BIOLOGY OF REPRODUCTION 38, 921–934 (1988)

Purification of the Guinea Pig Sperm PH-20 Antigen and Detection of a Site-Specific Endoproteolytic Activity in Sperm Preparations that Cleaves PH-20 into Two Disulfide-Linked Fragments[1]

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ABSTRACT

Previous work has indicated that the guinea pig sperm membrane protein, PH-20, functions in sperm-egg adhesion and that its surface expression is regulated by the acrosome reaction. The PH-20 protein was purified by monoclonal antibody affinity chromatography. Sixty-seven to one hundred percent of the PH-20 antigenic activity present in an octylglucoside (OG) extract of sperm was recovered in the purified protein. From 10^{10} sperm, ∿ 0.4 mg of PH-20 protein was obtained, which was about 0.24% of the total protein in the OG extract. The purified protein retained the ability to bind the three anti-PH-20 monoclonal antibodies we have isolated. Silver staining of purified PH-20 on overloaded sodium dodecyl sulfate (SDS) gels allowed the estimate that silver-stainable contaminants were present at a level of one part in 2000. The purified PH-20 protein exists in three forms separable on SDS-polyacrylamide gel electrophoresis: a major form with a molecular mass of 64 kDa, a minor form of 56 kDa, and an endoproteolytically cleaved form composed of two disulfide-linked fragments of 41–48 kDa and 27 kDa. Cleveland digests of the 64 kDa and 56 kDa polypeptides indicated that they were structurally related. A proportion of the 64 kDa polypeptide in each purified preparation had undergone endoproteolysis at a specific site, so that it was cleaved into the two disulfide-linked fragments, 41–48 kDa and 27 kDa. It is speculated that the site-specific endoproteolysis of PH-20 may occur during the acrosome reaction and have biological significance.

INTRODUCTION

We have evidence from previous experiments that the PH-20 antigen of guinea pig sperm has a role in sperm binding to the egg zona pellucida. Sperm binding to the zona is strongly inhibited by the PH-20 monoclonal antibody (MAb) that recognizes the PH-20 antigen. A control MAb, PH-22, also recognizes the PH-20 antigen, binds to sperm at the same level as the PH-20 MAb, but does not inhibit sperm-zona binding. Lack of inhibition by PH-22 indicates that PH-20 MAb inhibition of sperm-zona binding does not arise because PH-20 MAb simply coats the sperm and prevents sperm approach to the egg. Instead, the data suggest that the PH-20 MAb specifically affects an active site on the PH-20 protein essential to sperm-zona binding, while the PH-22 MAb does not affect this site (Primakoff et al., 1985).

The localization of the PH-20 protein is intriguing given its putative role in sperm-zona binding. There are two populations of the PH-20 protein in guinea pig sperm. One population, present on the plasma membrane, is termed $PH\text{-}20_{PM}$; the second, present on the acrosomal membrane, is termed $PH\text{-}20_{AM}$ (Cowan et al., 1986). On each of these membranes, the PH-20 protein is localized. $PH\text{-}20_{PM}$ is localized to the posterior head plasma membrane and $PH\text{-}20_{AM}$ is localized to the inner acrosomal membrane (IAM) (Fig. 1). The acrosome reaction is known to regulate the surface expression of the PH-20 protein: it changes PH-20's surface localization and increases the amount of PH-20 on the surface. The acrosome reaction results in the IAM becoming the anterior region of the sperm surface since it is joined by an exocytotic membrane fusion to the plasma membrane. After the acrosome reaction, $PH\text{-}20_{PM}$ migrates from the posterior head surface onto the IAM. $PH\text{-}20_{AM}$ is revealed on the sperm surface on the IAM, and this

Accepted November 24, 1987.
Received September 23, 1987.
[1]This work was supported by NIH Grants 21989 (to P.P.) and 16580 (to D.G.M.), a Rockefeller Foundation Grant (to P.P.), and an ACS Faculty Research Award No. 291 (to D.G.M.).
[2]Reprint requests.

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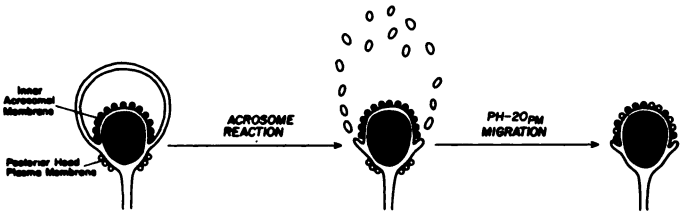

FIG. 1. Diagram representing the two populations of the PH-20 protein in acrosome-intact sperm (*left*) and acrosome-reacted sperm (*right*). PH-20_{AM} is shown with *black circles* and PH-20_{PM} is shown with *white circles*. After the acrosome reaction, PH-20_{PM} migrates from the posterior head plasma membrane to the inner acrosomal membrane, and PH-20_{AM} is exposed on the inner acrosomal membrane. (Modified from Cowan et al., 1986, and reprinted with permission of Rockefeller University Press).

increases about threefold the total amount of PH-20 protein on the surface (Fig. 1) (Primakoff and Myles, 1984; Cowan et al., 1986).

Although a variety of sperm surface antigens have been implicated by MAb inhibition experiments to function in fertilization, only a few of these have been purified for further study (e.g., O'Rand and Porter, 1979; Primakoff et al., 1987). We reasoned that a purified preparation of PH-20 protein will be required to provide direct evidence that PH-20 functions in sperm-zona adhesion and to define its activity. Studies with purified PH-20 protein may also provide insights to other questions. These include: what mechanisms target PH-20_{AM} and PH-20_{PM} to their respective membranes and then localize them to restricted domains during sperm development (Phelps and Myles, 1987), and what is the mechanism of PH-20_{PM} migration to the IAM after the acrosome reaction? To further our understanding of these various issues, we developed a single-step isolation procedure for the PH-20 protein. In this paper, we show that the procedure gives high yields of antigenically active, purified PH-20 protein. An unexpected finding is that the purified protein exists in three forms. We have been able to determine the interrelationships between the three forms and propose hypotheses about their biological significance.

MATERIALS AND METHODS

Antibodies

PH-20, PH-21, and PH-22, the monoclonal antibodies (MAbs) used in this study, have been previously described (Primakoff and Myles, 1983; Primakoff et al., 1985). They are of the immunoglobulin (Ig) Gl subclass and recognize at least two and probably three different epitopes on the PH-20 antigen (Primakoff et al., 1985).

Purification of the PH-22 MAb

The PH-22 MAb was purified from ascites fluid by affinity chromatography on Bio-Rad Affi-Gel Protein A MAPS II following the instructions of the supplier (Bio-Rad, Richmond, CA). The isolated MAb was >90% pure when examined on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and had roughly the same level of antigen-binding activity per μg MAb as found in the starting ascites fluid.

PH-20 Antigen Purification

The PH-20 antigen was purified by affinity chromatography on a PH-22 MAb Sepharose column. Of the three MAbs to the PH-20 antigen, PH-22 was selected for use in affinity chromatography because it gives the highest amount of precipitated ^{125}I-PH-20 antigen in immunoprecipitation experiments (Primakoff et al., 1985; Fig. 1). Good immunoprecipitation by a MAb is indicative of high affinity and is a property that correlates with the MAb's usefulness in affinity purification (Mescher et al., 1983; Parham, 1983; Johnson et al., 1985). The affinity column was prepared by coupling purified PH-22 MAb to cyanogen-bromide-activated Sepharose 4B (Sigma Chemical Co., St. Louis, MO). PH-22 MAb in coupling buffer (Mescher et al., 1983) was incubated with washed, activated Sepharose beads at a ratio of 2 mg MAb to 1 ml swollen beads; subsequent steps in the coupling and washing of the beads were performed according to Mescher et al. (1983).

