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Interaction of Chick Oviduct Progesterone Receptor with the 2′,3′-Dialdehyde Derivative of Adenosine 5′-Triphosphate†

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ABSTRACT: Avian oviduct progesterone receptor was treated with the 2′,3′-dialdehyde derivative of ATP (oATP) in an attempt to demonstrate the presence of nucleotide binding sites on the receptor. oATP, when added to cytosol, inhibited binding by transformed receptor to ATP–Sepharose, DNA–cellulose, phosphocellulose, or isolated nuclei in an irreversible manner. oATP did not disrupt the steroid–receptor complex, but it did alter the ionic properties of the receptor. This was demonstrated by an increased affinity of receptor for DEAE-cellulose and for hydroxylapatite. oATP mimicked the effect of ATP on progesterone receptor with regard to two properties: it altered the rate of receptor inactivation that occurs in the absence of progesterone, and it promoted receptor conversion from an 8S complex to lower sedimenting forms (4–6 S). The action of oATP on the receptor could be blocked by the addition of pyridoxal 5′-phosphate, which has been shown previously to interact with the progesterone receptor. A partial interference of oATP action was also observed when ATP was added. These results indicate that oATP interacts with the progesterone receptor and may be used as an affinity-labeling agent for receptor characterization.

Previous work in this laboratory has established that a relationship exists between the presence of ATP and the observed properties of the avian progesterone receptor. First, it was found that the receptor binds ATP–Sepharose (Moudgil & Toft, 1975, 1977) through a reaction that is reversed by high ionic strength or free ATP. The receptor must be in the transformed state[1] in order to bind to the ATP–Sepharose (Miller & Toft, 1978), and receptor transformation by a variety of methods including high ionic strength, elevated temperatures, increased pH, or dilution will yield the ATP binding form of the receptor (Toft et al., 1980). Additionally, high concentrations of ATP (greater than about 3 mM) enhance the temperature-induced transformation reaction while lower concentrations of ATP (less than 1 mM) inhibit this transformation reaction (Toft et al., 1977). Also, higher concentrations of ATP alone (about 10 mM) will tranform receptor to the ATP–Sepharose binding form in the absence of increased temperature (Moudgil et al., 1981; V. Moudgil et al., unpublished observations).

Several enzyme systems that interact with nucleotides have been characterized with respect to their nucleotide binding site(s) by use of the 2′,3′-dialdehyde derivative of the nucleotide as an affinity-labeling compound that binds covalently at the nucleotide binding site (Bragg et al., 1981; Easterbrook-Smith et al., 1976; Fayat et al., 1978; Gregory & Kaiser, 1979; King & Carlson, 1981; Kumar et al., 1979; Malcolm & Moffatt, 1978; Westcott et al., 1980). A survey of the data from these studies indicates that the usual effect of the dialdehyde on the enzyme is one of inhibition of the enzymatic activity.

While the avian progesterone receptor is affected by ATP, as mentioned above, it is not known whether the ATP exerts its effects directly on the receptor or whether other cytosolic

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[1] Transformation refers to the process by which steroid-bound receptor is converted from the cytoplasmic form to its nuclear counterpart. Inactivation refers to the process by which steroid-free receptor is converted to a form incapable of binding steroid.

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components may be involved. Therefore, we determined whether the 2′,3′-dialdehyde of ATP (oATP) could be used as a probe to characterize any ATP binding site(s) on the receptor, in a fashion analogous to that observed for certain nucleotide-dependent enzymes. Our results indicate that the dialdehyde of ATP interacts with the progesterone receptor to (1) inactivate steroid-free receptor, (2) alter the ionic characteristics and sedimentation properties of the transformed, labeled receptor, and (3) inhibit ATP–Sepharose, DNA–cellulose, phosphocellulose and nuclear binding of transformed labeled receptor.

Experimental Procedures

[1,2-$^3H_2$]Progesterone (50 Ci/mmol) and [^{14}C]formaldehyde (10 Ci/mmol) were obtained from New England Nuclear; ATP, Tris,[2] and sucrose (density-gradient grade) were from Schwarz/Mann; pyridoxal 5′-phosphate, dithiothreitol (DTT), cortisol, and diethylstilbestrol (DES) were from Sigma; glycerol was from Eastman Kodak. All other chemicals were from Fisher Scientific Co. Distilled/deionized water was used to prepare reagents.

Buffers. Two buffers, both pH 8.0 at 23 °C, were used predominantly with KCl or $MgCl_2$ added where indicated: (barbital buffer) 20 mM sodium barbital and 10% (v/v) glycerol; (Tris buffer) 10 mM Tris, 12 mM thioglycerol, 1 mM EDTA, and 10% (v/v) glycerol.

Preparation of [^{3}H]Progesterone-Labeled Receptor. Oviducts were excised from White Leghorn chicks pretreated with diethylstilbestrol for 2–4 weeks as described previously (Toft & O'Malley, 1972). The tissue was rinsed in normal saline and then homogenized in 4 volumes of barbital buffer with a Polytron homogenizer (Brinkmann). The temperature for this and subsequent preparative procedures was kept at 0–4 °C. The homogenate was centrifuged at 23000*g* for 10 min and then at 145000*g* for 60 min to prepare the cytosol fraction.

[^{3}H]Progesterone (10^{-6} M) plus cortisol (2×10^{-4}) in ethanol were each added to a test tube at $^1/_{50}$ of the cytosol volume and dried under N_2. The cytosol was added and incubated with the steroids (final concentration of [^{3}H]progesterone 20 nM) at 0–4 °C for 2–4 h, yielding labeled progesterone receptor.

Transformation of Labeled Receptor. Samples of labeled cytosol were incubated for 1 h at 4 °C with 0.25 volume of 2 M KCl in barbital buffer, resulting in a final concentration of KCl equal to 0.4 M. Following dialysis for 1 h in buffer without KCl, about 50–70% of the receptor was found to be transformed as detected by ATP–Sepharose binding (Miller & Toft, 1978). For certain experiments, the KCl treatment was extended to 18 h to give a higher proportion of transformed receptor. For some indicated experiments, transformation was effected by the ammonium sulfate precipitation of receptor (see below).

Preparation of Ammonium Sulfate Precipitated Receptor. [^{3}H]Progesterone-labeled receptor was prepared as described above. For removal of some extraneous ATP binding proteins, the sample was passed through an ATP–Sepharose column (bed volume equal to sample volume). The nontransformed receptor was recovered in the flow-through fractions and brought to 35% (v/v) ammonium sulfate with a saturated solution of ammonium sulfate in water, pH 8.0 at 23 °C. After 1 h, the receptor was pelleted by centrifugation for 20 min at 12000*g* and stored at −70 °C until use. For experimentation, the frozen pellets were dissolved in 25% of the original cytosol volume in barbital buffer and dialyzed against 100 volumes of the same buffer for 1 h.

