Structure of an Exophthalmos-producing Factor Derived from Thyrotropin by Partial Pepsin Digestion

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Previously reported experiments (Winand, R. J., and Kohn, L. D. (1970) J. Biol. Chem. 245, 967-975; Kohn, L. D., and Winand, R. J. (1971) J. Biol. Chem. 246, 6570-6575) have demonstrated that partial pepsin digestion of bovine thyrotropin preparation yields a fragment of the thyrotropin molecule which is exophthalmogenic but has negligible or no thyroid-stimulating activity. In the present report this exophthalmogenic derivative of the thyrotropin molecule is shown to contain two major polypeptide components with approximate molecular weights of 14,000 and 6,000. Amino acid analyses, carbohydrate analyses, and tryptic digestion experiments indicate that this exophthalmogenic factor is composed of an intact or nearly intact subunit of thyrotropin and an NH₂-terminal fragment of the subunit of thyrotropin.

Neither polypeptide component of the exophthalmogenic factor has the in vivo exophthalmogenic activity of the intact structure. In vitro the intact exophthalmogenic derivative of the thyrotropin molecule can bind to the thyrotropin receptor on thyroid membranes less efficiently than thyrotropin but significantly better than either of its own polypeptide components or the α or β subunits of thyrotropin. The exophthalmogenic factor and its parent thyrotropin molecule can stimulate adenylate cyclase activity in retro-orbital tissue membranes from guinea pigs, a mammalian model of exophthalmos; its polypeptide components have little or no such activity.

In previous reports (1, 2) we demonstrated that homogeneous bovine thyrotropin preparations were exophthalmogenic and that partial pepsin digestion of these preparations could yield an exophthalmogenic factor which lacks significant thyroid-stimulating ability. This exophthalmogenic derivative of the TSHe molecule was isolated by electrofocusing and was shown to have a molecular weight of 20,000 to 22,000 when evaluated by electrophoresis on gels containing sodium dodecyl sulfate; TSH analogously evaluated had a molecular weight of 27,000 to 30,000. Whereas the exophthalmogenic factor appeared to be composed of two polypeptide chains, one with a molecular weight of approximately 14,000 and the other with a molecular weight of approximately 6,000, TSH is composed of two 13,000 molecular weight polypeptide chains, the α and β subunits, which have different amino acid sequences (3). These data suggested that the exophthalmogenic derivative was composed of a major portion of one TSH subunit but only of a fragment of the second.

In the present report we have established that the exophthalmogenic derivative obtained by partial pepsin digestion of the TSH molecule is composed of an intact or nearly intact β-TSH subunit and the NH₂-terminal fragment of the α-TSH subunit.

Based on the primary sequence of the α subunit of TSH previously reported by Liao and Pierce (3), we suggest that the COOH terminus of the fragment of the α-TSH subunit remaining in the exophthalmogenic factor structure is between arginine 46 and valine 53.

Materials and Methods

The sources of all materials were the same as previously reported (1, 2) or are noted in the text. Preparation of Purified TSH and Exophthalmogenic Factor Derived from TSH by Partial Pepsin Digestion—Crude TSH was obtained from Ambinon (Oss, Holland) or Miles-Pentex. The crude TSH was purified by CM-cellulose and DEAE-cellulose chromatography as previously described (1, 2) plus an additional Sephadex G-100 chromatographic step to eliminate any residual TSH subunits and the β subunit of luteinizing hormone (4). The exophthalmogenic derivative of TSH, which has negligible thyroid-stimulating ability was prepared by the partial pepsin digestion of purified TSH preparations (2); it was isolated by electrofocusing (2). The subunits of TSH were prepared and isolated as described by Liao and Pierce (4); analogous procedures were adapted to isolate the polypeptide components of the exophthalmogenic derivative. Studies used both unlabeled TSH and [3H]TSH, i.e. preparations in which terminal galactose residues had been tritiated by a procedure previously described (1, 5).

