Partial Characterization of Chorionic Gonadotropin-Like Binding Sites from the Bacteria *Xanthomonas maltophilia*

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The gram-negative bacterium, *Xanthomonas maltophilia*, has low- and high-affinity luteinizing hormone/chorionic gonadotropin (LH/CG)-binding sites, similar to the LH/CG receptor found in mammals. Although the low-affinity site binds both LH and human CG (hCG), the high-affinity site is specific for hCG. In the current investigation, these two binding sites were independently isolated from *X. maltophilia* for further characterization. To isolate functional binding sites, we developed a solubilization method using the detergent zwittergent 3,14 and high glycerol concentrations that allowed for the maintenance of ligand-binding integrity. Gel filtration experiments established molecular weights of 170 and 11.5 kDa for the two binding sites, which were supported by data from photoaffinity labeling and ultracentrifugation experiments. Gel filtration data also suggested the presence of a third binding site of 5.4 kDa. The 170-kDa site had a binding affinity of Kd = 12×10^{-6} and bound both LH and hCG. The small molecular weight site had an affinity of Kd = 9.4×10^{-8} and was CG specific. Collectively, these data demonstrate the presence of multiple hormone binding sites in *X. maltophilia* that differ in molecular size, binding affinity, and ligand specificity. Exp Biol Med 228:926–934, 2003

Key words: *Pseudomonas maltophilia*; hCG; receptor; luteinizing hormone; binding assay

The mammalian luteinizing hormone/chorionic gonadotropin (LH/CG) receptor is a member of a subfamily of glycoproteins that belong to a 7-transmembrane, G-coupled protein receptor family (1, 2). All mammalian LH/CG receptors have a heterogeneous specificity for LH and CG. Both of these hormones contain two subunits, an α and a β. The α subunit is identical for all pituitary glycoprotein hormones (including CG, LH, follicle-stimulating hormone, and thyrotropin-stimulating hormone) within a species (3). Differences in the β subunits allow for receptor specificity. LH and CG have the highest homology of β subunits among the glycoprotein hormones with CG containing a 31-amino acid extension at the carboxyl-terminal, which is not present in LH. Mammalian LH/CG receptors bind CG with affinities ranging from Kd = 4.5×10^{-10} *M* to 5×10^{-12} *M* and have been isolated from ovaries (4), uterus (5), fallopian tube (6), prostate (7), and brain (8). The mammalian LH/CG receptor primarily functions in corpus luteum maintenance (CG), stimulating production of steroids, and gametogenesis (LH).

Surprisingly, some strains of bacteria and yeast express LH/CG-binding sites, which we refer to as binding sites rather than receptors because specific signal coupling and function for bacterial LH/CG-binding sites have not been completely established. The most thoroughly studied LH/CG-binding sites in a bacterium have been with the gram-negative *Xanthomonas maltophilia*. In 1977, Richert and Ryan (9) reported an LH/CG high-affinity binding site on *Pseudomonas (Xanthomonas) maltophilia* (Kd = 2.3×10^{-9} *M*). Later, Carrell and Odell (10) identified a second binding site of higher affinity that was specific for human CG (hCG; Kd = 1.3×10^{-10} *M*). This high-affinity binding site in *X. maltophilia* was the first site in any species that was entirely specific for hCG, showing neither reaction with human LH nor any other glycoprotein hormone. Presumably, the high-affinity, CG-specific bacterial binding site has a unique structure or binding domain with ligand/binding properties different from all other LH/CG receptors, making it advantageous to isolate and characterize this binding site to understand what grants this specificity.

Functionally, binding to the CG-specific site in *X. maltophilia* mediates autocrine/paracrine functions by stimulating cell proliferation and altering cell morphology (11). These changes were stimulated by hCG and xCG, the native bacterial ligand, which was also isolated and partially characterized in our laboratory (12). Activation of yeast

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(*Candida albicans*) LH/CG-binding sites (13–15) demonstrated a shift in the yeast from blastospore to the more virulent and pathogenic mycelium form (14, 16). This developmental shift was seen when using LH, hCG, and even xCG as the ligand (14). If yeast and bacterial LH/CG-binding sites are indeed involved in stimulating growth, antagonists for these sites might prove medically useful by inhibiting yeast transformation and reducing *X. maltophilia* proliferation. The resistance of *X. maltophilia* to many antibiotics also makes an alternative form of drug therapy attractive (17).

The evolutionary relationship between the mammalian receptors and bacterial binding sites is unclear. It was once suggested that bacterial incorporation of the human LH/CG receptor gene might account for bacterial competence because most of the bacterium containing LH/CG-binding sites were isolated from patients with cancer (18). However, the LH/CG-binding site has been identified on bacterium not recovered from humans (19) and a bacterial ligand has been isolated from *X. maltophilia*, xCG, that binds the native LH/CG-binding site with high affinity (Kd = 1.3×10^{-10} *M*). The entire genomic sequence for xCG has been characterized and shown to be similar but not identical to the hCG sequence, with portions of the gene having a 46% homology to the carboxyl-terminal and other regions of the β subunit (20). Antibodies against the β subunit of hCG but not LH recognize the bacterial ligand (12). Considering this information, the evolutionary relationship between mammalian and bacterial LH/CG-binding sites appears to favor a highly conserved ancestral gene or convergent evolution over theories of bacterial incorporation of a mammalian gene.