Caudae epididymides were removed from male guinea pigs and placed immediately at 4°C in Mg^{2+}-4-(2-hydroxyethyl)-piperazineethanesulfonic acid (HEPES) medium (Green, 1978) containing a mix of 8 protease inhibitors: 10 mM ethylenediaminetetraacetate (EDTA), 70 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 2 μg/ml antipain, 10 μg/ml benzamidine, 1 μg/ml chymostatin, and 1 μg/ml pepstatin. All procedures were done at 4°C. Sperm were expressed from the caudae into the medium and washed in 200 ml of the medium. The sperm were resuspended at 1 × 10^8/ml in solubilization buffer containing 0.01 M tris(hydroxymethyl)aminomethane (Tris), pH 8.0, 0.14 M NaCl, 30 mM n-octyl glucoside (OG), and the mix of 8 protease inhibitors, and were incubated for

15–20 min. The lysed sperm suspension was centrifuged at 100,000 × *g* for 45 min to remove nuclei, axonemes, and particulate matter, and the resulting supernatant was passed through a 45-μm Nalgene filter.

We used a 10-ml PH-22 MAb Sepharose column routinely loaded with 60–115 ml of the centrifuged, filtered OG extract of sperm. The extract was first applied to a 10-ml pre-column of Sepharose 4B to remove material that would bind nonspecifically to Sepharose; the flow-through from this column was applied to the 10-ml column of PH-22 MAb Sepharose 4B. The extract was loaded onto the column at a flow rate of 20 ml/h, and the column was washed with 50 ml of solubilization buffer maintaining a flow rate of 50 ml/h. After the column was washed, the PH-20 antigen was eluted with 50 mM diethylamine, pH 11.5, containing 30 mM OG. After elution was begun, fractions were collected in 5-ml volumes and immediately neutralized with 0.5 ml 2 M Tris, pH 7.6. The first 5.5 ml of eluted, neutralized fraction was called E1; the second 5.5 ml of eluted, neutralized fraction was E2, and so on.

The starting lysate and other column fractions were applied to microtiter plates and assayed in the radioactive solid-phase binding assay for ability to bind the PH-20, PH-21, and PH-22 MAbs. The fractions were also assayed for antigenic activity by measuring their ability to inhibit the solid-phase assay, since an inhibitory assay can give a quantitative measure of the amount of antigen present relative to the starting extract (Brown et al., 1981; Mescher et al., 1983; Johnson et al., 1985).

Solid-Phase Radioactive Binding Assay

This assay was performed essentially as previously described (Primakoff and Myles, 1983). Briefly, a 30-mM OG extract of 1×10^8 sperm/ml was prepared (Myles et al., 1984), diluted 1/8 in phosphate-buffered saline (PBS), and applied to a 96-well microtiter plate. The plate was washed, unbound sites were blocked with 3% ovalbumin in PBS, and the MAb to be tested was incubated in the wells. After washing, bound MAb was detected with ^{125}I-rabbit anti-mouse IgG (New England Nuclear, Waltham, MA).

Assay of Antigenic Activity

The amount of antigenic activity in cell fractions (i.e., OG extracts) or column fractions was measured by determining the ability of the fraction to inhibit MAb binding in the radioactive solid-phase binding assay. For the inhibition experiments, the PH-22 MAb was diluted so that it showed binding five- to tenfold above background, but was not saturating in the solid-phase assay. One hundred microliters of diluted PH-22 MAb was preincubated for 1 h at room temperature with 100 μl of serial twofold dilutions of the fraction to be tested for inhibition. Aliquots of the preincubated MAb-fraction mixture were used in the solid-phase binding assay to determine residual MAb binding to the solid-phase antigen. A curve of percentage of inhibition versus dilution of fraction was plotted (for example, see Fig. 2). According to Brown et al. (1981), one unit of antigenic activity is defined as the amount of antigen needed to give 50% inhibition of the solid-phase assay.

Protein Determination

The amount of protein in the sperm extract and in the eluted fractions from the affinity column was determined with the Pierce BCA protein assay reagent, according to the supplier's instructions (Pierce Chemical, Rockford, IL).

Surface Labeling and Immunoprecipitation

Sperm surface proteins were labeled with ^{125}I as previously described (Primakoff and Myles, 1983). Since we observe site-specific endoproteolysis of the immunoprecipitated PH-20 antigen (see Results), the procedures relevant to inhibition of proteolysis will be detailed here.

Mix of two protease inhibitors. In our initial immunoprecipitation experiments, steps taken to prevent proteolysis were relatively limited because control experiments had shown ^{125}I-labeled BSA was not proteolyzed during the procedures (Primakoff and Myles, 1983). In one procedure used in the current study (identical to the original, Primakoff and Myles, 1983), removal of the caudae, sperm washes, and subsequent iodination—procedures taking 2–3 h—were all done at room temperature (18–21°C) in the absence of protease inhibitors. After iodination, the sperm were washed twice at room temperature in Mg^{2+}-HEPES containing the protease inhibitor leupeptin (10 μM) and then lysed at 4°C in solubilization buffer containing 0.14 M NaCl, 1% bovine hemoglobin, 10 mM Tris, pH 8.0, 1% Triton X-100, and two protease inhibitors—leupeptin (10 μM) and phenylmethylsulfonyl fluoride (PMSF, 1 mM). The remaining procedures were done at 4°C in the presence

of 10 μM leupeptin and 1 mM PMSF. In the current paper, we term this protocol the "mix of two protease inhibitors" procedure.

Mix of five protease inhibitors. In a second procedure, we used a total of five protease inhibitors. The caudae were removed from the animal at room temperature and placed in Mg^{2+}-HEPES containing the protease inhibitors iodoacetamide (1 mM), PMSF (1 mM), and leupeptin (10 μM). The sperm were released from the cauda into this medium, washed once, resuspended, and incubated for 20 min at 37°C in the same medium containing the permeant and irreversible protease inhibitor, diisopropyl-fluorophosphate (2 mM), and 1 mM iodoacetamine, 1 mM PMSF, and 10 μM leupeptin. The cells were washed two more times in Mg^{2+}-HEPES containing 1 mM iodoacetamide, 1 mM PMSF, and 10 μM leupeptin, iodinated in this medium, and washed two more times in this medium at room temperature. They were then lysed in the solubilization buffer mentioned above containing the protease inhibitors iodoacetamide (70 mM), PMSF (1 mM), leupeptin (10 μM), and EDTA (10 mM) at 4°C. The remaining procedures were done at 4°C with medium containing 70 mM iodoacetamide, 1 mM PMSF, 10 μM leupeptin, and 10 mM EDTA. We term this protocol the "mix of five protease inhibitors" procedure.

In both procedures, prior to Triton lysis, the sperm were always maintained at room temperature rather than 4°C, because guinea pig sperm are easily damaged at 4°C and the surface labeling with ^{125}I requires a very high percentage of intact cells. Immunoprecipitation was done with either the PH-20 or PH-22 MAb at 4°C, as previously described (Myles et al., 1981; Primakoff and Myles, 1983), in the presence of the same protease inhibitors as were in the solubilization buffer for the particular preparation.

