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EFFECT OF DISULFIDE-BOND REDUCING AGENTS ON THE SPECIFIC BINDING OF GROWTH HORMONE TO MICROSOMAL MEMBRANE PREPARATIONS FROM RABBIT LIVER

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Abstract—The effect of the disulfide-bond reducing agents, mercaptoethanol (ME) and dithiothreitol (DTT), on the specific binding of ^{125}I-labeled human growth hormone (hGH) to microsomal membrane preparations from rabbit liver was investigated. The presence of ME or DTT caused a time- and dose-related inhibition of [^{125}I]hGH binding to both particulate and solubilized somatotrophic receptors of rabbit liver membranes. Maximum inhibition was 20–30%. Disulfide bond reduction also caused a marked increase in the extent of reversibility of [^{125}I]hGH binding. These effects were not due to effects on the GH, itself, but appeared to be directed at the receptor. Scatchard analysis showed that ME and DTT caused a change in the nature of the binding interaction, with at least partial conversion of receptors into sites with reduced affinity. These data together suggest that the partial effect of reducing agents on somatotrophic receptors of rabbit liver may reflect two types of receptors within the microsomal membrane preparations—one (~30% of total receptors) involving disulfide bonding and the remaining type (70%) being independent of disulfide bonds—or, alternatively, that the state of reduction affects a single class of receptors in a rather more complex manner. These studies provide further intriguing insights into the overall mechanism(s) involved in the interaction of GH with its target cell receptors.

For several polypeptide hormone receptors, including those for insulin [1–3], insulin-like growth factors [4, 5] and lactogenic hormones [6–8], recent data have indicated a clear role for disulfide bonds in both the structural organization of the receptor, as well as the actual hormone–receptor interaction. For growth hormone (GH), however, the situation is not so clear. The GH receptors of rat liver [9, 10] and rat adipocytes [11] have been shown to involve disulfide bonds, whereas some disagreement exists for the GH receptor of rabbit liver. Covalent cross-linking studies on detergent-solubilized [12, 13] and aqueous-solubilized rabbit liver GH receptors [14] each have indicated that the receptor is made up of oligomers and/or dimers of a primary GH binding subunit of mol. wt 53,000–57,000 with no apparent inter-subunit or intra-chain disulfide bonds. More recent studies on aqueous-solubilized receptor in this laboratory, however, indicated that intra-chain disulfide bonds may be present within the primary GH binding subunit (S.I. Ymer and A. C. Herington, unpublished observation). Similarly, Haeuptle *et al.* [7] reported the involvement of disulfide bonds in a GH receptor structure involving a subunit(s) of mol. wt 69,000, and Ymer *et al.* [15] demonstrated that a specific cytosolic GH binding protein from rabbit liver [16] has intra-chain disulfide bonds within the binding subunit.

In an attempt to clarify these apparent discrepancies, we undertook direct studies on the effects of disulfide-bond reducing agents (mercaptoethanol and dithiothreitol) on the specific binding of ^{125}I-labeled human and bovine GH to both particulate and soluble rabbit liver membrane preparations.

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MATERIALS AND METHODS

Reagents. Mercaptoethanol (ME) was obtained from Koch-Light Laboratories, Colnbrook, England, and dithiothreitol (DTT) and *N*-ethylmaleimide (NEM) were from Calbiochem-Behring, La Jolla, CA, U.S.A. Human GH for iodination (NIH-GH-H52160E) and bovine GH (NIH-GH-B-18) for unlabeled standards were provided by the National Hormone and Pituitary Program of the National Institutes of Arthritis, Diabetes and Digestive and Kidney Disease (Bethesda, MD, U.S.A.). Bovine GH for iodination was the gift of Dr. M. Sonenberg, Memorial Sloan-Kettering Cancer Center, New York, NY, U.S.A. Human GH for unlabeled standards was obtained from the Commonwealth Serum Laboratories, Melbourne, Australia. Ultrogel AcA54 chromatography gel was obtained from LKB Produkter, Bromma, Sweden.

