Covalent Stabilization of the Nontransformed Chick Oviduct Cytosol Progesterone Receptor by Chemical Cross-Linking†

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ABSTRACT: The nontransformed forms of the chick oviduct cytosol progesterone receptor of sedimentation coefficient ~8 S (8S-PR) are heterooligomers including one hormone binding molecule, either B, ~110000, or A, ~79000, and two non-hormone binding subunits recently identified as heat-shock protein M_r ~90000 (hsp 90) [Renoir, J. M., Buchou, T., Mester, J., Radanyi, C., & Baulieu, E. E. (1984) *Biochemistry 23*, 6016–6023]. In the crude cytosol, bisimidates reacted under mild conditions and gave rise to complexes, binding progesterone and reacting with BF4, an anti-hsp 90 monoclonal antibody. These complexes have a sedimentation coefficient of 8.4 S and R_s of 8.1 nm in the presence of 0.4 M KCl and in the absence of molybdate ions, i.e., in conditions that would transform non-cross-linked 8S-PR to R_s ~ 5 nm forms of ~4-S sedimentation coefficient. All bisimidates tested, of an effective reagent length between 0.73 and 1.09 nm, gave comparable results in the cytosol prepared with or without molybdate ions, confirming that the latter were not responsible for the formation of the cross-linked 8S complexes. It was found that the dimethyl pimelimidate cross-linked 8S-PR was more resistant to inactivating conditions, urea, or heat treatment than the non-cross-linked 8S-PR. The 8S-PR cross-linked in the cytosol was purified by affinity chromatography in the absence of molybdate ions. After purification, it also reacted with the monoclonal antibody BF4 and had the same R_s (8.0 nm), sedimentation coefficient (~8.5 S), and thus M_r (~290000) as the original cytosol. Molybdate-stabilized, nontransformed PR was purified by affinity chromatography, and values of ~7.1 nm and 7.9 S were found as before. When treated with dimethyl pimelimidate, BF4-positive progesterone binding complexes of ~7.5-S sedimentation coefficient showed Stokes radius of 6.3 and 5.8 nm on AcA 34 and HPLC, giving M_r ~ 200000 and 184000, respectively, therefore suggesting the loss of one hsp 90 unit from 8S-PR under these conditions. We conclude that cross-linking confirms the natural occurrence of the receptor–hsp 90 containing 8S-PR complexes in cytosol, including in the absence of molybdate ions. It also provides a means to stabilize the heterooligomer 8S-PR experimentally exposed to dissociating agents (e.g., urea and KCl).

The oligomeric structure of the chick oviduct cytosol progesterone receptor of sedimentation coefficient ~8 S (8S-PR)[1] has been a matter of controversy. Dimeric or tetrameric structures have been suggested (Schrader et al., 1975; Sherman et al., 1983a) with AB or A_2B_2 formulas, where A and B stand for the two related progesterone binding proteins of M_r 79000 and 110000 found in the chick oviduct cytosol. Purified preparations of the molybdate-stabilized nontransformed 8S-PR contain a protein of M_r 90000 that appears to be in association with either form A or form B of the PR (Baulieu et al., 1983; Renoir et al., 1984a; Dougherty et al., 1984). This protein is present in different tissues in a relatively large amount and is included in the structure of other nontransformed steroid receptors (Joab et al., 1984; Tai & Faber, 1985; Housley et al., 1985; Okret et al., 1985; Groyer et al., 1985; Sullivan et al., 1985; Renoir et al., 1986; Mendel et al., 1986; Redeuilh et al., 1987). However, it is unable to bind steroid and has recently been identified as the 90000 heat shock protein (hsp 90) (Catelli et al., 1985a,b; Sanchez et al., 1985). It has been suggested that the presence of the hsp 90 in purified 8S-PR preparations is an artifact, due to its abundance in cytosol and the presence of molybdate ions in the buffers, used to stabilize the receptor in its untransformed state (Birnbaumer et al., 1984). However, a complex containing hsp 90 and A or B receptor can be observed in the absence of molybdate ions (Joab et al., 1984). Quantitative analysis of the purified, molybdate-stabilized, 8S-PR suggested that two molecules of hsp 90 were associated with one molecule of A or B (Renoir et al., 1984a).

In this paper, we demonstrate that 8S-PR can be stabilized in the absence of molybdate ions by means of chemical cross-linking with bisimidates. Bisimidates have been utilized previously to assess the need of subunit separation for activation of the glucocorticosteroid receptor (Arányi, 1984). These reagents are especially useful since they react under mild conditions specifically with lysines and are hydrolyzed rapidly (half-life ~40 min), a feature that reduces the probability of formation of fortuitous linkages.

Materials and Methods

Chemicals. [2,4,6,7-^{3}H]Progesterone ([^{3}H]P), specific activity 82–118 Ci/mmol, was from the Radiochemical Centre (Amersham Bucks, U.K.). Nonradioactive cortisol and progesterone (>95% pure) were obtained from Roussel-Uclaf (Romainville, France). Dimethyl adipimidate, dimethyl pimelimidate, and dimethyl suberimidate were purchased from

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[1] Abbreviations: PR, progesterone receptor; IgG, immunoglobulin G; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; Trisacryl, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-2-propenamide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Sigma (St. Louis, MO). Dimethyl succinimidate and dimethyl sebacimidate were gifts from Dr. G. Redeuilh. DEAE-Sephacel was from Pharmacia (Uppsala, Sweden). All other chemicals were of reagent grade and obtained from Merck (Darmstadt, FRG), except when noted.

