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A Specific Subunit of Vitellogenin That Mediates Receptor Binding†

John W. Woods‡ and Thomas F. Roth*

ABSTRACT: Vitellogenin, an estrogen-induced serum protein synthesized in the liver, is composed of two M_r 250K polypeptides. It is specifically transported by a receptor-mediated endocytic process into the developing oocytes of virtually all oviparous animals. Following endocytosis, in the chicken, vitellogenin is specifically processed to yield several smaller products including the phosvitins (PV) and the lipovitellins (LV). These products are then stored within the oocyte until they are degraded during embryogenesis to provide nutrients for the developing embryo. Direct binding studies using iodinated vitellogenin demonstrate that vitellogenin binds to isolated oocyte membranes with a K_D of 2.5 μM. Competition studies indicate that PV is a competitive inhibitor of vitellogenin binding. This leads us to propose that the PV portion of the circulating vitellogenin molecule mediates binding and uptake. Direct binding studies using iodinated PV show that PV binds to isolated oocyte membranes with a K_D of 2.4 μM. Competition studies also demonstrate that 3.1 μM vitellogenin inhibits 50% of control ^{125}I-PV binding, but IgG and bovine serum albumin at concentrations up to 10 μM have no effect on ^{125}I-PV binding. Another series of competition experiments using a constant amount of vitellogenin and increasing amounts of ^{125}I-PV indicate that vitellogenin acts as a competitive inhibitor of PV binding and has a K_I of 2-3 μM. These results support our hypothesis that the receptor which mediates vitellogenin binding and uptake recognizes determinants on the PV portion of the native vitellogenin molecule.

Selective protein transport, mediated by specific receptors in association with coated pits and coated vesicles, is a fundamental cellular process (Roth & Porter, 1964; Goldstein et al., 1979; Anderson & Kaplan, 1983). The vital role of this receptor-mediated endocytic process is particularly manifested during reproduction. The selective transport of maternal immunoglobulins into the offspring provides the newborn with passive, maternally derived immunity until it becomes immunocompetent (Brambell, 1970). In addition to IgG, the specific transport of other maternal proteins is also essential for successful reproduction. A particularly graphic example occurs in all oviparous animals, where vitellogenin is selectively incorporated into the developing oocytes of these animals. Vitellogenin is synthesized in the liver in response to estrogen, carried by the circulation to the ovary, and incorporated into the developing oocyte by receptor-mediated endocytosis (Bergink & Wallace, 1974; Paterson et al., 1962; Schjeide et al., 1963; Wallace & Dumont, 1968). After its internalization, vitellogenin is specifically processed to yield phosvitin (PV) and lipovitellin (LV) (Deeley et al., 1975; Christmann et al., 1977). Two species of phosvitin have been identified from egg yolk with M_r's of 28K and 34K (Clark, 1970). Following fertilization, vitellogenin is degraded to provide nutrients for the developing embryo.

The developing chicken oocyte provides an excellent model system in which to study receptor-mediated endocytosis. During the last 5 days of development, a single oocyte will internalize up to 1 g of protein/day. Vitellogenin makes up the major portion of this transported protein. We (Yusko & Roth, 1976; Yusko et al., 1981) and others (Wallace & Jarad, 1976; Opresko et al., 1980; Engelmann, 1979) have shown that the first step in vitellogenin uptake is the receptor-mediated binding of vitellogenin to the oocyte plasma membrane surface. In this report we describe experiments that explore the binding properties of the vitellogenin receptor and show that "in vitro" the vitellogenin receptor recognizes determinants on its phosvitin moiety.

Experimental Procedures

Materials

Bovine serum albumin, chicken serum albumin, and phosvitin were purchased from Sigma Chemical Co. Sepharose CL-6B was obtained from Pharmacia. Acrylamide, bis(acrylamide), Enzymobeads, Bio-Gel P-10, and Bio-Gel A-1.5m were from Bio-Rad Laboratories. DE-52 ion-exchange resin was from Whatman. All other chemicals were of reagent grade and were purchased from commercial sources. Live white Leghorn laying hens, roosters, and developing oocytes were obtained from a local slaughter house.

Chicken IgG, kindly provided by Dr. John Soper, was purified from egg yolks by a modification of the method of Bernardi & Cook (1960) (Linden & Roth, 1978).

All experimental procedures were carried out in an incubation buffer consisting of 0.01 M 2-(*N*-morpholino)ethanesulfonic acid (MES),[1] pH 6.0, 0.14 M NaCl, 5 mM KCl, 0.83

† From the Department of Biological Sciences, University of Maryland Baltimore County (UMBC), Catonsville, Maryland 21228. *Received December 5, 1983.* This work was supported by National Institutes of Health Grants HD-09549 and HD-11519 and National Science Foundation Grant PMC-8118717.

* Author to whom correspondence and reprint requests should be addressed.
‡ Present address: Department of Cell Biology, School of Medicine, Yale University, New Haven, CT 06510.

[1] Abbreviations: IB, incubation buffer; IB-BSA, incubation buffer plus 10 mg/mL bovine serum albumin; PV, phosvitin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; LV, lipovitellin; M_r, molecular weight; MES, 2-(*N*-morpholino)ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride.

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mM $MgSO_4$, 0.13 mM $CaCl_2$, and 0.02% sodium azide. Where indicated, bovine serum albumin was added to a final concentration of 10 mg/mL.

