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ENZYMIC SYNTHESIS OF STEROID SULPHATES

XI. STUDY OF THE OESTROGEN BINDING SITE OF OESTROGEN SULPHOTRANSFERASE BY AFFINITY LABELLING WITH 4-MERCURI-17β-OESTRADIOL

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Summary

Oestrogen sulphotransferase (3′-phosphoadenylylsulphate: oestrone sulphotransferase, EC 2.8.2.4) contains a single sulphydryl group thought to be at, or near, the oestrogen-binding site. 4-Mercuri-17β-oestradiol, synthesised by Chin and Warren (Chin, C-C. and Warren, J.C. (1968) J. Biol. Chem. 243, 5056—5062) as an affinity label for oestrogen-binding sites on proteins, was used to gain further information.

Upon reaction of enzyme with 4-mercuri-17β-oestradiol, the activity of the enzyme decreased with increasing concentration of the oestrogen derivative. However, some 40% of the activity remained when all the sulphydryl had reacted to form mercaptide. Formation of mercaptide was only marginally decreased in the presence of the substrate 17β-oestradiol. Other steroids, such as 11-deoxycorticosterone and testosterone, which are non-substrates for the enzyme, were more effective than 17β-oestradiol in inhibiting mercaptide formation. Bovine serum albumin also reacted with 4-mercuri-17β-oestradiol and the effects of various steroids on mercaptide formation by the affinity label closely paralleled those found for the enzyme.

It is concluded that the single sulphydryl group in the enzyme is not directly involved in the binding of oestrogen at the active site but is perhaps in closer proximity to a second site capable of binding certain non-substrate steroids.

Introduction

Previous work with oestrogen sulphotransferase (3′-phosphoadenylylsulphate: oestrone sulphotransferase, EC 2.8.2.4) has shown that a single sulp-

hydryl group is present at, or near, the active site [1]. Although the enzyme is sensitive to sulphydryl blocking agents such as *p*-hydroxymercuribenzoate, the effectiveness of the latter as inhibitor is related to the concentration of oestrogen used in assaying activity. Synthesis of 4-mercuri-17β-oestradiol by Chin and Warren [2], and its use as an affinity label for the so-called oestrogen-allosteric sites on such enzymes as pyruvate kinase and glutamate dehydrogenase, was considered an ideal reagent for further exploring the possible involvement of the single sulphydryl group in the oestrogen-binding area of oestrogen sulphotransferase.

Bovine serum albumin closely resembles oestrogen sulphotransferase, both in amino acid analyses and other properties such as molecular weight, the lack of subunit structure and the possession of a single sulphydryl group in conjunction with a large number of disulphide bonds [3]. These common properties suggest that the two proteins may be genetically related. In order to examine further this possibility, bovine serum albumin has also been investigated in parallel studies.

Materials

Pyruvate kinase from rabbit muscle was purchased from Calbiochem. Crystalline bovine serum albumin and the various steroids employed were purchased from Sigma. Other materials were as described previously.

4-acetatomercuri-17β-oestradiol

This was prepared by the method of Chin and Warren [2]. Following two crystallisations from ethanol, the product had a melting point of 237° C (decomposition). Chin and Warren give a melting point of 235° C. The compound gave a single spot on paper chromatography in two Zaffaroni systems: (i) employing 50% methanol formamide as a stationary phase and chloroform as a mobile phase and (ii) using chloroform acetone (95 : 5) as a mobile phase. The R_f values, and mobilities relative to 17β-oestradiol, were identical to those quoted by Chin and Warren [2]. Aqueous solutions of the oestrogen derivative were obtained by dissolving the powdered material in 0.1 M NaOH, adjusting to pH 7.5 with 0.1 M NaH_2PO_4, and finally diluting to the desired volume with 0.1 M sodium phosphate buffer, pH 7.5. In such solutions the compound will be referred to as 4-mercuri-17β-oestradiol.

Methods

Reaction of 4-mercuri-17β-oestradiol with thiols and proteins

This was carried out as described by Chin and Warren [2]. However, the final reaction volume was 1 ml and the temperature 30° C. A value of $\Delta\epsilon = 3.4 \times 10^3\ M^{-1} \cdot cm^{-1}$ for the mercaptide formed from 4-mercuri-17β-oestradiol and reduced glutathione, as quoted by Chin and Warren [2], was used in calculations of mercaptide formation with the proteins investigated. All spectrophotometric studies were carried out on a Varian Techtron instrument, model 635.

Protein determination

The method of Lowry et al. [4], using bovine serum albumin as standard, was employed.

