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AFFINITY LABELING OF THE COFACTOR-BINDING SITE OF ESTRADIOL 17β-DEHYDROGENASE OF HUMAN PLACENTA BY 5′-*p*-FLUOROSULFONYLBENZOYL ADENOSINE

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Summary—An analogue of adenosine nucleotide, 5′-*p*-fluorosulfonylbenzoyl adenosine (5′-FSB-Ado), appears to interact irreversibly with the cofactor-binding site of estradiol 17β-dehydrogenase of human placenta. This conclusion is based on the following observations: (1) The estradiol 17β-dehydrogenase is inhibited by 5′-FSB-Ado. When NAD^+ is the variable component in the presence of saturated amount of steroid, the type of the inhibition is competitive in nature. When the steroid is the variable component, mode of the inhibition becomes non-competitive. The results suggest reversible binding of 5′-FSB-Ado to the cofactor-binding site of the dehydrogenase. (2) 5′-FSB-Ado inactivates irreversibly the estradiol 17β-dehydrogenase in time- and concentration-dependent manners, following pseudo-first-order kinetics. But, no inactivation is observed in the presence of *p*-fluorosulfonylbenzoic acid, suggesting that adenosine moiety of 5′-FSB-Ado is essential for the affinity labeling of estradiol 17β-dehydrogenase. (3) $NADP^+$ protects completely estradiol 17β-dehydrogenase from the inactivation of 5′-FSB-Ado, whereas NAD(H) is partially protective against the inactivation, suggesting that phosphate moiety at 2′-position of $NADP^+$ disturbs the covalent binding of 5′-FSB-Ado at or near the cofactor-binding site of the enzyme. (4) 2′,5′-ADP shows the significant protection against the inactivation by 5′-FSB-Ado, but less effect is observed in the presence of nicotinamide mononucleotides. These results suggest that 5′-FSB-Ado is an affinity ligand for binding-site of adenosine nucleotide moiety of the cofactor.

INTRODUCTION

Estradiol 17β-dehydrogenase of human placenta catalyzes the oxidoreduction between estradiol-17β and estrone in the presence of pyridine nucleotide cofactors. Affinity labeling studies using haloacetoxylated $NAD(P)^+$ analogues have shown that this dehydrogenase possesses cysteine residues at the cofactor-binding site [1–4]. Also, lysine residues have been identified at the NAD^+-binding site of isocitrate dehydrogenase by 2′,3′-dialdehyde derivative of 5′-ADP [5] and of malate dehydrogenase by 5′-FSB-Ado [6]. 5′-FSB-Ado is a reagent useful for affinity labeling of ATP-, cAMP- or $NAD(P)^+$-dependent enzymes. The sulfonylfluoride moiety of 5′-FSB-Ado is capable of functioning as an electrophilic agent in covalent reactions with several classes of amino acids. This paper presents some evidence that 5′-FSB-Ado binds covalently at or near the cofactor-binding site in human placental estradiol 17β-dehydrogenase.

Abbreviations: 5′-FSB-Ado, 5′-*p*-fluorosulfonylbenzoyl adenosine, FSB, *p*-fluorosulfonylbenzoic acid, FSB-Cl, *p*-fluorosulfonylbenzoyl chloride, DTNB, 5,5′-dithio-*bis*(2-nitrobenzoic acid), 5′-Br-acetamide-5′-dAdo, 5′-bromoacetamido-5′-deoxyadenosine, NMN, nicotinamide mononucleotide (oxidized form) NMNH, nicotinamide mononucleotide (reduced form) and 5′-ADP-Rib, 5′-adenosine diphosphoribose.