PAGE and Autoradiography

One-dimensional SDS-PAGE and autoradiography were performed as previously described (Myles et al., 1981; Primakoff and Myles, 1983). Silver staining was done by the method of Oakley et al. (1980). All molecular mass determinations for reduced and nonreduced samples were based on comparison to reduced standards. In previous studies, we reported that the ^{125}I-labeled, immunoprecipitated PH-20 antigen migrates under nonreducing conditions at ∿ 70 kDa, based on comparison to nonreduced standards (Primakoff and Myles, 1983), or in the range 59–66 kDa compared to reduced standards (Primakoff et al., 1985). Comparison of nonreduced samples to nonreduced standards gives less accurate determinations of molecular mass because the routinely used standards, particularly BSA, contain one or many intrachain disulfide bonds, and such standards migrate anomalously under nonreducing conditions (Fairbanks et al., 1971; Allore and Barber, 1983; Samelson, 1985).

The percentage of the protein in individual bands on Coomassie Brilliant Blue-stained gels was quantified with an SL-TRFF soft laser scanning densitometer interfaced with an Apple IIe computer for automated peak integration. ^{125}I-labeled bands were quantified by scanning the autoradiogram with the densitometer and/or using the autoradiogram to locate the bands on the dried gel, excising the bands and counting them in a gamma counter.

Two-dimensional gel electrophoresis used nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension and SDS-PAGE in the second dimension, according to the procedures described by O'Farrell et al., 1977. Cleveland digests of selected polypeptides from the gels were done using *Staphylococcus aureus* V8 protease (Sigma Chemical Co.) according to the published protocol (Cleveland et al., 1977; Cleveland, 1983).

RESULTS

Isolation of the PH-20 Protein by Affinity Chromatography

The affinity isolation procedure subjects the PH-20 protein to potential denaturation during elution with high pH and detergent (50 mM diethylamine, pH 11.5, and 30 mM OG), so we first tested whether the isolated protein retained antigenic activity. Retention of antigenic activity indicates that the PH-20 protein after elution has some degree of native structure and could be tested for zona binding-related activities inhibitable by the PH-20 MAb. The PH-20 antigen eluted from the column had the ability to bind similar amounts of all three PH-20-specific MAbs—PH-20, PH-21, and PH-22. Each of these MAbs binds to a heat-sensitive and thus potentially denaturable epitope (Primakoff, Hyatt and Myles, unpublished results). In the direct solid-phase assay, the eluted antigen bound higher levels of the three MAbs than were bound by the initial OG extract (Table 1), presumably because the PH-20 antigen in the OG

TABLE 1. Monoclonal antibody (MAb) binding in the solid-phase assay to PH-20 protein eluted from the PH-22 MAb-Sepharose column.

Antigen	cpm bound[b]		
	PH-20	PH-21	PH-22
Starting octylglucoside extract[a]	892	819	1168
Column flow-through[a]	110	35	233
Combined eluted fractions[a] (E2, E3, and E4)	1894	1757	2112

[a]The initial octylglucoside extract, the flow-through from the PH-22 MAb-Sepharose column, and the combined eluted fractions (E2, E3, and E4) were used as antigen in the solid-phase assay. E2, E3, and E4 were the second, third, and fourth 5.5-ml fractions obtained after initiating elution of the column with diethylamine. Each showed the same pattern on SDS gels, identical to the pattern of *Figure 3, Lanes 6–9*.

[b]cpm bound are the average of duplicate determinations.

extract must compete with many other proteins for binding to the solid phase.

To have a quantitative measure of the amounts of antigenic activity recovered, we tested the OG extract applied to the column and also the eluted fractions for their ability to inhibit PH-22 MAb binding in the solid-phase assay. In Figure 2 are data from a preparation of PH-20 protein from an OG extract of 115 ml of sperm. The OG extract at a dilution of about 1/4.8 gave 50% inhibition of PH-22 MAb binding (Fig. 2). The three peak diethylamine-eluted, neutralized fractions from the affinity column (5.5 ml each)—E2, E3, and E4—gave 50% inhibition of PH-22 MAb binding at dilutions of 1/28 (E2), 1/40 (E3), and 1/10.2 (E4) (Fig. 2). From these values, it is calculated that the initial OG extract contained 1.77×10^5 units of antigenic activity and E2, E3, and E4 together contained 1.38×10^5 units of antigenic activity. Thus, 78% of the antigenic activity loaded onto the column was recovered in the eluted fractions (Table 2). Other column preparations produced similar results, with final yields of antigenic activity in the range 67–100%.

The amount of protein obtained in the isolated PH-20 antigen was 0.46 mg/1.15×10^{10} sperm (Table 2). This is about 0.24% of the total protein in the ultracentrifuged OG lysate. In the single-step isolation procedure, the protein has been purified about 330-fold (Table 2).

Structural Characterization of the PH-20 Protein

The combined eluted fractions that contained PH-20 antigenic activity (Table 2) were concentrated and analyzed by gel electrophoresis under nonreducing and reducing conditions. Under nonreducing conditions, the isolated PH-20 protein showed a major polypeptide band centered at 62 kDa (range 59–66 kDa) and a minor band at 54 kDa (Fig. 3, Lane 2, Coomassie Brilliant Blue stain, and Lane 3, silver stain). (In the silver-stained nonreducing gel, a thin line can be seen just beneath the major 62 kDa band (Fig. 3, Lane 3) and the same very faint, thin line can be discerned on the Coomassie Brilliant Blue-stained gel (Fig. 3, Lane 2). This thin line was

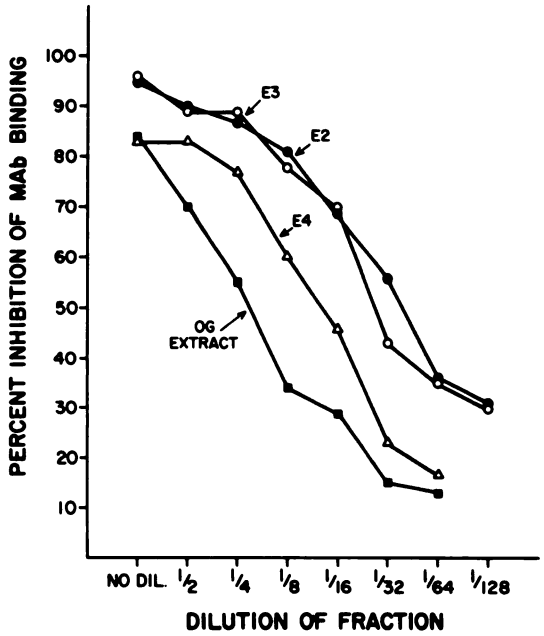

FIG. 2. Assay of antigenic activity. The ultracentrifuged, filtered octylglucoside (OG) extract of sperm applied to the PH-22 monoclonal antibody (MAb)-Sepharose column, and the eluted fractions were compared for their ability to inhibit PH-22 MAb binding in the solid-phase radioactive assay. The OG extract was from 115 ml of sperm at 1×10^8/ml; the three eluted fractions—E2, E3, and E4—each contained 5.5 ml. The OG extract and the E2, E3, and E4 fractions were each diluted first to 1/8 to reduce the level of OG; this is termed the "no dilution" level in the assay. The cpm bound using PH-22 MAb preincubated with buffer alone gave a measure of maximal binding. The percentage of inhibition = 100% minus (the ratio cpm bound with MAb preincubated with fraction/maximal binding × 100). All dilutions of the fractions were tested in duplicate. The eluted fractions E1 and E5 did not contain significant levels of antigenic activity.

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TABLE 2. Yield of purified PH-20 protein.

