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THE INTERACTION OF RADIOIODINATED THYROTROPIN WITH PLASMA MEMBRANES

EVIDENCE FOR HIGH AFFINITY BINDING SITES IN THE THYROID

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SUMMARY

The binding of biologically active [^{125}I]thyrotropin to purified plasma membranes prepared from bovine thyroid glands was studied. At 4 °C, specific binding reached a maximum after 2 h of incubation and a plateau was maintained for up to 20 h. Degradation of [^{125}I]thyrotropin was undetectable after 2 h of incubation and was only 10 % of the total after 20 h.

At pH 6.0, at which binding was maximal, a single class of binding sites, having a dissociation constant of approx. 25 nM, was evident. Dissociation studies revealed first order kinetics with a half-time of 2–3 min. At pH 7.5, binding curves were complex, suggesting two orders of binding sites with dissociation constants of approx. 200 nM and 80 pM. Further, at this pH, dissociation of the thyrotropin from its receptor was also complex, suggesting the presence of two first order reactions, one with a half-time similar to that seen at pH 6.0 and another with a half-time of 4 h. At both pH 6.0 and 7.5, insulin, glucagon, growth hormone, and prolactin were without effect on [^{125}I]thyrotropin binding.

Similar high affinity and low affinity binding sites were seen with porcine thyroid membranes, but only low affinity sites were seen with either rat liver membranes or human cultured lymphocytes.

INTRODUCTION

It is generally accepted that the initial event in the action of many polypeptide hormones, including thyrotropin, is binding to specific receptors on target cells [1–3]. As a result of technical advances in both the preparation of labeled hormones and the isolation of target cells and their plasma membranes, this binding process can now be studied directly in vitro [1–3]. Binding sites of high affinity, which react at physio-

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logical hormone concentrations, have been demonstrated for several pituitary and non-pituitary polypeptide hormones [1–3]. Most likely, these binding sites seen in vitro are those operative in vivo and thus play a major role in hormone action.

Previous studies, employing highly purified plasma membranes from bovine thyroid glands and labeled bovine thyrotropin, have demonstrated the presence of specific binding sites for this hormone; however, high affinity binding sites were not seen [4–6]. We have recently described a preparation of high specific activity bovine [^{125}I]thyrotropin that retains biological potency [7]. Because bovine thyroid glands are readily available in large quantities and because membranes from these glands have been studied extensively [4, 5, 7–12], we have investigated the binding of this [^{125}I]thyrotropin to bovine thyroid membranes. In the present study we confirm the presence of low affinity, high capacity binding sites similar to those seen previously. In addition, employing physiological concentrations of thyrotropin, we find evidence for another class of binding sites of higher affinity and lower capacity which are saturated in vitro over the range of thyrotropin concentrations found in vivo.

MATERIALS AND METHODS

Materials

Purified thyrotropin was isolated from partially purified hormone (NIH-TSH-B6) donated by the Hormone Distribution Office of the National Institute of Arthritis, Metabolism and Digestive Diseases. After sequential chromatography on CM-cellulose and DE-52 cellulose, followed by gel filtration on G-100 Sephadex [7, 13], the final product had a specific activity of 20 units/mg as determined by McKenzie bioassay [14]. Purified thyrotropin was radioiodinated with ^{125}I by a stoichiometric chloramine-T method [7] to specific activities of 0.95–2.0 iodine atoms per thyrotropin molecule (71–150 Ci/g) [7].

Beef-pork glucagon was a gift of Eli Lilly and Company. Bovine growth hormone (NIH-GH-B17) and bovine prolactin (NIH-P-B3) were gifts of the Hormone Distribution Office of the National Institute of Arthritis, Metabolism and Digestive Diseases. Pork insulin (26 units/mg) was purchased from Elanco, and partially purified thyrotropin (Thytropar) was purchased from Armour Pharmaceuticals. Thyroid plasma membranes (thyroid membranes) were prepared from frozen or fresh bovine glands or frozen porcine glands by differential and sucrose density centrifugation [4]. The content of adenylate cyclase, as well as other enzymes, in those membranes has been previously described [4]. Purified rat liver membranes were prepared by the method of Lesko [15]; human cultured lymphocytes (IM-9) were a gift of Dr. Jesse Roth and were grown as previously described [16].

