Detection of Conformational Changes in Human Chorionic Gonadotropin upon Binding to Rat Gonadal Receptors*

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After binding to rat testicular or ovarian luteinizing hormone (LH) receptors, human chorionic gonadotropin (hCG) and mammalian LH can be detected with monoclonal antibodies directed against a conserved epitope on the β subunit of the hormones. Two such anti-hCG/anti-LH monoclonal antibodies, known as B105 and B110, compete with one another for binding to this epitope region on free and receptor-bound hormone. By comparing the affinities of B105 and B110 for these two forms of hCG, we have detected apparent changes in the structure of the hormone which develop subsequent to receptor binding. Whereas the affinity of B105 for receptor-bound hCG is approximately 10-fold lower than that for free hCG, the affinity of B110 for receptor-bound hCG is nearly 20-fold greater than that for free hCG. Both B105/hCG and B110-hCG complexes bind to the receptor; however, they have approximately 25 and 50% lower affinity than hCG. Thus, although B110 binds better to the form of hCG which is bound to receptors, binding of B110 to hCG does not appear to induce a conformational change in the hormone which facilitates hormone-receptor binding. Consequently, both B105 and B110 partially inhibit binding of hCG to its receptors. Fab fragments of B105 and B110 are as effective as intact B105 and B110 in inhibiting the binding of labeled B105 and B110 to hCG-receptor complexes, suggesting that circular complexes which might be formed by the interaction of divalent antibody, two molecules of hCG, and two membrane-bound receptors or one divalent receptor are not contributing to the affinity of the antibodies for receptor-bound hCG. Alternatively, formation of circular complexes can explain an increase in apparent affinity of B105 for ovine or bovine LH-receptor complexes. Data obtained with B105 suggest either that the structure of the epitope is altered following binding or that a portion of the epitope is partially obscured when hCG binds to the receptor. In contrast, the data obtained using B110 are not explained by models in which steric factors reduce the affinity of the antibody for the hormone-receptor complex. Therefore, as a minimal explanation for these observations, we postulate that the conformation of the B105/B110 epitope region is altered following binding of the hormone to receptors. The nature of the conformational change and its relationship to LH/hCG action is unknown.

Human chorionic gonadotropin (hCG) is a glycoprotein hormone which has been used to model the interaction of glycoprotein hormones and their receptors (reviewed in Ref. 1). Although the binding of hCG to gonadal receptors has been studied extensively, few attempts have been made to characterize the structure of the hormone when it is bound to receptors. Most studies of the interaction of gonadotropins with receptors are based on measurements made using labeled hormone. Whereas this is a powerful approach for examining the kinetics of binding, it is not well suited to detecting reversible changes in the structure of the hormone which might occur as a result of receptor binding. The structure of hCG is not modified irreversibly as a consequence of binding to receptors. Labeled hCG can be recovered from the receptor, and the eluted hormone appears to rebind to receptors at least as well as fresh hCG (2). Other methods are needed to detect more subtle changes, if any, in the structure of the bound hormone. One method which we shall describe here employs monoclonal antibodies which can recognize hCG-receptor complexes.

Indirect evidence supports the concept that the structures of hCG and other glycoprotein hormones are not rigid in spite of the large number of disulfide bonds in each subunit (i.e. five in α and six in β). For example the kinetics of dissociation and reassociation of the subunits are complex and can be explained by models which incorporate an intermediary semistable state (3, 4). Further, the common α subunit of one glycoprotein hormone can recombine with most β subunits regardless of their mammalian origin to produce the four different classes of hormones, namely CG, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (5). Finally, many monoclonal antibodies have different affinities for intact heterodimer, and either isolated α or β subunit (6-8). These results are consistent with the idea that the subunits have the capacity to "flex" when forming dimer. Conceivably the conformation of hCG

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The abbreviations used are: hCG, human chorionic gonadotropin; LH, luteinizing hormone; FSH, follicle-stimulating hormone; o/bLH, ovine-bovine luteinizing hormone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
also changes following receptor binding.