EXPERIMENTAL

Materials

Estradiol 17β-dehydrogenase was purified by the same procedure as described by Karavolas *et al.* [7], together with the heating step [8]. The purified enzyme appeared as homogeneous by a SDS-polyacrylamide gel electrophoresis [9]. NAD^+, NADH, $NADP^+$, 5′-AMP, 5′-ADP, 5′-ATP and NMN^+ were obtained from Boehringer (Mannheim, F.R.G.). Steroids were purchased from Makor Chem. (Jerusalem, Israel). 5′-FSB-Ado, FSB-Cl, 2′-AMP, 2′,5′-ADP and NMNH were purchased from Sigma (St Louis, MO). FSB was obtained from Aldrich Chem. (Millwaukee, WI). Dithiothreitol was purchased from Nakarai Chem. (Kyoto, Japan).

Assays

All assay for estradiol 17β-dehydrogenase activity was performed at 25°C in 1 ml of 50 mM phosphate buffer (pH 7.2) containing 20% (v/v) glycerol, 760 μM NAD^+ and 50 μM estradiol. The enzymatic production of NADH during the incubation was monitored at 340 nm, using a spectrophotometer (Union Giken, SM-401, Osaka, Japan).

Reaction of estradiol 17β-dehydrogenase with 5′-FSB-Ado

5′-FSB-Ado (Fig. 1) was freshly dissolved in ethanol to give 4 mM solution. Estradiol 17β-

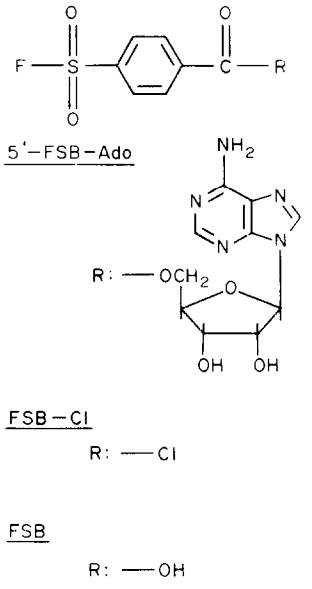

Fig. 1. The chemical formulae of the compounds used in this study.

dehydrogenase was incubated with 5′-FSB-Ado in 50 mM phosphate buffer (pH 7.2) containing 20% (v/v) glycerol at 25°C for various times as indicated in Results. $t_{1/2}$ and k_{app} were obtained from plotting log activity as a percentage of the control against time, and protection rate was expressed as its percentage obtained as follows:

$$\% \text{ Protection} = \frac{k_{app}\ (\text{control}) - k_{app}\ (\text{experiment})}{k_{app}\ (\text{control})} \times 100$$

RESULTS

Inactivation of estradiol 17β-dehydrogenase by fluorosulfonylbenzoyl derivatives

When estradiol 17β-dehydrogenase was incubated with 0.8 mM FSB-Cl in 50 mM KH_2PO_4-20% (v/v) glycerol at 25°C, the enzyme activity was rapidly inactivated ($t_{1/2}$ = 5 min). On the other hand, 0.8 mM FSB did not significantly inactivate the dehydrogenase. However, the dehydrogenase was inactivated by 5′-FSB-Ado slower ($t_{1/2}$ = 25 min) than by FSB-Cl in the same concentration (Fig. 2).

Evidence that 5′-FSB-Ado binds at the cofactor-binding site of estradiol 17β-dehydrogenase

Estradiol 17β-dehydrogenase was incubated at 25°C with 0.2 mM 5′-FSB-Ado in the presence of variable concentrations (30.4–76.0 μM) of NAD^+ and 50 μM estradiol in 50 mM phosphate buffer (pH 7.2) containing 20% (v/v) glycerol. The enzyme activity was markedly inhibited by 5′-FSB-Ado. Mode of the inhibition by 5′-FSB-Ado appeared to be competitive type against the cofactor, as shown in Fig. 3a.

In the presence of variable concentrations (4–10 μM) of estradiol and 760 μM NAD^+, estradiol 17β-dehydrogenase was incubated with 0.4 mM 5′-FSB-Ado in the phosphate buffer. By double reciprocal plots, it was found that 5′-FSB-Ado inhibited the estradiol 17β-dehydrogenase non-competitively (Fig. 3b).