Buffers. Buffer A: 10 mM potassium phosphate, 1.5 mM EDTA, 10% (v/v) glycerol, and 12 mM 1α-thioglycerol, pH 7.4 at 25 °C. Buffer B: buffer A supplemented with 20 mM sodium molybdate. Buffer C: buffer A supplemented with 400 mM KCl.

Chickens and Oviduct Cytosol Preparation. The estrogen stimulation schedule of the Warren chickens, as well as preparation of chick oviduct cytosol, has been described elsewhere (Wolfson et al., 1980). Assay for specific progesterone binding was done as in Renoir et al. (1984b).

Antibodies. The monoclonal antibody BF4, reacting specifically with the non-progesterone-binding hsp 90 (Joab et al., 1984), was prepared as described previously (Radanyi et al., 1983) but with the following modifications. Ascitic fluid obtained after injection of BF4 cells into nude mice was precipitated twice with ammonium sulfate (50% saturation) and then dialyzed against 25 mM Tris and 35 mM NaCl, pH 8.8. After the mixture was loaded on a DEAE-Trisacryl column (IBF, France) equilibrated in the same buffer, the flow-through fractions were pooled and used as a partially purified BF4 preparation.

Covalent Cross-Linking. The conditions were optimized by using pilot experiments not detailed here. The best procedure was the following: To cytosol or purified receptor preparations was added $^1/_{10}$ volume of 2.2 M triethanolamine, pH 8, to adjust the pH. Then $^1/_5$ volume of 0.1 M of cross-linker solution freshly prepared in 0.2 M triethanolamine, pH 8, buffer (whose pH had been readjusted with NaOH) was added. The test tube containing the reaction mixture was then immersed in a 10 °C water bath. The reaction was allowed to proceed for 30 min. Excess reagent was neutralized with 50 mM hydroxylamine.

Density Gradient Ultracentrifugation. Sedimentation coefficients were measured by gradient analysis as in Renoir et al. (1982) using either a 10–35% (w/v) glycerol gradient or a 5–20% (w/v) sucrose gradient with fungal glucose oxidase (GO; Boehringer, Mannheim, $s_{20,w} = 7.9$) and peroxidase (PO; Sigma, type VI, $s_{20,w} = 3.6$) as internal markers.

Gel Filtration. Analytical filtration on Ultrogel AcA 34 (IBF, France) was carried out at 4 °C on cytosol or purified receptor samples in a 40 × 1.5 cm column equilibrated in buffer C. The flow rate was 8 mL/h, and 0.69-mL fractions were collected. The following proteins (5–10 mg/mL) were used for the calibration of the column: (1) bovine thyroglobulin (Sigma, type I, $R_s = 8.6$ nm); (2) β-galactosidase from *Escherichia coli* (Sigma, grade IV, $R_s = 6.9$ nm); (3) ferritin from horse spleen (Boehringer, Mannheim, $R_s = 6.1$ nm); (4) catalase from bovine liver (Sigma, $R_s = 5.2$ nm); (5) aldolase from rabbit muscle (Boehringer, Mannheim, $R_s = 4.8$ nm); (6) bovine serum albumin (Sigma, $R_s = 3.6$ nm); (7) human hemoglobin (Sigma, $R_s = 3.1$ nm); and (8) cytochrome *c* from horse heart (Sigma, $R_s = 1.7$ nm). All the proteins were followed by their absorbance at 280 nm. Void volume (V_0) and total volume (V_t) were determined by filtration of dextran blue and potassium bichromate and were 21.4 and 59.9 mL, respectively. Standard curves were plotted according to the method of Porath (1963). $K_d^{1/3} = \alpha - \beta R_s$ with $K_d = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume.

The Stokes radius of the receptor was calculated from linear regression of $K_d^{1/3}$ vs the R_s of the standard proteins. The molecular weight (M_r) of the receptor and the frictional ratio due to shape (f/f_0) were calculated according to the method of Sherman et al. (1983b). R_s values of standard proteins are given in Radola (1968), Miller et al. (1975), and Andrews (1969).

High-Performance Liquid Chromatography (HPLC) Analysis. Analytical filtration of purified samples was done by using a Hewlett-Packard 1082 B liquid chromatograph and a TSK-G 4000 SW column (7.5 × 300 mm) equilibrated in buffer C. The flow rate was 0.5 mL/min, and 0.25-mL fractions were collected. The column was calibrated with the following standard proteins: (1) thyroglobulin; (2) ferritin; (3) catalase; (4) aldolase; (5) bovine serum albumin; and (6) cytochrome *c* from horse heart. The void volume (V_0) and total volume (V_t) were, respectively, 7.35 and 15.6 mL and were determined as described above.