Antigen	Protein[a] (mg)	Units of antigenic activity ($\times 10^{-5}$)[a]	Relative specific activity	Yield
Octylglucoside extract applied to column (from 1.15×10^{10} cells)	195	1.77	1	(100%)
Combined eluted fractions (E2, E3, and E4)	0.46	1.38	330	78%

[a]The values are from a typical purified preparation of the PH-20 protein. Amounts of protein and antigenic activity were determined as described in *Materials and Methods*. The units of antigenic activity were calculated using 50% inhibition values obtained from the inhibition curves in *Figure 2*.

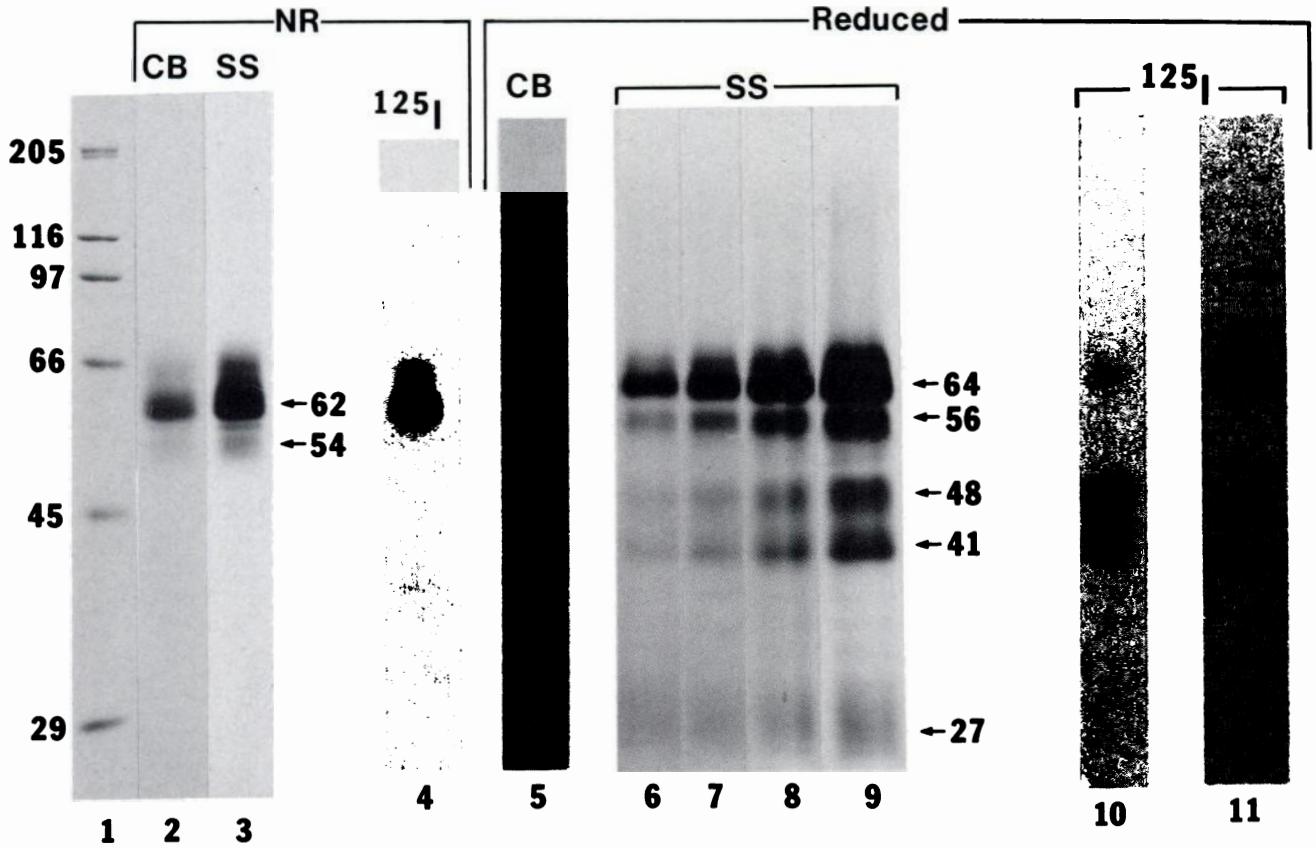

FIG. 3. SDS-PAGE analysis of the affinity-purified PH-20 protein and the ^{125}I-labeled, immunoprecipitated PH-20 protein. Eluted fractions from the PH-22 monoclonal antibody (MAb) affinity column that showed antigenic activity (*Fig. 2*) were pooled, concentrated, and examined on 10% polyacrylamide gels using Coomassie Brilliant blue or silver staining. The PH-20 protein from sperm surface-labeled with ^{125}I was immunoprecipitated and examined on 10% polyacrylamide gels, which were dried and autoradiographed.

Lane 1, reducing conditions, molecular mass standards: myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa.

Lanes 2–4, nonreducing conditions: *Lane 2*, purified PH-20 protein stained with Coomassie Brilliant Blue; *Lane 3*, purified PH-20 protein stained with silver; *Lane 4*, ^{125}I-labeled, immunoprecipitated PH-20 protein; procedure done with mix of two protease inhibitors.

Lanes 5–11, reducing conditions: *Lane 5*, purified PH-20 protein stained with Coomassie Brilliant Blue.

Lanes 6–9, increasing loads of purified PH-20 protein stained with silver: *Lane 6*, 1 μg protein; *Lane 7*, 5 μg protein; *Lane 8*, 10 μg protein; *Lane 9*, 20 μg protein.

Lane 10, ^{125}I-labeled, immunoprecipitated PH-20 protein; procedure done with mix of two protease inhibitors.

Lane 11, ^{125}I-labeled, immunoprecipitated PH-20 protein; procedure done with mix of five protease inhibitors.

seen in every lane of some gels, including those lanes loaded only with sample buffer, and corresponds in molecular mass to human "skin protein", possibly keratin, a frequently found contaminant of solutions and/or reagents used in SDS-PAGE (Ochs, 1983).

The major 62 kDa and minor 54 kDa bands obtained with the nonreduced sample showed, under reducing conditions, a reproducible ∿ 2 kDa increase in apparent molecular mass to 64 kDa (range 61–68 kDa) and 56 kDa (Fig. 3, Lanes 2–3 compared to Lanes 5–9). The ∿ 2 kDa magnitude of this difference was directly demonstrated by running the nonreduced and reduced samples on opposite sides of the same gel, which allowed precise comparison of polypeptide migration under the two conditions. The increase in apparent molecular mass under reducing conditions suggests that the nonreduced 62 kDa and 56 kDa may contain intra-chain disulfide bonds. A polypeptide migrating further (to lower molecular mass) under nonreduced conditions is diagnostic of a more compact conformation resulting from intra-chain disulfides (Fairbanks et al., 1971; Allore and Barber, 1983; Samelson, 1985).

Under reducing conditions, in addition to the major (64 kDa) and minor (56 kDa) band, three more minor bands were seen: two minor bands at 41 kDa and 48 kDa as well as material between 41 and 48 kDa, and a fifth species at about 27 kDa were obtained (Fig. 3, Lanes 5–9). These five polypeptides were the only bands readily detected on silver-stained reducing gels at loads of 1, 5, 10, and 20 μg of isolated PH-20 protein (Fig. 3, Lanes 6–9). Some additional faint bands could be discerned in the overloaded samples, 10 and 20 μg isolated protein, particularly in the region between 27 and 41 kDa (Fig. 3, Lanes 8 and 9).