Further characterization of the LH/CG sites on *X. maltophilia* will be required if we are to understand their evolutionary relationship with mammals, define the unique structure of the CG-specific binding site, and investigate the possibility of therapeutic treatments. Although a 342-base pair DNA sequence cloned from *X. maltophilia* with 73% homology to portions of the human receptor is likely a portion of a bacterial LH/CG-binding site (21), the sequence is incomplete and could be for either the high- or low-affinity binding site. In addition, neither the LH/CG- nor CG-specific binding sites, initially identified using binding assays to whole bacteria, have been isolated from the bacterial membrane or assigned a molecular weight.

To further characterize these binding sites, we have developed a solubilization technique to isolate them while maintaining ligand-binding integrity. After isolating the binding sites, dose-response assays were used to distinguish the high- from low-affinity sites and for determination of CG specificity. Photoaffinity labeling, ultracentrifugation, and gel filtration chromatography in conjunction with standard binding assays permitted the estimation of molecular weights for both the high- and low-affinity sites, and resulted in the identification of a third potential LH/CG-binding site.

Materials and Methods

Bacterial Cultures. Bacteria (*X. maltophilia*) were obtained from the American Type Culture Collection (no. 13637; Rockville, MD). To prepare the bacteria for storage and culturing, they were incubated in 1 to 2 ml of trypticase soy broth (TSB; Difco, Sparks, MD) for 3 to 4 hr at 30°C in a rotating incubator. This bacterial solution was mixed 1:1 with freezing buffer (FB), snap frozen, and stored at −80°C for no more than 6 months. Aliquots of frozen bacteria were thawed, plated on eosin methylene blue (EMB) agar, and incubated for 24 hr at 30°C. Five to seven colonies were lifted from the plate and incubated in 5 ml of TSB for 18 hr at 30°C in a rotating incubator. The 5-ml culture was then inoculated into 500 ml of TSB and incubated until the bacteria had grown to stationary phase (approximately 48 hr) in a 30°C rotating water bath (120 oscillations/min).

Solubilization of LH/CG-Binding Sites. The bacteria were harvested by centrifugation at 4500 rpm for 10 min. The bacterial pellet was washed in 100 ml of washing buffer (WB), repelleted, and either resuspended for cross-linking experiments or solubilized for separation techniques. To solubilize the bacterial pellet (4–5 g wet weight), it was resuspended in 10 ml of solubilization buffer (SB; adapted from Ref. 22) with 20% glycerol and sonicated on ice. Glycerol was added to all solubilization solutions to minimize binding site damage that can occur to glycoprotein receptors during solubilization (23). Without glycerol, total binding from solubilized bacteria was reduced by 55% (data not shown). After sonication, 30 ml of SB with 20% glycerol and 2.18 g of zwittergent 3,14 (Z 3,14; Bio-Rad, Hercules, CA) was added. The suspension was further solubilized for 75 to 90 min at 4°C on an orbit shaker (150 rpm), forming a crude bacterial homogenate

Separation of LH/CG-Binding Sites by Ultracentrifugation. Ultracentrifugation of the crude bacterial homogenate was used to separate binding sites for characterization. The crude bacterial homogenate was centrifuged (Beckman, Fullerton, CA) at either 2,500 (834*g*), 5,000 (3,330*g*), 10,000 (13,814*g*), 20,000 (48,384*g*), 40,000 (116,480*g*), 60,000 (278,852*g*), or 70,000 (356,720*g*) rpm for 30 min. For gel filtration, cross-linking, and pH experiments, the supernatant of crude bacterial homogenate centrifuged at 20,000 rpm (supernatant no. 1) was used. This centrifugation resulted in the removal of whole cells and large cell fragments. For dose-response binding assays, supernatant no. 1 was centrifuged again to create three other fractions used in this study. The supernatant after centrifugation at 40,000 rpm (supernatant no. 2) and at 70,000 rpm (supernatant no. 3), and the resuspended pellet (pellet no. 1; in SB with 20% glycerol and 5% Z 3,14) after a 70,000 rpm centrifugation.

Receptor Binding Assay. The standard binding assay for this study measured total and nonspecific binding as follows. To measure total binding, 170 to 200 μl (900–1000 μg of bacterial protein) of supernatant no. 1 was added to

100 μl of ^{125}I-hCG (25–40 pg hCG/1000 cpm; specific activity of 15-25 μCi/μg) in phosphate-buffered saline (PBS) and sufficient assay buffer (AB) with 0.1% bovine serum albumin (BSA) to bring the total volume to 1 ml. Nonspecific binding assays were identical to total binding assays except that 55 μg of unlabeled hCG was added to displace specific binding of ^{125}I-hCG. Both assays were carried out for 18 hr at 4°C in triplicate. To determine bound ^{125}I-hCG, an equal volume of 30% polyethylene glycol in double-deionized H_2O was added to the assay to separate it from free ^{125}I-hCG (24). After incubation for 1 hr, the solution was centrifuged at 10,000 rpm for 30 min and the supernatant was aspirated. The pellet containing the ^{125}I-hCG/binding-site complex was then placed for 10 min in a gamma counter (Beckman). To calculate specific binding, nonspecific binding was subtracted from total binding. All of the assays containing solubilized bacteria were carried out at pH 6.95 (as measured at 4°C). Dose-response binding assays of LH/CG and CG-specific binding sites were identical to standard nonspecific assays except that varying amounts of hCG (0.001–67 μg) or LH (0.001–10 μg) were added to the assay. Woolf plots were used to analyze dose-response binding data (25).