Methods

Studies of thyrotropin binding to membranes [7] were conducted in 400 μl plastic microfuge tubes (Beckman) containing labeled and unlabeled thyrotropin (purified or partially purified) and plasma membranes in 300 μl of buffer (0.01 M sodium phosphate, pH 6.0, or 0.01 M Tris · HCl, pH 7.5) enriched with 5 mg/ml of bovine serum albumin. Since binding of thyrotropin to bovine thyroid membranes was found to be much greater at pH 6.0 than at pH 7.5 (see above), higher concentrations of membrane (23–110 μg protein/ml) were employed at the latter pH than at

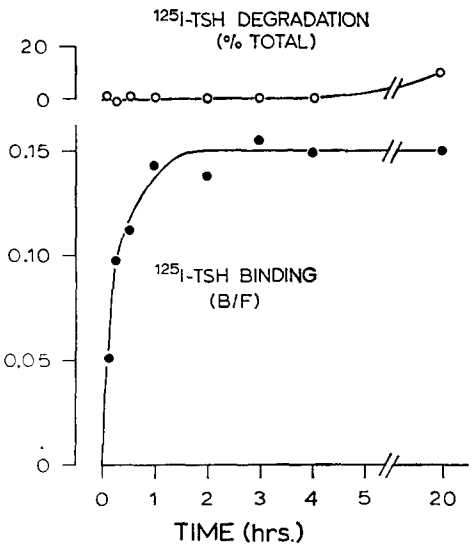

Fig. 1. Binding and degradation. [^{125}I]thyrotropin (^{125}I-TSH) (0.6 ng/ml) was incubated at 4 °C with thyroid membranes (54 μg protein/ml), and at appropriate times bound and free hormones were separated by centrifugation (see Materials and Methods). The hormone bound to membranes was measured (B/F, bound/free ratio). To assess degradation of [^{125}I]thyrotropin under these conditions, radioactive supernatants containing the unbound hormone or degradation products were recovered These, as well as control aliquots of [^{125}I]thyrotropin that had been incubated at 4 °C for 20 h in the absence of membranes, were then allowed to react with fresh membranes during a second incubation at 4 °C for 20 h. Degradation of [^{125}I]thyrotropin during the original exposure to membranes was assessed by comparing binding of the radioactivity in the original supernatants to that seen in the case of the control aliquots of [^{125}I]thyrotropin. Each point on the binding curve is the mean of triplicate determinations; each point on the degradation curve is the mean of duplicate determinations.

the former pH (0.8–6.0 μg protein/ml). After the desired period of incubation at 4 °C, free hormone was separated from hormone bound to membranes by centrifugation in a Beckman 152 Microfuge at room temperature (23–24 °C for 5 min at 10 000 × *g*). After aspiration of the supernatant radioactivity, the tips of the tubes containing the membranes were cut off and the bound radioactivity in the pellet was measured. Identical results were obtained whether centrifugation was carried out for 5 or 10 min at 4 °C. Further, an alternative method for separating bound hormone, using HAWP Millipore filters (0.45 μm), gave very similar results.

The binding of thyrotropin to human cultured lymphocytes was performed at 15 °C in a buffer containing 25 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.5, 50 mg/ml dextrose, and 5 mg/ml bovine serum albumin. Bound and free hormones were separated by centrifugation in a Beckman 152 Microfuge, as described for growth hormone binding [16].

To evaluate "non-specific" binding, tubes were also prepared with [^{125}I]-thyrotropin and an excess of either purified thyrotropin (200 μg/ml) or crude thyrotropin (10 units/ml). This binding, which was a small fraction of total binding (see above), was subtracted from the total to yield values for "specific" binding [7].