Time- and concentration-dependent inactivation of estradiol 17β-dehydrogenase by 5′-FSB-Ado

Estradiol 17β-dehydrogenase was incubated with excess of 5′-FSB-Ado (50 and 100-fold on molecular basis) in 40 μl of 50 mM phosphate buffer containing 20% (v/v) glycerol (pH 7.2) at 25°C. The control experiment was carried out under the identical condition except absence of 5′-FSB-Ado. It can be seen from Fig. 4 that inactivation of estradiol 17β-dehydrogenase by 5′-FSB-Ado progressed in a concentration-dependent manner of the ligand, and followed a pseudo-first order reaction. The pseudo-first-order inactivation rate constant (k_3) was calculated as 7.7×10^{-3} s^{-1} from Kitz and Wilson plot [10].

Modification of estradiol 17β-dehydrogenase by DTNB and 5′-FSB-Ado

Since the estradiol 17β-dehydrogenase has cysteine residues at or near the cofactor-binding site [1–4], we

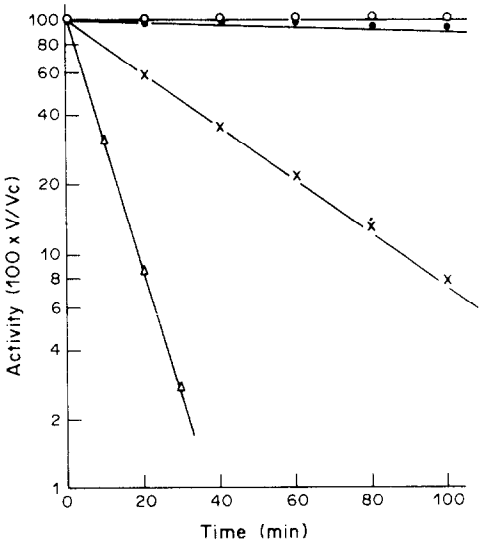

Fig. 2. Inactivation of estradiol 17β-dehydrogenase by *p*-fluorosulfonylbenzoyl derivatives. Estradiol 17β-dehydrogenase (13.2 μg protein) was incubated with 0.8 mM 5′-FSB-Ado (×), FSB-Cl (△) or FSB (●) at 25°C in 40 μl of 50 mM KH_2PO_4-20% (v/v) glycerol (pH 7.2). Aliquots (6 μl each) of the mixture were sampled at indicated times, and estradiol 17β-dehydrogenase activity was assayed. Control (○) was carried out by the same procedure without the ligand. Enzyme activity is expressed as % of the control values, where Vc is the control enzyme activity and V is the enzyme activity at the indicated time.