Preparation of 2′,3′-Dialdehyde of Adenosine 5′-Triphosphate (oATP) and Adenosine 5′-Monophosphate (oAMP). ATP was oxidized essentially by the method of Easterbrook-Smith et al. (1976). Briefly, ATP (0.2 mmol) and $NaIO_4$ (0.24 mmol) were dissolved separately in 2 and 4 mL of H_2O, respectively, and adjusted to pH 7.1 and 6.9, respectively. Following cooling on ice for 10 min, the solutions were mixed and incubated in the dark on ice for 1 h. The resulting reaction mixture (6 mL) was applied to a 75 × 1.6 cm column of Sephadex G-10 and eluted with H_2O to separate the nucleotide from IO_3^-. The eluted fractions were sampled for absorbance at 258 nm to detect the nucleotide and sampled by a starch–iodide test for the presence of IO_3^-. The G-10 column fractions containing nucleotide but no IO_3^- (slightly in excess of the lead half of the nucleotide peak) were combined and lyophilized. The lyophilized powder was redissolved in H_2O and the concentration of nucleotide determined by measuring its OD at 258 nm with an extinction coefficient of 14900 (Easterbrook-Smith et al., 1976). For verification that the reaction conditions were adequate for complete oxidation of the nucleotide, selected samples were analyzed by poly(ethylenimine) thin-layer chromatography in 0.4 M NH_4HCO_3 as the developing solvent. oAMP was prepared identically but was less well separated from IO_3^- on the Sephadex G-10 column so that only about the first 0.25 of the nucleotide peak was utilized.

ATP–Sepharose, DNA–Cellulose, and Phosphocellulose Column Chromatography. ATP–Sepharose was prepared as described previously (Moudgil & Toft, 1975) by the method of Lamed et al. (1973). Our preparations contained 6–13 μmol of ATP/mL of packed Sepharose. The ATP–Sepharose was washed with 1 M KCl in barbital buffer and then with barbital buffer before use; used columns were regenerated by similar washes.

DNA–cellulose was prepared by the method of Alberts & Herrick (1971) with Cellex N-1 (Bio-Rad) cellulose and calf thymus DNA (Sigma). The final resin contained about 1 mg of DNA/g of cellulose and was equilibrated with 1 M KCl in barbital buffer overnight and then washed extensively with barbital buffer before use.

Phosphocellulose (Whatman, PII) was precycled with 0.5 M NaOH and 0.5 M HCl before equilibration with 50 mM Tris, 12 mM thioglycerol, and 0.01 M KCl buffer (pH 8.0 at 23 °C). Prior to use, columns of phosphocellulose were washed with about 20 volumes of barbital buffer.

All columns contained 1–2-mL packed volume of resin that was equilibrated with barbital buffer prior to sample application. In general, treated or control samples equivalent to a 0.5-mL volume of original cytosol were layered on the resin and eluted at about 1 mL/min. The columns were washed with 20 mL of barbital buffer at about 2 mL/min to remove unbound radioactivity. Elution of bound radioactivity was achieved with 15 mL of 1 M KCl in barbital buffer, and the resulting eluate was sampled and counted for radioactivity.

DEAE-cellulose and Hydroxylapatite (HAP) Binding Assays. DEAE-cellulose (Whatman, DE-52) and hydroxylapatite (Bio-Rad, Bio-Gel HTP) were washed and then equilibrated with Tris buffer (see Buffers) overnight before use. Treated or untreated samples of cytosol in barbital buffer (1.5 mL) were dialyzed for 3 h against Tris buffer and applied to DEAE-cellulose or HAP columns of 5- or 2-mL packed volumes, respectively. Columns were washed with low-salt

[2] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

buffer and bound radioactivity was eluted either with stepwise or with linear salt gradients as indicated in figure legends.

Nuclear Binding Assays. Chick oviduct nuclei were isolated by the method of Spelsberg et al. (1974), omitting the Triton X-100 wash. Binding of receptor to the nuclei was performed as described previously (Lohmar & Toft, 1975). Samples of 0.2 mL of cytosol were incubated with 0.225 mL of buffer plus 0.075 mL of suspended nuclei at 4 °C for 1 h. Following two washes with 2 mL of 20 mM barbital, 1 mM $MgCl_2$, and 0.1% Triton X-100, pH 8.0 at 23 °C, buffer, the nuclei were suspended in 0.5 mL of H_2O, and the nuclear-bound radioactivity was determined.

Charcoal-Adsorption Binding Assay. Progesterone–receptor complex was quantitated in samples by the charcoal-absorption method previously described (Toft & O'Malley, 1972; Moudgil & Toft, 1977). Briefly, 0.5 mL of dextran–charcoal suspension (0.25% charcoal and 0.025% dextran T-70 in barbital buffer) was mixed with 0.1–0.2 mL of sample. Following a 10-min incubation on ice, the samples were centrifuged at 800*g* for 5 min, and the radioactivity in the supernatant was determined.

Sucrose Gradient Analysis. Linear 5–20% (w/v) sucrose gradients (4.5 mL) in 50 mM potassium phosphate and 12 mM thioglycerol buffer (pH 7.0 at 23 °C), with or without 0.3 M KCl, were prepared by the layering diffusion method (Stone, 1974; Nishigori & Toft, 1979). Samples for analysis (0.25 mL) were mixed with 0.01 mL of [^{14}C]ovalbumin (Rice & Means, 1971), layered (0.2 mL), and centrifuged at 145000*g* for 16 h at 4 °C. Fractions were collected by piercing the tube bottoms and the radioactivity from [^{3}H]progesterone was first determined in 5 mL of scintillation cocktail I where [^{14}C]ovalbumin remained in the aqueous phase. Ten milliliters of scintillation cocktail II was added to each vial for the detection of dissolved [^{14}C]ovalbumin. Appropriate channel selection assured quantitative determination of both ^{3}H and ^{14}C without cross interference.

Hydroxylapatite (HAP) Assay To Identify oATP-Affected Receptor. Hydroxylapatite (Bio-Rad, DNA grade) was washed and suspended in 10 volumes of barbital buffer. To 1 mL of this suspension was added 0.1 mL of the test sample, and the resulting mixture was incubated on ice for 30 min. The HAP was pelleted by centrifugation for 5 min at 800*g* and washed 3 times with barbital buffer containing 0.15 M potassium phosphate. The radioactivity associated with this washed pellet was extracted with 2 mL of ethanol and represented receptor with an increased affinity for HAP.