Methods

Isolation of Oocyte Membranes. The ovaries were removed from freshly killed white Leghorn laying hens and placed in IB at 0 °C. Oocytes approximately 1.5–2 cm in diameter were then slit, drained of yolk, and returned to ice-cold IB. Adherent yolk was removed by gentle shaking in IB. The membrane complex consisting of the oocyte plasma membrane, a fibrous vitelline layer, a monolayer of follicular epithelial cells, and an acellular basement lamella was dissected free of the overlaying connective tissue and placed in fresh IB. In some cases the membranes were fixed in freshly prepared 1% paraformaldehyde for 30 min. After 30 min of fixation, the membranes were washed 3 times in 10 mL of IB–BSA. Following the final wash the tissue was minced with scissors to yield pieces about 0.5 mm^2. Tissue fragments were harvested by centrifugation for 5 kg min and resuspended in a minimal volume of IB. In a typical experiment 20 oocytes were dissected and the minced tissue was resuspended in a final volume of 4 mL such that 100 μL of the final suspension was approximately equivalent to the tissue obtained from half an oocyte.

Purification of Ligands. Vitellogenin was purified from estrogen-treated rooster serum according to the method of Deeley et al. (1975). White leghorn roosters were injected with 17β-estradiol in cyclohexane at a dose of 20 mg/kg of body weight. Ten to fourteen days later the birds were exsanguinated, the blood was diluted with $^1/_{10}$th volume of 3.8% sodium citrate, and 25 μg of aprotinin and 100 μg of PMSF were added per milliliter of diluted blood. After cells and lipoprotein were removed by centrifugation, 2 mL of serum was applied to 50 mL of sterile DE-52 (Whatman), washed, and then eluted with 50 mL of 0.25 M NaCl in 0.1 M sodium citrate, pH 5.5 (Deeley et al., 1975). Our vitellogenin preparations gave a single homogeneous peak at M_r 500K on a Sepharose CL-6B column. SDS–PAGE analysis (Laemmli, 1970) indicated that 90% of the total protein was intact M_r 250K subunits.

Phosvitin was purified from egg yolks according to the method of Mecham & Olcott (1949) as modified by Joubert & Cook (1958) and Clark (1970) or purchased from Sigma Chemical Co. SDS–PAGE analysis of purified phosvitin demonstrated that it was composed of a mixture of two polypeptides of apparent M_r 35K and 28K, in good agreement with the results of Clark (1970). In physiological salt solutions, phosvitin eluted as two closely spaced peaks centered around M_r 100K from a Bio-Gel A-1.5m column. However, phosvitin is highly charged and may, therefore, be expected to exist in an extended rodlike conformation. Thus, this apparent M_r of 100K is most likely a reflection of the extended conformation and not an indication that phosvitin exists in physiological solution as a dimer.

Determination of Vitellogenin and Phosvitin Concentration. Vitellogenin concentrations were determined by absorbance at 280 nm using a value of $E^{1\%}_{1\,cm}$ of 7.75. The value for the extinction coefficient was obtained by measuring the OD_{280} of various dilutions of stock vitellogenin solutions. The concentration of stock vitellogenin solutions was quantitated by using the Lowry protein assay with bovine IgG as a standard. Molar concentrations were based on an average M_r of 500K for vitellogenin (Deeley et al., 1975; Wang & Williams, 1980).

Phosvitin concentrations were determined by absorbance at 280 nm using a value of $E^{1\%}_{1\,cm}$ = 4.4, obtained by measuring the OD_{280} of various dilutions of 10 mg/mL solutions of phosvitin. The 10 mg/mL solutions were prepared by weighing lyophilized phosvitin. Molar concentrations were based on an average M_r of 30K for the two classes of phosvitin (Clark, 1970).

Iodination of Ligands. Vitellogenin and phosvitin were routinely iodinated by using Enzymobeads (Bio-Rad Laboratories). Prior to iodination, proteins were exhaustively dialyzed against 0.2 M phosphate, pH 7.2. For a typical iodination, 100 μL of protein solution containing 5–50 mg/mL protein was added to 50 μL of Enzymobeads, 50 μL of carrier-free ^{125}I in 0.1 N NaOH (typically 1.2 mCi), and 100 μL of 10% β-D-(+)-glucose in 0.2 M phosphate, pH 7.2, and incubated for 30 min at 25 °C. Iodinated protein was separated from other reactants by chromatography over a Bio-Gel P-10 column that had been prerun with the unlabeled protein. This procedure routinely yielded iodinated proteins with a specific activity of about 10^8 cpm/mg.

Binding Assays. For a typical binding assay, ligands were placed in a 12 × 75 mm glass tube in a final volume of 500 μL of IB. Incubations were initiated by adding 100 μL of membrane suspension in IB. Unless otherwise noted, 100 μL of suspension contained the membrane equivalent of half an oocyte. The contents of the tube were mixed by gentle swirling every 5 min throughout the incubation period. Details of individual variations, ligand concentrations, and length of incubations are included in the appropriate figure legends. Approximately 30 s prior to the end of each incubation, three 175-μL aliquots were removed from each tube and layered onto 200 μL of a mixture of 11 parts of butyl phthalate plus 10 parts of bis(2-ethyltheoxyl) phthalate in three 1.5-mL microfuge tubes (Segal & Hurwitz, 1977) and centrifuged in a Beckman Model B microfuge for 2 min. All incubation steps were carried out in a 4 °C environmental room. For the time-course experiments, incubations were considered to be terminated when centrifugation was begun. Following centrifugation the total ^{125}I activity in each tube was quantitated. After the fluid in each tube was removed by aspiration, the tube tip containing the pelleted material was cut away and its radioactivity determined. The bound and free iodinated ligand concentrations were calculated from the bound and total activities from each tube. Each datum point in the figures is the mean of triplicate determination, and the error bars show the standard deviation.