Preparation of particulate and solubilized GH receptor. Crude microsomal membranes (100,000 *g*) were prepared from liver of pregnant female rabbits (New Zealand White) by differential centrifugation (1,500 *g*; 15,000 *g*; 100,000 *g*) [17]. These membranes were either used directly for binding studies or were extracted in 1 mM Tris buffer (pH 9.0) for 48 hr at 4° prior to centrifugation at 200,000 *g* for 90 min [18]. The resulting supernatant fraction was filtered through a 0.22 μm Millipore filter and then used

as an aqueous-solubilized receptor preparation for binding studies. The properties and binding characteristics of the aqueous-solubilized receptor preparation have been described fully previously [18].

Binding studies. Human and bovine GH were iodinated to specific activities of 60–80 μCi/μg or 30–50 μCi/μg by the chloramine T or iodogen methods as described previously [16, 19]. Prior to use for binding studies, the tracers were purified on an Ultrogel AcA54 column (1 × 55 cm) to yield the monomeric form of labeled GH. Binding studies (using 20–40 pM [^{125}I]GH) were carried out at 21° by conventional means in 25 mM Tris–HCl buffer (pH 7.4) containing 10 mM $CaCl_2$, 0.02 (w/v) sodium azide, and 0.1% (w/v) bovine serum albumin as outlined previously for particulate membrane preparations [17] and aqueous-solubilized preparations [18]. Bound and free hormone were separated by centrifugation (particulate membranes) or by polyethylene glycol (12.5%, w/w) precipitation (solubilized membranes). Specific binding was measured as the difference between binding in the absence (total) and presence (non-specific) of an excess (100 nM) of unlabeled GH.

For time course studies, the hormone–receptor interaction mixture was set up in polypropylene 20-ml scintillation vials. The addition of ME, DTT or unlabeled GH, or the removal of aliquots for determination of specific binding, was made at appropriate times as indicated for individual experiments.

Pretreatment of membranes with reducing agent was carried out at 21° with ME [1% (v/v) for 3 hr or 3% for 1 hr] or DTT (20 mM, 30 min) followed by centrifugation (2500 *g*, 20 min), aspiration of the supernatant fraction, and resuspension of the membrane pellet in 25 mM Tris–HCl assay buffer (pH 7.4). Such treatment is known to cause an increase in free SH-groups in the membrane, as measured by the dithionitrobenzene method [6]. Pretreatment of ^{125}I-labeled hGH was carried out by incubating, at 21°, a concentrated aliquot of tracer (200,000 cpm/100 μl) in the presence of 1% (v/v) ME for 3 hr. Previous studies [10] have shown that major reduction of the disulfide bonds of hGH occurs with such treatment. Following this preincubation, the mixture was diluted with assay buffer up to 7.4 ml, which effectively reduced the ME concentration in subsequent binding studies to less than 0.01%. The effect of pretreatment on membranes or tracer was assessed by immediately using these preparations together with untreated tracer or membranes, respectively, in binding studies. Binding was assessed during a 1-hr incubation period at 21° immediately after pretreatment and following 1, 2, 3 and 4 hr post-treatment. These latter time points allowed an assessment of the potential recovery or reoxidation of disulfide bonds during subsequent longer-term, post-treatment binding incubations.

RESULTS

The effect on [^{125}I]hGH specific binding (association and dissociation) to rabbit liver membranes by the addition of 1% mercaptoethanol (ME) is shown in Fig. 1. ME added after 1 hr of association had

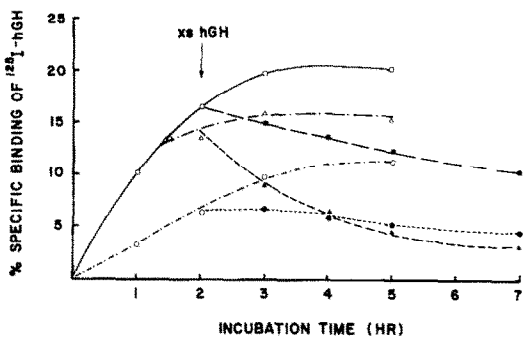

Fig. 1. Effect of mercaptoethanol (ME; 1%) on the association and dissociation of [^{125}I]hGH specific binding to particulate rabbit liver membranes. For the association curves ME was either absent (□—□) or added at the beginning of the incubation (○- - -○) or after 1 hr (△— · —△). Dissociation was measured following the addition at 2 hr of an excess (1 μg) of unlabeled hGH (■, ●, ▲ respectively) in a small volume (10 μl) to avoid any dilution effects. Similar data were obtained in two such experiments.