Enzyme-Linked Immunosorbent Assay (ELISA). The hsp 90 in the purified receptor preparation was detected by a direct ELISA. Ninety six-well polystyrene microtiter plates were coated at 100 μL/well for 1 h at 37 °C. After the plates were washed with PBS containing 0.1% Tween, 100 μL of BF4 culture supernatant was added per well and allowed to incubate for 2 h at 37 °C. The plate was washed as above and 100 μL of alkaline phosphatase conjugated rabbit anti-rat IgG (Sigma) was added to each well for 2 h at 37 °C. After the plates were washed, antibodies bound to hsp 90 were detected by adding 100 μL of *p*-nitrophenyl phosphate (1 mM) in 1 M Tris, pH 8. Absorbances were read at 405 nm by a Microplate EL 308 reader (Bio-Tek Instruments).

Protein determination was done according to the method of Schaffner and Weissman (1973) using bovine serum albumin as standard.

Purification of the molybdate stabilized 8S-PR was performed as described before (Renoir et al., 1984a). In brief, cytosol prepared in buffer B, which was supplemented with 0.3 mM phenylmethanesulfonyl fluoride, was saturated with 1 μM hydrocortisone to avoid copurification of the glucocorticosteroid receptor and plasma transcortin. Then it was loaded on the affinity gel (100 mL of cytosol:20 mL of gel). It was then washed successively with buffer B, 0.3 M KCl in buffer B, buffer B, 2.5 M urea in buffer B, and finally buffer B again. Elution was performed with 2 μM [^{3}H]progesterone, 33 Ci/mmol, overnight. The eluate was chromatographed on a DEAE-Sephacel column, using a 0–0.5 M KCl gradient in buffer B for elution. Purification was ~1000-fold with a yield of ~10%.

Radioactivity Counting. Samples were mixed with 7 mL of Scintimix 0.4% in toluene and counted in a Packard liquid scintillation spectrometer (counting efficiency 50%).

RESULTS

Cross-Linking of Receptor in the Cytosol. (a) Characterization. Cytosol was prepared in the presence of 20 mM molybdate and [^{3}H]progesterone and covalently cross-linked by bisimidates of an effective reagent length of 0.73 nm or longer. Stabilization of the oligomeric structure was observed as indicated by sedimentation as 8S species in the absence of molybdate and in the presence of 0.4 M KCl either in glycerol (Figure 1) or in sucrose gradients (not shown). In all these conditions, the non-cross-linked receptor was transformed and sedimented at ~4 S (Yang et al., 1982). Covalent stabilization of the 8S structure was also observed when cytosol was prepared in the absence of molybdate (Figure 1C). Treatment of PR with dimethyl succinimidate or dimethyl adipimidate did not prevent dissociation of the subunits, as visualized by the peak of radioactivity recovered in the 4S region of the

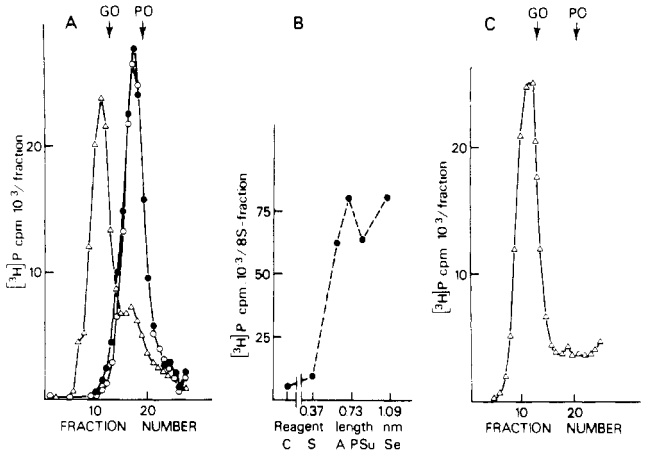

FIGURE 1: Sedimentation analysis of the cross-linked cytosol PR. Chick oviduct cytosol was prepared in buffer B and incubated with 20 nM [^{3}H]progesterone. Free steroid was removed by charcoal adsorption. Labeled cytosol was treated with different bisimidates for 30 min at 10 °C. Then 120 μL of each sample was layered on a 10–35% glycerol gradient in buffer C and run for 17 h at 40 000 rpm. (A) Sedimentation of control (○) and dimethyl succinimidate (●) and dimethyl pimelimidate (△) treated samples. (B) Radioactivity sedimenting in the 8S region for different cross-linkers as a function of effective reagent length: C, control; S, dimethyl succinimidate; A, dimethyl adipimidate; P, dimethyl pimelimidate; Su, dimethyl suberimidate; Se, dimethyl sebacimidate. (C) Cytosol was prepared in buffer A, incubated with [^{3}H]progesterone, and cross-linked with dimethyl pimelimidate. An aliquot of 200 μL was analyzed on a 10–35% glycerol gradient in buffer C and run for 17 h at 40 000 rpm.

