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DIETHYLPYROCARBONATE INHIBITION OF ESTROGEN BINDING TO RAT ALPHA-FETOPROTEIN: EVIDENCE THAT ONE OR MORE HISTIDINE RESIDUES REGULATE ESTROGEN BINDING

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SUMMARY

Rat alpha-fetoprotein contains a site that both binds serine enzyme inhibitors and substrates and regulates estrogen binding. We report that mM concentrations of the histidine selective reagent, diethylpyrocarbonate, inhibit estrogen binding to rat alpha-fetoprotein and that this inhibition is reversed by hydroxylamine. We suggest that rat alpha-fetoprotein contains one or more histidine residues that regulate estrogen binding. We also find that either estrone or the chymotrypsin substrate, acetyl-tryptophan methyl ester, protects rat alpha-fetoprotein from diethyl-pyrocarbonate-mediated inhibition of estrogen binding. We infer that the protease substrate and estrogen binding sites contain histidine residue(s) essential for estrogen binding by alpha-fetoprotein.

"Serine" proteases like trypsin and chymotrypsin contain a histidine residue at the catalytic site which reacts with chloromethyl ketone substrate analogues like TLCK and TPCK (1,2). Our finding that TPCK, TLCK and other inhibitors and substrates of serine enzymes can inhibit the binding of adrenal and sex steroid hormones to their receptors (3-5) has formed the basis of a hypothesis that steroid hormone binding proteins contain a histidine residue that is essential for the binding of both steroids and protease inhibitors and substrates. In this report, we describe experiments designed to test this hypothesis with the estrogen binding protein, rat alpha-fetoprotein (AFP). Our experiments show that the histidine-selective reagent, diethylpyrocarbonate (DPC) (5-9) inhibits estrogen binding to AFP. Moreover, either estrone or the chymotrypsin substrate, N-acetyl-tryptophan methyl ester (Ac-TrpOMe) can

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protect AFP from DPC-induced inhibition of estrogen binding. From these findings, we suggest that AFP contains one or more histidine residue(s) essential for the binding of both estrogen and protease substrates.

MATERIALS AND METHODS

The source of rat alpha-fetoprotein, serum from buffalo rats with Morris Hepatoma 7777, was a gift from Dr. Stewart Sell.

For each experiment, diethylpyrocarbonate (6.9M) was diluted to 200 mM into ice cold ethanol, then quickly diluted in ice cold 20 mM TES, 2 mM EDTA, 50 mM NaCl, 20% Glycerol (TEG) buffer, and then added to the AFP solutions. Control samples received a similar aliquot of TEG-ethanol. AFP (20-80 nM) was incubated with DPC for the indicated times and reaction terminated by diluting the AFP-DPC solution 1/40 into TEG @0°. This dilution would be expected to reduce the rate of a bimolecular reaction by 1600 fold. We also have direct experimental evidence that the reaction was terminated because 0.1 mM DPC, a DPC concentration at least 2 times that in the diluted solutions, is ineffective in inhibiting estrogen binding to AFP. The concentration of DPC in the stock solution was determined by the method of Melchior and Fahrney (6). This involves incubating DPC with 20 mM imidazole pH 7.2 for 10 minutes at room temperature and measuring the increase in optical density at 230 nm.

After the DPC-AFP solution was diluted, the binding of estrogen by AFP was determined by incubating the diluted AFP solution with 3-6 nM ^{3}H-estrone $\pm$ 200 fold unlabelled estrone for 2 hours at 0°. Bound steroid was separated from unbound steroid using a dextran-coated charcoal assay (10) which involves incubation of 2 ml sample solution with 0.2 ml of 100 mg/ml charcoal, 10 mg/ml dextran, 10 mg/ml ovalbumin for 1 minute, and then centrifugation at 6,000 xg for 15 minutes to remove the charcoal. The radioactivity in 1 ml of supernatant was determined in a liquid scintillation counter. Specifically bound estrone was determined by subtracting the amount of ^{3}H-estrone bound in the presence of a 200 fold excess concentration of non-radioactive estrone from the amount of ^{3}H-estrone bound in the absence of the non-radioactive estrone.