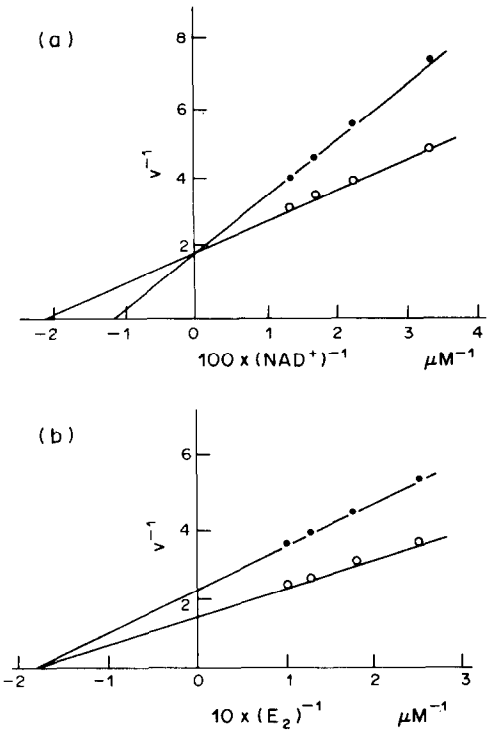

Fig. 3. Modes of inhibition of estradiol 17β-dehydrogenase by 5′-FSB-Ado. (a) Estradiol 17β-dehydrogenase (1.65 μg protein) was incubated with variable concentrations (30.4–76.0 μM) of NAD^+, 50 μM estradiol and 0 (○) or 0.2 (●) mM 5′-FSB-Ado in 1 ml of 50 mM KH_2PO_4–20% (v/v) glycerol (pH 7.2). From the kinetic analysis, K_m value for NAD^+ and V_{max} were calculated as 47 μM and 0.53 μmol·min^{-1}·protein^{-1}. K_i value for 5′-FSB-Ado was determined as 235 μM. (b) Estradiol 17β-dehydrogenase (1.65 μg protein) was incubated with variable concentrations (4–10 μM) of estradiol, 760 μM NAD^+ and 0 (○) and 0.4 (●) mM 5′-FSB-Ado in 1 ml of 50 mM KH_2PO_4-20% (v/v) glycerol. Velocity (v) is expressed in μmol·min^{-1}·mg protein^{-1}. K_m value for estradiol and K_i value for 5′-FSB-Ado were determined as 5.4 and 755 μM, respectively. V_{max} was estimated as 0.68 μmol·min^{-1}·mg protein^{-1}.

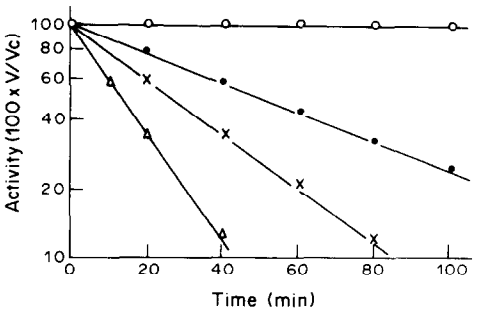

Fig. 4. Dose- and time-dependent inactivation of estradiol 17β-dehydrogenase by 5′-FSB-Ado. Estradiol 17β-dehydrogenase (13.2 μg protein) was incubated at 25°C in 40 μl of 50 mM KH_2PO_4-20% (v/v) glycerol containing 0 (○) as control, 0.4 (●), 0.8 (×) and 1.6 (△) mM 5′-FSB-Ado. Aliquots (6 μl each) of the mixture were sampled at the indicated time and the activity was assayed. Enzyme activity is expressed as % of the control values, where Vc is the control enzyme activity and V is the enzyme activity at the indicated time.

blocked its sulfhydryl (-SH) groups with 0.1 mM DTNB before the modification with 5′-FSB-Ado. Table 1 shows that the dehydrogenase activity was completely inactivated by modification of -SH groups with DTNB. But the enzyme activity was almost recovered to the initial level by further treatment with 5 mM dithiothreitol (Experiment III). The enzyme which was previously blocked -SH groups was treated with 5′-FSB-Ado. The DTNB and 5′-FSB-Ado treated enzyme which had no activity was then further treated with 5 mM dithiothreitol. The preparation obtained from the 3rd modification of Experiment IV had only 11% of activity of the native enzyme, suggesting that some amino acid residues except cysteine at or near the cofactor-binding site reacted to 5′-FSB-Ado. Almost all the enzyme activity was lost by treatment of the native enzyme with 5′-FSB-Ado. No significant reactivation of the product by dithiothreitol was observed, as shown in Experiment II.