Other Methods. Radioactivity from [^{3}H]progesterone in samples was determined by the addition of 5 mL of scintillation cocktail I (toluene and Scintiprep I, 95:4 v/v); for ^{14}C, an additional 10 mL of scintillation cocktail II (toluene, Triton X-100, and Scintiprep I, 190:99:8 v/v/v) was used. The counting efficiency for tritium was about 48% with cocktail I on a Beckman LS 250 liquid scintillation counter.

Results

Effect of oATP on ATP–Sepharose Binding by Labeled Transformed Progesterone Receptor. Transformed progesterone receptor binds ATP–Sepharose (Moudgil & Toft, 1975; Miller & Toft, 1978), indicating that the receptor probably contains an ATP binding site. If oATP were able to bind to this ATP binding site, an inhibition of receptor binding to ATP–Sepharose would be expected. So that this possibility could be tested, receptor labeled with [^{3}H]progesterone was transformed by KCl and treated with oATP. Shown in Figure 1A is the dose–response curve for the oATP effect on ATP–Sepharose binding by transformed labeled progesterone receptor. The oATP exhibits a concentration-dependent inhibition of binding to this resin with maximal inhibition observed at about 10 mM oATP. This inhibition is clearly not due to a disruption of the steroid–receptor complex (Figure 1A). In Figure 1B is the time course for the inhibition of ATP–Sepharose binding. A dose of 10 mM oATP gives a time-dependent inhibition of ATP–Sepharose binding; about 90% inhibition is seen by 3 h, and almost total inhibition is possible by 18 h.

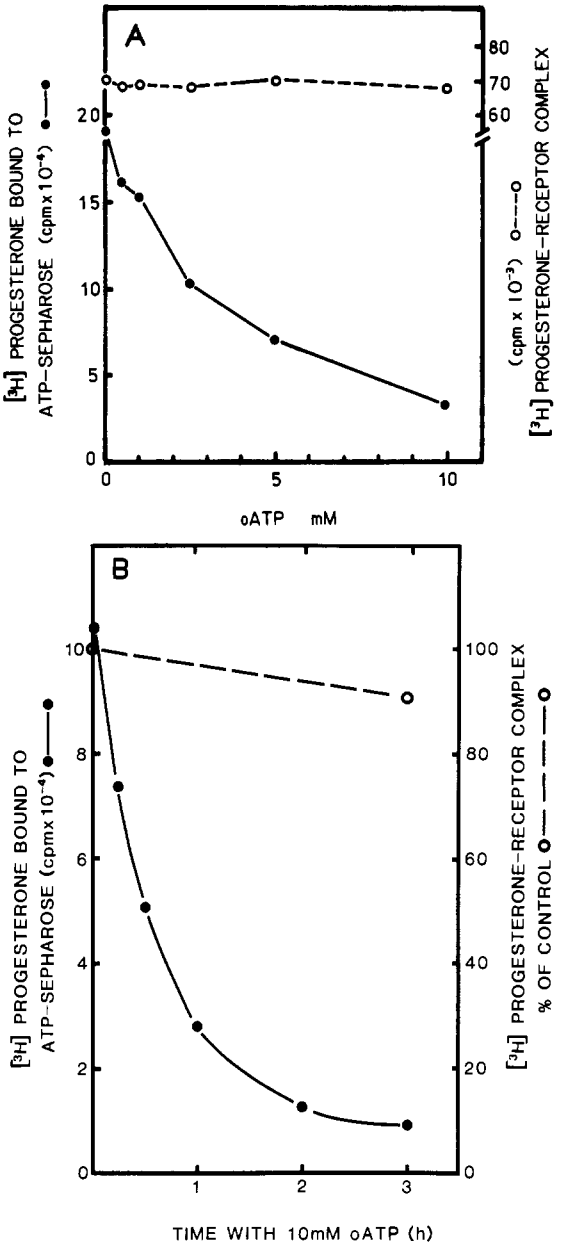

FIGURE 1: Dose–response relationship (A) and time course (B) for inhibition by oATP of ATP–Sepharose binding by transformed progesterone–receptor complex. Chick oviduct cytosol was prepared and labeled with [^{3}H]progesterone as described under Experimental Procedures. Receptor was transformed by exposure to 0.4 M KCl for 1 h at 0 °C, dialyzed 1 h against barbital buffer, and treated with oATP. For (A), increasing doses of oATP were reacted with receptor for 4 h. For (B), 10 mM oATP was reacted with receptor for increasing periods of time. The resulting samples were applied to 2-mL columns of ATP–Sepharose and washed with buffer, and bound receptor (●) was eluted with 1 M KCl as described under Experimental Procedures. The total amount of [^{3}H]progesterone–receptor complex was measured by the charcoal-adsorption assay (○).

In addition to ATP–Sepharose, the transformed progesterone receptor is able to bind to DNA–cellulose, phosphocellulose, and isolated oviduct nuclei. We tested the binding

Table I: Effect of Various Agents on oATP Activity toward Progesterone Receptor[a]

	[^{3}H]progesterone bound to ATP-Sepharose (% of control)	
	minus oATP	plus oATP
none	100 (control)	4
amino compounds		
lysine, 200 mM	140	80
lysine, 100 mM	122	72
Tris, 500 mM	155	40
ethanolamine, 200 mM	130	30
methylamine, 200 mM	104	12
diethylamine, 200 mM	114	4
reducing agents		
cysteine, 200 mM	116	124
dithiothreitol, 100 mM	115	3
$NaBH_4$, 10 mM	93	5
$NaCNBH_3$, 10 mM	91	3
$NaBH_4$, 10 mM[b]	94	110
$NaCNBH_3$, 10 mM[b]	99	2

[a] Cytosol receptor was prepared, labeled with [^{3}H]progesterone, and transformed with KCl as described under Experimental Procedures. The compounds indicated were added to cytosol samples for 30 min at 4 °C, and the samples were then incubated with or without 10 mM oATP for 17 h at 4 °C. ATP-Sepharose binding by receptor was measured as in Figure 1. [b] In these samples, the test compound was added at the same time as oATP, not 30 min beforehand.

of receptor to these following treatment with 10 mM oATP at 0 °C for 16 h. In all cases, greater than 90% of the binding activity was inhibited by oATP (results not shown).

It should be noted that care was taken to remove all iodate from our nucleotide preparations. This was a very important precaution since $NaIO_3$, when added to cytosol containing transformed receptor, also inhibited receptor binding to ATP–Sepharose in a dose-dependent manner with about 50% inhibition of binding at approximately 0.5 mM $NaIO_3$ (data not shown).