Gel Filtration Chromatography. To determine the molecular weights of bacterial LH/CG-binding sites and radioactive complexes formed by photoaffinity labeling experiments, a size-exclusion gel filtration column (Sephacryl S-300; Pharmacia, Peapack, NJ) was used to fractionate bacterial proteins of crude homogenates and the products of photoaffinity labeling. The inverted column (height, 90 cm; radius, 1.25 cm; volume, 442 cm^3) allowed for approximately 4.4 ml of homogenate to be applied. The eluent buffer was pumped through the column at a rate of 0.52 ml/min, and 3-ml fractions were collected. To elute radioactive proteins and complexes, SB with 0.01% sodium azide and 0.05% Z 3,14 was used as the eluent buffer. To elute bacterial proteins, SB with 0.01% sodium azide, 1% Z 3,14, and 20% glycerol was used. Molecular weight markers included ribonuclease A (13,700 Da), lysozyme (18,500 Da), ovalbumin (43,000 Da), BSA (67,000 Da), and human γ-globulin (171,000 Da).

Binding Assays of Gel Filtration Chromatography Fractions. To estimate the molecular weight of the LH/CG-binding sites, assays were carried out on bacterial proteins fractionated by gel filtration. Protein concentrations for assays were not calculated, although a 1:10 dilution of ^{125}I-hCG during gel filtration chromatography suggests a similar dilution of proteins in supernatant no. 1. Consequently, the assay volume was increased 10-fold (10 ml) to maintain the relative amounts of protein in each assay. To measure total binding, 2 ml of a gel filtration fraction was added to 1 ml of ^{125}I-hCG in PBS (1000 cpm) and 7 ml of AB (0.1% BSA). Nonspecific binding assays contained 55 μg of hCG.

Photoaffinity Labeling to LH/CG-Binding Sites. A photoaffinity labeling procedure was used to independently confirm the molecular weight of the LH/CG-binding sites. To conduct these experiments, we used a photoactivatable radioiodinatable heterobifunctional reagent (*N*-hydroxysuccinimide ester of 4-azidosalicylic acid; NHS-ASA) (26, 27) that was covalently bound to ^{125}I-hCG as follows. One microliter of an NHS-ASA solution (1.5 mg/100 μl of dimethyl sulfoxide) was added to 199 μl of 0.1 *M* PBS to make a 0.5 m*M* working solution that was mixed with radiolabeled hCG (1-2 × 10^6 cpm in PBS) at 25°C on an orbit shaker (125 rpm) for 20 to 30 min, forming a complex between the two. This complex was used in the following experiments.

In the first experiment, the NHS-ASA/^{125}I-hCG complex was cross-linked to the LH/CG-binding sites in supernatant no. 1. To do this, the supernatant was incubated with the NHS-ASA/^{125}I-hCG complex and AB (0.1% BSA) in volumes proportional to our standard assay (totaling 5 ml). The solution was left for 18 hr at 4°C, and was then cross-linked using 366 nm UV light (26) while mixing, with the homogenate no deeper than 0.5 cm and the UV source placed <5 cm above it (28). This solution was then fractionated by gel filtration to estimate the molecular weight of any radioactive complexes formed.

To be sure the cross-linked protein was located on the outer membrane of the bacteria, we performed a second experiment with whole bacteria. Approximately 0.3 g of whole bacteria in AB (0.1% BSA) was mixed with the NHS-ASA/^{125}I-hCG complex for 18 hr at 4°C. To separate any unbound NHS-ASA/^{125}I-hCG from the bacteria, the solution was centrifuged and the bacterial pellet was washed (in WB) and recentrifuged twice. The rinsed bacterial pellet was resuspended in 10 ml of SB with 20% glycerol and was cross-linked as previously described, solubilized, centrifuged (20,000 rpm), and the supernatant was analyzed by gel filtration.

In both of the previous experiments, total photoaffinity labeling to LH/CG sites was investigated using a low incubation pH (6.95), which maximized binding at these sites. Nonspecific photoaffinity labeling was determined by using a high incubation pH (8.0) to eliminate most specific binding to LH/CG sites.

To control for possible formation of undesired complexes between NHS-ASA and hCG, we radiolabeled NHS-ASA and covalently bound it to hCG (see "Results") following the protocol of Ji *et al.* (27). The 0.5 m*M* working solution of NHS-ASA was radioiodinated and mixed with 5 μg of hCG (National Institutes of Health, Bethesda, MD).