Membrane protein was measured by the method of Lowry et al. [17] using a bovine serum albumin standard.

RESULTS

Time course of binding and degradation. At 4 °C and pH 7.5, [^{125}I]thyrotropin rapidly bound to membranes (Fig. 1). Hormone binding, expressed as the bound/free ratio, was one-half maximal after 10–20 min of incubation and was maximal in 1–2 h; a plateau was maintained for up to 20 h.

Under these conditions there was very little degradation of the unbound thyrotropin as judged by the criterion of rebinding to fresh membranes (Fig. 1) [18]. No degradation was detected after 4 h and degradation was only 10 % at 20 h (Fig. 1).

At temperatures greater than 22 °C, there is a calcium-dependent autodegradation of the bovine thyrotropin receptor, which results in decreased binding of the hormone [19].

Effect of pH. When binding was performed with two concentrations of thyrotropin (0.1 and 30 ng/ml), no binding was seen below pH 4.0 and above 8.5 (Fig. 2). With both concentrations of thyrotropin, binding of labeled thyrotropin was the same from pH 4.0 up to and including pH 6.5 and was maximal at pH 6.0. However, over a pH range of 7.0–8.5, less binding was observed at the higher thyrotropin concentration, which suggested saturation of binding sites in this pH range.

Effect of membrane protein concentration. At pH 6.0, binding of [^{125}I]thyrotropin increased, in a nearly linear fashion, with increasing membrane concentrations up to 20 μg/ml, at which point a bound/free ratio of 0.95 was reached (Fig. 3). Binding was much less at pH 7.5. At this pH, binding was linear up to 110 μg/ml,

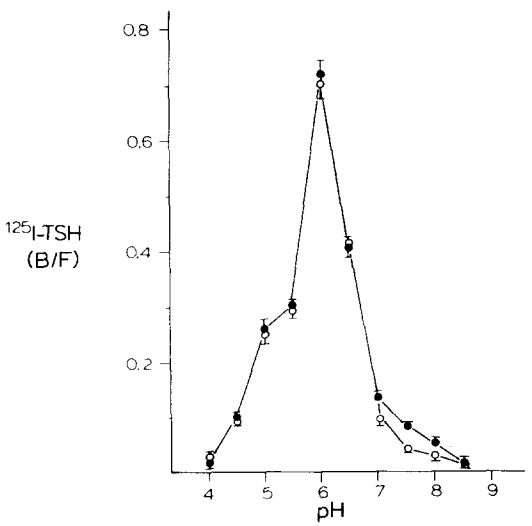

Fig. 2. Effect of pH. [^{125}I]thyrotropin (^{125}I-TSH) (●, 0.1 ng/ml or ○, 30 ng/ml) was incubated with thyroid membranes (100 μg protein/ml) for 20 h in several buffers (0.01 M) at the pH values shown. Acetate buffer was used at pH 4.0, 4.5 and 5.0; succinate buffer at pH 5.5; phosphate buffer at pH 6.0, 6.5 and 7.0; and Tris at pH 7.5, 8.0 and 8.5. Each point is the mean +S.D. of triplicate determinations. Non-specific binding has been subtracted (see Materials and Methods). Other experiments, in which the foregoing buffer systems were employed to obtain overlapping pH values, indicated that the pH value of 6.0 for maximal binding was independent of the buffer used. B/F, bound/free ratio.