Table I: Effect of Cross-Linking on Progesterone Binding of Cytosol PR

expt	cross-linker	[urea] (M)	residual binding (% of control)
1[a]	dimethyl succinimidate		86
	dimethyl adipimidate		83
	dimethyl pimelimidate		93
	dimethyl suberimidate		84
	dimethyl sebacimidate		20
2[b]	none	2	94
	none	3	67
	none	4	10
	dimethyl pimelimidate	2	96
	dimethyl pimelimidate	3	93
	dimethyl pimelimidate	4	91

[a] Cytosol was prepared in buffer B; it was cross-linked, and the remaining progesterone binding was determined thereafter. Control specific progesterone binding: 9.8×10^5 cpm/mL. [b] Cytosol was prepared in buffer A, incubated with [^{3}H]progesterone, and halved. One half was cross-linked; the other received the corresponding volume of buffer A. Then urea was added to the final concentration indicated. After 1 h at 0 °C, samples were diluted 6-fold with buffer containing urea to a final urea concentration of 0.66 M in all samples. Urea at that concentration does not interfere with the charcoal assay of steroid binding. The specific progesterone binding was determined. It was 2×10^6 cpm/mL in the non-cross-linked and 2.2×10^6 cpm/mL in the cross-linked samples that had no urea in them during incubation.

gradient (Figure 1A). On the contrary, dimethyl pimelimidate was found to be optimal, since in the presence of 0.4 M KCl all of the radioactivity bound to the PR was observed in the 8S region of the gradient (Figure 1B,C). Nevertheless, if cytosol PR has been transformed by KCl, subsequent treatment with dimethyl pimelimidate did not restore an 8S structure, suggesting that once dissociated the PR subunits cannot be cross-linked to each other under these conditions (data not shown).

The Stokes radius of cytosol PR cross-linked by dimethyl pimelimidate was determined (Figure 2). Ultrogel AcA 34

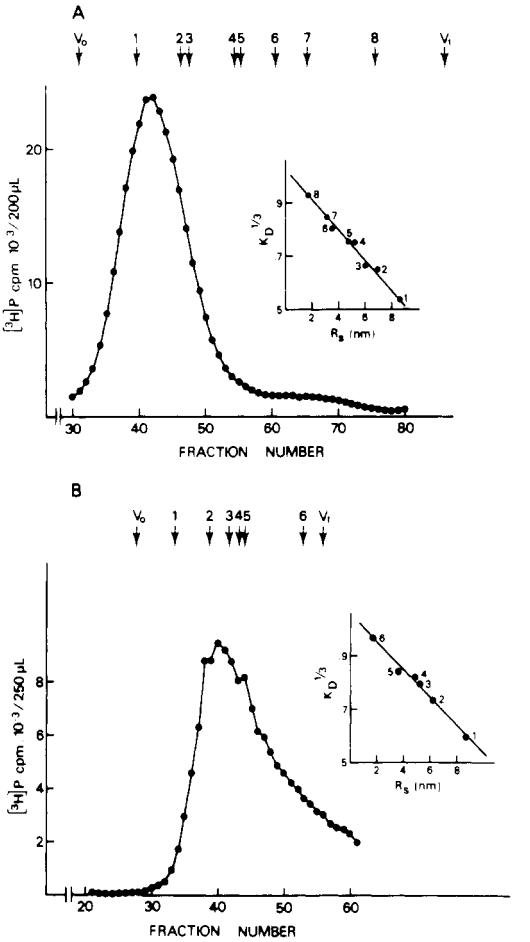

FIGURE 2: Gel permeation chromatography and HPLC analysis of the cross-linked cytosol PR. Cytosol prepared in buffer A was incubated with 20 nM [^{3}H]progesterone and cross-linked with dimethyl pimelimidate. (A) An aliquot of 500 μL was applied to an Ultrogel AcA 34 column equilibrated in buffer C, and filtration was performed as described under Materials and Methods. The arrows indicate void volume (V_0), thyroglobulin (1), β-galactosidase (2), ferritin (3), catalase (4), aldolase (5), serum albumin (6), hemoglobin (7), cytochrome c (8), and total volume (V_t). (B) An aliquot of 100 μL was injected on top of the TSKG column and chromatographed as described under Materials and Methods. The arrows indicate void volume (V_0), thyroglobulin (1), ferritin (2), catalase (3), aldolase (4), serum albumin (5), cytochrome c (6), and total volume (V_t). The Stokes radius of the PR samples was calculated by reference to the standard curves (inset).

gel permeation chromatography performed in the presence of 0.4 M KCl and without molybdate gave a value of 8.1 nm (Figure 2A), similar to the R_s of the nontransformed, non-cross-linked cytosol PR (Renoir & Mester, 1984). Surprisingly, the Stokes radius of the same sample, measured in identical conditions by HPLC, gave a value of only ~5.5 nm (Figure 2B); dissociation of the cross-linked oligomeric structure, under these conditions, is probable.