Oestrogen sulphotransferase

Enzyme was prepared from bovine placental tissue as described previously [1] and stored frozen at −20° C. Assay was carried out using the following incubation mixture: 0.2 mM 3′-phosphoadenylylsulphate; 10^5 cpm 3′-phosphoadenylyl [^{35}S]sulphate; 15 mM $MgCl_2$; 0.1 M Tris · HCl, pH 7.4; 0.06 mM oestrone added as 5 μl of a propylene glycol stock solution; enzyme 10—15 μg; total volume 0.15 ml. Incubation was carried out for 20 min at 37° C and the reaction stopped by placing the tubes in a boiling water bath for 1 min. The amount of oestrone [^{35}S]sulphate formed was determined as previously [5].

Results

Reactions of 4-mercuri-17β-oestradiol with glutathione and pyruvate kinase

Chin and Warren [2] demonstrated the ability of the affinity label 4-mercuri-17β-oestradiol to deliver an increased localised reagent concentration to oestrogen-binding sites such as those present in pyruvate kinase and glutamate dehydrogenase. Resultant formation of mercaptide, measured at 305 nm, was inhibited when 17β-oestradiol was also present, but not by other steroids such as cortisol. Proteins such as egg albumin, and peptides such as reduced glutathione, which possessed sulphydryl groups but no high-affinity oestrogen-binding sites, also formed mercaptide but in these cases the reaction was uninhibited in the presence of 17β-oestradiol [2]. These results were confirmed in the present work. In addition, lysozyme, which does not contain sulphydryl groups, did not absorb in the 305 nm region upon reaction with the oestrogen derivative.

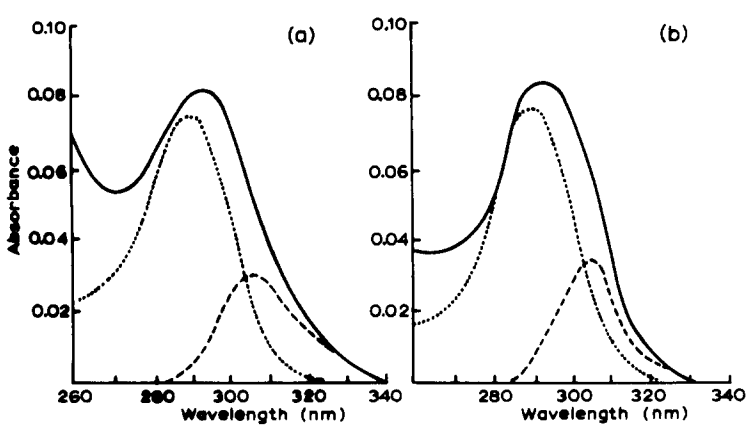

Fig. 1. (a) Reaction of 4-mercuri-17β-oestradiol with oestrogen sulphotransferase. Absorption spectra of 4-mercuri-17β-oestradiol (30 μM) alone (. . . . . .) and in the presence of enzyme (5 μM) (———) are shown. (b) Reaction of 4-mercuri-17β-oestradiol with bovine serum albumin. Spectra as in (a) but the concentration of the oestrogen derivative was 25 μM and serum albumin 10 μM. The broken lines represent the difference spectra and show maxima at 305 nm.

Reaction of 4-mercuri-17β-oestradiol with oestrogen sulphotransferase

Fig. 1a shows the formation of mercaptide, with absorption maximum at 305 nm, upon reaction of 4-mercuri-17β-oestradiol with oestrogen sulphotransferase. The yield of mercaptide was found to vary with age of the enzyme. For example, in one enzyme preparation, the value increased from 1.25 ± 0.13 mol 4-mercuri-oestradiol bound/mol enzyme (6 determinations) to 1.42 ± 0.02 (7 determinations) on aging. A separate, unaged enzyme preparation gave a value of 1.24 ± 0.14 (4 determinations).

The substrate 17β-oestradiol inhibited formation of mercaptide (Table I). Dehydroepiandrosterone was without effect, whereas 11-deoxycorticosterone and testosterone were more effective than 17β-oestradiol as inhibitors (Table I). Cortisol and corticosterone were less effective than 11-deoxycorticosterone.

Mercaptide formation and enzyme activity

Formation of mercaptide was accompanied by a loss in enzyme activity (Fig. 2a). However, the maximum loss in activity was about 60%, even when 4-mercuri-17β-oestradiol was present in excess. When activity was plotted against the actual amount of mercaptide formed per mol of enzyme (in a separate experiment), then the activity decreased rapidly to some 40% of its initial value when 0.6—0.7 mol of 4-mercuri-oestradiol per mol of enzyme were bound. The rate of change in activity then decreased markedly during the final titration of the sulphydryl group (Fig. 2b).