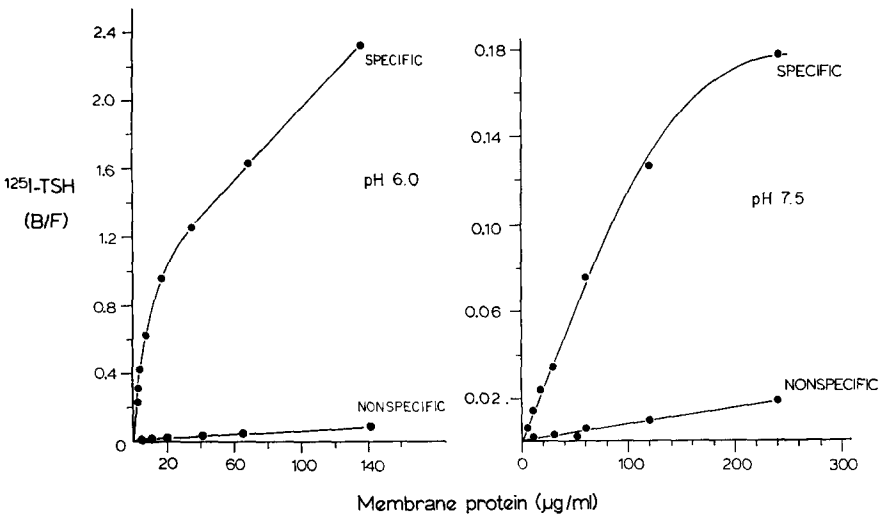

Fig. 3. Effect of membrane protein concentration. Increasing concentrations of thyroid plasma membranes were incubated at pH 6.0 and pH 7.5 for 4 h with [^{125}I]thyrotropin (^{125}I-TSH) (0.5 ng/ml) in the presence and absence of an excess of unlabeled thyrotropin (see Materials and Methods). B/F, bound/free ratio.

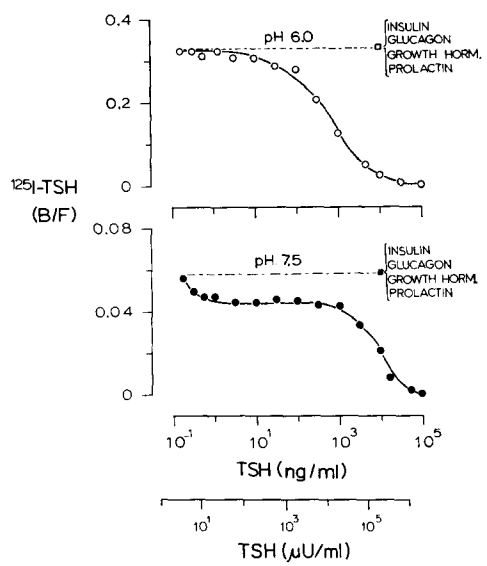

Fig. 4. Effect of increasing thyrotropin concentrations. Top, pH 6.0. [^{125}I]thyrotropin (^{125}I-TSH) (0.3 ng/ml) plus various concentrations of unlabeled thyrotropin were incubated with thyroid membranes (4 μg protein/ml) for 20 h. Bottom, pH 7.5. [^{125}I]thyrotropin (0.3 ng/ml) plus various concentrations of unlabeled thyrotropin were incubated with thyroid membranes (34 μg protein/ml) for 20 h. Open square (top) and solid square (bottom) are data points for the hormones listed at the indicated concentration. In both experiments the thyrotropin concentrations are the sum of the [^{125}I]thyrotropin and unlabeled thyrotropin, and each point is the mean of triplicate determinations. The scales shown for thyrotropin concentrations represent both the absolute protein concentration (ng/ml) and the equivalent concentration in terms of biological activity (units/ml). B/F, bound/free ratio.