Effect of cofactors and substrates on inactivation of estradiol 17β-dehydrogenase by 5′-FSB-Ado

By addition of 2 mM $NADP^+$, inactivation of estradiol 17β-dehydrogenase by 0.4 mM 5′-FSB-Ado was completely protected (Fig. 5). Also, NAD^+ and NADH (2 mM each) indicated 95 and 85% protection against the inactivation. When estradiol 17β-dehydrogenase was incubated with 0.4 mM 5′-FSB-Ado in the presence of 0.5 mM estrone, estradiol or estriol, the enzyme was inactivated slower than that in the absence of estrogens (Fig. 6). But the

Table 1. Effect of DTNB and 5′-FSB-Ado on activity of estradiol 17β-dehydrogenase from human placenta

	1st		2nd		3rd	
Expt	Modification	Velocity[a] (%)	Modification	Velocity (%)	Modification	Velocity
I	0 mM DTNB	1.60 (100)	0 mM 5′-FSB-Ado	1.57 (98)	5 mM Dithiothreitol	1.48 (93)
II	0 mM DTNB	1.60 (100)	1 mM 5′-FSB-Ado	0.12 (8)	5 mM Dithiothreitol	0.09 (6)
III	0.1 mM DTNB	0.04 (3)	0 mM 5′-FSB-Ado	0.04 (3)	5 mM Dithiothreitol	1.55 (97)
IV	0.1 mM DTNB	0.04 (3)	1 mM 5′-FSB-Ado	0.03 (2)	5 mM Dithiothreitol	0.17 (11)

In Experiment II, the estradiol 17β-dehydrogenase (23.1 μg protein) was treated with 1 mM 5′-FSB-Ado at 25°C for 30 min, and then 5 mM dithiothreitol was further added to the mixture. In Experiment IV, -SH groups of estradiol 17β-dehydrogenase were modified by 0.1 mM DTNB in 70 μl 50 mM KH_2PO_4-20% (v/v) glycerol (pH 7.2) for 10 min. After the DTNB-treated enzyme was further modified by 1 mM 5′-FSB-Ado for 30 min at 25°C, free -SH groups were regenerated from the DTNB-5′-FSB-Ado treated enzyme by addition of 5 mM dithiothreitol. Experiments I and III were respectively the same as Experiments II and IV, except treatment with 5′-FSB-Ado. [a]nmol·min^{-1}·0.1 nmol subunit of the enzyme^{-1}.

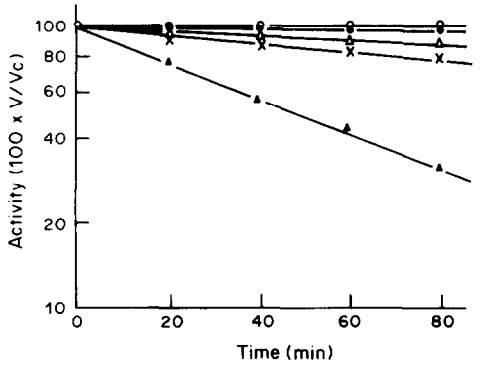

Fig. 5. Effect of cofactors on inactivation of estradiol 17β-dehydrogenase by 5′-FSB-Ado. Estradiol 17β-dehydrogenase (13.2 μg protein) was incubated at 25°C in 40 μl of 50 mM KH_2PO_4-20% (v/v) glycerol containing 0.4 mM 5′-FSB-Ado without (▲) and with 2 mM $NADP^+$ (●), NAD^+ (△) or NADH (×). Aliquots (6 μl each) of the mixture were sampled at the indicated times and the activity was assayed. Control (○) was carried out by the same procedure without the ligand. Enzyme activity is expressed as % of the control value, where Vc is the control enzyme activity and V is the enzyme activity at the indicated time.