Solutions. The AB contained 40 m*M* Tris-HCl (pH 6.1; measured at 4°C). This buffer brought the overall pH of an assay to approximately 6.95 (measured at 4°C). The FB contained 25 m*M* Tris-HCl, 0.1 *M* $MgSO_4$, and 65% glycerol (pH 8.0) (29). The PBS contained 137 m*M* NaCl, 2.68 m*M* KCl, 10.1 m*M* Na_2HPO_4, and 1.76 m*M* KH_2PO_4

(pH 7.4). The SB contained 10 m*M* Tris-HCl, 10 m*M* NaCl, and 1 m*M* $MgCl_2$ (pH 7.4). The WB contained 40 m*M* Tris-HCl (pH 7.4). All reagents were purchased from Sigma (St. Louis, MO) unless noted otherwise.

Results

Solubilization of Functional LH/CG-Binding Sites. A variety of bacterial solubilization techniques have been described previously (22, 30, 31). We compared and modified these techniques to optimize binding activity of solubilized *X. maltophilia* LH/CG-binding sites. The effectiveness of two detergents (triton X-100, and Z 3,14) and one enzyme (lysozyme) were assessed using two solubilization pHs (7.4 and 4.0), different assay incubation temperatures (24° and 4°C), and in the presence or absence of glycerol. Although 5% Triton X-100 (Table I; pH 4.0 at 4°C with 20% glycerol) was fairly effective, the highest specific binding came after solubilization with 5% Z 3,14 (pH 7.4 at 4°C with 20% glycerol). All subsequent studies used the latter solubilization technique. Binding assays with supernatant no. 1 indicated that the pH of incubation was very important for total binding, as shown previously for these binding sites in *X. maltophilia* and *C. albicans* (9, 10, 15). Maintaining a neutral assay pH (pH 6.95–7.05) gave a 10- to 20-fold increase in total binding over assays carried out at pH 8.0 (data not shown).

Molecular Weight Estimates of LH/CG-Binding Sites. *Ultracentrifugation.* Ultracentrifugation studies indicated the presence of two LH/CG-binding sites based on molecular weight. Glycerol was used during ultracentrifugation rather than density gradient fractionation, a common method to estimate molecular weight, to separate the two binding sites while maintaining binding site viability, which would be lost using the latter. Binding assays of homogenates ultracentrifuged at various speeds revealed a reduction in total binding from 40,000 to 60,000 rpm and a leveling off, but not elimination, of binding at 70,000 rpm (Fig. 1). Even centrifugation for up to 6 hr at 70,000 rpm did not further reduce binding activity in the supernatant (data not shown). These findings suggest that a higher-molecular-weight binding site or site associated with nonbinding proteins, pellets between 40,000 and 70,000 rpm, whereas a lower-molecular-weight binding site remains in the supernatant. Binding assays on a pellet centrifuged at 70,000 rpm

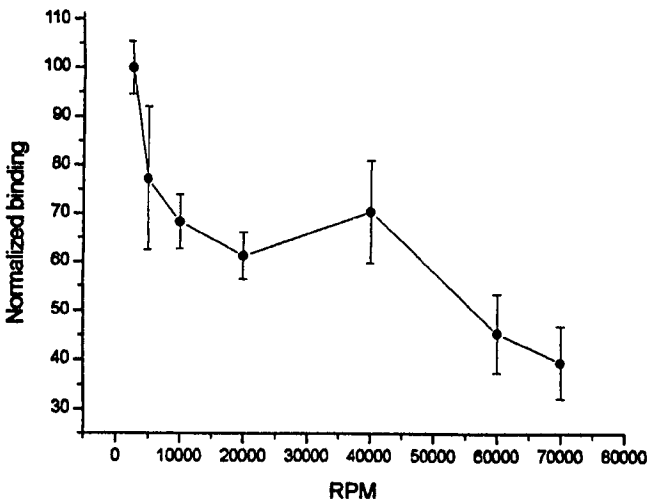

Figure 1. Ultracentrifugation experiments indicated the presence of a very large LH/CG-binding site or sites associated with other proteins that could be separated from a smaller one. After centrifugation of homogenized bacteria at various speeds (2,500; 5,000; 10,000; 20,000; 40,000; 60,000; and 70,000 rpm), LH/CG-binding activity of the supernatant was measured. Averages from five trials are plotted (±SD). The decrease in binding occurring at 2,500 to 20,000 rpm was due to the pelleting of whole cells and large membrane fragments. The large-molecular-weight site pelleted between 40,000 to 70,000 rpm. The pellet from a 70,000 rpm centrifugation contained a high-affinity CG-binding site (see Fig. 4), indicating that a large LH/CG-binding site was indeed being pelleted. The drop in binding between 40,000 and 70,000 rpm is significant ($P < 0.05$; paired *t* test). Normalized binding at 100% represents a total specific ^{125}I-hCG binding of approximately 15% (150–200 counts per million, cpm), after subtraction of nonspecific binding (50–70 cpm).

(pellet no. 1) indicated that there was indeed a large binding site being pelleted (see Fig. 4). Because ^{125}I-hCG did not pellet after centrifugation at 70,000 rpm for 30 min, the large binding site presumably has a weight greater than 55,000 Da.

Size-Exclusion Chromatography. To further refine the molecular weight estimates, we performed gel filtration chromatography on the supernatant after centrifugation at 20,000 rpm (supernatant no. 1), and then assayed each fraction collected. Three binding peaks were identified (Fig. 2). Peak binding activity corresponded to proteins with molecular weights of 170,500 ± 6,000 Da, 11,500 ± 3,200 Da, and 5,400 ± 400 Da ($n = 5$). Although the magnitude of the peak of the smallest binding site was less than that for the two larger molecular weight sites, its elution volume was very consistent in all assays.