Properties of the Reaction with oATP. We examined the effect of a number of compounds on the interaction of oATP with the receptor. Shown in Table I are the effects of amino and reducing compounds on the ability of oATP to inhibit the ATP–Sepharose binding of KCl-transformed receptor. The KCl treatment used (0.4 M KCl at 4 °C for 60 min) in these experiments transformed approximately 50% of the receptor to the ATP–Sepharose binding form indicated in the table as the 100% control value. The amines and cysteine, as well as dithiothreitol, caused a further transformation of the receptor as indicated by values of greater than 100%. As shown in Table I, treatment of receptor with 10 mM oATP for 17 h reduced ATP–Sepharose binding to 4% of the control binding. Lysine (100–200 mM) prevented about 50% of the oATP effect while the other amines were less effective. Cysteine (200 mM) completely blocked the oATP effect, and cyanoborohydride had no effect. Sodium borohydride was ineffective when added prior to the oATP. However, when added simultaneously with the oATP, it totally inhibited the oATP effect. The results with the borohydride are consistent with the idea that the preincubation allowed an inactivation of the borohydride through its interaction with various cytosol components while addition of BH_4 with the oATP led to a reduction of the dialdehyde to an inactive diol (Sonenberg & Shatkin, 1977). The lack of effect on oATP action by the reducing agent, cyanoborohydride, is consistent with the inability of this agent to reduce aldehydes (Borch et al., 1971; Jentoft & Dearborn, 1979). Cysteine, the most effective inhibitor of oATP action, when added subsequent to the oATP,

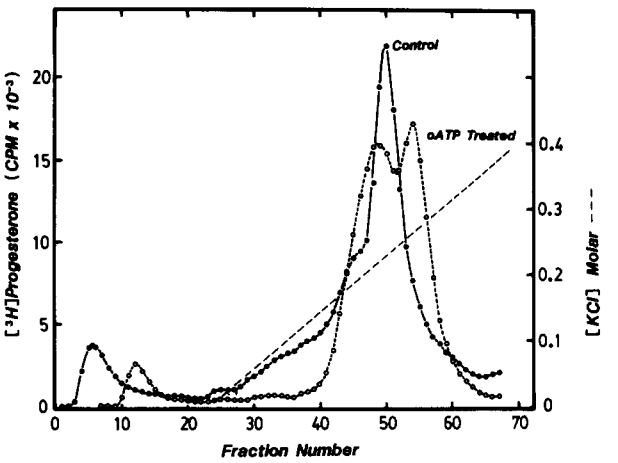

FIGURE 2: Effect of oATP on DEAE-cellulose binding by transformed progesterone–receptor complex. Transformed receptor prepared as in Figure 1 was exposed to 10 mM $NaCNBH_3$ with or without 10 mM oATP for 18 h at 0 °C. Samples were applied to parallel columns of DEAE-cellulose as described under Experimental Procedures, washed with barbital buffer, and eluted with a linear gradient of KCl. The salt gradient elution measured as conductivity (––) was identical for both columns; however, the initial buffer wash volume differed as indicated. Fractions eluted for the control (●) and oATP-treated (○) samples are shown.

was unable to reverse the oATP effect as measured by receptor binding to ATP–Sepharose (not shown). These results indicate that the oATP–receptor interaction is irreversible and involves a reaction with the dialdehyde.

Previous experiments in this laboratory with the aldehyde pyridoxal 5′-phosphate (PLP) have indicated that PLP interacts with progesterone receptor through Schiff-base formation (Nishigori & Toft, 1979). The PLP effect on receptor can be reversed by disrupting the Schiff base but cannot be reversed if the Schiff base is reduced by borohydride. We attempted to reverse the effect of oATP on receptor by exposing treated receptor to 100 mM Tris, a treatment known to reverse the PLP effect. Neither an 18-h dialysis against 100 mM Tris nor direct addition of the Tris to the cytosol was able to reverse the oATP effect (data not shown). In fact, in no experiment did we observe a difference in receptor treated with oATP alone vs. receptor treated with oATP plus sodium cyanoborohydride (to reduce Schiff bases), suggesting that the oATP interaction with receptor did not require reduction in order to become irreversible. It should be noted that in some of our earlier experiments, cyanoborohydride was added even though it was later found not to be required to achieve an irreversible interaction (see legends to Figures 2 and 3).

Evidence That oATP Treatment Chemically Alters Progesterone Receptor. If the oATP were covalently linked to the receptor, it is possible that certain measurable receptor characteristics would be altered. The effect of oATP on the ionic properties of the receptor was assessed by examining the binding to DEAE-cellulose and hydroxylapatite (HAP). In Figure 2, it can be seen that reaction with 10 mM oATP resulted in receptor forms having higher affinities for DEAE as indicated by the higher salt concentrations required for their elution. Also, when compared to the control, the oATP-treated receptor was more completely separated into two components during the elution from DEAE. These two components are probably the A and B receptor forms because a similar increase in receptor affinity for DEAE and separation into A and B forms have been observed when the receptor is reacted with pyridoxal 5′-phosphate (Nishigori & Toft, 1979).

The oATP treatment also resulted in a progesterone receptor that exhibited a higher affinity for HAP. The column elution

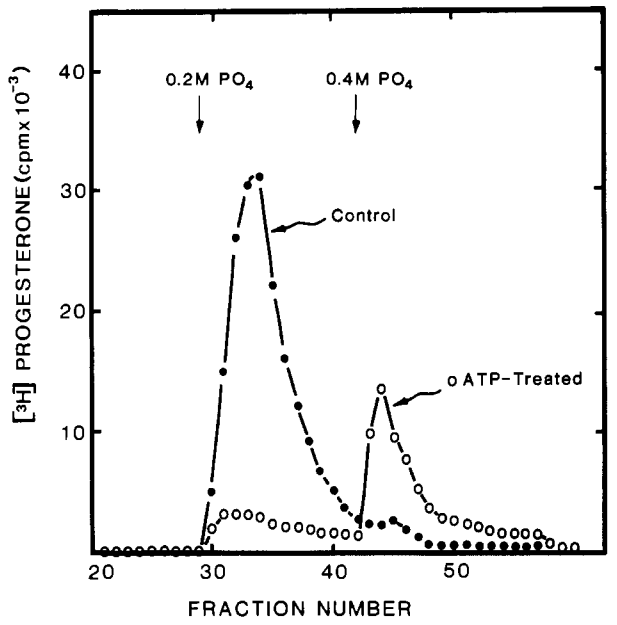

FIGURE 3: Effect of oATP on hydroxylapatite binding by transformed progesterone–receptor complex. Samples of labeled transformed receptor prepared as in Figure 1 were exposed to 10 mM $NaCNBH_3$ with or without 10 mM oATP for 18 h at 0 °C and then applied to columns of hydroxylapatite as described under Experimental Procedures. Columns were washed with buffer and eluted stepwise with phosphate as indicated. The elution of adsorbed receptor is shown for control (●) and oATP-treated (○) samples.