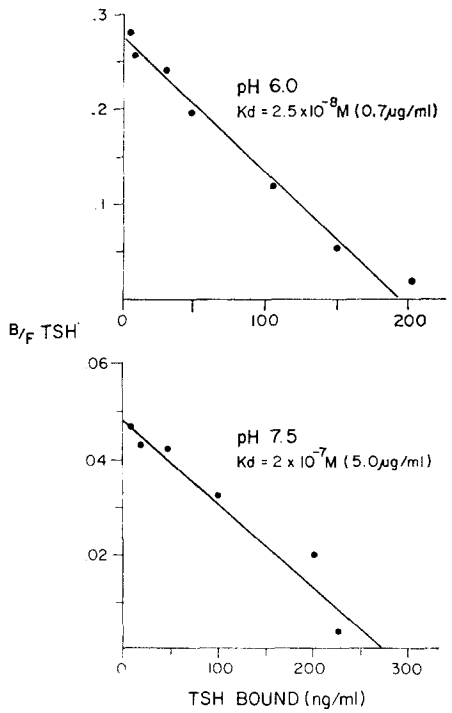

Fig. 5. Scatchard plots of the low affinity sites. Data from Fig. 4 have been plotted according to the methods of Scatchard [20]. Numbers in parentheses are the dissociation constants expressed in μg/ml. B/F, bound/free ratio; TSH, thyrotropin.

at which point the bound/free ratio was 0.12. The subsequent change in slope was a reproducible phenomenon which could be explained in part by increased hormone and receptor degradation. At both pH values, "non-specific" binding was less than 10 % of "total" binding.

Interrelation of thyrotropin concentration and pH. At pH 6.0, the binding of thyrotropin remained constant up to a thyrotropin concentration of 100 ng/ml, at which point it began to fall (Fig. 4). Binding was one-half maximal at 600 ng/ml and approached zero at 10 000 ng/ml. Scatchard plots [20] revealed a single class of binding sites. On the assumption that bovine thyrotropin has a molecular weight of 28 000 [13], the plots indicated a dissociation constant (K_d) of approx. 25 nM with a binding capacity of approx. 1.6 nmol thyrotropin/mg of protein (Fig. 5).

At pH 7.5, binding of [^{125}I]thyrotropin was more complex (Fig. 4). A small decrease in binding was evident at low thyrotropin concentrations, 0.3–10.0 ng/ml (6–200 μunits/ml). In nine separate experiments in which four membrane preparations were employed, the binding of [^{125}I]thyrotropin was inhibited 29±12 % (mean± S.D.) by 10–20 ng/ml of unlabeled thyrotropin. With progressively higher concentrations, no further decrease in the bound/free ratio was evident until a concentration of 1000 ng/ml (20 munits/ml) was reached; beyond that a progressive fall in binding occurred. One-half maximal inhibition of the bound/free ratio at these higher concentrations occurred at 10 000 ng/ml (200 munits/ml), and complete inhibition was

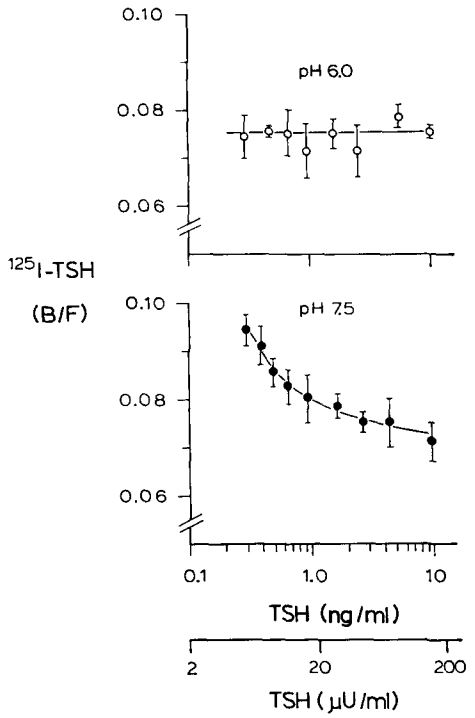

Fig. 6. [^{125}I]thyrotropin (^{125}I-TSH) binding at low hormone concentrations. Top, pH 6.0. [^{125}I]-thyrotropin (0.3 ng/ml) plus various concentrations of unlabeled thyrotropin were incubated with thyroid membranes (0.8 μg protein/ml) for 20 h. Bottom, pH 7.5. [^{125}I]thyrotropin (0.3 ng/ml) plus various concentrations of unlabeled thyrotropin were incubated with thyroid membranes (23 μg protein/ml) for 20 h. In both experiments the thyrotropin concentrations are the sum of the [^{125}I]-thyrotropin and unlabeled thyrotropin and each point is the mean ±S.D. for quadruplicate determinations. The scales shown for thyrotropin concentrations represent both absolute protein concentration (ng/ml) and the equivalent concentrations in terms of biological activity (μunits/ml). B/F, bound/free ratio.