To determine the relative amounts of the major and strongly stained minor bands, Coomassie Brilliant Blue-stained gels of the purified PH-20 protein were scanned with a densitometer. On a nonreducing gel (Fig. 3, Lane 2), 79% of the protein was in the 62 kDa major band and 21% was in the 54 kDa band (Table 3, Column 1). When the same purified protein sample was run on a reducing gel (Fig. 3, Lane 5), 64% migrated as the 64 kDa major band, 17% as 56 kDa, and 19% as 41–48 kDa (Table 3, Column 2). The 27 kDa band was detected on silver-stained, but not on Coomassie Brilliant Blue-stained gels, and thus was not quantified by the densitometry.

Relationship of the Minor Polypeptide Species to the Major 64 kDa Polypeptide

The experiments presented below indicated that all five molecular weight species seen on reducing gels (Fig. 3, Lanes 5–9) are structurally related: the four minor bands are either differently modified forms or proteolytic fragments of the major 64 kDa band.

To determine if the 56 kDa polypeptide might be structurally related to the major 64 kDa band, we did Cleveland digests of the two polypeptides (Cleveland et al., 1977; Cleveland, 1983; Peyrieras et al., 1985). If the smaller 56 kDa band was related to the larger 64 kDa band, their V8 protease digests would be

TABLE 3. Percentage of PH-20 protein in different polypeptides.[a]

Item	Purified protein (1)	Purified protein (2)	Immunoprecipitated ^{125}I-protein (3)	Immunoprecipitated ^{125}I-protein (4)
Gel conditions	Nonreducing	Reducing	Reducing	Reducing
Temperature of preparation	4°C	4°C	18–21°C	18–21°C
Protease inhibitors	Mix of 8	Mix of 8	Mix of 2	Mix of 5
64 kDa	79	64	8	40
56 kDa	21	17	Not detected	7
41–48 kDa	Not detected	19	92	53
27 kDa	Not detected	Not detected	Not detected	Not detected
Ratio $\frac{\%41-48\text{ kDa}}{\%64\text{ kDa}}$		0.3	11.5	1.3

[a]The percentage of protein in individual bands on Coomassie Brilliant Blue-stained gels (Columns 1 and 2) was determined by scanning *Figure 3, Lanes 2* and *5* with an SL-TRFF soft laser scanning densitometer inferfaced with an Apple IIe computer for automated peak integration. The percentage of ^{125}I-labeled protein in individual bands (*Columns 3* and *4*) was determined by analyzing *Figure 3, Lanes 10* and *11*. The autoradiogram was scanned with the densitometer to obtain the percentages shown; the bands were also excised from the dried gel and counted, which gave essentially the same values.

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expected to contain some unique peptides and a significant number of peptides in common. The common peptides in the 64 and 56 kDa digests are denoted by arrows in Figure 4. The V8 protease itself appeared as a triplet at about 27 kDa and a single faint band of about 11 kDa in Lanes 1–4. To show the position of these peptides, the V8 protease was run by itself (Fig. 4, Lane 5). Excluding the V8 protease peptides, the 64 kDa digest (Fig. 4, Lanes 2 and 3) and 56 kDa digest (Fig. 4, Lanes 1 and 4) contained at least eight peptides in common, suggesting that the 64 kDa and 56 kDa forms are structurally related.

The polypeptides in the 41–48 kDa and 27 kDa regions were present on reducing gels and absent on nonreducing gels (Fig. 3). We found these polypeptides could be obtained by reducing the major 62 kDa band taken from a nonreducing gel. An overload, 20 μg, of the isolated PH-20 protein, was first run on a nonreducing gel. This nonreducing gel was briefly stained and destained, using Coomassie Brilliant Blue and the brief staining protocol of Cleveland et al. (1977) to locate the bands. The nonreduced 62 kDa (59–66 kDa) band was cut into three gel slices representing the top of the band ∿ 64–66 kDa; the middle, ∿ 61–63 kDa; and the bottom, ∿ 59–62 kDa. The slices were soaked in SDS-PAGE sample buffer containing 10% β-mercaptoethanol and placed in the wells of a second gel with reducing sample buffer; subsequently, the protein was re-electrophoresed under the reducing condition. Each of the slices gave rise to a corresponding top, middle, or bottom band in the region 61–68 kDa (centered at 64 kDa) and to bands in the region 41–48 kDa, and to faint bands in the region 24–30 kDa (Fig. 5, Lanes 1–3). Two other bands can be seen between 41

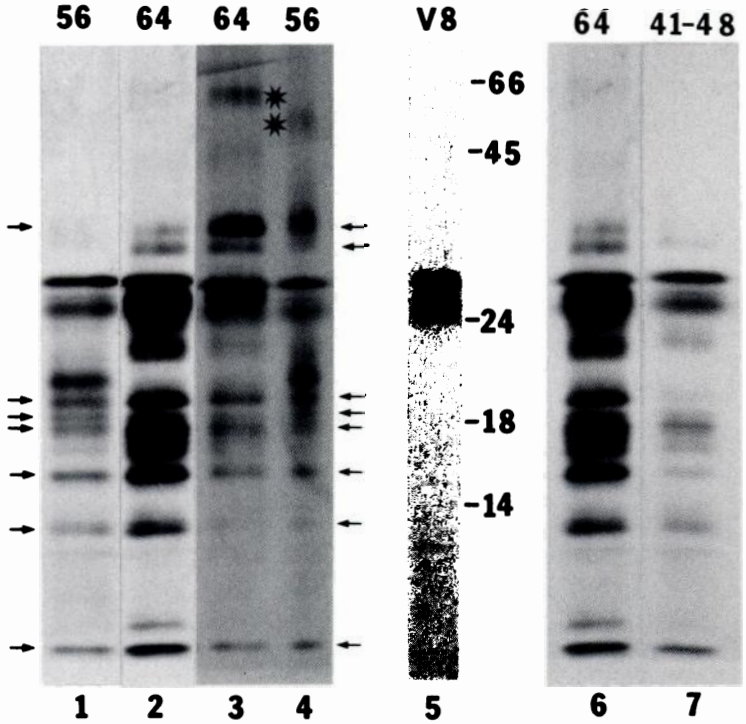

FIG. 4. Cleveland digest of the 56 kDa and 64 kDa polypeptides. Purified PH-20 protein was run under reducing conditions and briefly stained and destained (Cleveland et al., 1977). The 64 kDa, 56 kDa, and 41–48 kDa bands were excised from the first gel, placed in the wells of a second gel, and overlaid with sample buffer containing two different levels of *Staphylococcus aureus* V8 protease (*Lanes 1, 2, 6* and 7, 500 ng; *Lanes 3* and *4*, 200 ng). Proteolytic digestion during electrophoresis was done as described by Cleveland et al., 1977, and Cleveland, 1983. The second gel was silver-stained. *Lane 1*, 500 ng V8 protease, digest of 56 kDa polypeptide; *Lane 2*, 500 ng V8 protease, digest of 64 kDa polypeptide; *Lane 3*, 200 ng V8 protease, digest of 64 kDa polypeptide; *Lane 4*, 200 ng V8 protease, digest of 56 kDa polypeptide; *Lane 5*, 500 ng V8 protease alone; *Lane 6*, same as *Lane 2*, 500 ng V8 protease, digest of 64 kDa polypeptide; *Lane 7*, 500 ng V8 protease, digest of 41–48 kDa polypeptide. Peptides that appear in common in the 56 kDa and 64 kDa digests are marked with *arrows*. Two hundred nanograms V8 protease leaves some undigested 64 kDa and 56 kDa polypeptides; these intact polypeptides are marked with *stars*. Five hundred nanograms V8 protease leaves no detectable intact 64 kDa, 56 kDa, or 41–48 kDa polypeptides. The molecular mass standards used were bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; trypsinogen, 24 kDa; lactoglobulin, 18 kDa; and lysozyme, 14 kDa.