Elution of ^{125}I-Phosvitin from Membranes. The elution of ^{125}I-phosvitin from membranes that had been previously incubated with ^{125}I-phosvitin was assayed with a filter apparatus that consisted of a pump, a three-way valve, a 25-mm Swinnex filter holder with a 52 μm mesh nylon screen for a filter, and a fraction collector connected in series. Membranes were introduced into the filter apparatus via the three-way valve and washed with IB at a flow rate of 1 mL/min. Further experimental details are included in the figure legends.

Results

Time Course of Vitellogenin Binding. Receptor-mediated binding is a dynamic process. Thus, it is necessary first to assay the time course of binding to determine the length of incubation required to reach equilibrium.

The time course of binding of ^{125}I-vitellogenin to isolated oocyte membranes shows that the amount of vitellogenin bound initially increases very rapidly. Thereafter, the rate of increase gradually slows, and after 30 min, the amount bound increases with a slow linear rate for at least the next 40 min. In a parallel experiment to quantitate nonspecific binding, 100 μM unlabeled phosvitin was added in addition to labeled vitellogenin. The validity of this approach will be substantiated

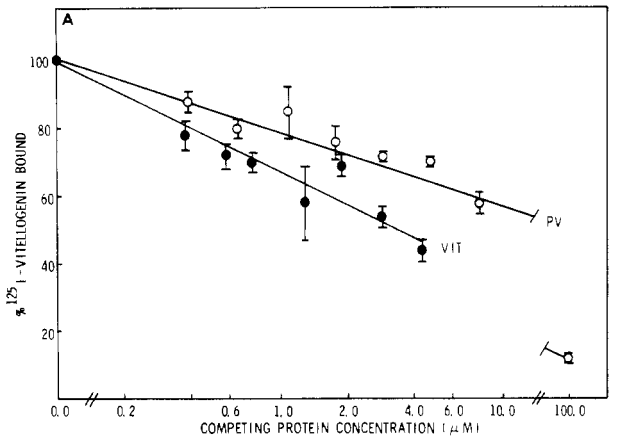

FIGURE 1: Competitive inhibition of ^{125}I-vitellogenin binding of unlabeled vitellogenin (●) or phosvitin (○). ^{125}I-Vitellogenin (0.1 μM) was incubated with the membrane equivalent of half an oocyte in a total volume of 600 μL of IB–BSA in the presence of from 0 to 4.3 μM unlabeled vitellogenin (●) or from 0 to 100 μM phosvitin (○). Incubations were carried out for 30 min. After incubation bound and free ^{125}I-vitellogenins were separated with the oil assay procedure.

in a later section. The binding of ^{125}I-vitellogenin in the presence of phosvitin was much lower, and the amount bound increased linearly from 0 to 120 min at a rate that was approximately equal to the rate of increase observed from 30 to 120 min in the absence of phosvitin. Thus, specific binding of vitellogenin appears to reach equilibrium within 30 min of incubation (data not shown).

In addition to the length of incubation, the time course of binding is also a function of both ligand and receptor concentration. Therefore, the ligand concentration used in the time-course experiments was at the lowest concentration used in subsequent equilibrium experiments. This ensures that the time to equilibrium is the maximum that may be expected in subsequent experiments. Total receptor concentration was held constant in all experiments to ensure that changes in receptor concentration would not affect the time to equilibrium.

Competition for Vitellogenin Binding. In order to determine the affinity and specificity of the vitellogenin receptor, a series of competition experiments were carried out. In these experiments 0.1 μM ^{125}I-vitellogenin and increasing concentrations of unlabeled vitellogenin or phosvitin were incubated with isolated oocyte membranes. Both unlabeled vitellogenin and phosvitin inhibited ^{125}I-vitellogenin binding. At the highest concentration of phosvitin tested, 100 μM, 89% of control binding was inhibited. At the highest concentration of unlabeled vitellogenin tested, 4.4 μM, 56% of control binding was inhibited. It was not possible to use higher concentrations of vitellogenin because concentrations greater than 10 μM formed insoluble aggregates. The results of these competition experiments suggest that phosvitin binds to the same receptor site as vitellogenin (Figure 1). These results also suggest that 100 μM unlabeled PV can be used to assess the extent of nonspecific binding of ^{125}I-vitellogenin.

The results of the competition experiments were replotted according to the Lineweaver–Burk format to yield more specific information on the affinity of the receptor–vitellogenin interaction. The data yield a straight line in a double-reciprocal plot, demonstrating that vitellogenin binds to isolated oocyte membranes with an apparent equilibrium dissociation constant, K_D, of 2.52 μM. In addition, these data indicate that there are approximately 2.4×10^{14} receptors per oocyte. Specific binding is defined as the difference between vitellogenin bound in the absence and presence of 100 μM unlabeled phosvitin.