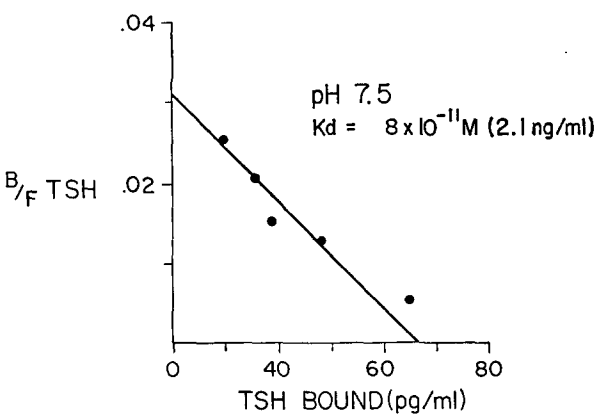

Fig. 7. Scatchard plot of the high affinity site at pH 7.5. Data from Fig. 6 have been plotted. The contribution of the low affinity site was subtracted according to the method of Kahn et al. [21]. The number in parentheses is the dissociation constant expressed in μg/ml. B/F, bound/free ratio; TSH, thyrotropin.

seen at 100 μg/ml (2 units/ml). Scatchard plots (see above) indicated the presence of two classes of binding sites. For the low affinity, high capacity site, the K_d was approx. 200 nM, with a binding capacity of approx. 0.28 nmol thyrotropin/mg of protein (Fig. 5).

Additional experiments were then performed in which special attention was directed at the low range of thyrotropin concentrations (0.3–10 ng/ml) (6–200 μunits/ml) (Fig. 6). Again, at pH 6.0 no inhibition of [^{125}I]thyrotropin binding by increasing concentrations of thyrotropin was evident. In contrast, at pH 7.5 there occurred a progressive fall in thyrotropin binding. Scatchard plots over this range of thyrotropin concentrations indicated a binding site with a K_d of approx. 80 pM and a binding capacity of approx. 0.1 pmol thyrotropin/mg of protein (Fig. 7).

At neither pH was the binding of thyrotropin decreased by high concentrations of insulin, glucagon, growth hormone, or prolactin.

Binding of thyrotropin to other tissues. To determine whether two orders of thyrotropin binding sites were unique to bovine thyroid membranes, binding was studied in porcine membranes prepared under identical conditions (Fig. 8). As with bovine membranes, approx. 30 % of the binding of labeled thyrotropin was inhibited by 200 μunits/ml of unlabeled thyrotropin, and complete inhibition was seen at 2–5 units/ml.