Complementary studies indicate that cross-linking reagents do not alter the hormone binding activity of the PR and do not increase the rate of dissociation of the complex. The unliganded PR could be cross-linked without a substantial loss of steroid binding capacity (except for the case of dimethyl sebacimidate) (Table I). We examined the heat inactivation kinetics of PR after cross-linking with dimethyl pimelimidate. The stability of the cross-linked PR in solutions of high ionic strength and without molybdate was higher than that of the 4S-PR, approaching that of the non-cross-linked 8S-PR in molybdate low-salt medium (Figure 3). It was found that the PR–progesterone complex was reasonably stable in 2.5–3

Table II: Purification of the Cross-Linked PR[a]

	vol (mL)	binding act. (*a*)[b] (pmol/mL)	proteins (*b*) (mg/mL)	sp act. (*a*/*b*)	yield (%)	purification (*x*-fold)
cytosol after cross-linking	67	44	18.4	2.4	100	
DEAE pool	12	40	0.015	2666	16.3	1111

[a] Four independent purifications were done. A representative experiment is documented in the table. [b] The binding activity is expressed in picomoles of bound [^{3}H]P, taking into account the isotopic dilution of the ligand used at the affinity chromatography elution step, on the basis of 82 Ci/mmol. In the purification reported in the table, the purity, calculated as (final purification factor)/("theoretical" purification factor) × 100 was 27%.

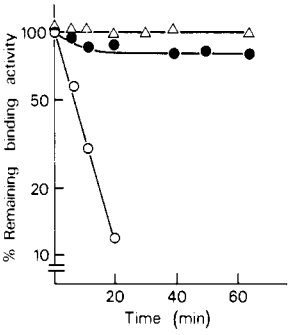

FIGURE 3: Heat inactivation of the cross-linked cytosol PR. Cytosol was prepared in buffer A and incubated with 20 nM [^{3}H]progesterone and divided into two parts. One part was cross-linked with dimethyl pimelimidate. After cross-linking, it was given KCl to a final concentration of 0.3 M (●). The other part was divided into two halves, one of which was made 0.3 M with respect to KCl (○), and the other was given sodium molybdate (△) to a final concentration of 20 mM but received no KCl. All samples were then incubated at 37 °C, and at the times indicated, aliquots were withdrawn and charcoaled to determine binding. Correction was made for nonspecific binding.

M urea solution (Buchou et al., 1983), an observation used in the purification procedure (Renoir et al., 1984a). Therefore, stability of the cross-linked cytosol progesterone–PR complexes was studied in the presence of varying concentrations of urea, and again it was found higher than that of the non-cross-linked counterpart (Table I).

(*b*) *Purification of Cross-Linked Cytosol 8S Receptor.* We attempted to purify the dimethyl pimelimidate cross-linked 8S-PR with the same methods used for the non-cross-linked 8S-PR. The purification procedure consisted of three steps, starting with the cortisol-supplemented cytosol prepared in buffer A (see Materials and Methods). The cross-linking resulted in a loss of ~15% of progesterone binding. Then KCl was added to a concentration of 0.3 M, and the preparation was loaded onto the affinity gel. About 80% of the receptor was fixed on the gel, which was washed thereafter successively with buffer A supplemented with 0.3 M KCl, buffer A, 3 M urea containing buffer A, and buffer A again. PR was eluted with 2 μM [^{3}H]progesterone (33 Ci/mmol) overnight. The affinity eluate was loaded on a DEAE-Sephacel column and eluted with a 0–0.5 M KCl gradient in buffer A. Cross-linked PR was eluted at KCl concentration of ~0.17 M (data not shown). At this step, the cross-linked PR had a specific progesterone binding activity of ~2–3 pmol/μg of protein calculated on the basis of one hormone-binding site per protein of 100 kDa, corresponding to 20–30% purity, purification of 1000–2000-fold, and yield 10–20 % (Table II).

The purified cross-linked PR was submitted to physicochemical and immunological analyses. It sedimented as an 8.5S species in the absence of molybdate and in the presence of 0.4 M KCl in sucrose gradient (data not shown). The Stokes radius of the PR, determined by gel filtration chromatography on AcA 34 performed as described above, was 8 nm (Figure 4A). Calculation according to the method of Sherman et al. (1983b) gave M_r ~290 000 and f/f_0 = 1.68.

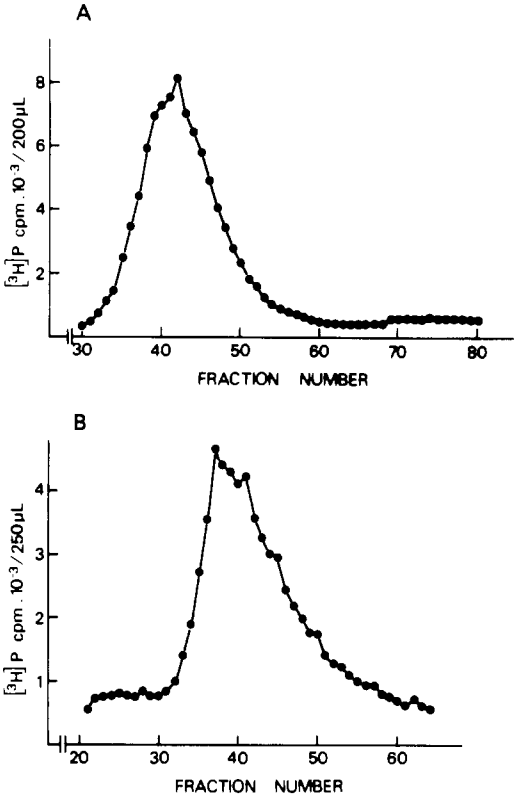

FIGURE 4: Gel permeation chromatography and HPLC analysis of the purified cross-linked PR. (A) An aliquot of 500 μL of the DEAE pool was applied onto an Ultrogel AcA 34 column equilibrated in buffer C. (B) An aliquot of 100 μL of the DEAE pool was injected on top of the TSKG column equilibrated in buffer C. Chromatography was performed as described under Materials and Methods, and the Stokes radii of the PR samples were calculated by reference to the standard curves depicted in Figure 2.