Thyroid-stimulating and exophthalmogenic activities were measured as previously described (1, 2, 6-8). Protein was determined colorimetrically with the use of recrystallized bovine serum albumin as the standard (9).
The purified TSH used in this report had a specific thyroid-stimulating activity of 24 ± 1 i.u./mg when initially prepared. The purified TSH used had a specific activity of 21 ± 3 i.u./mg. The purified TSH had an exophthalmogenic activity in the fish bioassay of 21 ± 6 units/mg and the purified TSH 19 ± 4 units/mg. The exophthalmogenic derivative, prepared by both partial pepsin digestion and electrofocusing (2), of either purified TSH or purified thyrotropin-stimulating activity of the preparations was always less than 0.05 i.u./mg. Both preparations were stored at -20°C as a lyophilized powder, the powder was distributed into individual 10 to 30-mg packets and into evacuated desiccator jars containing drying agents.

Ultracentrifugation—Meniscus depletion and low speed sedimentation equilibrium experiments were performed as described (10–13). Meniscus depletion experiments used a 4-hole titanium rotor and double sector Kel-F centriple. Low speed sedimentation equilibrium experiments were performed in a 2-hole aluminum rotor and in double sector annular spill cells with sapphire windows. Material for these experiments was prepared either by dialysis for 72 hours against several changes of buffer or by chromatography over Sephadex G-15 or Sephadex G-25 columns equilibrated with buffer.

Amino Acid and Carbohydrate Analyses—Amino acid analyses were performed as described (14, 15) and used a Beckman model 120C amino acid analyzer. Half-cystine residues were measured as cysteic acid in samples oxidized with performic acid prior to hydrolysis (16). Total sugars in the glycopolycarbonate preparations were quantitated by the anthrone reaction (17). Neutral sugars and amino acids were measured by colorimetric methods previously reported (1, 2, 17–19). Neutral sugars were measured by gas-liquid chromatography (20) as well as by enzymatic techniques (19).

Miscellaneous Procedures—After reduction and S-carboxymethylation, the α subunit of TSH and a polypeptide component of the exophthalmogenic factor derived from TSH were maleylated by the procedure of Butler et al. (21) as used by Liao and Pierce (3). Tryptic hydrolysis of the maleylated, carboxymethylated polypeptides and the separation of the resultant tryptic peptides used the procedure adapted by Liao and Pierce (3) to their sequence studies of TSH.

Binding assays of hormones to plasma membranes and adenylate cyclase assays used techniques previously detailed (22–25).

RESULTS

Structure of Exophthalmogenic Derivative Isolated from Partial Pepsin Digests of Purified TSH Preparations—When chromatographed on Sephadex G-100 (Fig. 1, top), the exophthalmogenic derivative of the TSH molecule eluted after TSH but before either of the TSH subunits. This finding was compatible with previous gel electrophoresis data obtained in the presence of sodium dodecyl sulfate which indicated that the derivative had a molecular weight of approximately 20,000 as compared to 27,000 to 28,000 for TSH and 13,000 to 14,000 for each of the subunits of TSH. When the exophthalmogenic derivative of the TSH molecule was chromatographed on Sephadex G-100 after being subjected to the propionic acid treatment used to separate TSH into its component subunits (4), two new peaks appeared (Fig. 1, bottom). One of these peaks (Peak I) eluted in the same position as the β subunit of TSH and the other (Peak II) in a position after the α subunit of TSH and just before the position where salts elute from the column. No protein peak was eluted coincident with the cy subunit of TSH, i.e. partial pepsin digestion appeared to have converted the α subunit to smaller peptide fragments.

Peaks I and II were rechromatographed on Sephadex G-25 and Sephadex G-15, respectively. In both cases equilibration and elution was at 2–4°C with 0.125 M ammonium bicarbonate, pH 7.4. An absorbance peak preceding Peak I on Sephadex G-25 appeared to be residual exophthalmogenic derivative which was either undissociated or had aggregated during the propionic acid treatment and subsequent manipulations, i.e. it chromatographed as exophthalmogenic factor under chromatographic conditions described in Fig. 1. The peak fractions of Peak I and Peak II were pooled and concentrated as described in Fig. 1 for analyses in the ultracentrifuge and for carbohydrate and amino acid analyses.

Meniscus depletion equilibrium ultracentrifugation studies (Fig. 2) indicated that the polypeptide material in Peaks I and II were homogeneous species by size criteria and had molecular weights of 14,000 ± 1,500 and 6,000 ± 1,500, respectively. Analogous molecular weights and linear plots were derived from low speed sedimentation equilibrium experiments, i.e. meniscus depletion of the Peak II component had apparently been obtained under the conditions utilized. These data (Fig. 2) coupled with the Sephadex G-100 chromatography data (Fig. 1), suggested that Peak I was an intact or nearly intact β subunit of the TSH molecule and that Peak II was a fragment of the α subunit of the TSH molecule.