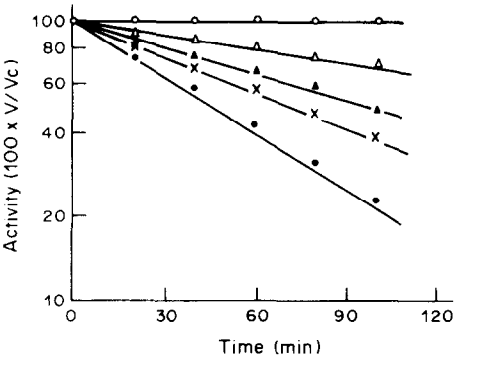

Fig. 6. Effect of steroids on inactivation of estradiol 17β-dehydrogenase by 5′-FSB-Ado. Estradiol 17β-dehydrogenase (13.2 μg protein) was incubated at 25°C in 40 μl of 50 mM KH_2PO_4-20% (v/v) glycerol containing 0.4 mM 5′-FSB-Ado without (●) and with 0.5 mM estrone (×), estradiol (△) or estriol (▲). Aliquots (6 μl each) of the mixture were sampled at the indicated time and the activity was assayed. Control (○) was carried out by the same procedure in the absence of the ligand. Enzyme activity is expressed as % of the control value, where Vc is the control enzyme activity and V is the enzyme activity at the indicated time.

inactivation by 5′-FSB-Ado in the presence of the steroids proceeded more rapidly than that in the presence of cofactors, suggesting that 5′-FSB-Ado binds preferentially the cofactor-binding site or its vicinity.

Effect of nucleotides on inactivation of estradiol 17β-dehydrogenase by 5′-FSB-Ado

Estradiol 17β-dehydrogenase was incubated with 0.4 mM 5′-FSB-Ado in the presence of several adenine or nicotinamide nucleotides at 2 mM as final concentration. As shown in Table 2, 2′,5′-ADP showed the most protective effect against the inactivation by 5′-FSB-Ado, and 2′-AMP and 5′-ATP were also significantly protective. Less protective effects were observed of 5′-AMP, 5′-ADP, 5′-ADP-Rib and NMN. No protective effect was found in the presence of NMNH.

Table 2. Protective effect of nucleotides on inactivation of estradiol 17β-dehydrogenase by 5′-FSB-Ado

Nucleotide	$t_{1/2}$ (h)	% Protection
Control	0.8	...
NMNH	0.8	0
NMN	1.3	35
2′-AMP	3.0	73
5′-AMP	1.0	14
5′-ADP	1.1	25
5′-ATP	4.1	81
5′-ADP-Rib	1.2	34
2′,5′-ADP	5.8	86

Estradiol 17β-dehydrogenase (13.2 μg protein) was incubated at 25°C with 0.4 mM 5′-FSB-Ado alone (control) or with 0.4 mM 5′-FSB-Ado and 2 mM nucleotides in 40 μl of 50 mM KH_2PO_4-20% glycerol (pH 7.2). Aliquots (7 μl each) of the mixture were sampled and estradiol 17β-dehydrogenase activity was assayed. $t_{1/2}$ Value was obtained from plotting log relative activity (%) against time, and % protection was calculated as described in the Experimental section.

DISCUSSION

Conversion of estradiol to estrone by estradiol 17β-dehydrogenase in the presence of NAD^+ was inhibited by 5′-FSB-Ado. The mode of inhibition by 5′-FSB-Ado was competitive type against NAD^+, but non-competitive one against estradiol. K_i value for 5′-FSB-Ado was 5 times as much as K_m value for NAD^+, when the cofactor was variable component. On the other hand, K_i value for the ligand was indicated 140-fold larger than K_m value for estradiol, when the cofactor was saturated. These results suggest a reversible binding of 5′-FSB-Ado at or near cofactor-binding site of the estradiol 17β-dehydrogenase.

Since 5′-FSB-Ado is a structural analogue of the cofactor, it would be possible that the ligand for affinity labeling can be preferentially and covalently bound at or near the active-site of the estradiol 17β-dehydrogenase. 5′-FSB-Ado inactivated the enzyme in time- and concentration-dependent manners, and $t_{1/2}$ value obtained from inactivation by 0.8 mM ligand was 25 min. But, the inactivation rate by 5′-FSB-Ado was slower than that by FSB-Cl ($t_{1/2} = 5$ min), because FSB-Cl has no site-specificity for lack of the adenosine moiety (Fig. 1). FSB-Cl is also a bifunctional reagent for covalently binding to a nucleophilic amino acid residue. No significant inactivation of the enzyme was observed in the presence of FSB, which is a monofunctional ligand in the absence of adenosine moiety. Inactivation of estradiol 17β-dehydrogenase by FSB-Cl progressed

by chemical modification without preference of cofactor-binding site. On the other hand, the enzyme activity was lost by 5′-FSB-Ado likely due to affinity labeling.