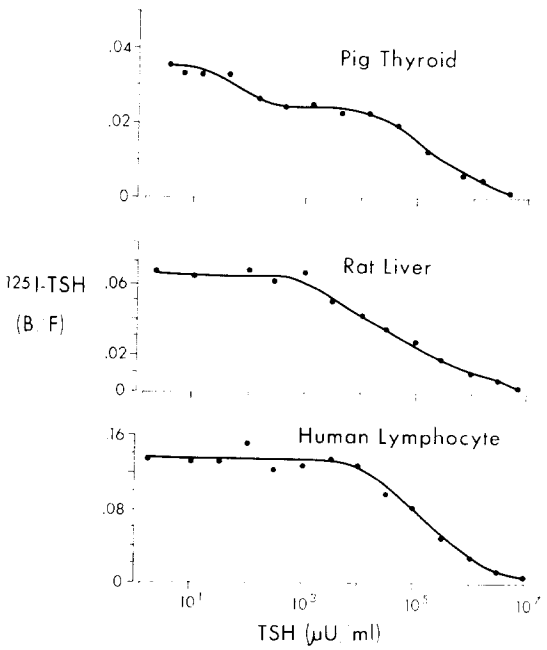

Fig. 8. Binding of thyrotropin to pork thyroid membranes, rat liver membranes, and intact human cultured lymphocytes. Top. Binding of [^{125}I]thyrotropin (0.5 ng/ml) to pork thyroid membranes (60 μg/ml). Middle. Binding of [^{125}I]thyrotropin (0.2 ng/ml) to rat thyroid membranes (40 μg/ml). Bottom. Binding of [^{125}I]thyrotropin (^{125}I-TSH) (0.3 ng/ml) to human cultured lymphocytes ($3.8 \cdot 10^6$/ml). The time of incubation was 2 h. In these studies partially purified unlabeled thyrotropin was employed. B/F, bound/free ratio.

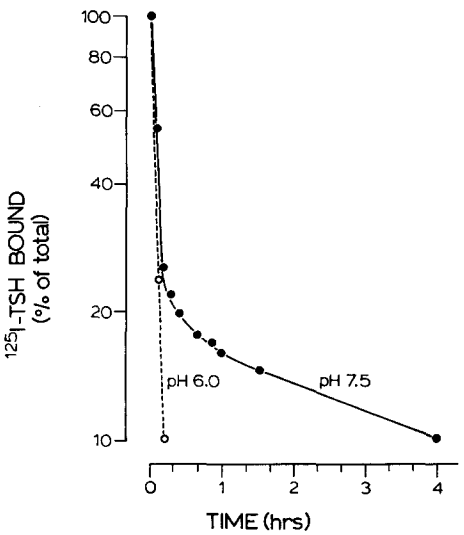

Fig. 9. Dissociation studies. [^{125}I]thyrotropin (^{125}I-TSH) (1.5 ng/ml) was incubated with bovine thyroid membranes (2.4 μg protein/ml) at pH 6.0 and (110 μg protein/ml) at pH 7.5 for 2 h at 4 °C. Then 250 μg/ml of unlabeled thyrotropin was added and the loss of binding was followed. The initial bound/free ratio at pH 6.0 was 0.184 and at pH 7.5 was 0.123. Each point is the mean of triplicate determinations. Similar patterns of dissociation were observed when [^{125}I]thyrotropin and membranes were incubated for 20 h before the addition of unlabeled thyrotropin.

Two other tissues known to bind thyrotropin, lymphocytes [5] and liver [10], were also studied (Fig. 8). In neither tissue was a comparable high affinity binding site observed; only low affinity binding sites were detected.

Dissociation of [^{125}I]thyrotropin from bovine thyroid membranes. [^{125}I]thyrotropin was allowed to bind to bovine thyroid membranes at pH 6.0 or pH 7.5 until steady state was achieved; a large excess of unlabeled thyrotropin was added (250 μg/ml) and dissociation of the thyrotropin-receptor complex was observed (Fig. 9). At pH 6.0, dissociation followed first order kinetics with a half-time of 2–3 min. At pH 7.5, the dissociation curve appeared to be the resultant of two first order reactions, one with a half-time similar to that seen at pH 6.0 and the other with a half-time of 4 h.

DISCUSSION

In the present study we measured the binding of physiological concentrations of [^{125}I]thyrotropin to bovine thyroid membranes and detected a class of high affinity, low capacity binding sites (K_d, 80 pM) that were saturated at thyrotropin concentrations between 0.3 and 10 ng/ml. Two lines of evidence suggest that this class of binding sites, which comprises a small fraction of the total number of binding sites, most likely plays a major role in thyrotropin action. First, the thyrotropin concentrations at which these sites were operative are similar to those thyrotropin concentrations normally present in the serum of cows and sheep (0–6 ng/ml) [22, 23]. Second, these high affinity binding sites for thyrotropin were also seen with pork

thyroid membranes, but these high affinity sites were not seen in other tissues such as liver or lymphocytes.