Over the past several years we have been using monoclonal antibodies to discern the overall topography of hCG and to determine how the hormones interact with receptors found on rodent gonadal tissues. As part of these studies, we observed that some epitopes on the β subunit remain exposed when the hormone binds to the receptors (9, 10). These comprise an epitope region on hCG and LH which can be detected readily using radiolabeled monoclonal antibodies. Of the several antibodies which bind to this epitope region (i.e. B102, B103, B105, B108, and B110), two (B106 and B110) had a high affinity binding that was a high affinity binding from that of the hormone to the receptors. If true, then some monoclonal antibodies might have higher affinity for receptor-bound hCG compared to free hCG. We found several such antibodies and characterized one extensively (i.e. B110). As discussed here, based on these observations, we conclude that the conformation of hCG is altered after the hormone binds to the receptor.

EXPERIMENTAL PROCEDURES

hCG purified by methods described previously (12) was a gift from Dr. Robert Canfield (Columbia University, New York, NY). Ovine LH was donated to us by Dr. Darrell Ward (University of Texas Cancer Center, Houston, TX). Bovine LH, and a hormone with the same amino acid sequence as ovine LH (13), was given to us by Dr. John Pierce (UCLA, Los Angeles, CA). Monoclonal antibody B105 was prepared by immunizing BALB/c mice with hCG and preparing hybridomas as previously described (14). The antibody was subsequently grown in ascites and purified by protein A affinity chromatography (15) using a kit (i.e. MAPS) obtained from Bio-Rad. Preparation of B110 is described in the next paragraph. Fab fragments of B105 and B110 were prepared by papain digestion (16), and the completeness of the digestion was confirmed by examining the molecular weights of the products using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (17). The affinity of the antibodies for hCG was measured using labeled antibody since antibody binding can be written as: b

m

is the influence of circular complex formation will increase the ordinate intercept by (1

2K') is twice the binding constant. When circular complexes, RH

A-HR bond. If K' = 0, circular complexes do not form, and the affinity measured using labeled antibody should be identical to that measured using Fab fragments. If K' > 0, then the affinity measured with Fab fragments will be lower than that determined using intact antibody since antibody binding can be written as: b = RHA + RHARH, and the Scatchard plot will become: b/f = 2K[b-h] + 2K'K[b-h]. This would create a hyperbolic Scatchard plot in which circular complex formation will increase the ordinate intercept by (1 + K' KB) without changing the abscissa intercept. Given the measurement errors caused by dissociation of hormone-receptor complex, nonspecific binding, etc., the observed Scatchard plot might appear to be a straight line and, depending on the value of K', the apparent affinity may be substantially overestimated.

Assume that antibody (A) binds to preformed hormone-receptor complexes (RH) as: RH + A ↔ RHA where RHA is bound antibody (b) and A is free bivalent antibody (f). We define B as the total hormone-receptor complex. The binding constant (K) for forming an A-HR bond can be defined: K = [RHA]/[R][A] = b/2[b-h]. Therefore, the Scatchard plot can be derived as h/f = 2K[b-h] + 2K'K[b-h]. This would create a hyperbolic Scatchard plot in which circular complex formation will increase the ordinate intercept by (1 + K' KB) without changing the abscissa intercept. Given the measurement errors caused by dissociation of hormone-receptor complex, nonspecific binding, etc., the observed Scatchard plot might appear to be a straight line and, depending on the value of K', the apparent affinity may be substantially overestimated.

Free hCG while its apparent affinity for receptor-bound hCG was confirmed by examining the molecular weights of the products using sodium dodecyl sulfate-polyacrylamide gel electrophoresis was published by our laboratory (17). The affinity of the antibodies for hCG was measured using labeled antibody since antibody binding can be written as: b

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A-HR bond. If K' = 0, circular complexes do not form, and the affinity measured using labeled antibody should be identical to that measured using Fab fragments. If K' > 0, then the affinity measured with Fab fragments will be lower than that determined using intact antibody since antibody binding can be written as: b = RHA + RHARH, and the Scatchard plot will become: b/f = 2K[b-h] + 2K'K[b-h]. This would create a hyperbolic Scatchard plot in which circular complex formation will increase the ordinate intercept by (1 + K' KB) without changing the abscissa intercept. Given the measurement errors caused by dissociation of hormone-receptor complex, nonspecific binding, etc., the observed Scatchard plot might appear to be a straight line and, depending on the value of K', the apparent affinity may be substantially overestimated.
RESULTS