Inactivation of estradiol 17β-dehydrogenase by affinity labeling with 5′-FSB-Ado was almost completely prevented by addition of $NADP^+$, but partially protected by NAD^+. These findings are in agreement with the previous results of ours that $NADP^+$ is bound to estradiol 17β-dehydrogenase with much greater affinity than NAD^+ [11]. 2′,5′-ADP and 2′-AMP were significantly protective against the inactivation. These results suggest that the phosphate moeity at 2′-position of $NADP^+$ disturbs the covalent binding of 5′-FSB-Ado at or near the cofactor-binding site of estradiol 17β-dehydrogenase. On the other hand, the appreciable protection (81%) by 5′-ATP may be explained from the speculation that the terminal phosphate moiety of the nucleotide can swing around to the position of phosphate in 2′-AMP. No protection by NMNH seems to exclude the major role of this portion of the cofactor for its binding to the estradiol 17β-dehydrogenase. These results presented in this paper were in agreement with our previous findings [12, 14].

Sweet and Samant have reported that incubation of 3α(20β)-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* with 5′-FSB-Ado [14] or 5′-Br-acetamido-5′-dAdo [15] caused time-dependent and irreversible loss in the enzyme activity. They obtained the results that those inactivations were prevented by NADH, but not by progesterone. 5′-FSB-Ado inactivated NADH-dependent enzymes, such as NADH-5α-hydrogenase and NADH-cytochrome *c* reductase, but not NADPH-dependent enzymes [16]. Affinity alkylation of human placental 20α-hydroxysteroid dehydrogenase by 5′-FSB-Ado suggested that the oxidoreductions both C-20 and C-17 positions represented dual activities at one active-site in the enzyme [17].

Estradiol interrupted significantly the inactivation of estradiol 17β-dehydrogenase by affinity labeling with 5′-FSB-Ado, in this paper. 3-(Arylazido-β-alanine)estrone, analogue of the substrate, irreversibly inactivated the estradiol 17β-dehydrogenase by affinity labeling at its substrate-binding site [18]. The inactivation by 3-(arylazido-β-alanine)estrone was strongly prevented by $NADP^+$. From these results of protection experiments, the cofactor-binding site of estradiol 17β-dehydrogenase would be close to the substrate-binding region, and both the binding sites in the enzyme molecule were mutually affected. When estradiol 17β-dehydrogenase modified with 3-(arylazido-β-alanine)estrone was incubated with NAD(P)H, estrone covalently bound at the substrate-binding site can be catalytically transformed to estradiol which still remained in covalently bound form [18]. The same phenomena were observed by Groman *et al.* [19], who used 3-bromoacetoxyestrone as an affinity ligand. Those substrate analogues which affinity labeled of estradiol 17β-dehydrogenase were probably located in a certain orientation to permit approach of the cofactors to its binding site.

Several amino acids, such as cysteine [1–4], tyrosine [20], arginine [12], acidic amino acid (aspartic acid or glutamic acid) [21] and histidine [22] were identified to localize at or near the catalytic site of estradiol 17β-dehydrogenase by chemical modification and affinity labeling. By affinity labeling with 5′-*p*-fluorosulfonylbenzoyl derivatives of nucleotides, tyrosine residues at GTP-site of glutamate dehydrogenase [23], cysteine residues of carbamyl phosphate synthetase [24] and lysine residues of malate dehydrogenase [6] were labeled. By pretreatment with DTNB, cysteine residues of estradiol 17β-dehydrogenase were tested as a possible site of modification by 5′-FSB-Ado. However, cysteine residue was excluded as the site of attack by 5′-FSB-Ado from the results of differential modification shown in Table 1.

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