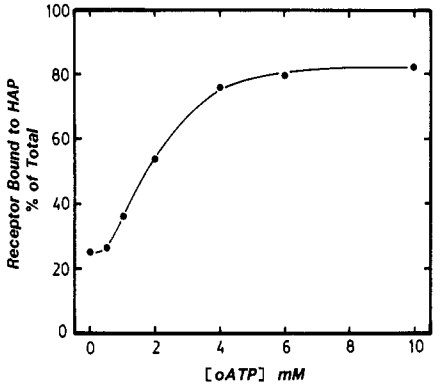

FIGURE 4: Dose-dependent relationship for ability of oATP to convert progesterone receptor to a form that remains bound to hydroxylapatite (HAP) in the presence of 0.15 M phosphate. Transformed progesterone receptor was prepared by ammonium sulfate precipitation (see Experimental Procedures), and the redissolved receptor was reacted with increasing concentrations of oATP for 2 h. The 0.15 M phosphate resistant binding to HAP was determined as described under Experimental Procedures.

profiles (Figure 3) indicate that the receptor in control cytosol was eluted with about 90% recovery by 0.2 M potassium phosphate. In contrast, the oATP-treated receptor was not eluted with 0.2 M phosphate and could only be recovered in lower yield (about 70%) by increasing the phosphate concentration to 0.4 M. A further wash with 1.5 M phosphate at 23 °C failed to release additional label from the hydroxylapatite (not shown). The difference in elution of untreated vs. treated receptor from HAP was so great that 0.15 M phosphate resistant HAP binding could be used to identify and quantify oATP-treated receptor.

Figure 4 illustrates the concentration dependency for the oATP-induced formation of progesterone receptor that binds to HAP in a 0.15 M phosphate resistant form. The effect was maximal at about 6 mM oATP. Interestingly, the reaction of receptor with the aldehyde, pyridoxal phosphate, yielded

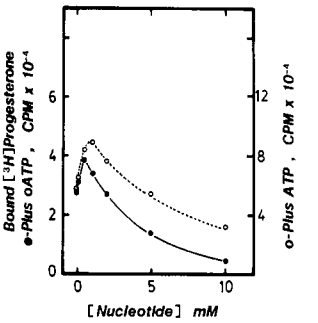

FIGURE 5: Effect of nucleotide concentration on inactivation of hormone-free receptor. Oviduct cytosol samples without progesterone were incubated at 37 °C for 5 min to promote inactivation (loss of hormone binding capacity). ATP (○) or oATP (●) was added prior to the 37 °C incubation period at the indicated concentrations. Following the 37 °C temperature exposure, samples were chilled in ice and incubated with 20 nM [3H]progesterone for 2 h. Bound hormone was determined by the charcoal-adsorption assay.

a receptor product with a lowered affinity for HAP (Nishigori & Toft, 1979)

Comparison of ATP and oATP Effects on Progesterone Receptor. Previous studies have shown an effect of ATP on the rate of progesterone receptor transformation (Toft et al., 1977; Moudgil et al., 1981). At low concentrations (<1 mM), ATP retarded receptor transformation, whereas at higher concentrations (>1 mM) transformation was accelerated. Since oATP blocks the acceptor binding processes that are characteristic of transformed receptor, we were unable to test its effect on receptor transformation. However, ATP has been shown to alter the stability of the glucocorticoid receptor and to retard and, in some cases, reverse the receptor inactivation that occurs when receptor is incubated at elevated temperatures in the absence of hormone (Sando et al., 1979). Furthermore, it has been suggested that receptor transformation and inactivation, as observed in vitro, may be the result of the same process (McBlain et al., 1981). This suggestion is supported by our results.

We first compared the effects of ATP on receptor transformation and inactivation. The influence of ATP on receptor transformation was determined by incubating [3H]progesterone–receptor complex at 23 °C for 30 min in the absence or presence of increasing concentrations of ATP. The samples were then chilled, and the extent of receptor transformation was measured by the amount that bound to ATP–Sepharose. As reported previously, dual effects were observed where low concentrations (0.5 and 1 mM) of ATP retarded and high concentrations (5 and 10 mM) of ATP accelerated transformation (results not shown).

To study receptor inactivation, we incubated cytosol samples at 37 °C for 5 min in the absence of hormone. The samples were chilled in ice and labeled with [3H]progesterone for 2 h, and the receptor binding activity was measured by charcoal assay. When hormone-free receptor is incubated at 37 °C, a loss of steroid binding activity occurs rapidly, and the inactivation is essentially complete by 15 min (results not shown). As shown in Figure 5, ATP had dual effects on this inactivation process. Low concentrations retarded inactivation, and higher concentrations enhanced the process. Thus, the effect of ATP concentration on receptor inactivation compares well with the influence of this nucleotide on receptor transformation.

Figure 5 also shows the effect of oATP on receptor inactivation. The results were very similar to those with ATP, although oATP appeared to be slightly more potent than ATP. That this is the case is shown more clearly in Figure 6A. In

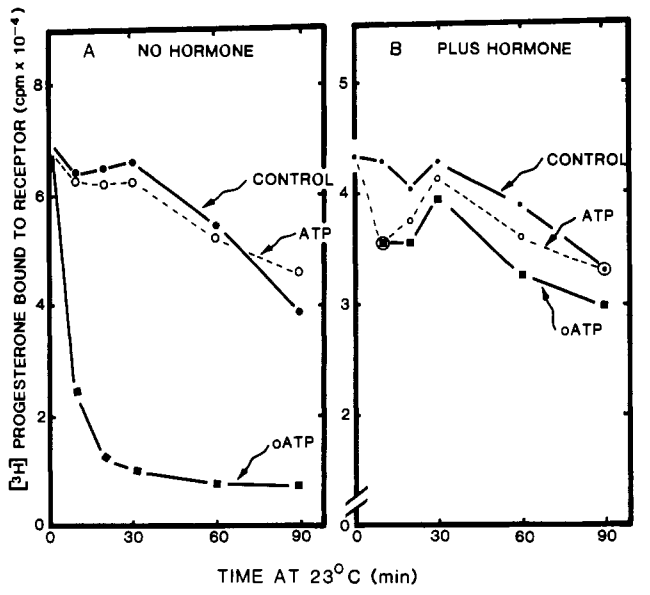

FIGURE 6: Temperature stability of unlabeled (A) and labeled (B) progesterone receptor in the presence of ATP or oATP. For (A), unlabeled cytosol with or without ATP or oATP was exposed to 23 °C, and at the times indicated samples were placed at 4 °C for 2 h in the presence of 20 nM [^{3}H]progesterone. For (B), cytosol labeled for 2 h with 20 nM [^{3}H]progesterone was incubated at 23 °C in the presence or absence of nucleotide for the times indicated. Receptor-bound radioactivity was measured by the charcoal-adsorption assay.