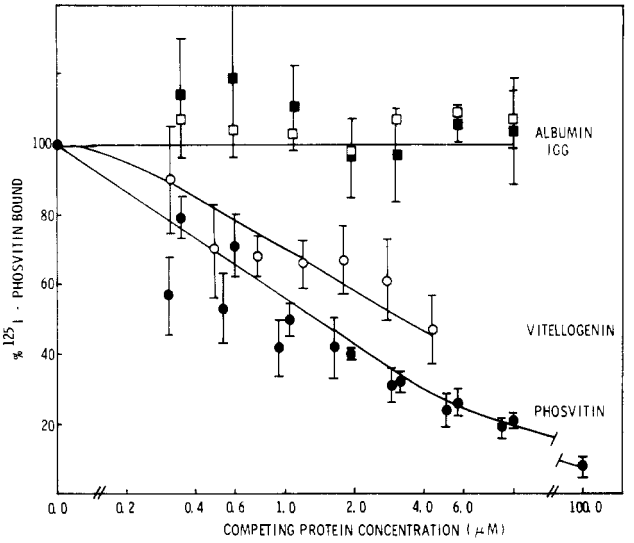

FIGURE 2: Competitive inhibition of ^{125}I-phosvitin binding. ^{125}I-Phosvitin (0.1 μM) was incubated with the membrane equivalent of half an oocyte in a total volume of 600 μL of IB–BSA in the presence of from 0 to 100 μM unlabeled phosvitin (●), 0 to 4.4 μM vitellogenin (○), 0 to 10 μM chicken IgG (■), or 0 to 10 μM chicken serum albumin (□). Incubations were carried out for 30 min. After incubation bound and free ^{125}I-phosvitins were separated with the oil assay procedure.

Time Course of Phosvitin Binding. To determine the time course of phosvitin binding to isolated oocyte membranes and ensure that subsequent experiments are incubated for a period sufficient to reach equilibrium, identical aliquots of isolated oocyte membranes were incubated with a low concentration (0.1 μM) of ^{125}I-phosvitin for various times. The results indicate that specific binding increases rapidly during the first 2 min of incubation. Thereafter, the rate of increase slowed significantly and was apparently linear from 2 to 32 min (data not shown). All further experiments were incubated for at least 30 min to ensure equilibrium of binding has been reached. In addition, all experiments were carried out at ligand concentrations equal to or greater than, and receptor concentrations approximately equal to, those used in this time-course determination.

Competition for Phosvitin Binding. The specificity of ^{125}I-phosvitin binding to isolated oocyte membranes was assayed by incubating a low concentration of ^{125}I-phosvitin (0.1 μM) with increasing amounts of either unlabeled phosvitin, vitellogenin, chicken serum albumin, or chicken IgG. The results (Figure 2) indicate that both the unlabeled phosvitin and vitellogenin competed with ^{125}I-phosvitin. Phosvitin appeared to be slightly more effective than vitellogenin since 1.4 μM unlabeled phosvitin inhibited 50% of control binding whereas 3.2 μM vitellogenin was required to achieve the same level of inhibition. In addition, the results showed that neither IgG nor albumin at concentrations up to 10.0 μM had any effect on ^{125}I-phosvitin binding.

Since the preceding experiments suggest that vitellogenin inhibits ^{125}I-phosvitin and phosvitin inhibits ^{125}I-vitellogenin binding, a further experiment designed to explore the nature of this inhibition was carried out. In this experiment a low concentration of ^{125}I-phosvitin plus increasing concentrations of unlabeled phosvitin was incubated in the presence of a constant amount of vitellogenin in order to determine the type of competition that is occurring between the two ligands. The data from this experiment, which were analyzed according to the Lineweaver–Burk format, yielded three straight lines, all of which intersect on the y axis (Figure 3). These results indicate the vitellogenin inhibits phosvitin binding by acting

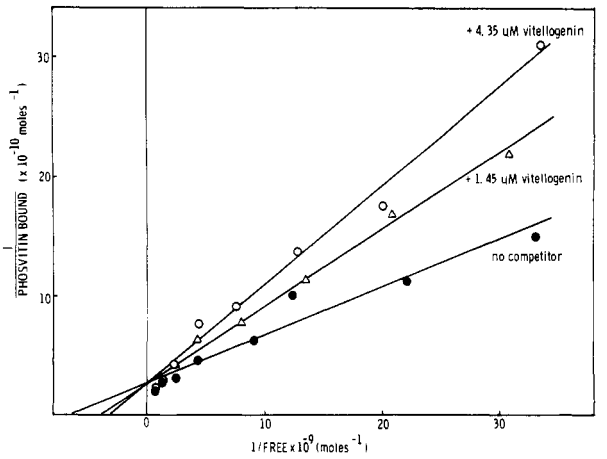

FIGURE 3: Competitive inhibition of ^{125}I-phosvitin binding by vitellogenin. ^{125}I-Phosvitin (0.1 μM) was incubated with the membrane equivalent to half an oocyte in a total volume of 600 μL of IB–BSA in the presence of from 0.0 to 10.0 μM unlabeled phosvitin (●). Parallel experiments were also carried out that included either 1.45 μM vitellogenin (▲) or 4.35 μM vitellogenin (○) in addition to phosvitin and membranes. Incubations were carried out for 30 min and free and bound ^{125}I-phosvitins were separated with the oil assay procedure. The data, which were plotted according to the Lineweaver–Burk format, yielded three straight lines by linear regression analysis. The correlation coefficients were 0.95 (no vitellogenin), 0.97 (1.45 μM vitellogenin), and 0.92 (4.34 μM vitellogenin).