Carbohydrate analysis (Table I) and amino acid analysis (Table II) further supported the idea that the glycopeptide in Peak I was an intact or nearly intact β subunit of the TSH molecule. Carbohydrate analysis (Table I) and amino acid analysis (Table II) of the material eluting as Peak II indicated that it was missing all of the carbohydrate residues of the α subunit; that it was missing all of the histidine, at least 5 of the 6 valine, 5 of the 10 lysine residues, and 5 of the 10 half-cystine residues.

![Fig. 1. Gel filtration chromatography of the exophthalmogenic factor (●) before (top) and after (bottom) exposure to conditions (1 M propionic acid) which separate the subunits of TSH. TSH (---) after exposure to these same dissociating conditions and after chromatography on the same column serves as a marker (top) for the elution of residual nondissociated or reaggregated TSH and for the elution of the α and β subunits of TSH. Denaturation and chromatography on Sephadex G-100 used conditions described by Liao and Pierce (4). Peak I (bottom) coelutes with the β subunit of TSH. Peak I and Peak II fractions were pooled, concentrated over an Amicon filter (UM 2) with a nominal retention of molecules greater than 1000 in molecular weight and were rechromatographed on Sephadex G-15. In this experiment 19 mg of the exophthalmogenic factor were exposed to propionic acid and applied to the column; 9 mg of protein were recovered in the Peak I concentrate. 4.8 mg were recovered in the Peak II concentrate, and 4.1 mg of peptide material lower than 1000 molecular weight (i.e. material passing through UM 2 Amicon filter) were recovered in fractions eluting after Peak II and coincident with the salt peak which was assayed by conductivity changes of the eluate. In these experiments unlabeled exophthalmogenic factor was used; Ht preparations gave analogous results.](http://www.jbc.org/)
terminal half, whereas proline is located almost exclusively in the NH$_2$-terminal half, whereas proline is located almost exclusively in the NH$_2$-terminal half; but that it was missing only 1 of the 7 proline residues of the $\alpha$ subunit. An examination of the primary sequence of the $\alpha$ subunit of bovine TSH as reported by Liao and Pierce (3) indicated that the carbohydrate, histidine, and valine residues are exclusively located in its COOH-terminal half, whereas proline is located almost exclusively in its NH$_2$-terminal half, and lysine and half-cystine are equally distributed throughout the molecule. On the basis of these results, Peak II could thus be predicted to be the NH$_2$-terminal portion of the $\alpha$ subunit extending to a residue in the area between lysine residue 48 and valine residue 53 (3).

In studies of the tryptic peptides of the maleylated $\alpha$ subunit of TSH, Liao and Pierce (3) have shown that 4 peptides could be isolated on Sephadex G-50 columns. Two of these, CY T(m)-1 and CY T(m)-2, encompass the COOH-terminal half of the molecule up to arginine 46, whereas the other two, CY T(m)-3 and CY T(m)-4, encompass the NH$_2$-terminal half of the molecule and contain the carbohydrate moieties of the $\alpha$ chain (3). Assuming as above, that Peak II is the NH$_2$-terminal portion of the $\alpha$ subunit up to at least lysine residue 48 and valine residue 53 (3). Assuming as above, that Peak II is the NH$_2$-terminal portion of the $\alpha$ subunit up to at least lysine residue 48 and valine residue 53 (3). Assuming as above, that Peak II is the NH$_2$-terminal portion of the $\alpha$ subunit up to at least lysine residue 48 and valine residue 53 (3). Assuming as above, that Peak II is the NH$_2$-terminal portion of the $\alpha$ subunit up to at least lysine residue 48 and valine residue 53 (3). Assuming as above, that Peak II is the NH$_2$-terminal portion of the $\alpha$ subunit up to at least lysine residue 48 and valine residue 53 (3). Assuming as above, that Peak II is the NH$_2$-terminal portion of the $\alpha$ subunit up to at least lysine residue 48 and valine residue 53 (3). Assuming as above, that Peak II is the NH$_2$-terminal portion of the $\alpha$ subunit up to at least lysine residue 48 and valine residue 53 (3). Assuming as above, that Peak II is the NH$_2$-terminal portion of the $\alpha$ subunit up to at least lysine residue 48 and valine residue 53 (3). Assuming as above, that Peak II is the NH$_2$-terminal portion of the $\alpha$ subunit up to at least lysine residue 48 and valine residue 53 (3). Assuming as above, that Peak II is the NH$_2$-terminal portion of the $\alpha$ subunit up to at least lysine residue 48 and valine residue 53 (3).