As we have reported previously (10), B105 bound to hormone-receptor complexes which had been formed when either ovarian or testicular membranes were incubated with hCG. Antibodies which inhibited B105 binding to hCG-receptor complexes would themselves be expected to bind to receptor complexes. The preliminary screening of the culture supernatants from one fusion (Fig. 1) illustrate the variability in the types of antibodies which were found following immunization and preparation of hybridoma lines. Some of the antibodies had a high affinity for free hCG and low ability to compete with B105 for hCG-receptor complex and vice versa. A large percentage of the hybridomas secreted antibodies which competed with B105 for free hCG. Many, but not all, also bound o/bLH.

In view of the procedures used to select antibody B110, we anticipated that B110 would map to a similar location as B105. B110 was able to bind to hCG at the same time as antibodies to the B101, B107, and B109 epitopes on the β subunit or antibodies to the A102, A104, A105, and A109 epitopes on the α subunit. B110 did not bind to hCG simultaneously with antibodies to β subunit epitopes such as B102, B103, B105, and B106. These latter antibodies also bind to the same epitope region on hormone-receptor complexes (1). The affinities of B105 and B110 for hCG and hCG-receptor complexes differ considerably (Table I). Thus, B110 has a much lower affinity than B105 for free hCG, however, it has higher affinity than B105 for receptor-bound hCG. The apparent affinity of B105 for o/bLH receptor complexes is greater than that for the free hormone.

### Table I

| Hormone                  | B105 Ratio | B110 Ratio |
|--------------------------|------------|------------|
| Free hCG                 | (3–10) x 10^6 | 3 x 10^6  |
| hCG-receptor complex     | 4 x 10^6   | <0.13      |
| Free o/bLH               | 8 x 10^6   | 20         |
| o/bLH-receptor complex   | 4 x 10^6   | >8         |

Note all values were obtained with ovarian receptors except as noted and are expressed as M^-1 rounded to one significant figure. In the case of B105 for free hCG, the affinity was high and fell within a wide range as noted. The column ratio represents the ratio of affinities for receptor-bound hormone versus free hormone.

**FIG. 2. Inhibition of labeled hCG binding to ovarian luteal receptors by hCG, B105, and B110.** Radiolabeled hCG (2 ng) was preincubated with hCG, B105, or B110 for 1 h at 37°C. Washed ovarian luteal homogenates equal to 5 mg of luteal tissues were added and the incubation continued for an additional hour. The suspensions were then diluted to 4 ml with ice-cold 0.9% NaCl solution containing 1 mg of bovine serum albumin/ml and the membranes sedimented at 1,500 X g for 15 min. Results illustrated refer to the amount of radiolabeled bound to the pellet above the blank. The blank values were determined by incubating the radiolabeled hCG in the presence of 1 μg of unlabeled hCG and were 1,188 cpm. At 100%, 24,070 cpm hCG were bound. Similar results were observed with testes homogenates (not shown). In addition, the ability of B105-Fab to inhibit labeled hCG binding was indistinguishable from that of B106 (not shown). B106-Fab was not tested in this type of experiment.

Based on our previous observations that antibodies B102 and B103 were poor inhibitors of hCG binding and steroidogenesis (1) and the locations of the B105 and B110 epitopes, we anticipated that B105 and B110 would not inhibit or, at most, poorly inhibit hCG binding and stimulation of steroidogenesis. Unexpectedly, both antibodies partially inhibited the binding of hCG to gonadal receptors (Fig. 2). Unlike inhibition caused by antibodies to the B101 epitope region, inhibition in
the presence of excess B105 or B110 was never as great as that caused by an excess of unlabeled hCG. B105 did not potentiate the dissociation of hCG which had been bound (not shown), and the degree of inhibition described in Fig. 2 was observed only if the antibody and the hormone were mixed together prior to measuring receptor binding. Similarly, B105 was only partially able to suppress hCG-induced steroidogenesis (Fig. 3A). Inhibition of steroidogenesis could not be ascribed to an effect of B105 on the efficacy of bound hCG per se since the antibody was unable to block testosterone synthesis once hCG had been permitted to bind to the receptors (Fig. 3B). Similar studies were not performed using B110 since this antibody inhibited hCG binding less than B105.