Table I. Influence of Bacterial Solubilizations under a Variety of Conditions on Specific Binding of ^{125}I-hCG

	pH 7.4, 4°C	pH 7.4, 24°C	pH 4.0, 4°C	pH 4.0, 24°C
Triton X-100 (0.5%)	<1%	2.2%	10.1%	NT
Triton X-100 (5%)	<1%	1.5%	12.5%	NT
Zwittergent 3,14 (0.5%)	2.0%	<1%	ND	1.3%
Zwittergent 3,14 (5%)	15.2%	ND	ND	<1%
Lysozyme (0.5%)	ND	ND	ND	ND
Lysozyme (5%)	ND	ND	ND	ND

Note. Listed is the percentage of specific binding, which was calculated as the difference between total and nonspecific binding (ND, not detectable; NT, not tested; $n = 3$ for each).

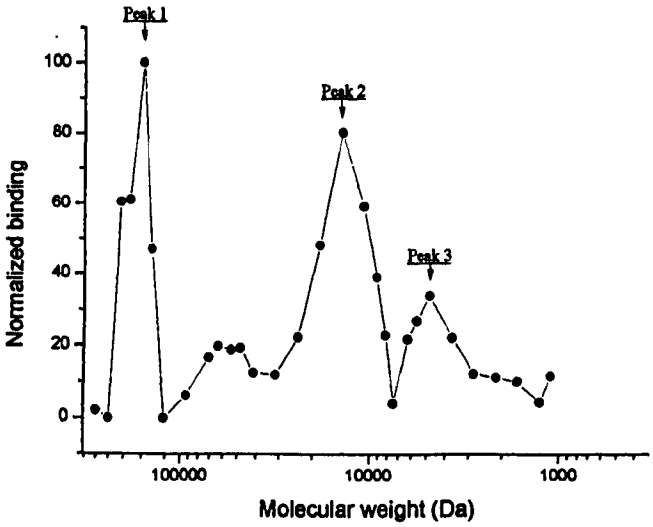

Figure 2. Size-exclusion chromatography indicated the presence of three LH/CG-binding sites within *X. maltophilia*. Data from one representative preparation illustrates the three peaks of CG binding corresponding to molecular weights of 163 kDa (peak 1), 14 kDa (peak 2), and 4.5 kDa (peak 3). Normalized binding at 100% represents a total specific ^{125}I-hCG binding of approximately 10% to 20% (150–240 cpm), after subtraction of nonspecific binding (40–60 cpm). Molecular weights were determined using linear regression of a calibration curve.

Photoaffinity Labeling of a Small-Molecular-Weight LH/CG-Binding Site to HCG. In another effort to independently confirm molecular weights estimated from gel filtration and ultracentrifugation studies, we attempted to photoaffinity label the bacterial LH/CG-binding sites. If photoaffinity labeling is successful, we would expect that some of the NHS-ASA/^{125}I-hCG complex would be linked to the binding site and gel filtration analysis would indicate two peaks, with one peak corresponding to the molecular weight of ^{125}I-hCG or NHS-ASA/^{125}I-hCG (NHS-ASA = 276 Da) and the other to NHS-ASA/^{125}I-hCG/binding site. The difference between the two peaks would indicate the size of the LH/CG-binding site. However, the result demonstrated only a broadening of the curve, with a shift to the right rather than two distinct peaks (Fig. 3). By subtracting molecular weights of nonspecific photoaffinity labeling from total photoaffinity labeling products (at approximately 75% of their total cpm), we estimated a binding site corresponding to a molecular weight of 10,000 to 13,000 Da. This difference in molecular weight is too small for the column to resolve into two distinct peaks. This weight is also similar to estimates of an 11.5-kDa binding site from gel filtration experiments and might indicate that NHS-ASA/^{125}I-hCG is being cross-linked to it. However, the larger site did not appear to have been cross-linked. Photoaffinity labeling of LH/CG-binding sites with whole bacteria yielded a similar shift in the curve corresponding to a molecular weight of 13,000 to 17,000 Da (n = 3; data not shown).

Affinities of High- and Low-Molecular-Weight LH/CG-Binding Sites. For comparison with previously published data, we used dose-response assays to determine dissociation constants of the LH/CG-binding sites identified in this study. This was done by assaying binding sites present in supernatant no. 2, supernatant no. 3, and pellet no. 1 (Fig. 4). Ultracentrifugation fractions rather than gel filtration fractions were used to determine affinities because they yielded volumes sufficient for binding assay studies. The raw data are very similar to that noted previously from competition dose-response studies using intact bacteria (9, 10). Binding affinity of the two smaller binding sites identified by gel filtration assays could not be resolved because both sites are found in supernatant no. 3. Dose-response assays of this supernatant demonstrated that hCG displaced ^{125}I-hCG over a range of 0.04 to 0.47 μg, and raw data indicate an estimated Kd of 9.4×10^{-8} *M*. The Woolf plot (32) was linear, indicating that there was only one binding site in the supernatant or that both small binding sites have similar binding affinities (data not shown). In pellet no. 1, hCG displaced ^{125}I-hCG over a range of 2 to 47 μg with a Kd of 12×10^{-6} *M*. Its Woolf plot was also linear. Supernatant no. 2 contained all binding sites and hCG displaced ^{125}I-hCG over a range of 0.67 to 5.4 μg. Its Woolf plot was curved, indicating the presence of two or more binding sites (data not shown).