However, as with crude preparations, smaller values were obtained again after elution from HPLC column (two peaks corresponding to R_s = 6.8 and 5.1 nm) (Figure 4B). The purified cross-linked PR reacted with BF4 monoclonal antibody as shown by displacement on sucrose gradient, confirming the presence of hsp 90 in this purified species (Figure 5).

Cross-Linking of the Purified 8S-PR. Molybdate-stabilized 8S-PR was purified by affinity chromatography followed by DEAE chromatography as described in Renoir et al. (1984a) and then cross-linked. Glycerol gradient studies performed in the presence of 0.4 M KCl revealed that the 8S structure was not preserved in the case of the shortest (succinic) and longest (sebacic) bisimidates but that the analogues of intermediate effective reagents lengths stabilized substantially the 8S form (Figure 6). Dimethyl pimelimidate (effective reagent length 0.73 nm) proved the best as visualized by the presence of a single peak of bound radioactivity sedimenting at 7.5 S (Figure 7). Sebacic bisimidate treatment was detrimental to progesterone binding in cases of nonoccupied receptor (see Table I) and purified preparations, and this may explain why the 8S form could not be detected after cross-

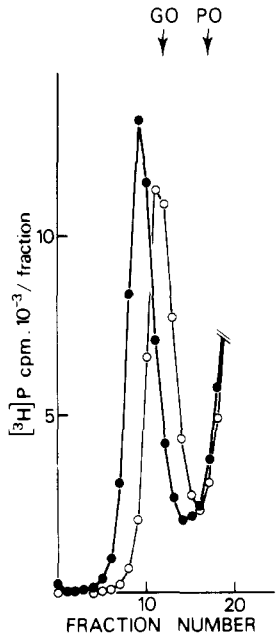

FIGURE 5: Immunological recognition of the purified cross-linked 8S-PR. An aliquot of 100 μL of the DEAE pool was incubated for 4 h at 4 °C with 50 μg of monoclonal antibody BF4 (●) or nonimmune rat IgG (○). The incubates were then layered on a 5–20% sucrose gradient in buffer B and run for 15 h at 47000 rpm.

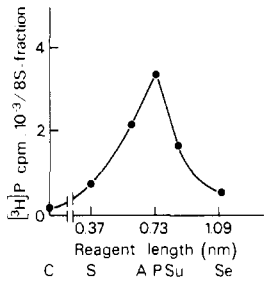

FIGURE 6: Effect of cross-linking on the sedimentation behavior of the purified molybdate-stabilized PR. Molybdate-stabilized 8S-PR was purified as described under Materials and Methods. Aliquots were then cross-linked with different cross-linkers and analyzed on 10–35% glycerol gradients containing 0.4 M KCl but no molybdate as in Figure 1. Radioactivity recovered in the 8S region is plotted versus the effective reagent length (cf. Figure 1B).

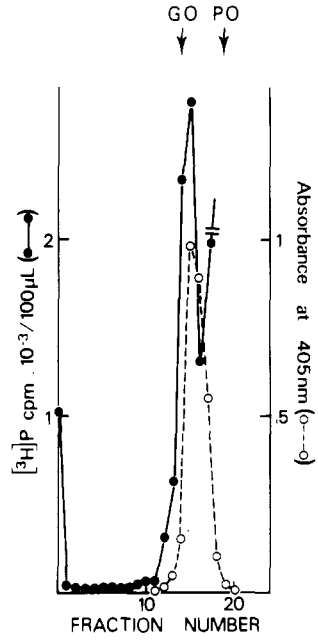

FIGURE 7: Sucrose (5–20%) gradient ultracentrifugation of the purified cross-linked 8S-PR. Molybdate-stabilized 8S-PR was purified as described under Materials and Methods and cross-linked by dimethyl pimelimidate. An aliquot of 100 μL was layered on a 5–20% sucrose gradient in buffer C and run for 15 h at 47000 rpm. At the end of the run, aliquots of 100 μL were assayed for radioactivity (●), and a 96-microwell plate was coated with 100 μL of each fraction to detect the hsp 90 by the ELISA technique (○) as described under Materials and Methods.