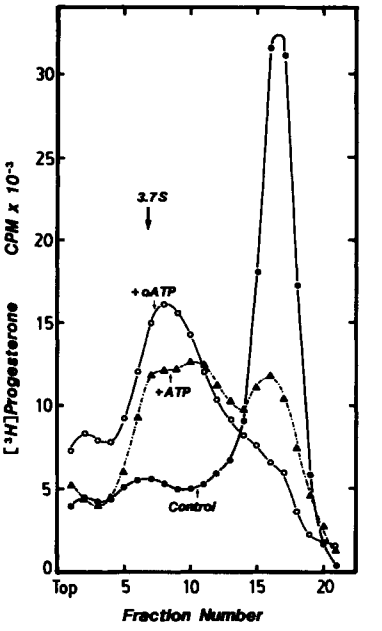

FIGURE 7: Effect of ATP and oATP on sedimentation of cytosol [^{3}H]progesterone–receptor complex. Oviduct cytosol was prepared in 20 mM barbital buffer, pH 8, and incubated with 20 nM [^{3}H]-progesterone plus 10^{-6} M cortisol (to eliminate binding to serum proteins) for 1 h at 0 °C. A control sample (●) and samples containing either 10 mM ATP (△) or oATP (○) were prepared and incubated for a further 2 h at 0 °C. They were then layered onto 5–20% sucrose gradients in phosphate buffer without KCl (see Experimental Procedures) and centrifuged at 145000g for 16 h at 4 °C. [^{14}C]Ovalbumin (3.7 S) was used as the internal standard.

this experiment, inactivation of receptor was measured at 23 °C. At this temperature, inactivation was much more gradual and was insensitive to ATP. However, 10 mM oATP caused a rapid inactivation of the receptor. On the other hand, in the presence of hormone, loss of binding activity was less apparent and was relatively unaffected by either nucleotide (Figure 6B).

When transformed receptor was exposed to oATP and sedimented into a sucrose gradient containing 0.3 M KCl, the receptor migrated as an approximately 4S species, as did the control receptor (not shown). Thus, oATP does not alter the sedimentation of transformed receptor. When oATP was reacted with nontransformed receptor, it had an effect similar to that of ATP as shown in Figure 7. That is, both ATP and oATP converted the receptor from the larger 8S form to forms sedimenting at about 4–6 S.

Comparison of Multiple Effects of oATP. We have shown that oATP influenced three different properties of the receptor: ATP–Sepharose binding, hydroxylapatite affinity, and receptor inactivation. The oATP concentration dependencies for these effects were very similar. We also compared the time courses for the three reactions as shown in Figure 8 and found them to be remarkably similar. It should be noted that, in this experiment, receptor inactivation was measured at 4 °C to conform with the other parameters that were measured. oATP does promote inactivation at this temperature although the process is much slower than at elevated temperatures. The results shown in Figure 8 would suggest that oATP causes the three observed alterations in receptor properties through a single mechanism.

Inhibition of oATP Action by Pyridoxal 5′-Phosphate. The previously characterized interaction of PLP with progesterone receptor apparently involved a reaction of the PLP aldehyde group with receptor lysine residues (Nishigori & Toft, 1979). Since oATP is reactive because of its aldehyde groups, it was of interest to see if PLP and oATP would compete for their effects on receptor. It was possible to test this by using binding to hydroxylapatite as an assay since oATP increases receptor

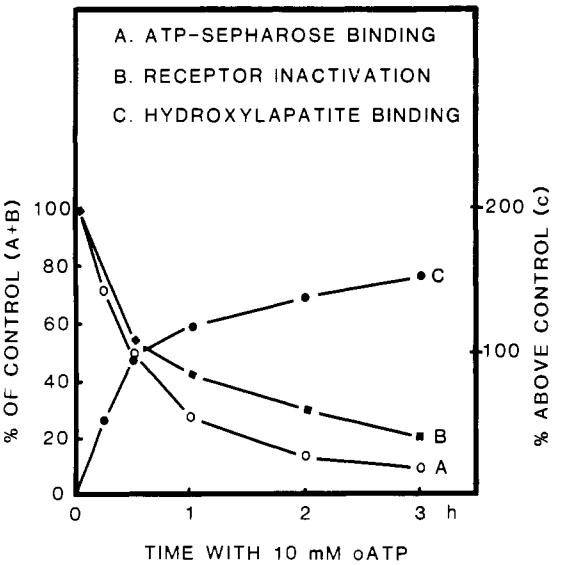

FIGURE 8: Relative time courses for effect of oATP on inhibition of ATP–Sepharose binding by transformed progesterone receptor, on inactivation of unlabeled receptor, and on modification of receptor for high-affinity binding to hydroxylapatite. The ATP–Sepharose binding (○) experiments were performed as shown in Figure 1B. The inactivation experiment shown (■) is similar to that of Figure 6A except that it was performed at 4 °C in this case. The quantity of 0.15 M phosphate resistant receptor binding to HAP (●) was determined by exposing receptor to 10 mM oATP for the times indicated; the oATP was inactivated by the addition of 10 mM $NaBH_4$, and receptor with an increased affinity for HAP was detected as described under Experimental Procedures and Figure 4.

affinity for hydroxylapatite and PLP has the opposite effect (Figure 3; Nishigori & Toft, 1979). Shown in Figure 9 is a concentration curve for the inhibition by PLP of the oATP-induced alteration of receptor to the high-affinity form on

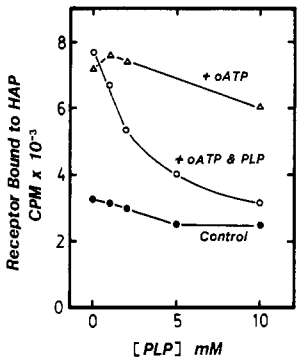

FIGURE 9: Effect of pyridoxal 5′-phosphate on action of oATP. Cytosol [^{3}H]progesterone–receptor complex was first fractionated by ammonium sulfate precipitation, and three sets of samples were then prepared: the control samples (●) contained pyridoxal 5′-phosphate (PLP) at the concentrations indicated; the second set of samples contained 3 mM oATP (△); and the third set contained 3 mM oATP and PLP at the concentrations indicated (○). After all samples were incubated at 4 °C for 30 min, PLP was added to the second set of samples containing oATP alone (△). PLP did not reverse the effects of oATP but was a control measure for any effect of PLP on the hydroxylapatite (HAP) assay. All samples were then tested for receptor binding to HAP in the presence of 0.15 M phosphate (see Experimental Procedures).