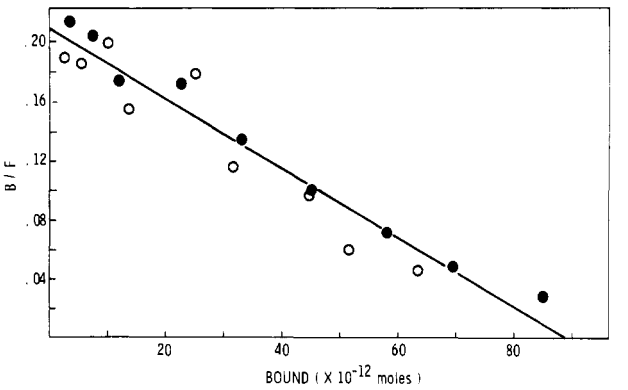

FIGURE 4: Concentration dependence of binding of native or iodinated phosvitin to isolated oocyte membranes plotted according to the Scatchard format. Phosvitin, at a final concentration from 0.1 to 15 μM, was incubated with the membrane equivalent of one-sixth an oocyte in a total volume of 600 μL of IB for 30 min. In one set of experiments increasing amounts of ^{125}I-phosvitin of a constant specific activity were added to each tube to yield the desired final concentration (●). Alternately, increasing amounts of unlabeled phosvitin were added to 0.1 μM ^{125}I-phosvitin (5×10^4 cpm) to yield the desired final phosvitin concentration (○). Nonspecific binding (lower line) (○, ●), quantitated in a parallel series of experiments, was subtracted from each data point to yield specific binding (upper line). Following incubation free and bound ^{125}I-phosvitins were separated with the oil assay procedure.

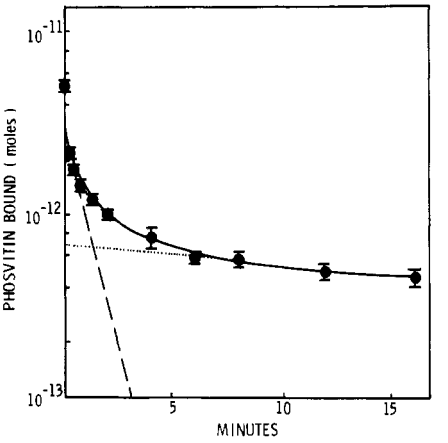

FIGURE 5: Time course of dissociation of bound phosvitin. ^{125}I-Phosvitin, final concentration 1 μM, was incubated with the membrane equivalent of half of an oocyte in a total volume of 600 μL of IB. After 30 min of incubation 50 μL of unlabeled phosvitin in IB was added to a final concentration of 100 μM. At various times after the addition of unlabeled phosvitin, three 220-μL aliquots were withdrawn and bound and free ^{125}I-phosvitins were separated with the oil assay procedure. Binding at $t = 0$ was calculated by adding IB containing no unlabeled phosvitin and immediately assaying for free and bound ^{125}I-phosvitin. The dashed line represents the fast component of dissociation and was calculated by subtracting out the effect of the slow component (dotted line) from the early time points.

as a competitive inhibitor. Further, from the x intercept and vitellogenin concentrations, the inhibition constant K_I was calculated as being 2.2 μM.

The effect of phosvitin concentration on phosvitin binding to isolated oocyte membranes at equilibrium was determined by incubating increasing concentrations of phosvitin with a constant amount of membranes. The results of this experiment show that specific phosvitin binding is saturable at about 15 μM, thus indicating that the number of binding sites is finite. Nonspecific binding as that binding which remains in the presence of 100 μM unlabeled phosvitin, shows a slow linear increase with increasing ligand concentration. Nonspecific binding never exceeded 20% of specific binding. A plot of the data for specific binding according to the Scatchard formate (Figure 4) indicates that the apparent equilibrium dissociation constant, K_D, is 2.24 μM. From the x intercept the total number of receptors in each assay tube can be determined by inspection as being 5.3×10^{13}. Since each assay tube contained the tissue equivalent of $^1/_6$th of an oocyte, there must be at least 3×10^{14} receptors per oocyte. Also, since the data produced a straight line, we infer that phosvitin binds to only a single class of receptor.

An additional aspect of these experiments demonstrates that native and ^{125}I-labeled phosvitins bind to the receptor with equal affinities. In these experiments the concentration of phosvitin varied in one of two ways. Either an increasing amount of ^{125}I-phovitin was added to each incubation tube or a low (0.1 μM) constant amount of ^{125}I-phosvitin plus an increasing amount of unlabeled phosvitin was added. In one method only the binding of ^{125}I-phosvitin was monitored, whereas in the second method, the competitive inhibition of ^{125}I-phosvitin by unlabeled phosvitin was monitored. The results when using a constant specific activity (open symbols) are indistinguishable from those obtained when specific activity varies (closed symbols). Thus, the iodination of phosvitin has no effect on its affinity for the receptor.