CG Specificity of Binding Sites. Competition binding assays using LH indicated that the small binding site(s) are CG specific. Although in dose-response assays of supernatant no. 3 (containing the small binding site), ^{125}I-hCG could be displaced by unlabeled hCG, serial dilutions of human LH, LH α subunit, or LH β subunit did not displace ^{125}I-hCG (Fig. 5). The largest binding site was LH/CG specific as indicated by the ability of 10 μg of LH to displace approximately one-half of the ^{125}I-hCG from pellet no. 1 using a standard assay (data not shown). Dose-response assays of this pellet were not be run because of the large amount of LH required.

Discussion

The mammalian LH/CG receptor has been well characterized, but many properties of its bacterial counterpart are unknown. Herein, we report the first molecular weight estimates for the LH/CG low- and high-affinity binding sites in *X. maltophilia* as 170,500 and 11,500 Da, respectively, the latter being CG specific.

The molecular weight of mammalian LH/CG receptors isolated from various tissues ranged from 86 to 93 kDa (7, 33–35). Ascoli and Segaloff (36) report that the mammalian LH/CG receptor can also form a 180,000-Da dimeric complex. As far as we know, the molecular weight of an LH/CG-binding site has only been reported for one other nonmammalian eukaryote, yeast (*C. albicans*), where two sites of 65,000 and 11,000 Da were found (15). The latter site was thought to be a fragment of the larger produced

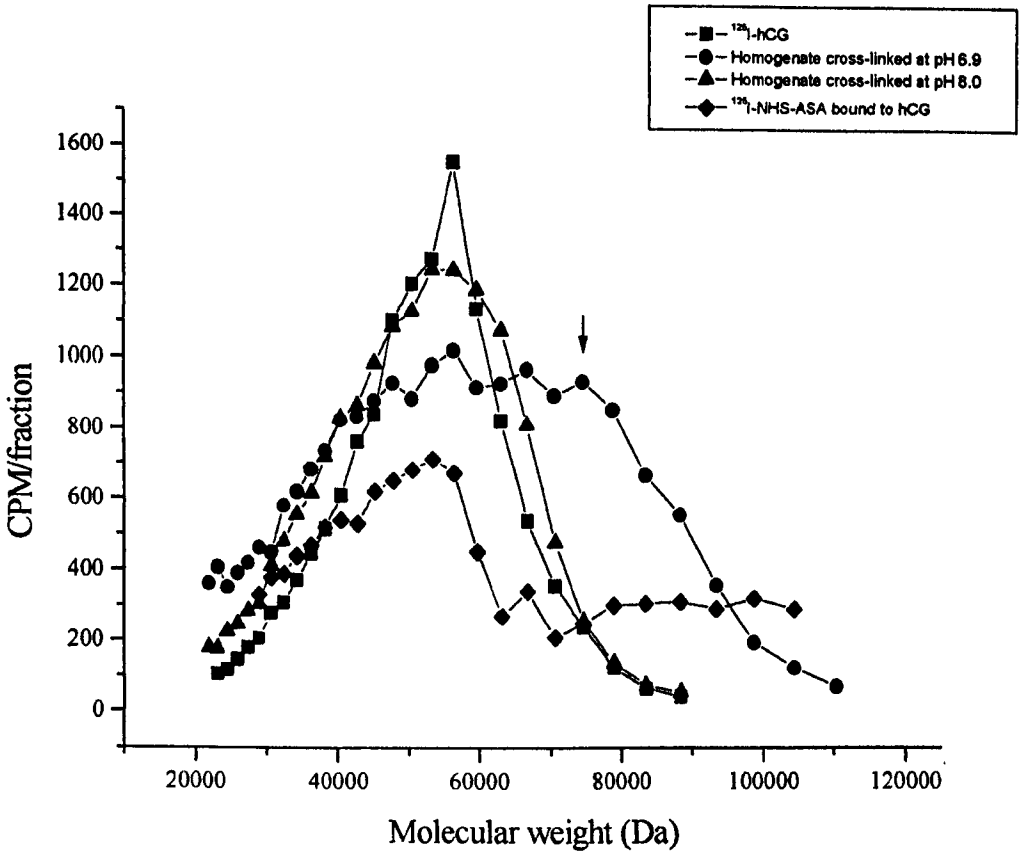

Figure 3. Photoaffinity labeling confirmed the presence of a small-molecular-weight LH/CG-binding site by cross-linking ^{125}I-hCG to a 10,000- to 13,000-Da protein using NHS-ASA (n = 4). Labeled bacteria homogenates and controls were separated by size using gel filtration. Nonspecific photoaffinity labeling (incubated at pH 8.0) and controls: ^{125}I-hCG and ^{125}I-NHS-ASA/hCG all gave a single peak in the area of 55,000 Da corresponding to the weight of hCG. However, total photoaffinity labeling (pH 6.95) led to a broadening and a shift of the curve to the right. The shift right in this individual experiment (see arrow) corresponded to a molecular weight of approximately 13,400 Da. The control ^{125}I-NHS-ASA/hCG was performed to make sure the shift right was not due to NHS-ASA forming large undesired complexes (^{125}I-NHS-ASA covalantly bound approximately 35%–40% of the hCG).