Reaction of 4-mercuri-17β-oestradiol with bovine serum albumin

Bovine serum albumin reacted with 4-mercuri—oestradiol to form a mercaptide with an absorption maximum at 305 nm (Fig. 1b). From the difference spectrum, it was calculated that 0.89 ± 0.16 mol 4-mercuri—oestradiol were bound per mol of protein (6 determinations). For purposes of comparison with oestrogen sulphotransferase, various steroids were examined for their effects on mercaptide formation with 4-mercuri—oestradiol. It can be seen from Table II that 17β-oestradiol and the other steroids examined gave results remarkably

TABLE I

EFFECT OF STEROIDS ON MERCAPTIDE FORMATION WITH OESTROGEN SULPHOTRANSFERASE AND 4-MERCURI-17β-OESTRADIOL

Values represent mean ± S.D. or actual values for each experiment. Lack of enzyme prevented additional experiments with some steroids. Enzyme (5.8 μM) was present in 0.1 M phosphate buffer, pH 7.5. The concentration of 4-mercuri-17β-oestradiol was 30 μM. Steroid was added in 40 μl of propylene glycol prior to addition of enzyme. Spectra were recorded immediately at 30° C.

Steroid (60 μM)	Mercaptide formed (nmol/ml)	No. of determinations
—	6.4 ± 0.4	7
17β- Oestradiol	5.2 ± 0.6	7
Dehydroepiandrosterone	6.3 ± 0.5	5
11-Deoxycorticosterone	3.8 ± 0.5	6
Testosterone	4.4, 4.7	2
Cortisol	5.7	1
Corticosterone	5.7	1

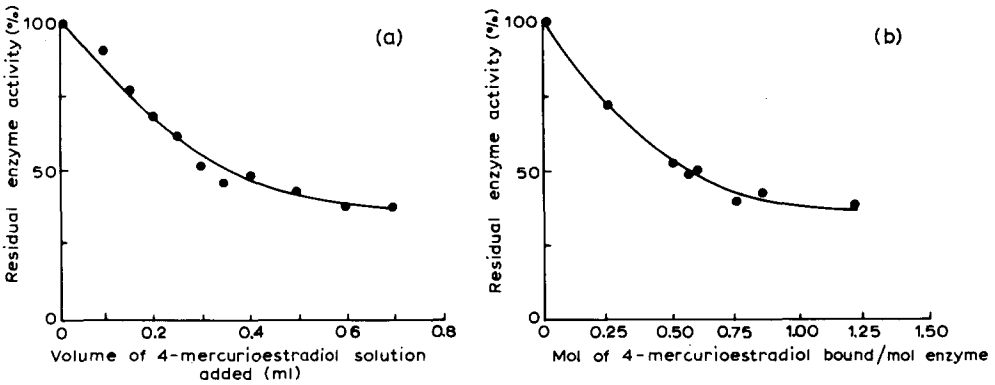

Fig. 2. (a) Effect of increasing concentration of 4-mercuri-17β-oestradiol on enzyme activity. Increasing vols of the oestrogen derivative (25 μM in 0.1 M phosphate buffer, pH 7.5) were added to oestrogen sulphotransferase (2 μM in the same buffer). Final vol was 1.0 ml. Aliquots (50 μl) were taken for enzyme assay. (b) Relationship between enzyme activity and number of mol of mercaptide formed per mol of enzyme. Conditions as above. Spectrophotometric readings were determined prior to assay.

similar to that exhibited with oestrogen sulphotransferase. Again, 11-deoxycorticosterone and testosterone were most effective in decreasing mercaptide formation.

Inhibition by 4-mercuri-17β-oestradiol and p-hydroxymercuribenzoate

Fig. 3 shows the inhibition of enzyme activity exhibited by these compounds in an experiment in which oestrogen was varied from 0—15 μM. The wave-like nature of the kinetics when oestrogen is the variable substrate [1] can be seen. The two compounds differed considerably in their inhibitory action; 4-mercuri-17β-oestradiol being some one hundred times as effective. The oestrogen derivative also caused the peaks to be shifted to the right i.e. to higher concentrations of oestrone. This effect was not observed with *p*-hydroxymercuribenzoate.

TABLE II

EFFECT OF STEROIDS ON MERCAPTIDE FORMATION WITH BOVINE SERUM ALBUMIN AND 4-MERCURI-17β-OESTRADIOL

Experimental details as per Table I.