The binding of labeled hCG to receptors (i.e. Fig. 2) at high antibody concentration could be explained if either B105-hCG and B110-hCG complexes were able to bind to gonadal receptors or if the antibody-hCG complexes dissociated and the resulting free hCG bound to the receptors. To distinguish these possibilities, we incubated an excess of radiolabeled B105 or B110 with limiting amounts of unlabeled hCG and subsequently monitored the binding of the radiolabeled B105 or B110 to receptors in the presence or absence of excess unlabeled B105 or B110. When unlabeled antibody was added to the mixture of labeled antibody and hCG, it failed to block binding of the labeled antibody to the membranes (Table II). In contrast, when unlabeled antibody was added to hCG before radiolabeled antibody, it inhibited binding of the labeled antibody to the receptor-containing membranes. These observations are consistent with the premise that antibody-hCG complexes bind to the receptor and that dissociation of the antibody-hCG complex was not necessary to observe hCG binding. Binding of labeled antibody-hCG complex was also inhibited by a massive excess of unlabeled hCG (not shown).

To confirm the observation that intact B105-hCG complexes could bind to receptors, we measured the rate of exchange of B105 for labeled-B105 in labeled B105-hCG complexes when both were in solution, when hCG was bound to membrane receptors, and when hCG was adsorbed to a plastic surface.

All such studies revealed that the exchange was limited by the dissociation of labeled B105 from hCG and that at least 29 min at 37 °C were required for half of the complex to dissociate. Indeed, the rate is probably much lower. In four of four studies in which hCG was adsorbed to a plastic surface, the half-life averaged nearly 100 min (minimum observed value, 86 min). In one study in which hCG was bound to membrane receptors, the half-life was 130 min. In two studies in which both labeled B105 and hCG were in solution, the half-life values were estimated to be 85 and 29 min. These data suggested that B105-hCG complex could bind to receptors and that a requirement for dissociation prior to binding would be highly unlikely.

Since B105-hCG and B110-hCG complexes appeared to bind to the receptor without first dissociating, we compared the affinity of the complexes and hCG for the receptor (Fig. 4). In this study, we added a massive antibody excess (i.e. at least 100-fold at the highest concentration of labeled hCG) to different concentrations of radiolabeled hCG and monitored the binding of hCG to ovarian luteal membranes. Based on the slopes of the lines, we calculated that B110-hCG and B105-hCG complexes had one-half and one-quarter the affinity of hCG. No attempt was made to use concentrations of antibody-hCG complex which would saturate the receptors since these high concentrations the antibody excess would be reduced, and the effects of antibody-hCG dissociation would be magnified. Even under these nonsaturating conditions, the relative estimates of the binding constant are clearly maximal values since they include the small amount of labeled hCG binding which occurs due to dissociation of the antibody-hCG complex. We routinely found that B105-hCG complexes had 20–25% of the affinity as hCG (cf. maximal inhibition in Fig. 2, inhibition in Fig. 4, and displacement of the dose response curve in Fig. 3A) and that B110-hCG complexes had 50% of the affinity as hCG (cf. maximal inhibition in Fig. 2 and inhibition in Fig. 4).

Previous studies have shown that the affinity for an antigen for an antibody can be greatly enhanced if an antigen-antibody circular complex can form (21, 22). This type of complex would occur if the receptor were bivalent or if the antibody promoted cross-linking of surface-bound antigens. Although the type of complex would not have been expected in the case of B105 since the affinity of B105 for hCG-receptor complexes is lower than that for free hCG, it could account for the increased affinity of B110 for hCG-receptor complexes relative to free hCG. To detect circular complexes or cross-links between receptors, hCG, and B105 or B110, we compared the influence of Fab fragments and intact B105 or B110 on the binding of radiolabeled B105 and B110. Both B105 and B110-Fab fragments as well as B110 and B110-Fab fragments had equivalent ability to inhibit the binding of labeled B105 or B110 (Fig. 5, A and B) to hCG-receptor complexes. These observations suggested that circular complexes did not form and were not responsible for the ability of B105 or B110 to bind to hCG-receptor complexes. In contrast, B105 was considerably more effective than B105-Fab in inhibiting the interaction of labeled B105 with o/LH receptor complexes (Fig. 6). This finding, coupled with the tendency of B105 to slightly potentiate binding of labeled o/LH (not shown) is consistent with the notion that the enhanced ability of B105 to bind to o/LH-receptor complexes is due to formation of circular complexes. Thus, hCG- and o/LH-receptor complexes are recognized differently by B105.