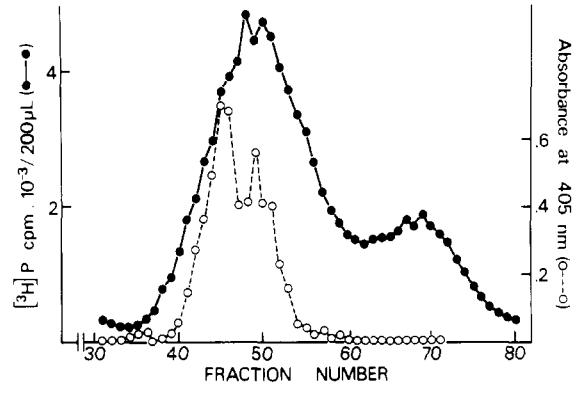

FIGURE 8: Gel permeation chromatography of the purified cross-linked 8S-PR. An aliquot of 500 μL of purified molybdate-stabilized 8S-PR cross-linked by dimethyl pimelimidate was applied onto an Ultrogel AcA 34 column equilibrated in buffer C. Two hundred microliters was counted for radioactivity (●), and a 96-microwell plate was coated with 100 μL of each fraction to detect the hsp 90 by the ELISA technique (○) as described under Materials and Methods. The Stokes radii of the proteins were calculated by reference to the standard curves depicted in Figure 2A. The same results have been obtained after HPLC analysis of the purified cross-linked samples (data not shown).

linking with this reagent. The Stokes radius of the [^{3}H]P–PR complexes cross-linked by dimethyl pimelimidate was measured by gel filtration on AcA 34 or HPLC. In both cases, identical values corresponding to R_s = 6.3 nm and R_s = 5.8 nm were obtained giving M_r of 200000 and 184000, respectively (Figure 8). Since, before cross-linking, hydrodynamic parameters of this purified PR were similar to the values previously published, $s_{20,w}$ = 7.9, R_s = 7.1 nm (Renoir & Mester, 1984), these results suggest either dissociation of one non-hormone binding component from the original purified structure or conformational change of the receptor upon cross-linking. In parallel fashion, hsp 90 was revealed in the fractions of ultracentrifugation and gel filtration experiments, by the ELISA technique, and with the BF4 monoclonal antibody (Figures 7 and 8). The values obtained, $s_{20,w} \sim 7$, R_s = 6.9 nm and 6.1 nm, are not compatible with the presence of hsp 90 monomer in the preparation analyzed. From these results, calculation of the M_r according to the method of Sherman et al. (1983b) gave ~200000 for both radioactive complexes as well as for BF4 positive protein. Receptor-containing complexes likely include one molecule of receptor and one molecule of hsp 90, indicated by interaction with BF4 assessed in sucrose gradient experiment (Figure 9). The occurrence of this 200000 structure implicates the loss of one molecule of hsp 90 from the 8S-PR, and since it is known that "free" (non-receptor-linked) hsp 90 is preponderantly found as a dimer, the most logical interpretation is that we have observed both PR–hsp 90 complexes and the hsp 90 dimer, which do not separate with the techniques used here.

Discussion

Bisimidates have been used to covalently stabilize the 8S structure of the chick oviduct cytosol PR. The cross-linked structure resembles closely the native 8S-PR. No gelation or precipitation was observed in the samples during or after

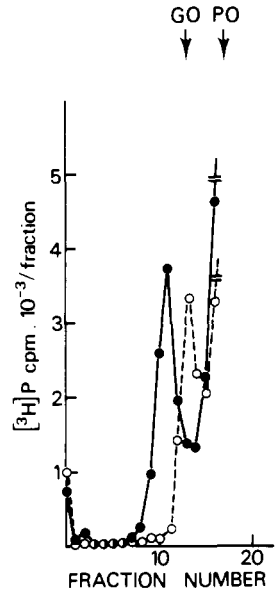

FIGURE 9: Immunological recognition of the purified cross-linked 8S-PR. An aliquot of 100 μL of purified cross-linked 8S-PR was incubated for 4 h at 4 °C with 50 μg of monoclonal antibody BF4 (●) or nonimmune rat IgG (○). The incubates were layered on a 5–20% sucrose gradient in buffer B and run for 15 h at 47 000 rpm.

cross-linking. The cross-linked PR still displayed progesterone binding capacity, including when the binding site was not occupied by hormone during the cross-linking procedure. The non-hormone binding component was still recognized by the monoclonal antibody BF4, confirming identification of this protein as hsp 90 (Catelli et al., 1985a). These results compare favorably with those obtained with glutaraldehyde, which was used earlier for cross-linking the chick oviduct PR (Baulieu et al., 1983; Renoir et al., 1984a).

Cytosol PR is an 8S heterooligomer, and sedimentation analysis of the cross-linked preparation in sucrose or glycerol gradients containing 0.4 M KCl and no molybdate confirmed that the formation of this complex is not dependent on the presence of molybdate ions. The specificity of cross-linking of PR subunits as opposed to nonspecific cross-linking with other proteins is indicated by the values of hydrodynamic parameters identical with those of the non-cross-linked 8S-PR (Renoir & Mester, 1984) and by the lack of 8S formation by cross-linking treatment after KCl exposure, which dissociates 8S-PR.