during purification. However, if the 11,000-Da site was not a fragment, it is very similar in size to the 11,500-Da site that we identified in bacteria. Similarities in *X. Maltophilia* and yeast binding sites would not be completely unexpected because xCG, isolated from *X. Maltophilia*, which does not bind mammalian LH/CG receptors, can bind to yeast LH/CG binding sites, causing developmental changes (14). Because the 11,500-Da site in our studies was CG specific and had a high affinity, it is not likely a fragment from the 170,500-Da binding site. It is also of interest to note that the partial 342-base pair sequence cloned from *X. maltophilia* (21) correlates to just over 11,000 Daltons, similar in size to our reported CG-specific site and, therefore, may be a complete sequence for this receptor.

The hCG-binding affinities estimated in our study for the large-molecular-weight LH/CG-binding site of *X. maltophilia* was about 125-fold lower than for the small-molecular-weight site. Previously reported Kds were lower than we determined currently (9, 10), however, the amount of hCG required to displace binding in our study was very similar to these reports for both the high- and low-affinity binding sites using competition assays. The difference in reported Kds is possibly due to binding site damage that can occur during solubilization (37), the manner in which Kds were determined, or the fact that Richert and Ryan determined affinity using a curve plotted bound ^{125}I-hCG as a function of its concentration, rather than from competition assays (9) as we did. However, our results are consistent with binding sites of two different affinities with the higher affinity site being CG specific and thus we report that the 170.5-kDa site is the LH/CG site and the 11.5-kDa site is the CG-specific site. Previous work on this high-affinity site demonstrated that neither glycosylated follicle-stimulating hormone, thyrotropin-stimulating hormone, or leutinizing hormone displaced CG-specific binding nor the hCGα and hCGβ subunits applied independently, indicating that the site binds the whole molecule (10) of which the hCGβ subunit carboxyl terminal specifically binds it (12).

The smallest binding site (5.4 kDa), not reported previously, may represent one of several possibilities. First, it could be a member of one of many small protein gene families reported in bacteria (38) and could represent a site independent of the 11.5-kDa site, although having similar binding properties to it. Second, the 5.4-kDa site could be a

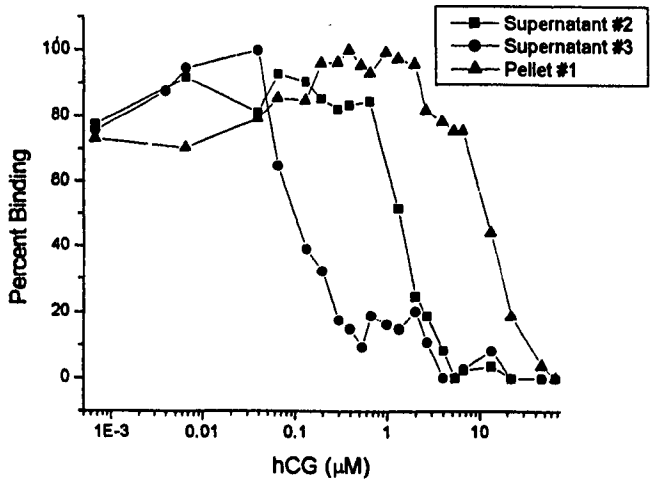

Figure 4. Affinities of LH/CG-binding sites were assessed from dose-response binding assays of ultracentrifuged bacterial homogenates. Illustrated is a graph from a representative experiment of ^{125}I-hCG binding with supernatants no. 2 and no. 3, and pellet no. 1. The three fractions were assayed with increasing concentrations of hCG (0.001–67 μg). Supernatant no. 3, containing the low-molecular-weight binding site(s), had the highest binding affinity. Pellet no. 1, containing the high-molecular-weight binding site, had the lowest binding affinity. Supernatant no. 2, containing all binding sites, had an intermediate binding affinity. The rise in binding from 1 to 4 μg in supernatant no. 3 could reflect a small amount of the larger binding site that was not pelleted. Average affinities for each fraction were $9.4 \pm 0.4 \times 10^{-8}$ M (±SD; $n = 4$) for supernatant no. 3, $1.4 \pm 1.2 \times 10^{-6}$ M (±SD; $n = 3$) for supernatant no. 2, and $12 \pm 0.8 \times 10^{-6}$ M (±SD; $n = 4$) for pellet no. 1. Normalized binding at 100% represents a total specific ^{125}I-hCG binding of approximately 10% to 15% (150–200 cpm) for supernatants no. 2 and no. 3, and 15% to 30% (200–400 cpm) for pellet no. 1, after subtraction of nonspecific binding (50–70 cpm).

subunit of the 11.5-kDa site with, perhaps, two 5.4-kDa subunits forming a dimer. Third, this site could be a fragment of the 11.5-kDa binding site, such as mediated by protease activity.