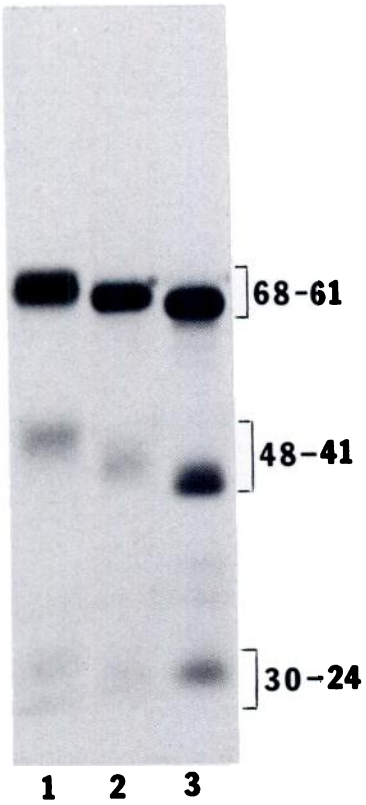

FIG. 5. Analysis of the nonreduced 62 kDa and 54 kDa polypeptides by re-electrophoresis under reducing conditions. Purified PH-20 protein (20 μg) was electrophoresed under nonreducing conditions, briefly stained with Coomassie Brilliant Blue, and briefly destained. The major, nonreduced 62 kDa (59–66 kDa) band was excised in three slices, a top, middle, and bottom slice, representing ∿ 64–66 kDa material, ∿ 61–63 kDa material, and ∿ 59–62 kDa material, respectively. The three slices were soaked for 45 min at room temperature in SDS-PAGE sample buffer containing 10% β-mercaptoethanol, placed in wells of a second gel with sample buffer containing 5% β-mercaptoethanol, and re-electrophoresed under the reducing condition. The second gel was silver-stained. *Lane 1,* top slice; *Lane 2,* middle slice; *Lane 3,* bottom slice.

and 27 kDa in the reduced material derived from the nonreduced slice of 59–62 kDa (Fig. 5, Lane 3). These may be minor proteolytic fragments of the 41 kDa form detectable in a 20-μg load and may correspond to some of the faint bands between 41 and 27 kDa seen in the reduced 20-μg load in Figure 3, Lane 9.

To confirm that the PH-20 antigen's nonreduced 62 kDa polypeptide upon reduction gives rise to both 64 kDa and polypeptides of lower molecular mass, we isolated the antigen by a second method. The alternative method was surface labeling with ^{125}I followed by immunoprecipitation. On a nonreducing gel the ^{125}I-labeled, immunoprecipitated PH-20 protein showed a major polypeptide at 62 kDa, range 59–66 kDa (Fig. 3, Lane 4, and Primakoff et al., 1985). The ^{125}I-labeled 62 kDa band often had a diffuse top like the column-purified PH-20 (Fig. 3, Lane 4, compared to Lanes 2 and 3). On a two-dimensional (2D) nonreducing gel, this ^{125}I-labeled polypeptide ran as one spot with a diffuse top in the range 59–66 kDa (Fig. 6A,B). When the ^{125}I-labeled PH-20 protein was first run nonreduced, cut from the gel, reduced and re-electrophoresed, a result analogous to the result with the purified antigen was found. The major nonreduced band 62 kDa (59–66 kDa) from a one-dimensional (1D) gel was cut into six slices, the top slice representing ∿ 66 kDa material and the bottom slice representing ∿ 59 kDa material. Protein from the six gel slices was re-electrophoresed under reducing conditions (Fig. 7, Lanes 1–6) and compared to a sample of the ^{125}I-labeled antigen run under reducing conditions in an adjacent lane (Fig. 7, Lane 7). Each slice from the nonreduced gel yielded protein in the 61–68 kDa region and in the 41–48 kDa region. Thus, the ^{125}I immunoprecipitated PH-20 antigen was like the column-purified antigen in that upon reduction, the major, nonreduced 62 kDa bands in both cases produced polypeptides in the 61–68 kDa and the 41–48 kDa regions. A difference between the ^{125}I immunoprecipitated PH-20 and the column-purified PH-20 was that with the ^{125}I-labeled antigen, polypeptides in the 27 kDa region were not observed, possibly because the 27 kDa form is not labeled by ^{125}I.

Endoproteolysis of 64 kDa as the Source of the 41–48 kDa and 27 kDa Polypeptides

The results so far indicate that 56 and 64 kDa polypeptides are related and the major nonreduced 62 kDa (59–66 kDa) polypeptide appears to be a single protein that exists in two forms. One form under reducing conditions migrated at 61–68 kDa (64 kDa) and a second form migrated at 41–48 kDa and 27 kDa. The two forms might arise if the PH-20 protein were subject to endoproteolysis. One form, 61–68 kDa, was uncleaved and the second form was cleaved by an endoproteolytic activity to a 41–48 kDa fragment and a 27 kDa fragment. As shown in the model presented in Figure 8, the 41–48 kDa and 27 kDa fragments are linked by at least one disulfide bond. Thus they were seen under reducing conditions but not under nonreducing conditions. A finding

FIG. 6. Two-dimensional gel analysis of the ^{125}I-labeled, immunoprecipitated PH-20 protein under nonreducing conditions. The ^{125}I-labeled PH-20 was immmunoprecipitated using the "mix of two protease inhibitors" procedure and examined under nonreducing conditions using NEPHGE in the first dimension and SDS-PAGE in the second dimension. The acidic side of the pH gradient is on the *right* and the basic side is on the *left*. The pH along the first dimension was determined by slicing an identical tube gel run at the same time (without a protein sample) and measuring the pH of the slices according to O'Farrell et al., 1977. Molecular mass standards (same as in *Fig. 3*) were electrophoresed on the second dimension after being placed in a well cut by a spatula on one edge of the second dimension gel. (*A*) A typical exposure time (2½ days) for the autoradiogram shows one spot in the pH range 7.0–7.6. (*B*) A very long exposure time (31 days) for the autoradiogram shows one spot with a diffused top.

supporting this interpretation was that the relative amounts of 64 and 41–48 kDa forms varied depending upon the conditions for inhibition of proteolysis. When the ^{125}I surface-labeled PH-20 protein was isolated by immunoprecipitation, the sperm were maintained at room temperature for 2–3 h in procedures that used either two or five protease inhibitors (Materials and Methods). With the mix of two protease inhibitors, a typical ratio of the amount of the 41–48 kDa fragment to the intact 64 kDa polypeptide was 11.5 (Fig. 3, Lane 10; Table 3, Column 3), whereas with the mix of five protease inhibitors, the ratio of the 41–48 kDa fragment to the 64 kDa polypeptide was 1.3 (Fig. 3, Lane 11; Table 3, Column 4). When the PH-20 protein was purified on the PH-22 MAb Sepharose column, it was possible to hold the sperm at 4°C throughout and use a mix of eight protease inhibitors, procedures that gave a ratio of the 41–48 kDa to 64 kDa bands as 0.3 (Fig. 3, Lane 5; Table 3, Column 2).

An additional experiment which indicated that 41–48 kDa band represents a proteolytic fragment of 64 kDa polypeptide was a comparison of Cleveland digests of the two polypeptides. The V8 protease digests of 41–48 kDa and 64 kDa bands showed numerous peptides in common (Fig. 4, Lanes 6 and 7).