RESULTS AND DISCUSSION

Reaction of alpha-fetoprotein with diethylpyrocarbonate. Initial studies showed that incubation of ~40 nM AFP with 0.5 to 5 mM DPC inhibited estrogen binding to AFP. Figure 1 shows results of one of several experiments where inhibition of estrone binding to AFP by DPC at pH 7.2, 22° was demonstrated. The deviation from pseudo-first order kinetics can be attributed to a decreasing concentration of DPC during the reaction caused by the spontaneous hydrolysis of DPC that occurs at neutral pH (6,8). Two further findings are consistent with our inference that this inhibition is due to covalent reaction of DPC with AFP. First, the DPC had been diluted prior to the estrogen binding assay to a nominal concentration of 0.05 mM DPC, which does not inhibit estrogen

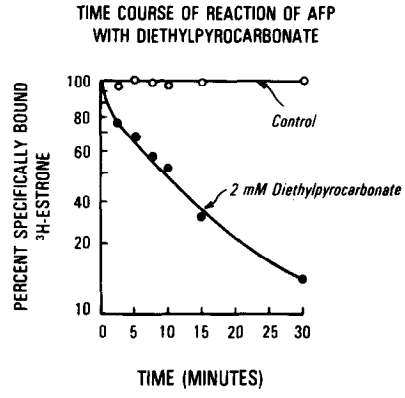

Figure 1: Time course of reaction of rat alpha-fetoprotein with diethylpyrocarbonate. AFP was incubated alone or with 2 mM DPC at pH 7.2, 22°. At indicated time intervals 0.1 ml aliquots were diluted 1/40 and incubated with 3 nM ^{3}H-estrone $\pm$ 6 x 10^{-7}M estrone for 2 hours at 0°. Then ^{3}H-estrone specifically bound to AFP was determined using the Dextran-coated charcoal assay. Specifically bound ^{3}H-estrone in the control sample was 45,600 cpm/ml.

binding to AFP. Second, we also found that inhibition of estrogen to AFP does not reverse after 20 hours at 0°, a time sufficient for spontaneous hydrolysis to reduce the concentration of DPC substantially from 0.05 mM and for any DPC reversibly bound to AFP to have dissociated.

Diethylpyrocarbonate inhibits estrogen binding by AFP by reacting with histidine residue(s). Even though at 0.5 - 2.0 mM, DPC usually reacts with histidine residues, reaction with serine or tyrosine residues are also possible (6-8). For example, Melchior and Fahrney (6) found that DPC reacted with the catalytic serine on chymotrypsin at pH 4. However, they also found that the reaction product was unstable at neutral pH ($t_{1/2}$=30 min at 25°) and brief incubation at neutral pH restored chymotrypsin activity. We find that DPC inhibition of estrogen binding to AFP at neutral pH is stable for at least 1.5 hours @37°, 3 hours @25°, and 20 hours at 0° which makes it unlikely that DPC is inhibiting estrogen binding by AFP by reacting with serine.

If DPC is reacting with a histidine residue(s) on AFP to inhibit estrogen binding, then incubation of the DPC-reacted AFP with hydroxylamine should

TABLE 1: Hydroxylamine Reverses Diethylpyrocarbonate Inhibition of ^{3}H-Estrone Binding to Rat Alpha-Fetoprotein

Condition	Specifically Bound ^{3}H-Estrone (CPM/ml)
control	120,000
2 mM DPC (1 hr, 22°)	6,800
2 mM DPC (1 hr, 22°) then 0.4 M NH_2OH (1 hr, 37°)	77,000

Rat AFP was incubated alone or with 2 mM DPC for 1 hr at pH 7.2, 22°. Then aliquots from the control and DPC incubated samples were 1) assayed for ability to specifically bind 4.5 nM ^{3}H-Estrone and 2) incubated with .4 M NH_2OH for 1 hr at 37° and assayed for ability to specifically bind 4.5 nM ^{3}H-Estrone. Results for each condition are the average of duplicate samples. Variation between samples was less than 10%. 28,000 CPM/ml = 3 x 10^{-10}M ^{3}H-Estrone.

restore the ability of AFP to bind estrogen. As seen from table 1, incubation with 0.5M hydroxylamine after inhibition by DPC restores most of the estrogen binding capability. This result suggests that histidine residue(s) are essential for binding of estrogen by AFP.