**DISCUSSION**

To our knowledge, B110 is the first antibody which has a higher affinity for glycoprotein hormone-receptor complexes...
Influence of antibodies on the ability of labeled antibody-hCG complex (Ab-hCG) to bind to ovarian LH receptors

Values are the amount (uncorrected cpm) of labeled-antibody ± S.E. (triplicates) bound to ovarian homogenates. Labeled-B105-hCG or B110-hCG complexes were prepared by incubating radiolabeled B105 or B110 (≈2 ng, 150,000 cpm) with hCG (0.2 or 1 ng) for 1 h at 37 °C. Since, based on the relative molecular weights of antibodies and hCG, 1 ng of antibody is expected to bind 0.5 ng of hCG, we anticipated these amounts of hCG would provide approximately 20 and 100% saturation of either radiolabeled antibody. Labeled antibody-hCG complexes were added to membranes which had been incubated with 0 or 1 μg of unlabeled B105 or B110 and incubation was continued at 37 °C for 30 min. Binding was terminated by diluting the incubation medium 40-fold (i.e., 4 ml) with ice-cold 0.9% NaCl solution containing 1 mg of bovine serum albumin/ml, and the membranes were collected by sedimentation at 1,500 × g. In some cases unlabeled B105 and B110 were added to hCG prior to addition of labeled antibody (i.e., denoted Ab-Ab-hCG; row 3). This served as a control and illustrated the amount of labeled antibody which would be bound if the labeled antibody were to dissociate from hCG completely prior to binding to receptor or if the labeled antibody in the labeled antibody-hCG-receptor complex were to exchange with unlabeled antibody. Values in rows 1 and 2 were found to be greater than those in the corresponding positions of row 3 (p < 0.02) using a t test.

Table II

| Addition | Inhibitor | Antibody B105 | | | Antibody B110 | | |
|----------|-----------|---------------|-----------------------------|-----------------------------|-----------------------------|
|          |           | 0.2 ng hCG | 1 ng hCG | | 0.2 ng hCG | 1 ng hCG | |
| Ab-hCG   | 0         | 15,013 ± 702 | 20,837 ± 957 | | 6,871 ± 109 | 10,192 ± 266 |
| Ab-hCG   | Ab        | 14,310 ± 779 | 21,202 ± 841 | | 6,153 ± 164 | 7,164 ± 181 |
| Ab-Ab-hCG| 0         | 10,618 ± 250 | 11,724 ± 600 | | 4,822 ± 172 | 4,658 ± 193 |

Fig. 4. Binding of radiolabeled hCG, B105-radiolabeled hCG complex, and B110-radiolabeled hCG complex to ovarian membranes. Buffer (10 μl), 0.1 μg B105 (10 μl), or 1 μg B110 (10 μl) were added to varying amounts of radiolabeled hCG (10 μl) for 1 h at 37 °C. These amounts of B105 and B110 are expected to reduce the free hCG concentrations by more than 300- and 30-fold, respectively. Ovarian homogenate (2.5 mg of luteal tissue equivalents/100 μl) was added and binding measured at the end of an additional hour of incubation. The maximal concentration of radiolabeled hCG used (approximately 4 ng/120 μl) is not sufficient to saturate the receptors under these conditions and Scatchard plots (18) of the binding were not prepared. Since the concentration of receptors in each case is identical, the relative binding constants can be determined from the ratios of the slopes of the lines at low hCG concentrations.

than for the free hormone. This antibody was produced in response to hCG-o/bLH immunization and does not bind to the receptor in the absence of hormone. Had we immunized with hormone-receptor complex, then we would have expected to find some antibodies which bound to the hormone-receptor complex better than free hormone because they bound to epitopes on both the hormone and the receptor. The strategy used to screen for the antibody is general and should facilitate rapid selection and cloning of hybridoma lines which produce high affinity antibodies to other ligand-receptor or ligand-acceptor systems. We cannot make similar claims for the immunization procedure and have not studied the effects of different immunization schedules. We chose the procedure described because we had preliminary data showing that B105 bound to exposed epitopes in every mammalian LH/CGR-receptor complex tested3 including LH from whales, dogs, rodents, cattle, and horses, to name a few. By immunizing mice with hCG, one of the most antigenic glycoprotein hormones, and boosting with o/bLH, we anticipated that we would facilitate development of antibodies to this site. Indeed, most hybridoma lines which secreted antibodies to hCG competed with B105 for binding.