HAP. In this experiment, 3 mM oATP was used to yield the high-affinity HAP-binding form of the receptor, and PLP was used as a competitor for the reaction. PLP at 10 mM inhibited about 83% of the oATP effect but was unable to reverse the reaction when added 30 min after oATP (top line in Figure 9).

Ability of Other Nucleotides To Inhibit oATP Effect on Receptor. If oATP were acting at a specific nucleotide binding site, the oATP effect should by subject to inhibition by ATP and should exhibit nucleotide specificity. Both of these properties have been difficult to demonstrate. First, we prepared oAMP (see Experimental Procedures) and reacted this modified nucleotide with receptor. We found the time-course and dose–response curves for oAMP to be virtually identical with those for oATP (data not shown). Second, we reacted receptor with oATP in the presence of ATP and examined the oATP effectiveness by the HAP affinity method. As shown in Figure 10, ATP did protect receptor from 3 mM oATP in a dose-dependent fashion, giving about 36% protection at 30 mM (10-fold excess of ATP). However, at this concentration of ATP, ionic effects are possible. We have found that KCl and potassium phosphate also inhibit the oATP effect with a 50% inhibition occurring at about 0.4 and 0.15 M, respectively (not shown).

Discussion

Since transformed avian progesterone receptor interacts with ATP, we have used oATP in an attempt to probe the nucleotide binding of the receptor and to assess the effect the nucleotide binding may have on receptor properties. We have used three approaches to study the interaction of oATP with receptor. First, we have determined that the nucleotide inhibits the ATP–Sepharose binding of transformed receptor. Second, we have found that oATP can mimic ATP in such reactions as the inactivation of steroid-free receptor and the dissociation of receptor to more slowly sedimenting forms. oATP is, in fact, more potent than ATP. Third, we have utilized the ability of oATP to increase the affinity of receptor for HAP in order to further characterize the oATP interaction with receptor, especially with respect to competitors for the oATP interaction with receptor.

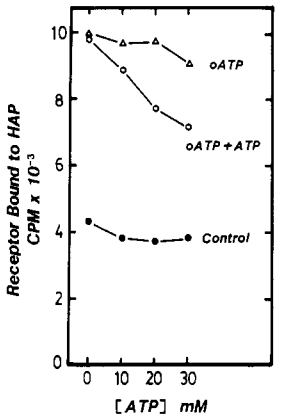

FIGURE 10: Effect of ATP on action of oATP. The protocol for this experiment was identical with that in Figure 9, but ATP at the indicated concentrations was used in place of PLP as the competitor, and the concentration of oATP was 5 mM.

oATP inhibits transformed receptor binding to ATP–Sepharose in a dose- and time-dependent fashion. It is generally assumed that during the transformation reaction, positively charged regions in the receptor are exposed, thereby facilitating binding to nuclei and anionic resins such as ATP–Sepharose, DNA–cellulose, and phosphocellulose. Since oATP blocks receptor binding to all these resins, it would appear either that the oATP interacts directly at the exposed, positively charged regions or that oATP alters the receptor properties sufficiently to mask the nuclear binding sites. The findings regarding binding to DEAE-cellulose and HAP indicate that the receptor ionic properties are altered by oATP treatment; however, it is not clear whether the alterations take place at nuclear binding sites or at more distant sites that affect receptor characteristics through conformational changes.

One of the positively charged groups that could be exposed during receptor transformation is lysine. It has been proposed that pyridoxal 5′-phosphate (PLP) inhibits nuclear binding of receptor by interacting with exposed lysine groups (Nishigori & Toft, 1979). Our data support a possible involvement of lysine in the binding of oATP to receptor; lysine itself inhibits the oATP effect and PLP inhibits the oATP-induced high-affinity binding to HAP.

It is interesting that both oATP and PLP (Nishigori & Toft, 1979) alter the ionic properties of the receptor, resulting in forms that bind more tightly to DEAE-cellulose. However, the alterations caused by these two agents are not identical. oATP increases the affinity of receptor for hydroxylapatite and promotes inactivation of steroid-free receptor, whereas PLP decreases receptor affinity for hydroxylapatite and stabilizes the steroid-free receptor (Nishigori & Toft, 1979).

Three mechanisms have been proposed to explain the interaction of oATP with enzymes. The aldehyde groups of oATP could form a Schiff base with exposed lysine residues, and reduction of this linkage would yield a covalently bound nucleotide (Kumar et al., 1979). The Schiff-base linkage is reversible prior to the reduction. Second, the dialdehyde of ATP could spontaneously become covalently linked to the enzyme through a morpholine-type adduct (Gregory & Kaiser, 1979). Third, the dialdehyde might covalently bind proteins through cysteine residues (King & Carlson, 1981). These last two mechanisms would not be reversible. For the enzymes studied, the oxidized nucleotide interaction has been reported to be both reversible (Kumar et al., 1979) or irreversible (Fayat et al., 1978; Gregory & Kaiser, 1979; King & Carlson, 1981) in the absence of reduction, suggesting differing interactions for different enzymes. Most attempts to reverse the effects

have involved treatment with Tris, gel filtration, or extensive dialysis to either remove the nucleotide or disrupt Schiff bases. Other workers have not examined reversibility (Bragg et al., 1981; Malcolm & Moffatt, 1978) or presented data for which reversibility is established on the basis of competitive inhibition of enzymatic activity (Easterbrook-Smith et al., 1976; Westcott et al., 1980).

For our study of oATP with progesterone receptor, the reaction was not readily reversible as attempted by treatment with Tris or dialysis. Thus, while lysine could compete for the oATP effect, Schiff-base formation seems unlikely. Cysteine could compete for the oATP effect, and this finding might support the theory of interaction through receptor cysteine residues. This observation could possibly be extended through the use of sulfhydryl reagents in order to determine if cysteine is essential for the reaction or if a morpholine-type adduct with lysine residues could explain the irreversibility of the reaction.