Steroid (60 μM)	Mercaptide formed (nmol/ml)	No. of determinations
—	9.4 ± 0.7	5
17β-Oestradiol	8.0 ± 0.3	4
Dehydroepiandrosterone	8.7 ± 0.2	3
11-Deoxycorticosterone	5.8 ± 0.1	4
Testosterone	7.5 ± 0.1	4
Corticosterone	9.1, 9.0	2
Cortisol	8.4, 8.4	2
Progesterone	8.8, 8.9	2

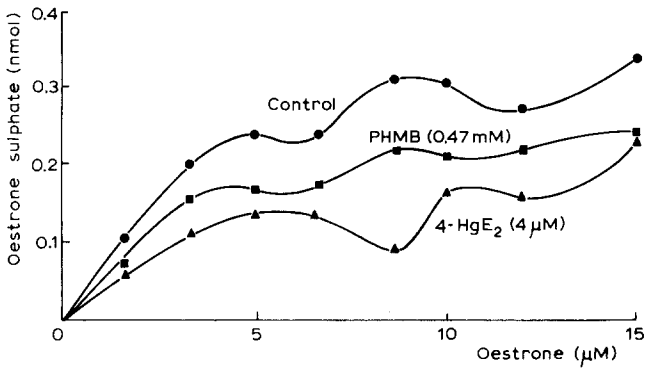

Fig. 3. Effect of the sulphydryl blocking agents *p*-hydroxymercuribenzoate (PHMB) and 4-mercuri-17β-oestradiol (4-HgE_2) on kinetics of oestrogen sulphotransferase. Enzyme was assayed as described in the Methods except that an incubation time of 10 min was employed. PHMB and 4-HgE_2 were added from stock solutions made in Tris/HCl or phosphate buffers, respectively.

Discussion

When 4-mercuri-17β-oestradiol was utilised to probe the possible involvement of the single sulphydryl group in oestrogen binding to oestrogen sulphotransferase, it became immediately apparent that the oestrogen derivative was "delivered" to the vicinity of this sulphydryl group. In previous studies it was necessary to add detergent in order to titrate the sulphydryl group, using sulphydryl reagents such as *p*-hydroxymercuribenzoate or Ellman's reagent [1]. In the absence of detergent, only 0.18 sulphydryl residues/mol of enzyme were titrated with the latter reagent [1]. As is seen from Fig. 1a and Fig. 2, 4-mercuri-17β-oestradiol reacts completely with the sulphydryl group of the enzyme in its native state. However, the activity of the enzyme was not completely inhibited when the sulphydryl group was fully converted to mercaptide (Fig. 2b). This suggested that the sulphydryl was in close proximity to, but not directly involved in, the binding of oestrogen at the active site of the enzyme. Such an interpretation is in keeping with results of the experiments recorded in Table I where various steroids were examined for their effects on mercaptide formation by 4-mercuri-17β-oestradiol. Although 17β-oestradiol apparently decreased mercaptide formation, it was not as effective as other steroids such as 11-deoxycorticosterone and testosterone. Kinetic experiments, carried out in the presence of various non-substrate steroids, showed that inhibition of enzyme activity occurred. Due to the complexity of the kinetics obtained when oestrogen is varied at constant 3'-phosphoadenylylsulphate [1], unequivocal interpretation of the nature of the inhibition was difficult. However, all the results strongly suggested that the inhibition was of the noncompetitive type. Binding of these steroids to the enzyme could then involve regions which are closer to the single sulphydryl group than are the regions concerned with the binding of oestrogens at the active site. The enzyme is known to be highly specific for oestrogens [6, 7] and will not sulphurylate such steroids as dehydroepiandrosterone, etiocholanolone, 11-deoxycorticosterone, testosterone and pregnenolone [6].

Finally, the close similarities exhibited by a group of steroids on mercaptide formation upon reaction of 4-mercuri-17β-oestradiol with oestrogen sulphotransferase and bovine serum albumin (Tables I and II), further strengthens the data which are accumulating to link these proteins genetically. Some of the similarities in composition and properties of the proteins were mentioned in the Introduction. To this we could add the fact that bovine serum albumin has a single oestrogen-binding site [8]. A peptide containing the single sulphydryl group has been isolated from bovine serum albumin [9]. Its amino acid sequence was identical to that recently reported by King and Spencer [10]. A similar peptide has been isolated from oestrogen sulphotransferase and its amino acid sequence shows a close homology with that of the sulphydryl-containing peptide of bovine serum albumin [9]. The parallel effects of steroids in altering mercaptide formation upon reaction of these proteins with 4-mercuri-17β-oestradiol (Tables I and II) could then reflect the conservation of sequences responsible for oestrogen and/or steroid binding in these proteins.

Acknowledgements

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