Where is the diethylpyrocarbonate binding site(s)? DPC inhibition of estrogen binding by AFP could be non-specific effects on the tertiary structure of AFP due to reaction of DPC with some of the approximately 15 histidine residue(s) on AFP (11) or it could be due to reaction with histidine residue(s) at the protease substrate and/or estrogen binding sites. We tested these possibilities by testing for protection of AFP from DPC inhibition of estrogen binding by incubation with estrone or with the chymotrypsin substrate, Ac-TrpOMe, which is also a competitive inhibitor of estrogen binding to rat AFP (10).

The experiments with estrone protection of AFP from DPC inhibition of estrogen binding are complicated by the rapid off-rate of estrone from AFP in TEG buffer ($t_{1/2}$ ~ 25 minutes @ 0°). Therefore, even though greater than 99% of the AFP molecules in our sample would be occupied by 10^{-6}M estrone, the rapid exchange of estrone in the presence of 2 mM DPC at room temperature would not cause complete protection, because of the 10^{3} greater concentration ratio of DPC to estrone. We did find in preliminary experiments, that 2 mM

TABLE 2: Estrone Protects Rat Alpha-Fetoprotein From Diethylpyrocarbonate Inhibition of ^{3}H-Estrone Binding.

	Condition	Specifically Bound ^{3}H-Estrone (CPM/ml)
a)	control	43,000
	+2mM DPC (1hr, 24°)	5,700
	+2mM DPC + 7.5 μM Estrone (1 hr, 24°)	18,500
b)	control	40,600
	+5mM DPC (1hr,0°)	16,600
	+5mM DPC + 0.75 μM Estrone (1hr,0°)	29,400

a) Rat AFP was incubated with 7.5 μM estrone in TEG buffer (40% glycerol) for 1 hr at 24° and then with 2 mM DPC for 1 hr. The reaction with DPC was stopped by sample dilution into TEG (20% glycerol) at 0°. This solution was treated twice with Dextran-coated charcoal (10 minutes, 0°) to remove unbound estrone. Then the solution was incubated with 3.0 nM ^{3}H-estrone + 1.5 x 10^{-6}M estrone for 2 hours, 0°, and specifically bound ^{3}H-estrone was determined using the Dextran-coated charcoal technique.

b) Rat AFP was incubated with 0.75 μM estrone for 30 minutes @22° and then 40 minutes @0°, in TEG buffer (40% glycerol) and then with 5 mM DPC for 1 hr @0°. The reaction with DPC was stopped by sample dilution into TEG (20% glycerol) at 0°. This solution was then treated once with Dextran-coated charcoal (20 minutes, 0°) to remove unbound estrone and then incubated with 4.5 nM ^{3}H-estrone $\pm$ 1.5 x 10^{-6}M estrone for 2 hours at 0°, and specifically bound ^{3}H-estrone was determined using the Dextran-coated charcoal technique.

DPC (1 hr, 24°) reduced specifically bound ^{3}H-estrone from 61,500 cpm to 4,500 cpm, whereas AFP protected with 6 x 10^{-5}M estrone specifically bound 17,000 cpm.