Dissociation Rate of Phosvitin. To determine the dissociation rate constant for phosvitin, isolated oocyte membranes were incubated with 1 μM ^{125}I-phosvitin (50% of K_D concentration) and allowed to come to equilibrium. Then a large excess (100 μM) of unlabeled phosvitin was added, and the amount of iodinated phosvitin bound after various times was determined. The results indicate that the half-time of dissociation is 0.35 min; thus $k_{-1} = 2$ min^{-1}. This interpretation of the data assumes that the straight line derived in Figure 5 representing the initial rate of dissociation reflects the dissociation of bound ^{125}I-phosvitin from the receptor.

Dilution of the specific activity of the labeled ligand by the addition of excess unlabeled ligand should give the same results as dilution by the addition of excess incubation buffer. Although it was experimentally difficult to determine an accurate dissociation rate in a large volume of solution, qualitative data were obtained when membranes were washed free of ligand by a flow of buffer (Figure 6). Membranes were equilibrated

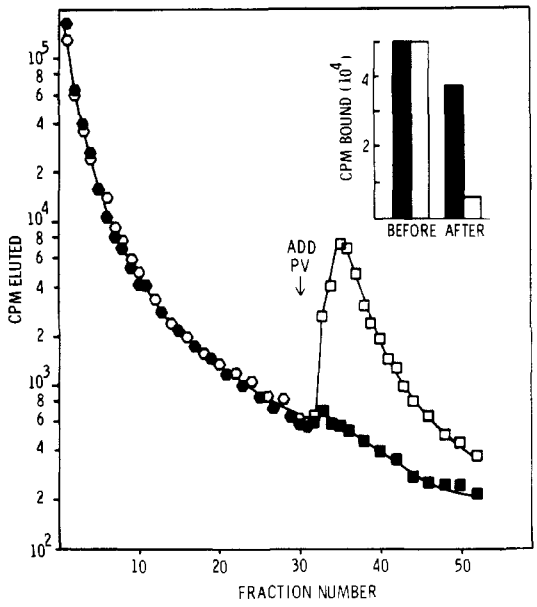

FIGURE 6: Elution of ^{125}I-phosvitin from preincubated membranes. For each experiment 1.1 mL of membrane suspension containing the membranes of one oocyte was incubated with 0.1 μM ^{125}I-phosvitin for 30 min in a total volume of 1.6 mL. One milliliter was applied to each of two identical filter apparatus (see Experimental Procedures) and washed with IB at a flow rate of 1 mL/min. One-milliliter fractions were collected and assayed for ^{125}I (●, ○). After 30 min of washing, the wash buffer in one apparatus was replaced by IB containing 100 μM unlabeled phosvitin plus a trace amount of vitamin B (□). After this phosvitin began to elute, the flow to both filters was stopped for 5 min. Washing was then resumed in both apparatus with normal IB (■, □). (Inset) The level of bound ^{125}I-phosvitin before and after washing was determined by assaying membrane aliquots with the oil assay procedure. Closed bars represent ^{125}I-phosvitin bound without unlabeled phosvitin. Open bars represent ^{125}I-phosvitin bound after incubation with unlabeled phosvitin.

with 0.1 μM ^{125}I-phosvitin (5×10^4 cpm bound) and then placed in a filter chamber. Buffer was then pumped through the chamber. The first eluate fraction contained 1.5×10^5 cpm; subsequent fractions declined rapidly to 600 cpm. The flow was then stopped, and the membranes were incubated for 5 min with or without 100 μM unlabeled phosvitin. When flow was resumed, 7000 cpm of ^{125}I-phosvitin eluted from those membranes incubated with excess phosvitin, whereas buffer alone eluted only 700 cpm of ^{125}I-phosvitin. After a further 20 min of washing, eluate fractions from both contained approximately 300 cpm. The amount of ^{125}I-phosvitin remaining bound was quantitated with the oil assay; buffer elution left 3.7×10^4 cpm or 74% of that initially bound, whereas incubation with 100 μM phosvitin left 6×10^3 cpm or only 12% of that initially bound. These results indicate that excess unlabeled phosvitin enhances the apparent dissociation rate of bound ^{125}I-phosvitin from the receptor.

Calculation of the Phosvitin Association Rate Constant. The association rate constant, k_1, can be estimated by using the value obtained for k_{-1} and the time course of binding data. By assuming an initial value for k_1, a time course of binding curve can be calculated on the basis of the rate equation

$$d[RL]/dt = k_1[R][L]k_{-1}[RL]$$

The calculated rate curve was then compared to the actual experimental data. On the basis of this comparison, the assumed value of k_1 was adjusted and the process reiterated until the "best fit" of the experimental data was obtained. By use of this procedure, it was calculated that an association rate constant of between 1.2 μM^{-1} min^{-1} and 1.5 μM^{-1} min^{-1} yields a range of rate curves that best approximate the experimental data (data not shown). It should also be noted that in order to solve the rate equation, it was necessary to calculate the total receptor concentration. This was accomplished by using the previously derived value for K_D of 2.2 μM and the phosvitin bound at equilibrium of 4.7×10^{-12} mol.