Most reports regarding the interaction of oATP with enzymes demonstrate a protection by ATP against the oATP inhibition of enzymatic activity (Easterbrook-Smith et al., 1976; Fayat et al., 1978; Gregory & Kaiser, 1979; King & Carlson, 1981; Kumar et al., 1979; Malcolm & Moffatt, 1978; Westcott et al., 1980). Careful examination of these studies reveals that usually large excesses of ATP are required and that the inhibition of the oATP effect is often incomplete (Easterbrook-Smith et al., 1976; Gregory & Kaiser, 1979; King & Carlson, 1981; Westcott et al., 1980). The interaction of oATP with progesterone receptor is poorly inhibited by ATP. Three reasons for the limited competition by ATP are suggested. The effective doses of oATP are so large that large excesses of ATP cannot be used without ionic effects. For the inactivation of receptor, oATP is more potent than ATP, indicating that the efficacy of oATP vs. ATP is such that only large excesses of ATP could inhibit oATP. The difference in the effectiveness of ATP and its derivative, oATP, in this regard, should not be surprising since the detailed chemical properties of oATP are unknown (Mehler et al., 1981). Third, oATP may interact in more than one way with receptor and the nucleotide competition may be difficult to observe by the indirect hydroxylapatite assay because of the complexity of the reaction of oATP with the receptor. It should be emphasized that the ability of oATP to act as a suitable affinity-labeling agent cannot be assumed without a substantial amount of analysis. In some cases, this compound has been shown to bind to multiple sites on a protein (Mehler et al., 1981; Hinrichs & Eyzaguirre, 1982) and to bind nonspecifically (Mehler et al., 1981). Thus, it is still possible to explain the present results by the interaction of oATP with sites on the receptor that are not involved in binding nucleotides.

While the interaction of oATP and ATP with a specific site on the receptor remains uncertain, the two nucleotides have very similar effects on the receptor. The dual effects of ATP in retarding or promoting receptor transformation were reported previously (Toft et al., 1977; Moudgil et al., 1981). However, the mechanisms involved in these events remain unknown. More recently, the transformation of receptor by high ATP concentration has been demonstrated with glucocorticoid and estrogen receptors (Moudgil & John, 1980; Moudgil & Eessalu, 1980). Since we were unable to test the effects of oATP on receptor transformation, we analyzed its effects on the inactivation of steroid-free receptor. Our results show that the avian progesterone receptor does undergo an in vitro inactivation process, as was first demonstrated with receptors for glucocorticoids (Nielsen et al., 1977; McBlain & Shyamala, 1980). The effects of ATP concentration on this process indicate that it is related to the receptor transformation process, as suggested previously (McBlain et al., 1981). It is interesting that oATP mimics both the low and the high nucleotide effects of ATP in this system.

It is not possible to say with certainty that oATP interacts directly with progesterone receptor rather than through other components of the cytosol. However, the oATP effect is consistent with that expected if it were binding directly to a site on the receptor. That is, it is difficult to conceive of cytosolic interactions of oATP that could yield the variety of observations of receptor properties reported herein. However, very pure preparations of receptor would be required to determine with certainty the minimal effective dose of oATP and the stoichiometry of the reaction. If one assumes that oATP does react directly with receptor, this nucleotide derivative should be useful for affinity labeling of steroid receptors, for characterizing nuclear binding sites for steroid receptors, and for elucidating the role of nucleotides in steroid-receptor function.

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Registry No. Progesterone, 57-83-0; ATP 2′,3′-dialdehyde, 54970-91-1; ATP, 56-65-5; pyridoxal 5′-phosphate, 54-47-7; 5′-AMP 2′,3′-dialdehyde, 13011-02-4.

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Action of Lipoprotein Lipase on Phospholipid Monolayers. Activation by Apolipoprotein C-II†

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ABSTRACT: Action of lipoprotein lipase and its activation by apolipoprotein C-II (apoC-II) were studied with monomolecular films of 1,2-didodecanoyl-*sn*-glycero-3-phosphoglycerol as a substrate. The enzyme velocity and the specific activity of the interface-bound enzyme show a bell-shaped curve as a function of lipid packing, both in the presence and absence of apoC-II. Above critical surface pressure of 20 dyn cm^{-1}, lipoprotein lipase alone is no longer able to hydrolyze a monolayer of 1,2-didodecanoyl-*sn*-glycero-3-phosphoglycerol. However, lipoprotein lipase readily penetrates into the phospholipid interface up to surface pressures exceeding 40 dyn cm^{-1}, without any effect by apoC-II. Activation of lipoprotein lipase by apoC-II can be assigned to be due to two specific effects. Below the critical surface pressure of 20 dyn cm^{-1}, apoC-II merely increases the turnover number of lipoprotein lipase 4-fold. The minimal sequence region required to produce this effect is contained in the carboxyl-terminal residues 55–78 of the activator, as determined with synthetic peptide fragments apoC-II(43–78), -(50–78), -(55–78), -(60–78), and -(66–78). Above the surface pressure of 20 dyn cm^{-1}, apoC-II activates in an all-or-none manner a noncatalytic enzyme already bound to the substrate interface, causing the critical surface pressure to increase from 20 to 25 dyn cm^{-1}. The presence of the phospholipid-associating residues 43–50 in the carboxyl-terminal synthetic activator peptide is mandatory for the latter effect. The main conclusions are that the expression of the catalytic activity of lipoprotein lipase can be regulated by the physical state of the substrate interface and that apoC-II can affect this regulation.

Lipoprotein lipase (EC 3.1.1.34) is a triacylglycerol hydrolase located at the capillary endothelium in peripheral tissues such as heart, muscle, and adipose tissue [for reviews, see Smith et al. (1978) and Kinnunen et al. (1983)]. Its action appears to be the rate-limiting step in the removal of circulating plasma triacylglycerol transported in chylomicrons and very low density lipoproteins (Garfinkel et al., 1967; Kompiang et al., 1976; Bensadoun & Kompiang, 1979). For maximal activity lipoprotein lipase requires the presence of a small protein cofactor, apolipoprotein C-II, which is a component of the surface film of substrate lipoproteins, chylomicrons and VLDL[1] (La Rosa et al., 1970; Havel et al., 1973). Direct protein–protein interaction between lipoprotein lipase and apoC-II occurring at 1:1 stoichiometry has been established (Smith et al., 1982; Miller & Smith, 1973; Chung & Scanu, 1977; Fielding & Fielding, 1977). Extensive studies with native and synthetic peptide fragments have identified functionally distinct regions in the activator (Kinnunen et al., 1977; Musliner et al., 1977; Catapano et al., 1979; Smith et al., 1980). We have recently proposed the apoC-II-induced in-

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[1] Abbreviations: VLDL, very low density lipoprotein; apoC-II, apolipoprotein C-II; $diC_{12}PG$, 1,2-didodecanoyl-*sn*-glycero-3-phosphoglycerol; π, surface pressure; v, enzyme velocity; Tris, tris(hydroxymethyl)aminomethane.

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