Structural Distinction between PH-20$_{AM}$ and PH-20$_{PM}$

Since we have previously found, using immunofluorescence microscopy, that there are two populations of the PH-20 protein in sperm, PH-20$_{AM}$ and PH-20$_{PM}$ (Cowan et al., 1986), we asked if 2D gel analysis of purified PH-20 would show two distinct isoelectric forms of the major 64 kDa polypeptide. As noted above, under nonreducing conditions, the major PH-20 polypeptide migrated as a single spot in the pH range 7.0–7.6 (Fig. 6). Under reducing conditions, the major polypeptide in purified PH-20 (polypeptide No. 1, Fig. 9), also migrated as one spot at 64 kDa (61–68 kDa), and in a broader, more acidic pH range (pH 5.0–6.6). Polypeptide No. 2 migrated as two spots at 56 kDa, one relatively acidic (pH ∿ 5.2) and one neutral (pH ∿ 7.0).

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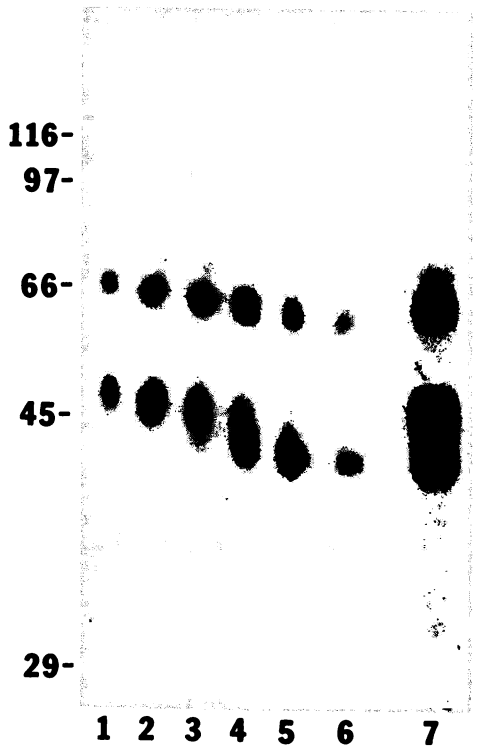

FIG. 7. Analysis of the ^{125}I-labeled, nonreduced 62 kDa (59–66 kDa) band by re-electrophoresis under reducing conditions. The PH-20 protein was immunoprecipitated from an extract of sperm surface-labeled with ^{125}I and run on a nonreducing gel. The gel lane was cut into 45 slices that were counted to identify the peak of ^{125}I radioactivity where the 62 kDa band is located. The six slices that constituted the 62 kDa band were soaked for 30 min in SDS-PAGE sample buffer containing 10% β-mercaptoethanol and re-electrophoresed under reducing conditions as described in *Figure 5*. *Lane 1*, top slice; *Lane 2*, second slice; *Lane 3*, third slice; *Lane 4*, fourth slice; *Lane 5*, fifth slice; *Lane 6*, bottom slice; *Lane 7*, the ^{125}I-labeled, immunoprecipitated PH-20 protein initially placed into reducing sample buffer and run in this lane for comparison to *Lanes 1–6*. It shows the positions on the gel of the 61–68 kDa region, centered at 64 kDa, and the 41–48 kDa region. Molecular mass standards are the same as in *Figure 3*.

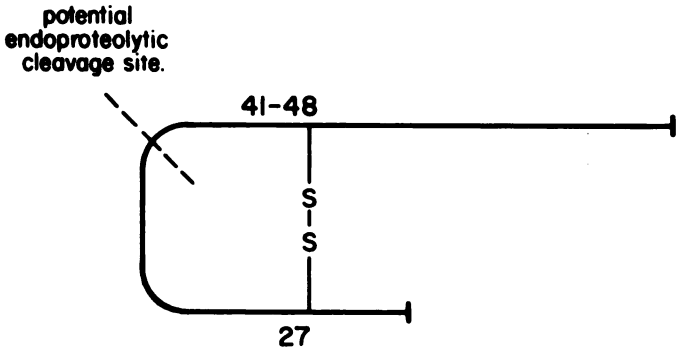

FIG. 8. A model of the structure of the PH-20 protein. The molecule is ∿ 61–68 kDa and contains at least one intra-chain disulfide bond between a 27 kDa segment and a 41–48 kDa segment. It can undergo endoproteolytic cleavage at a specific site. If this cleavage has been made and the protein is examined under reducing conditions on SDS-PAGE, the two segments of the initial protein are separated on the gel and migrate at 41–48 kDa and 27 kDa.

Polypeptides Nos. 3 and 4 are the 48 and 41 kDa forms and polypeptide No. 5 is the 27 kDa form (Fig. 9). Although the major PH-20 polypeptide showed considerable charge heterogeneity under nonreducing and reducing conditions, two clearly distinct isoelectric forms that might correspond to PH-20$_{AM}$ and PH-20$_{PM}$ were not seen.

DISCUSSION

In the current paper, we report a one-step procedure for the purification of the PH-20 antigen, a guinea pig sperm membrane protein for which previous evidence indicates a function in sperm binding to the zona pellucida (Primakoff et al., 1985). Silver-stained reducing gels of purified PH-20 reveal five strongly stained bands that are the interrelated PH-20 polypeptides. In addition, on gels overloaded with 10 or 20 μg of isolated protein, a few other faint, silver-stainable bands can be detected. These may be either minor proteolytic fragments of PH-20 or contaminating polypeptides. We have found that the silver-

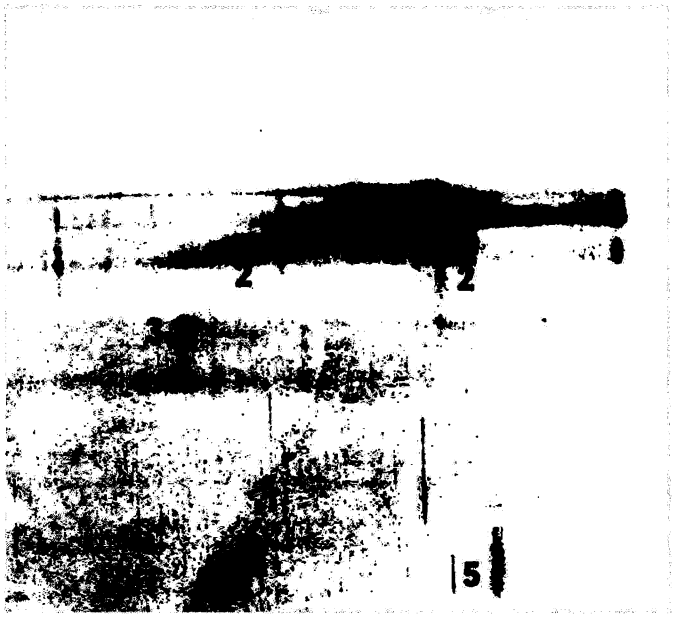

FIG. 9. Two-dimensional gel analysis of the purified PH-20 protein under reducing conditions. The fractions eluted from the PH-22 monoclonal antibody (MAb) affinity column that showed antigenic activity (*Fig. 2*) were pooled, concentrated, and examined under reducing conditions with NEPHGE used in the first dimension and SDS-PAGE in the second dimension. The acidic side of the pH gradient is on the *right* and the basic side is on the *left*. The pH gradient along the first dimension and position of molecular mass standards in the second dimension were determined as in the *legend* to *Figure 6*. The gel was silver-stained.

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staining procedure in our hands can detect the molecular mass standards down to a level of 5 ng of protein, suggesting the rough estimate that silver-stainable contaminants or minor proteolysis products are present at a level of 5 ng in 10 μg of isolated PH-20 protein, i.e., one part in 2000.