Thermal inactivation studies of the cross-linked complex also revealed that the higher stability of 8S-PR compared to that of 4S-PR in terms of progesterone binding is again due to its oligomeric structure rather than to the presence of molybdate, suggesting a stabilizing effect of hsp 90 on receptor, as already observed by T. Buchou and J. Mester (unpublished experiments). By use of the stabilizing properties of bisimidates, cross-linked cytosol 8S-PR was purified in the absence of molybdate and washed with 3 M urea on the affinity gel. Under these conditions, conversion of the non-cross-linked 8S-PR to the 4S form has been previously reported (Buchou et al., 1983). Its hydrodynamic parameters and M_r were similar to the values found for crude non-cross-linked 8S-PR in the cytosol, even in the presence of 0.4 M KCl. The molybdate-stabilized purified 8S-PR (R_s = 7 nm) was smaller than the previously cross-linked PR (R_s = 8 nm), as reported in Baulieu et al. (1983). Whether this small but significant difference reflects a real change of M_r during purification (i.e., loss of an unidentified small component of the non-cross-linked native molecule) or only a change in the conformation of the receptor due to low protein concentration in the medium remains uncertain. Analysis of cross-linked purified 8S-PR by SDS–PAGE as well as attempts to obtain Western blots were unsuccessful (too low intensity of diffuse and fuzzy bands). In addition, as for cross-linked molecular weight markers for proteins (Sigma kit), anomalous migration of the cross-linked PR in the Laemmli system was observed, thus leading to an uncertain determination of molecular weights. The purified cross-linked 8S-PR was studied in nondenaturing conditions. The presence of the hsp 90 moiety was demonstrated by monoclonal antibody recognition and excluded dimeric structures such as AA, BB, or AB (Birnbaumer et al., 1979; Shermal et al., 1983a).

It should be pointed out that the molecular weight calculated from the hydrodynamic parameters of the PR (~200 000 and 184 000) is smaller if cross-linking was performed with purified 8S-PR rather than in crude cytosol; however, interaction with the monoclonal anti-hsp 90 antibody was still observed. From these results and given the apparent molecular weight of the purified (non-cross-linked) 8S-PR (~240 000), it is difficult to tell unequivocally at present if one subunit (presumably one hsp 90) is removed during the process of cross-linking, giving rise to A-hsp 90 and B-hsp 90 heterodimers, or if cross-linking of purified PR induces a change of conformation of the receptor. However, identification of hsp 90 dimers and of progesterone binding units–hsp 90 complexes by ELISA favors the former hypothesis, assuming that, once removed from the 8S-PR structure, two molecules of hsp 90 can reassociate.

In contrast, M_r ~290 000 calculated from the hydrodynamic parameters of PR obtained after purification of receptor, cross-linked in the cytosol, is compatible with the A_1-hsp 90_2 or B_1-hsp 90_2 previously suggested structures (Renoir et al., 1984a) and consistent with the fact that free hsp 90 is mostly present as a dimer in the cytosol.

In summary, these results demonstrate that cytosol 8S-PR can be stabilized by specific covalent cross-linking in the absence of molybdate ions, suggesting an already organized structure, in contrast to a possible artifactual interaction occurring during one of the purification steps. This stabilized structure can be treated with 3 M urea during purification and studied in the presence of 0.4 M KCl without dissociation of the complex.

ACKNOWLEDGMENTS

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Registry No. Dimethyl succinimidate, 51534-06-6; dimethyl adipimidate, 13139-70-3; dimethyl pimelimidate, 36875-25-9; dimethyl suberimidate, 29878-26-0; dimethyl sebacimidate, 54377-13-8.

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Reaction of Phosphoenolpyruvate Carboxylase with (*Z*)-3-Bromophosphoenolpyruvate and (*Z*)-3-Fluorophosphoenolpyruvate†

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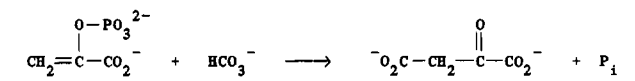
ABSTRACT: (*Z*)-3-Bromophosphoenolpyruvate inactivates phosphoenolpyruvate carboxylase from maize in the presence of HCO_3^- and either Mg^{2+} or Mn^{2+}. The inactivation rate follows saturation kinetics. Inactivation is slower in the presence of phospholactate or epoxymaleate, both of which are inhibitors of the enzyme, or dithiothreitol. Inactivation is completely prevented by the presence of lactate dehydrogenase and NADH, and 3-bromolactate is formed during this treatment. If the reaction is conducted by using $HC^{18}O_3^-$, the inorganic phosphate produced contains ^{18}O. This and other evidence indicate that phosphoenolpyruvate carboxylase catalyzes conversion of bromophosphoenolpyruvate into bromopyruvate by way of the usual carboxyphosphate–enolate intermediate, and bromopyruvate is the species responsible for enzyme inactivation. (*Z*)-3-Fluorophosphoenolpyruvate is transformed by the enzyme into a 6:1 mixture of 3-fluoropyruvate and 3-fluorooxalacetate, presumably by the same mechanism. The enzyme is not inactivated during this treatment.

Phosphoenolpyruvate (PEP)[1] carboxylase (EC 4.1.1.31) catalyzes the reaction



The enzyme is widely distributed in plants but is apparently absent from animal tissues (Vennesland & Mazelis, 1958;

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