Table I: Kinetic and Equilibrium Binding Parameters of Vitellogenin Receptor

vitellogenin binding	K_D = 2.52 μM
	$R_T = 2.44 \times 10^{14}$ receptors per oocyte
phosvitin binding	K_D = 2.24 μM
	$R_T = 3.18 \times 10^{14}$ receptors per oocyte
	k_1 = 1.3 μM^{-1} min^{-1}
	k_{-1} = 2 min^{-1}
	$k_{-1}/k_1 = K_D$ = 1.53 μM
vitellogenin inhibition of phosvitin binding	K_I = 2.2 μM

Discussion

The numerical parameters representing the binding constants for vitellogenin and phosvitin binding to isolated oocyte membranes are summarized in Table I. Direct binding studies indicate that both vitellogenin and phosvitin bind with essentially similar affinities to the membranes. In both cases, these ligands bind with equilibrium dissociation constants, K_D, of about 2 μM. In addition, the results demonstrate that a similar number of receptors exist for both vitellogenin and phosvitin. When ^{125}I-phosvitin was used to probe the kinetic parameters of the vitellogenin receptor, the association and dissociation rate constants were 1.3 μM^{-1} min^{-1} and 2 min^{-1}, respectively. These give a calculated K_D of approximately 1.5 μM since the $K_D = k_{-1}/k_1$. As expected, this is in good agreement with the value for the K_D calculated from the equilibrium experiments. Finally, the results show that both vitellogenin and phosvitin compete with each other for binding and, in particular, that vitellogenin acts as a competitive inhibitor of ^{125}I-phosvitin binding with a K_I of 2 μM. These results suggest that the determinants on vitellogenin that are recognized by the vitellogenin receptor are present on the phosvitin moieties.

Cutting & Roth (1973) obtained data that support this hypothesis. They injected ^{125}I-phosvitin, as well as other labeled proteins, directly into the circulation of the laying hen. Iodinated phosvitin had a half-life in the circulation of 1–2 h, whereas injected iodinated IgG had a half-life of 10–20 h. Further, they report that 4 h after injection the oocytes had accumulated much more ^{125}I-phosvitin when normalized to weight than any other tissue, indicating that the oocytes were responsible for the rapid clearance of ^{125}I-phosvitin from the circulation. These results demonstrate that not only is phosvitin recognized by the receptor but also it is endocytosed into the developing oocytes in the laying hen.

Additional circumstantial evidence supporting the hypothesis comes from the observation that the experimentally determined K_D value is in good agreement with the serum concentration of vitellogenin. Cuatrecasas & Hollenberg (1976) stated the "the observed blood level (of the ligand) can ofen serve as an indicator of the ligand-receptor affinity" on the premise that for a receptor to have a physiological effect the receptor must be able to bind the ligand at physiological ligand concentrations. Since Christmann et al. (1977) reported the vitellogenin plasma concentration is approximately 2 μM, we expected that vitellogenin would bind to its receptor with a K_D of about 2 μM. This is in excellent agreement with our experimentally determined value.

As a further control to test the hypothesis, it would have been of interest to assay the effect of lipovitellin on vitellogenin and phosvitin binding. Unfortunately, these experiments could

not be carried out because lipovitellin is extremely hydrophobic and is insoluble in the normal physiological buffers used in these experiments (Franzen & Lee, 1970). However, in view of its extreme hydrophobicity, it seems likely that lipovitellin would be buried in the interior of the vitellogenin molecule and thus unlikely to be involved in the receptor recognition process.

Significance of Anomalous Dissociation Rates. The dissociation rate of bound ligand from the receptor should be constant. However, dissociation in the presence of excess unlabeled ligand is much faster than dissociation in the presence of infinite dilution. There are at least two likely mechanisms.

(*1*) *Multivalent Ligand Hypothesis.* In this hypothesis each ligand binds to more than one receptor.

$$R + L \leftrightarrow RL + R \leftrightarrow RLR$$

When excess unlabeled ligand is added, it enhances dissociation relative to simple dilution because excess ligand perturbs both steps of the reaction by binding to free receptors. In contrast, simple dilution perturbs only the first step of the reaction because it lowers the free ligand concentration but leaves the second step unaffected since the receptors are associated. In addition, if the receptors are closely spaced, the second reaction could be fast relative to the first and follow the mechanism $2R + L \leftrightarrow RLR$ to give a linear Scatchard plot and a 2-fold lower estimate of the receptor number. Our data indicate that there are 2.4×10^{14} receptors per oocyte and morphometry gives a surface area of between 1×10^{16} and 10×10^{16} nm^2 for each oocyte (unpublished observation). Thus, assuming a uniform distribution of receptors, each receptor is within 7–20 nm of its nearest neighbor, a distance that could be spanned by vitellogenin. Thus, the postulated second binding reaction requires little or no diffusion of receptors and could be very fast relative to the first reaction. However, near saturation, vitellogenin binding might be constrained by steric considerations.

This multivalent binding is much like that seen in other systems. For instance, epidermal growth factor (EGF) mediates its response by cross-linking surface receptors since low concentrations of EGF, which do not induce a response, initiate one when antibody cross-links the bound ligand (Schechter et al., 1979). Similarly, divalent antibodies against the insulin receptor mimicked the insulin effect, whereas monovalent antibodies do not (Kahn et al., 1978). Insulin binding has a direct parallel to our system in that excess insulin enhances dissociation whereas dilution does not. However, unlike our system, insulin binding exhibits a curvilinear Scatchard plot that was interpreted as indicating negative cooperativity (De Meyts et al., 1973, 1976) or a change in aggregation of the receptor (Ginsberg et al., 1976). Multivalent binding is also involved in IgG Fc binding to macrophages (Segal & Hurwitz, 1977; Dower et al., 1981a,b) where dimers and trimers of IgG bind with a much higher affinity than monomeric IgG. They suggest this increase in apparent affinity is due to the multivalent interactions and allows cells to preferentially internalize antigen–antibody complexes (Segal et al., 1983a,b).