In addition to the high affinity site detected in the present study, we also found a class of thyrotropin binding sites of lower affinity (K_d, 200 nM) and higher capacity which are probably the same as, or similar to, those sites seen previously. Similar low affinity sites were also detected in rat liver membranes and human cultured lymphocytes. The physiological role of this class of thyrotropin binding sites in thyroid and other tissues is not known.

In the present study the two rates of dissociation observed for the thyrotropin-thyroid receptor complex provided additional evidence for two orders of binding sites in this tissue. However, it should be pointed out that the two rates of dissociation were not sufficiently different to explain the 10 000-fold difference in the two apparent dissociation constants, which suggests the presence of either two rates of association or site-site interactions [24]. That both the high and low affinity sites in our studies were specific for thyrotropin was indicated by the failure of other hormones at high concentrations to inhibit the binding of labeled thyrotropin.

In addition, we found, as did Moore and Wolff [5], that the optimum pH for the binding of thyrotropin to bovine thyroid membranes is well below the normal pH values found in plasma. At pH 6.0, the optimum pH in our studies, only a single class of specific binding sites of low affinity was detected. This single site was detected by both steady-state analysis and dissociation studies. The broad shape of the pH curve, which extended beyond pH 7.5, and the similarity of the single component of dissociation seen at pH 6.0 to the rapid component of dissociation seen at pH 7.5 suggest that the low affinity sites seen at these two pH values are the same or very similar. Studies employing selective enzymatic digestion of bovine thyroid plasma membranes [19] also suggest that these two binding sites are very much alike.

The binding of thyrotropin to receptors has been studied in thyroid tissues prepared from other species. In particulate fractions of normal guinea pig glands, a single order, high affinity binding site was reported (K_d, 40 pM) [25]; whereas in particulate fractions of hyperplastic (propylthiouracil-induced) guinea pig glands, a lower affinity constant (K_d, 600 pM) was seen [26]. In membranes from cultured porcine thyroid tissue, a single order of binding sites (K_d, 1.8 nM) was present [27]. In contrast, membranes from human thyroid glands showed two orders of binding sites (K_d, 200 pM and 2 nM) [28]. Since the methods for preparing receptors were different in all of the above studies, it is not possible to decide whether these dissimilar affinity constants are due to differences amongst the several species studied or to variations in the types of receptor preparations employed.

Because a large body of evidence indicates that the effects of thyrotropin on the thyroid are mediated through cyclic AMP, the question arises as to the relationship between the binding of thyrotropin to the high affinity binding sites in bovine thyroid tissue and the activation of adenylate cyclase. In plasma membranes prepared from cultured porcine thyroid tissue, Lissitzky and co-workers have shown that there is a very good correlation between binding and the activation of this enzyme [29]. However, with bovine thyroid membranes prepared by sucrose density centrifugation, a direct correlation between hormone binding and the activation of adenylate cyclase is not presently possible [5]. We have found, as have others, that activation of this enzyme in vitro occurs only with concentrations of thyrotropin that far exceed values

found in vivo [4, 5, 8–12]. Although these bovine thyroid membranes are of high purity, such observations suggest that when bovine thyroid membranes are prepared by current methods and assayed under routine conditions [5], the high affinity thyrotropin receptors do not activate adenylate cyclase. This lack of sensitivity may be due either to the insensitivity of the adenylate cyclase assays available or to changes in the plasma membrane which occur during preparation. Marshall et al. have suggested that such alterations may uncouple the high affinity thyrotropin receptor from the catalytic unit of the enzyme [11]. These observations indicate, therefore, that plasma membranes prepared from bovine thyroid tissue may not be as suitable as those prepared from cultured porcine tissue [27, 29] for studies correlating the binding of physiologic concentrations of thyrotropin with the subsequent stimulation of adenylate cyclase.

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