no effect on binding during the next 30 min but subsequently prevented any further binding of [^{125}I] hGH to the particulate membrane receptors. In ten such experiments, the magnitude of the inhibition of binding after 5 hr of exposure to ME was 31.1 ± 3.5% (mean ± S.E.). In similar experiments with 10 mM DTT, [^{125}I]hGH binding was inhibited by 29.3 ± 4.8% (N = 6). No effect of ME was seen on the level of non-specific binding. ME itself did not cause any dissociation of [^{125}I]hGH already bound to receptor but it did enhance the extent of reversibility of [^{125}I]hGH binding when initiated by the addition of an excess of unlabeled hGH at 2 hr. Five hours after addition of excess hGH, binding was reduced by 76% and almost reached non-specific binding levels, whereas in control tubes (no ME) specific binding was reduced by only 38% and was still well above the non-specific level. Similar association and dissociation data were obtained when studies were carried out using ^{125}I-labeled bovine growth hormone as ligand (data not shown).

The effect of ME on the binding of [^{125}I]hGH to aqueous-solubilized GH receptor is shown in Fig. 2. A pattern very similar to that for the particulate receptor preparation was observed for both association and dissociation. Again, addition of ME at 1 hr prevented any further binding of [^{125}I]hGH, and the extent (although not the rate) of dissociation was enhanced. Of particular note in these experiments was the restriction of binding following delayed addition of ME (i.e. at 1 hr) to exactly the same level of binding reached in the continual presence of ME (i.e. added at 0 hr). This suggested the possibility that the GH binding interaction may involve two components—one being sensitive and one being insensitive to the effect of disulfide bond reduction.

To examine this further, a more comprehensive study of the effect of the time of ME addition was undertaken in particulate membranes (Fig. 3). ME was added at 0, 1, 2, and 3 hr, and the level of specific binding was determined hourly over a 7-hr period. In two such experiments, one of which is shown in Fig. 3, no matter at which time during incubation

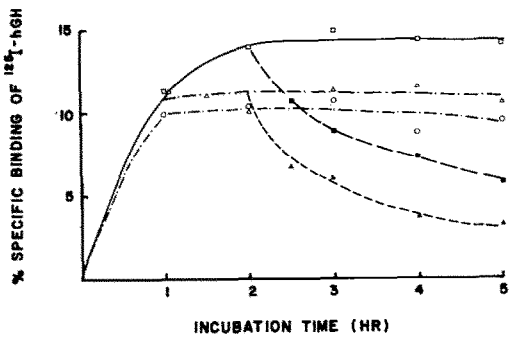

Fig. 2. Effect of ME (1%) on the association and dissociation of [^{125}I]hGH specific binding to solubilized rabbit liver membranes. For the association curves ME was either absent (□—□) or added at the beginning of the incubation (○- - -○) or after 1 hr (△— · —△). Dissociation was measured following addition at 2 hr of an excess (1 μg) of unlabeled hGH (■, ▲ respectively) in a small volume (10 μl) to avoid any dilution effects. Similar data were obtained in three such experiments.

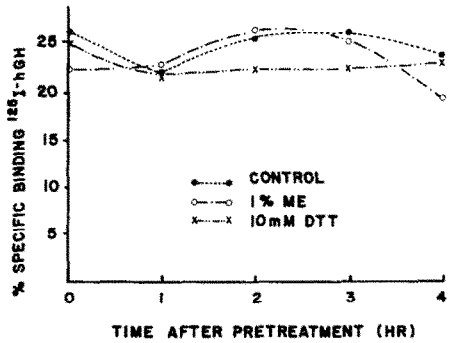

Fig. 4. Effect of pretreatment of [^{125}I]hGH with ME (1%, 3 hr) and DTT (10 mM, 1 hr) on the subsequent binding of hGH. Pretreatment was carried out as described under Materials and Methods. Pretreated [^{125}I]hGH was bound to untreated rabbit liver membranes during a 1-hr incubation at 0, 1, 2, 3 and 4 hr after ME or DTT pretreatment and removal. Similar data were obtained in four experiments.