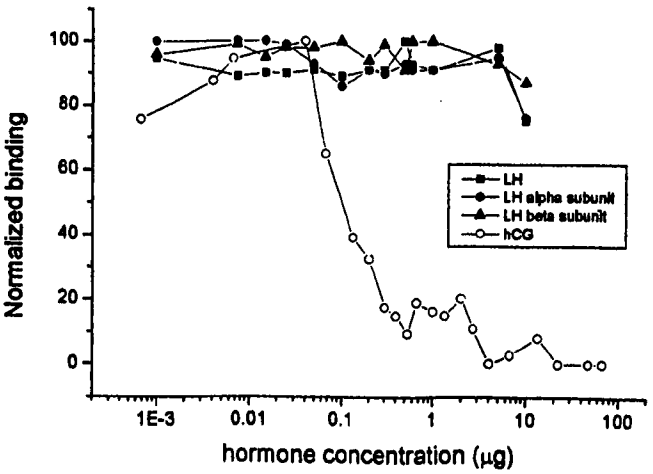

Figure 5. The CG specificity of the small binding site(s) was determined by the ability of hCG but not human LH to displace ^{125}I-hCG in dose-response binding assays ($n = 3$). A representative trace when assaying binding sites of supernatant no. 3 demonstrates that the displacement of ^{125}I-hCG occurs using hCG dilutions of 0.054 to 0.47 μg, but did not occur with similar concentrations of LH, LH α subunit, or LH β subunit. Total and nonspecific binding are similar to that mentioned in Figure 4 for supernatant no. 3.

Although it should be noted that protease inhibitors were not included in these studies, our laboratory previously demonstrated no effect of protease activity on hCG binding to *X. maltophilia* extracts solubilized with zwittergent detergent or on hCG itself (12). In addition, although the smallest binding site (5.4 kDa) could be an artifact of protease activity, this is less likely for the 11.5-kDa site because it is CG specific and has a higher affinity than the 170-kDa site. However, we cannot exclude the possibility of protease activity altering our estimated molecular weights.

Using photoaffinity labeling, we identified the presence of a small-molecular-weight binding site (approximately 10,000–13,000 Da) that presumably correlates with the 11.5-kDa site identified by gel filtration. Photoaffinity labeling did not detect the large site, possibly due to stereochemical restrictions that prevented the cross-linker from approaching the binding site (28). A similar sized protein was photoaffinity labeled with intact cells, suggesting that the binding site is membrane bound and extracellular as proposed previously (9). To our knowledge, we are the first to successfully photoaffinity label a native membrane glycoprotein hormone-binding site in bacteria. This was also the first demonstration of a solubilization technique that maintains bioactivity of bacterial glycoprotein-binding sites. This was done using the detergent Z 3,14, which has been effective for extracting membrane proteins from gram-negative bacteria previously (39), although many other detergent and nondetergent-based techniques have been tested with varying degrees of success for membrane-bound proteins (40–42).

The probability that these LH/CG-binding sites in bacteria are an ancestral gene of the mammalian LH/CG receptor or a result of convergent evolution has been considered in several papers (15, 19, 20, 43). The conservation of ancestral genes from bacteria to mammals is not novel and has been reported for several other proteins. These proteins include a cAMP responsive promoter element (44), heat shock proteins or chaperones (45), and a prokaryotic glutamate receptor (46). However, to positively determine the relationship between the bacterial LH/CG-binding sites and the mammalian LH/CG receptor, a protein and complete DNA sequence will be needed. Recently, two Xanthomonas genomes were completely sequenced, which should assist in identifying and cloning the binding sites we have characterized (47).

The presence of LH/CG-binding sites in bacteria and yeast has been well documented. Their potential role in growth stimulation also has been described, as previously noted (11, 14, 16). Their involvement in growth could be especially useful to the medical field where inhibiting yeast transformation or reducing bacterial cell proliferation might reduce infection. In yeast, this is particularly true because hCG secreted by the placenta is postulated to increase yeast transformation to its pathogenic form (13). Also, the ability to retard growth of *X. maltophilia* by developing selective antagonists to the LH/CG-binding sites could be an effec-

tive alternative strategy to fighting this antibiotic-resistant bacterium, which is increasingly found in hospitalized patients (17). The 11.5-kDa CG-specific site should be targeted because the autocrine/paracrine functions mediated by hCG binding in *X. maltophilia* likely are through the CG-specific site because hCG, and not LH, affected cell proliferation (10). It is also interesting to note that conversion of gonococci to a more invasive phenotype in humans is mediated by a membrane-bound ribosomal 13-kDa protein with immunological similarities to hCG after it is bound by lutropin receptors in reproductive cells (48, 49).

In conclusion, our findings support previous evidence for the presence of two binding sites in *X. maltophilia* and the CG specificity of one of these sites. These binding sites are likely to be distinct because of the ability to photoaffinity label the 11.5-kDa site, but not the 170-kDa site, the 125-fold difference in binding site affinities, and evidence of the 11.5-kDa site being CG specific. This study is the first to report an estimated molecular weight for these binding sites and describes a novel solubilization technique to maintain the integrity of glycoprotein-binding sites. Our further characterization of these binding sites should assist in future work to isolate and purify sufficient quantities of protein needed for sequence analysis of both the LH/CG- and CG-specific sites.

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