to the biological activity, and the role (if any) of this particular cysteine residue in the catalytic mechanism of the enzyme, remains to be established. The fact that the substrate (17β-oestradiol) does not protect the thiol group against reaction with NEM argues against its involvement in the substrate binding site. The coenzyme (NADP) on the other hand does afford a high degree of protection [4] suggesting that the cysteine and its immediate neighbours in the primary structure may be part of the coenzyme binding site in 17β-oestradiol dehydrogenase.

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