With the goal of decreasing the rate of dissociation of estrone from AFP and, therefore, reducing the estrone concentration required to protect AFP from DPC inhibition of estrone binding, we increased the glycerol concentration in the buffer to 40%. This lengthened the dissociation time of estrone from AFP to about 110 minutes @ 0° (data not shown). Table 2 shows that, at 24°, 7.5 x 10^{-6}M estrone significantly protected AFP from DPC reaction at the site(s) that control estrogen binding. Table 2 also shows that at 0°, 7.5 x 10^{-7}M estrone protected AFP from DPC inhibition of estrone binding even though the

TABLE 3: N-Acetyl-L-Trptophan Methyl Ester Protects Rat Alpha-Fetoprotein from Diethylpyrocarbonate Inhibition of ^{3}H-Estrone Binding.

Condition	Specifically Bound ^{3}H-Estrone (CPM/ml)
control	55,500
+2.75mM DPC (1 1/2 hr, 0°)	13,000
+2.75mM DPC + 0.6 mM Ac-TrpOMe (1 1/2 hr 0°)	23,000

Rat AFP was incubated alone or with 0.6 mM Ac-TrpOMe for 1 hr at 0°, in pH 7.2 TES + 2 mM EDTA + 50 mM NaCl + 40% glycerol buffer. Then samples were incubated alone or with 2.75 mM DPC for 1 1/2 hr @ 0° and aliquots diluted into TEG (20% glycerol) buffer and incubated with 4.5 nM ^{3}H-Estrone $\pm$ 3 μM Estrone for 2 hours at 0°. Specifically bound ^{3}H-Estrone was determined using the Dextran-coated charcoal technique. Results for each condition are the average of duplicate samples. Variation between samples was less then 10%.

DPC concentration was increased to 5 mM. These results suggest that the estrone binding site on AFP contains a histidine residue(s) that is required for estrogen binding.

The chymotrypsin substrate, TrpOMe, is a competitive inhibitor of estrone binding to AFP (10). Therefore, we would expect this substrate to protect AFP from DPC inhibition of estrogen binding. In the protection experiment, we used N-Ac-L-TrpOMe instead of N-Ac-D-TrpOMe or D-TrpOMe for several reasons. First, the L isomers of TrpOMe or Ac-TrpOMe bind AFP about 40% better than the D isomer (10). Second, Rosen et al (12) and our unpublished studies show that the alpha amino group of tryptophan will react with DPC, and that N-acetyl-tryptophan does not react with DPC in aqueous solution. And third, while at pH 8.5 Ac-TrpOMe binds AFP as well as TrpOMe, the binding of Ac-TrpOMe to AFP does not decrease as the pH is lowered (unpublished results)as does TrpOMe (10).

In an initial experiment 1 mM Ac-TrpOMe significantly protected AFP from inhibition of ^{3}H-E_1 binding in TEG (20% glycerol) buffer pH 7.2 by 2 mM DPC at 0° and 20°. We then employed the strategy of increasing the glycerol concentration in the buffer to slow down the dissociation of Ac-TrpOMe from AFP. As shown in Table 3, using this strategy we could lower the Ac-TrpOMe concentration to 0.6 mM (which could occupy about 75% of the substrate binding sites (10)), increase the DPC concentration to 2.75 mM and still get significant protection

by Ac-TrpOMe of AFP against inhibition of estrogen binding by DPC. Our results suggest that the protease substrate binding site on AFP contains histidine residue(s) that are essential for estrogen binding.

Based on our observation that protease inhibitors and substrates can inhibit the binding of steroid hormones to receptors, we have formulated a hypothesis that steroid hormone binding proteins contain spatially adjacent histidine and serine residues which can bind protease inhibitors and substrates and regulate steroid hormone binding (3). The results of our studies on DPC inhibition of estrogen binding by AFP and the protection of AFP by Ac-TrpOMe and estrone provide an independent line of evidence to support the hypothesis that a histidine residue(s) is essential for binding of protease substrates and estrone by AFP.

We have also suggested that the estrone and TrpOMe binding sites on AFP are spatially close and may even overlap, because TrpOMe is a competitive inhibitor of estrone binding to AFP (10). The protection against DPC inhibition of estrone binding of AFP by Ac-TrpOMe and estrone supports the hypothesis that the protease substrate and estrone binding sites are spatially close.

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