An unexpected finding in this study was that the purified PH-20 protein exists in three structural forms separable on reducing SDS-PAGE: the major 64 kDa form, the lower molecular mass 56 kDa form, and the cleaved 41–48 kDa and 27 kDa forms. Cleveland digests indicated that the 56 kDa form is related to the 64 kDa form and could be a structurally similar protein, a differently modified form, or a proteolytic fragment of the 64 kDa form. The 56 kDa form apparently has two distinct isoelectric forms (Fig. 9). We have not excluded the unlikely possibility that by coincidence one 56 kDa spot is a contaminant in the preparation that migrates at 54 kDa nonreduced, 56 kDa reduced, and a distinct pH from the 56 kDa form that is related to the 64 kDa form. Although the 56 kDa polypeptide was seen at about the same level (15–20%) in all column-purified samples, it was only detected in PH-20 immunoprecipitates when proteolysis was more stringently controlled (mix of five protease inhibitors). Though it cannot be seen in the photograph of the autoradiogram (Fig. 3, Lane 11), a faint 56 kDa band was visible on the original autoradiogram that could be quantified by scanning densitometry as 7% of the total ^{125}I-labeled protein (Table 3, Column 4). The 56 kDa form was not detected in PH-20 immunoprecipitates using the mix of two protease inhibitors (Table 3, Column 3). These findings suggest that the 56 kDa form can be surface-labeled with ^{125}I to some degree, but may be susceptible to complete proteolysis in the two protease inhibitors procedure.

The 41–48 kDa and 27 kDa forms are concluded to be endoproteolytic fragments of the 64 kDa form that are linked to each other by disulfide bonds (Fig. 8). Three lines of evidence support this interpretation. First, on nonreducing gels, the 64 kDa and 41–48 kDa and 27 kDa forms migrated as a single polypeptide at 62 kDa (Figs. 5, 6, 7). Second, when conditions to block proteolysis were not stringent, the 41–48 kDa/64 kDa ratio was relatively high, whereas with more stringent conditions to block proteolysis, the 41–48 kDa/64 kDa ratio was relatively low. Third, Cleveland digests of the 41–48 kDa and 64 kDa forms showed related patterns.

The 27 kDa form stains diffusely and perhaps poorly. It would be anticipated to be present in stoichiometric amounts with the 41–48 kDa form, but was not detected with Coomassie Brilliant Blue and was weakly detected with silver. If 27 kDa (24–30 kDa) and 41–48 kDa are taken as precise determinations of molecular mass and added together, obviously the total is more than 61–68 kDa. One or more of the bands may run anomalously, possibly because each of them contains N-linked carbohydrate (Primakoff and Myles, unpublished results), so that precise additivity for the apparent molecular mass of the two fragments is not observed.

Possible Physiological Significance of the Endoproteolysis

Since the PH-20 antigen undergoes endoproteolytic cleavage, this cleavage may well have physiological significance. The endoproteolytic cleavage of PH-20$_{PM}$ may affect its ability to migrate to the IAM or cleavage of both PH-20$_{PM}$ and PH-20$_{AM}$ may affect their activity in sperm-zona binding. The hemagglutinin (HA) protein of influenza virus and F protein of Sendai virus are integral membrane proteins that, like PH-20, are cleaved by endoprotease into two disulfide-linked fragments. The endoproteolytic cleavage converts both HA and F from an inactive to an active form (White et al., 1983).

However, several possibilities exist to explain the endoproteolytic cleavage we have observed. (1) The endoprotease may be a contaminant of epididymal origin and act on the PH-20 protein before or after sperm lysis. (2) The endoprotease may be a sperm protease, of which many have been described (McRorie and Williams, 1974; Zaneveld et al., 1975; de Lamirande et al., 1983; Johnson et al., 1985; Talbot and Dicarlantonio, 1985; de Lamirande and Gagnon, 1986), and may be released from damaged sperm in the initial sperm isolation and released in higher amounts after sperm lysis. In both these possibilities, endoproteolysis of the PH-20 antigen may be of no physiological significance. (3) The endoprotease may be acrosin or some other acrosomal protease that is activated or released during the acrosome reaction and cleaves the PH-20 protein. In this case, the endoproteolysis may be physiologically relevant. This third hypothesis would be consistent with our finding that a fairly high level of PH-20 protein is already endoproteolytically cleaved even though all purification steps were completed at 4°C and inhibitors were

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present of a broad spectrum of known proteases. Some cleavage of PH-20 antigen could occur on sperm in the animal, since cells may have lost their acrosomes in the animal; "acrosome-intact" populations of sperm, immediately after removal from the male guinea pig, normally contain about 10–15% cells without acrosomes.

In preliminary experiments to examine when the endoproteolytic cleavage of the 64 kDa polypeptide occurs, we found that the cleavage can occur on the cell surface during the acrosome reaction. When sperm, isolated and washed at 4°C in the presence of the mix of eight protease inhibitors, were induced to acrosome-react at 37°C for 10 min in the absence of protease inhibitors, almost all of the purified PH-20 protein was cleaved to the 41–48 kDa and 27 kDa fragments and very little of the uncleaved 64 kDa form remained (Primakoff, Hyatt and Myles, unpublished results). This experiment indicates that the endoproteolysis of native PH-20 can occur on the cell surface during the acrosome reaction. Thus, it appears not to be a proteolytic event that is possible only as an artifact after the protein's removal from the membrane by detergent. However, this experiment does not determine whether the endoprotease is a residual, contaminating protease in the sperm preparation or a protease that is specifically released or activated during the acrosome reaction. Further work using the purified 64 kDa polypeptide as substrate will be required to define the cellular origin of the endoprotease and the possible effects of the site-specific cleavage on PH-20 activity during sperm-zona binding.

Structural Identity of PH-20$_{AM}$ and PH-20$_{PM}$

One of our goals in studying the behavior of the PH-20 protein on 1D and 2D gels was to see if we could distinguish two forms of the protein that might correspond to PH-20$_{AM}$ and PH-20$_{PM}$. Since site-counting experiments on sperm show twice as many PH-20$_{AM}$ sites as PH-20$_{PM}$ sites (Cowan et al., 1986), one might expect to find about two times as much PH-20$_{AM}$ as PH-20$_{PM}$ in purified PH-20. Distinct isoelectric forms of the major band (62 kDa non-reduced, Fig. 6; 64 kDa reduced, Fig. 9) were not separated by isoelectric focusing in the first dimension on 2D gels. Two different forms of purified PH-20 were found—one of 64 kDa and one of 56 kDa—present in the ratio of about 4:1, not 2:1. However, a differential recovery of the two forms during isolation could result in a 64 kDa/56 kDa ratio in purified PH-20 that might not accurately reflect their ratio in intact sperm. We found that 20–25% of the PH-20 antigen in the initial OG extract from sperm remains in the 100,000 × *g* pellet from the extract and thus is never applied to the affinity column. If this pelleted antigen is, for some reason, predominantly of the 56 kDa polypeptide, relatively reduced amounts of the 56 kDa form would be obtained in the protein eluted from the column. Another possibility is that the 56 kDa form is very sensitive to complete proteolysis and thus could be differentially lost during purification procedures that fail to entirely block proteolysis. Thus, it is plausible that the 64 kDa form is PH-20$_{AM}$ and the 56 kDa form is PH-20$_{PM}$. Other hypotheses about the structural identity of PH-20$_{AM}$ and PH-20$_{PM}$ are also consistent with our current evidence, and further work will be necessary to test them.

ACKNOWLEDGMENTS

We thank Dr. Alfred Goldberg, Department of Physiology, Harvard Medical School, for advice on protease inhibitors and blocking proteolysis.

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