(*2*) *Surface Topology Hypothesis.* An alternate interpretation of our dissociation rate data is that the receptors are closely packed in deep crypts and invaginations that typify the oocyte plasma membrane (Roth et al., 1976; Perry et al., 1978). Thus, as a bound ligand dissociates from one receptor deep in a crypt, it is likely to rebind sequentially to other receptors close by before it reaches the surface. Because of this rebinding, simple dilution will not perturb the equilibrium to the degree that excess ligand does, since excess ligand competitively binds to all unoccupied receptors, preventing iodinated ligand from rebinding. In this case the apparent off rate is only diffusion limited. This could explain why there is an enhanced off rate in the presence of excess unlabeled ligand from receptors that are restricted environments such as coated pits, even more so for those localized in deep folds of a membrane.

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Electron-Transfer Kinetics in Photosynthetic Reaction Centers Cooled to Cryogenic Temperatures in the Charge-Separated State: Evidence for Light-Induced Structural Changes†

D. Kleinfeld,‡ M. Y. Okamura, and G. Feher*

ABSTRACT: We have compared the electron-transfer kinetics in reaction centers (RCs) cooled in the dark with those cooled under illumination (i.e., in the charge-separated state). Large differences between the two cases were observed. We interpreted these findings in terms of light-induced structural changes. The kinetics of charge recombination $D^+Q_A^- \rightarrow DQ_A$ in RCs containing *one quinone* were modeled in terms of a distribution of donor–acceptor electron-transfer distances. For RCs cooled under illumination the distribution broadened and shifted to larger distances compared to the distribution for RCs cooled in the dark. The model accounts for the nonexponential decay observed at low temperatures [McElroy, J. D., Mauzerall, D. C., & Feher, G. (1974) *Biochim. Biophys. Acta 333*, 261–277; Morrison, L. E., & Loach, P. A. (1978) *Photochem. Photobiol. 27*, 751–757]. A possible physiological role of the structural changes is an enhanced charge stabilization. For RCs with *two quinones*, the recombination kinetics $D^+Q_AQ_B^- \rightarrow DQ_AQ_B$ were found to be strongly temperature dependent. This was interpreted in terms of temperature-dependent transitions between structural states [Agmon, N., & Hopfield, J. J. (1983) *J. Chem. Phys. 78*, 6947–6959]. This interpretation requires that these transitions occur at cryogenic temperatures on a time scale $t \gtrsim 10^3$ s. The electron transfer from Q_A^- to Q_B was found to not take place in RCs cooled in the dark ($\tau_{AB}^{dark} > 10^{-1}$ s). In RCs cooled under illumination, we found $\tau_{AB}^{light} < 10^{-3}$ s. We suggest the possibility that the drastic decrease in τ_{AB} observed in RCs cooled under illumination is due to the trapping of a proton near Q_B^-.

The primary process in photosynthesis involves the conversion of light into electrochemical energy through the formation of oxidized and reduced molecules. In photosynthetic bacteria, this process occurs in the reaction center (RC),[1] a protein complex that spans the plasma membrane. The RC consists of three polypeptide subunits and a number of cofactors associated with the electron-transfer chain: four bacteriochlorophylls, two bacteriopheophytins, two ubiquinones (UQ-10), and one non-heme iron (Fe^{2+}) [for a review, see Feher & Okamura (1978)]. The light induces a charge separation with an electron leaving the donor, D, a specialized bacteriochlorophyll dimer, and passing via intermediates to the primary and secondary quinone acceptors, Q_A and Q_B, respectively [for a review, see Parson & Ke (1982)].

In this work we address two questions concerning the structural dynamics of RCs: (1) Is the light-induced charge separation accompanied by a change in the structure of the RC? (2) Do the RCs have a unique structure, or are they distributed over a range of structural states?

The possibility of a light-induced structural change was discussed by McElroy et al. (1974). They found that the oxidized donor (i.e., D^+) was trapped in RCs cooled to cryogenic temperatures after a long period of illumination and suggested that this could result from immobilizing RCs in a conformation favoring the charge-separated state. Similar findings were reported by Noks et al. (1977, and references therein) and by Ke et al. (1979) for photosystem I of chloroplasts [for a review of early work, see Ke et al. (1976)]. Evidence for bulk structural changes comes from the calorimetric studies of Arata & Parson (1981a,b). Their data suggested that the light-induced charge separation is accompanied by a decrease in the volume of the RC–solvent system. However, Kirmaier et al. (1983) found from photodichroism measurements that the bacteriochlorophylls and bacteriopheophytins did not move significantly with respect to each other in the time interval between 2 ns and 10 ms after excitation.

A search for specific light-induced structural changes using protein modification techniques was performed by Noks et al. (1977). They found that incubation of chromatophores with glutaraldehyde, a cross-linker of amino groups, affected the electron-transfer kinetics in RCs only when the incubations were performed under illumination. Since the kinetics are

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[1] Abbreviations: LDAO, lauryldimethylamine *N*-oxide; LN_2, liquid nitrogen; RC, reaction center; Tris, tris(hydroxymethyl)aminomethane; UQ, ubiquinone.

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