the ME was added, the final level of [^{125}I]hGH binding was the same, being considerably but not totally reduced compared to control. In general, binding proceeded normally, even in the presence of ME, until reaching an apparently ME insensitive level, whereas if binding was already greater than this level before ME addition, e.g. at 2 and 3 hr, then ME caused a subsequent, partial dissociation. Superficially, these data would seem to support the existence of two components of the GH binding site itself or of the overall binding mechanism, which differ in their susceptibilities to the actions of disulfide bond reducing agents. However, since such delayed and uniform effects of added reducing agent were not always seen (e.g. Fig. 1), such a simple explanation for this observation is difficult to substantiate.

Since hGH contains two internal disulfide bonds [20], one possible explanation for the ME effect on binding is an effect on GH itself rather than the receptor *per se*. This was tested in two ways—by pretreating either the [^{125}I]hGH or the membrane preparation with reducing agents, washing or diluting out the ME/DTT, and then measuring specific binding to either untreated membranes or [^{125}I]hGH tracer as appropriate.

When [^{125}I]hGH was pretreated with ME (1%, 3 hr) or DTT (10 mM, 1 hr), no consistent change (inhibition) in the binding of [^{125}I]hGH to rabbit liver membranes was observed during a 1-hr incubation performed 0, 1, 2, 3 or 4 hr post-treatment (Fig. 4). This suggests that, even though under such conditions the hormone was reduced [10], the effect of ME or DTT observed on hGH binding resulted from an action at the receptor level and not at the hormone

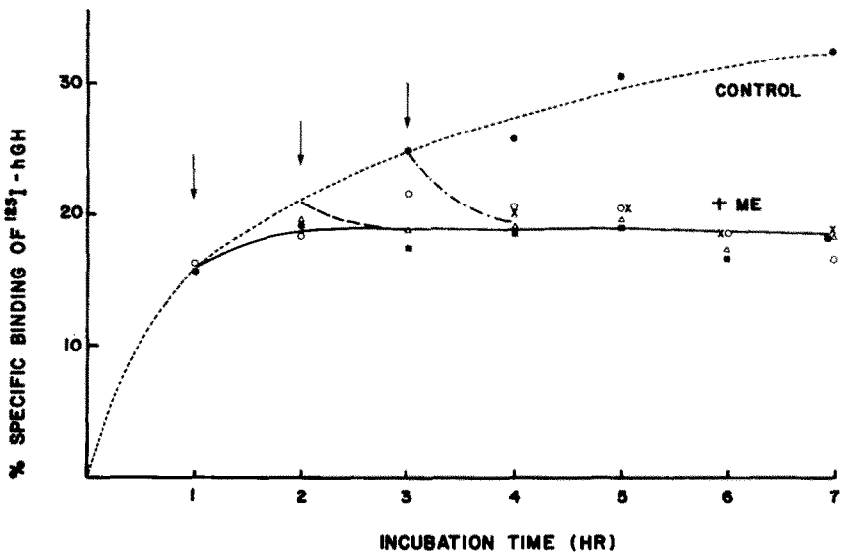

Fig. 3. Effect of the time of addition of ME (1%) on [^{125}I]hGH specific binding to particulate rabbit membranes. ME was either absent (●) or added at 0 hr (○), 1 hr (△), 2 hr (■) or 3 hr (×). Similar data were obtained in two experiments.