The finding that the affinity of antibody B110 is higher for receptor-bound hCG than free hCG suggests that the conformation of at least one epitope is different in solution than when the hormone is bound to receptors. Since B110 binds to

Fig. 5. Influence of Fab fragments on antibody binding to hCG-receptor complexes. Panel A, comparison of B105 and B105-Fab fragments. hCG (25 μg/5 ml; 220 μg of tissue equivalent) was incubated with ovarian homogenate for 15 min at 37 °C and washed three times in ice-cold buffer C as described under "Experimental Procedures" to remove nonbound hCG. The tissue was resuspended in 5-ml and 100-μl aliquots incubated with labeled B105 (93,000 cpm; ~1.5 ng) and varying amounts of B105 or B105-Fab. After 1 h at 37 °C, 4 ml of buffer C was added, the mixture centrifuged at 1,500 × g, the supernatant aspirated, and the radiolabel remaining in the pellet analyzed in a gamma counter. Values illustrated represent means of triplicate incubations and vertical bars extend to the limits of the S.E. Panel B, comparison of B110 and B110-Fab fragments. hCG (0.5 μg/5 ml; 220 μg of tissue equivalent) was incubated with ovarian homogenate for 1 h at 37 °C. The remainder of the procedure was as in panel A except that 157,000 cpm (~3 ng) B110 was used in place of radiolabeled B105.

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that antisera can facilitate the binding of FSH to its receptors. Fab fragments of the antisera do not have this same effect and, in contrast, are much more inhibitory to FSH binding. The reason for the differences in hCG and o/bLH to form circular complexes is unknown and may be related to the conformational change of the hormone to an altered conformation. One explanation for the increased apparent affinity of B105 for bound relative to free o/bLH is formation of circular complexes with receptor-bound o/bLH. Although we cannot exclude the possibility that a conformational change is also involved, the finding that Fab fragments of B105 have much lower ability to inhibit binding of radiolabeled B105 to o/bLH-receptor complexes provides strong support for the circular complex explanation.

The reason for the differences in hCG and o/bLH to form circular complexes are unknown and may be related to the greater mobility of LH-receptor complexes (23) or to the very slowly reversible (26). The difference between o/bLH and hCG appeared to be the activation energy required for the apparently irreversible step. In view of the studies presented here, these earlier results might be explained by a conformational change in the hormone. Thus, binding of either form of the hormone would occur. However, stabilization of the bound form would require a conformational change. In the case of hCG, this would appear to have a low activation energy requirement whereas for o/bLH this would appear to require considerable energy. Since both the loosely bound and tightly bound forms can stimulate steroidogenesis (1), change in the structure of the hormone may not be essential for biological activity. Buettner and Ascoli (27) have shown that alterations in the sodium content of the medium can facilitate the binding of o/bLH to the receptors. Perhaps this is due to an effect of these ions on the conformation of the hormone. The notion that gonadotropins may be able to flex in solution is supported by observations on the structures of the hormones during subunit association and dissociation studies. Thus, whereas the subunits can bind one another rapidly, acquisition of the circular dichroism spectrum of the native molecule requires longer incubations (3, 4).

Studies of hCG binding in other laboratories indicate that binding is complex (28, 29). Although the Scatchard plot appears to be linear within the limits of precision which can be obtained, the dissociation of hCG from the receptors is at least biphasic. The model we have provided in this paper is consistent with these observations. Thus, one can show (1) that measurements of bound hormone will produce a linear Scatchard plot given a sequential model of binding (i.e. formation of hormone-receptor complex followed by conversion of the hormone to an altered conformation).

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