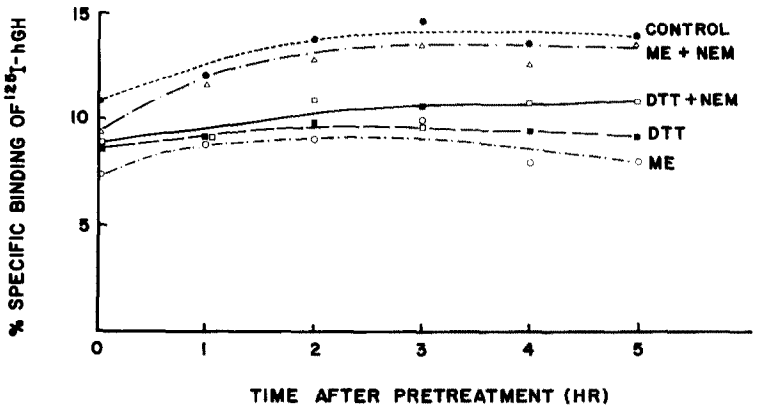

Fig. 5. Effect of pretreatment of rabbit liver membranes with ME (1%, 3 hr) or DTT (10 mM, 30 min) on the subsequent specific binding of [^{125}I]hGH. Pretreatment was carried out as described under Materials and Methods. Binding of untreated [^{125}I]hGH to pretreated membranes was carried out for 1 hr at 0, 1, 2, 3, 4 and 5 hr after ME/DTT treatment. In some cases, the alkylating agent N-ethylmaleimide (NEM, 20 mM) was also added. Similar data were obtained in six experiments.

itself. This was supported by the pretreatment of membranes with ME (1%, 3 hr or 3%, 1 hr) or DTT (20 mM, 30 min) which resulted in a subsequent decrease (24.7 ± 4.6%, mean ± S.E., N = 7) of [^{125}I]hGH specific binding (Fig. 5). In some experiments there was a slight but certainly not complete recovery of binding during the subsequent 4 hr after ME (or DTT) treatment, despite the fact that the ME or DTT was no longer present. As might be expected the effect of ME was prevented by the inclusion in the pretreatment period of the alkylating agent NEM. Surprisingly, NEM had a lesser effect on DTT action.

The dose–response effects of ME and DTT on the specific binding of [^{125}I]hGH to particulate membranes are shown in Fig. 6. ME had no effect at 0.1% but caused a dose-related inhibition of [^{125}I]-hGH binding at 0.3 and 1%. DTT showed a very similar dose–response effect between 0.3 and 1 mM with the maximum effect (observed between 1 and 10 mM) being the same as that seen with 1% ME.

Scatchard analysis of [^{125}I]hGH binding to rabbit liver membranes in the absence of ME or DTT gave linear plots (Fig. 7) as reported previously [18]. This suggests the presence of a single class of binding site with K_a 4.13 ± 0.32 × 10^9 M^{-1} (mean ± S.E., N = 3). Disulfide bond reduction with either ME (1%) or DTT (10 mM) caused a decrease in [^{125}I]hGH specific binding as expected (control 100%; ME 67.4 ± 4.3%; DTT 63.1 ± 4.0%), which was associated with a change in shape of the Scatchard plot to yield atypical curvilinear plots. This change in shape reflects a change in the nature of the GH–receptor interaction. If one analyses the curvilinear plots of Fig. 7 on the basis of a two-site binding model (according to the method of Weder *et al.* [21]), then for both ME and DTT treatment the data indicate that the high-affinity sites (5.5 and 3.5 × 10^9 M^{-1} respectively) were reduced in binding capacity from 1400 fmoles/mg protein (control) to 560 (ME) and 700 fmoles/mg (DTT). This would be accompanied

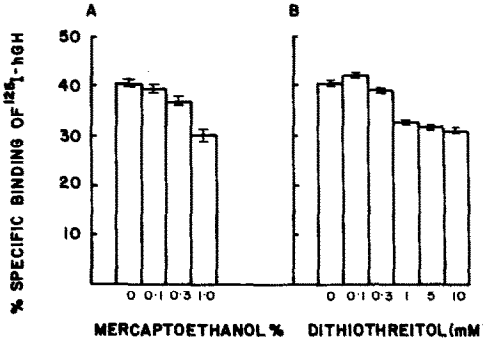

Fig. 6. Dose–response effects of ME and DTT on the specific binding of [^{125}I]hGH to particulate rabbit liver membranes. Binding was measured 5 hr after the addition of ME or DTT. Similar data were obtained in three experiments.

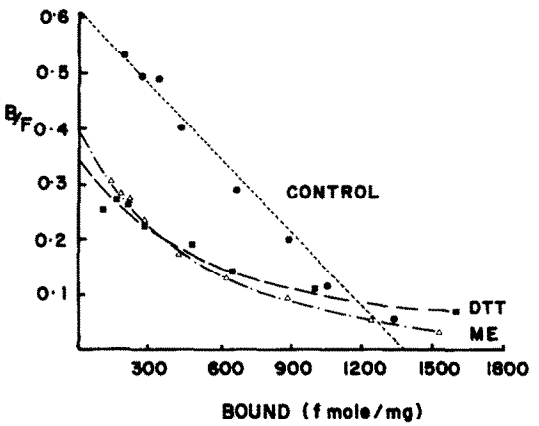

Fig. 7. Representative Scatchard plots of [^{125}I]hGH binding to rabbit liver membranes in the absence (●) or presence of ME (1%, △) or DTT (10 mM, ■). Analyses were performed on data obtained from conventional competitive displacement curves. Similar data were obtained in three experiments, each incubated for 5 hr which was usually quite sufficient to attain a binding equilibrium.

by a concomitant appearance of sites [capacity: 1500 fmoles/mg (ME) and 2000 fmoles/mg (DTT)] of much lower affinity (0.39 and $0.32 \times 10^9\,M^{-1}$ respectively). The total binding capacity (a reflection of the number of available binding sites) would be greater in the presence of reducing agents than in the control sample.

Alternatively, if one assumes that the curvilinear plots result from induction of a negative cooperative situation, then analysis can be made according to the method of de Meyts and Roth [22]. In this instance, the calculated maximum affinity (K_e, i.e. when all sites are empty) was reduced from $4.37 \times 10^9\,M^{-1}$ (control) to 1.76×10^9 (ME) and $1.09 \times 10^9\,M^{-1}$ (DTT). Again it appeared that the total binding capacity in each case (2100 fmoles/mg and 2900 fmoles/mg respectively) was increased over control. The induction of a negative cooperative situation could involve either changes in the receptor itself, or changes in neighbouring membrane proteins which lead to a subsequent conformation change (and therefore affinity change) in the receptor. Since a similar effect was seen with Scatchard plots of solubilized receptor preparations (data not shown), it would seem that a direct effect on the receptor itself, rather than on neighbouring membrane proteins, is the more likely explanation.

No simple interpretation of these Scatchard data can be made at this time. No matter which model (two-site versus negative cooperativity) proves to be correct, the observations of lowered affinity coupled with an apparent increase in the total binding capacity (or number of binding sites) suggest that the mechanism of action of reducing agents in this system is complex and that probably at least two different processes take place, which may or may not be related.

DISCUSSION

This study has investigated the direct effects of ME and DTT, disulfide-bond reducing agents, on the binding of [^{125}I]hGH to rabbit liver GH receptor preparations. In both particulate and aqueous-solubilized systems, ME and DTT caused a dose- and time-dependent reduction in specific binding of [^{125}I]-hGH. This reduction was only partial, accounting for ~30% of the specific binding capability, and was associated with a complex lowering of the binding affinity of the receptor. Under the reducing conditions used in this study, it is known that ME and DTT are effective and cause a rise in the number of free SH-groups in the membrane preparation [6]. Thus, the reduction in GH binding clearly resulted from an effect at the level of the membrane proteins, and presumably the GH receptor, since there was no detectable effect of ME or DTT on the binding ability of the hGH itself, despite its two internal disulfide bonds [20]. This latter observation is consistent with previous reports (6, 23, 24] which also showed that reduction (and alkylation) of GH disulfide bonds has no effect on its receptor binding potency. The effects of ME and DTT on the GH receptor and/or other membrane proteins were reflected not only in the association process but also in the dissociation of hGH from the receptor. This observation is consistent with the lowering of the binding affinity. Addition of ME caused an increase in the degree of dissociation of previously-bound [^{125}I]hGH, such that binding became almost totally reversible compared to only about 50% reversibility under normal circumstances. These data are very similar to those reported for the effects of ME and DTT on the lactogenic receptor of rat liver membranes [6]. They differ somewhat from a recent study [10], where DTT caused opposite effects on the binding of [^{125}I]hGH (decreased) and [^{125}I]rGH (rat GH) (increased) to the GH receptor of isolated rat hepatocytes. This dichotomy suggested either that rGH and hGH bound to separate somatotrophic receptors or to a single receptor by separate mechanisms. These possibilities obviously require further elucidation in the rat model.

Returning to the present study, one cannot be certain that the effect of the disulfide-bond reducing agents is a direct one on the receptor itself, rather than an indirect effect via neighbouring membrane proteins which may subsequently cause alterations in the receptor. However, our data showing that ME (Figs. 1 and 2) and DTT (not shown) have essentially the same effect on both particulate and solubilized membranes support the contention that the reducing agents act directly at the receptor itself.

The partial reduction in association, together with the increased ability for dissociation in the presence of disulfide reducing agents, clearly indicate that disulfide bonds are involved in regulation of at least part of the GH binding reaction. This partial effect may be explained by the existence of two classes of binding site (albeit with a very similar binding affinity)—one involving disulfide bonds and one not. This might arise from the presence of two classes of receptor within the same membrane type or may be due to the relative crudeness of the microsomal preparation and reflect the presence of receptors from various cellular fractions (e.g. plasma and Golgi membranes). Alternatively, the explanation may lie with the existence of a more complicated effect of reducing agents on a single class of GH receptors, e.g. induction of negative cooperativity. The complex nature of the changes observed upon Scatchard analysis would support this latter view. The data also suggest that the fraction of hGH which becomes increasingly irreversibly bound to its receptor with increasing incubation time [6, 25, 26], and which might be destined for internalization, involves a mechanism dependent on disulfide bond interactions. This situation would perhaps be analogous to the covalent disulfide binding of insulin to its receptor [1], with GH also undergoing a time-dependent, covalent binding reaction through disulfide bonding to its receptor. Further studies are currently under way to assess this possibility.

The involvement of inter-subunit disulfide bonds in GH receptor structure has been reported using disulfide reducing agents [10] or chemical cross-linking and SDS-PAGE techniques in liver and adipocytes of rats [9, 11] but not in human IM-9 lymphocytes [13]. In rabbit liver, however, there are conflicting data on the presence [7] or not [12–14] of inter- and/or intra-subunit disulfide bonds. In our own cross-linking studies, although initially reporting

an absence of disulfide bonds in aqueous-solubilized preparations [14], more recently using similar preparations or using liver cytosol GH binding protein [15, 16] we have detected the presence of intra-subunit disulfide bonds (data to be published elsewhere). This was evidenced by the increase in subunit mol. wt from 60,000 to 66,000 upon reduction with DTT. The reasons for these anomalies are not known, although the possibility exists that during purification and/or cross-linking procedures the receptor may be variably subject to reduction, even without the overt presence of a reducing agent such as ME or DTT.

Nevertheless, the current study has clearly shown that at least part (~30%) of the binding of [^{125}I]hGH to the somatotrophic receptor can be inhibited by reduction of disulfide bonds. The implication that disulfide bonds are therefore involved at least partially in GH receptor structure suggests that this situation is similar to that reported for receptors for other protein/polypeptide hormones such as insulin [1], prolactin [6] and TSH [27]. Our data suggest that the disulfide bonds involved are intrinsic to the receptor itself and not simply to GH, raising the possibility that the degree of irreversibility of GH binding noted in several studies [6, 12, 17, 18, 25, 26] may be due to the involvement of GH-receptor disulfide-bond formation as described for insulin and its receptor [1]. On the other hand, our data do not yet adequately explain why approximately 70% of GH binding is not affected by disulfide bond reduction. The possibility that two classes of GH receptor exist—one dependent on disulfide bonds and one independent of disulfide bonds—is suggested but cannot be confirmed by our current data. Our data may alternatively reflect that the state of reduction affects the binding ability of all GH receptors in some quite complex manner. Thus, although the process of GH binding to its receptor is still relatively poorly understood, these studies have provided additional insight into the possible mechanisms involved and have helped to clarify the uncertainty generated by covalent cross-linking structural studies regarding disulfide bond involvement in GH receptor function.

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