The structure outlined in Fig. 4 raised the possibility that either fragment alone, Peak I or Peak II (the $\beta$ subunit of TSH or the NH$_2$-terminal fragment of the $\beta$ subunit of TSH,
respectively), could have all of the exophthalmogenic activity of the intact molecule, i.e. the exophthalmogenic fragment of the TSH molecule before it was dissociated by propionic acid into its component polypeptides. When tested, however, Peak I had only limited exophthalmogenic activity, i.e. 10% that of TSH or the exophthalmogenic fragment, and Peak II had no exophthalmogenic activity. By comparison, both Peak I and Peak II had less than 0.1% of the thyroid-stimulating activity of the intact TSH molecule.

During the course of these studies it was noted that although the exophthalmogenic derivative isolated from pepsin digests of purified unlabeled TSH and purified [3H]TSH gave the same results in the experiments detailed above, the yield of exophthalmogenic factor from partial pepsin digests of these two preparations was different; [3H]TSH preparations and unlabeled TSH preparations gave 54 to 74% and 29 to 35% yields, respectively. Since tritiation of the molecule involves a reductive procedure, since disulfide bonds could be rearranged in the course of this and subsequent manipulations, and since consequent conformational changes could alter the molecule's sensitivity to pepsin as well as the ratio of thyroid-stimulating to exophthalmogenic activities, disulfide bonds and their position may be critical to the formation of the exophthalmogenic derivative of the TSH molecule.

Binding and Adenylate Cyclase-stimulating Activity of Exophthalmogenic Derivative of TSH Molecule—With the presumption that differences in structure are expressed functionally, the activity of the exophthalmogenic derivative of the TSH molecule was compared to the activity of TSH and to the activity of its component subunits, using both binding and adenylate cyclase assays. In vitro binding data using thyroid plasma membranes (Fig. 5) demonstrated a closer structural relationship between TSH and its exophthalmogenic derivative than between TSH and its subunits, i.e. the exophthalmogenic factor was a better competitive inhibitor of binding than either the α or β subunits of TSH. As expected, the polypeptide material in Peak I (Fig. 1) behaved as a competitive inhibitor in the binding assay exactly like the β-TSH subunit, and the polypeptide material in Peak II (Fig. 1) had no effect on TSH binding.

In vitro studies of adenylate cyclase stimulation in plasma membranes from guinea pig retro-orbital tissue supported the conclusions of the binding data. The exophthalmogenic factor stimulated adenylate cyclase activity less effectively than TSH but significantly better than either of the subunits of TSH.

Fig. 4. Theoretic primary sequence of the exophthalmogenic factor derived from pepsin digests of TSH preparations (2). This postulated sequence is an assumption based on the primary sequence of bovine TSH described by Liao and Pierce (3) and on the data presented in Fig. 1 and Tables I and II. These studies cannot define the exact COOH-terminal end of the α subunit fragment; the italic residues are those encompassing the most likely area of pepsin cleavage.
TSH cy subunit indicate that a disulfide bond would have to be pending sequence studies. Distribution make this a most likely "working" structure.

Present work by Pierce localizing the disulfide links of the TSH molecule and its component subunits is conformationally distinctly different. In this regard, the binding and adenylate cyclase activation data presented in Figs. 5 and 6 suggest that the exophthalmogenic derivative of the TSH molecule characterized in this report is in turn conformationally distinct from both TSH and the subunits of TSH. Immunological data support this view since antisera made against the exophthalmogenic derivative react either poorly or not at all with TSH or β-TSH subunit preparations (Fig. 7).

**DISCUSSION**

The present report describes the subunit structure and a possible primary sequence of an exophthalmogenic derivative of the TSH molecule which can be formed by partial pepsin digestion of TSH preparations. The deduction of this depends on comparisons of data reported by Lia and Pierce (3) with the data described herein. Although we have inferred an intact or nearly intact β subunit as the structure of the Peak I component of the exophthalmogenic factor, the exact nature of the β subunit primary sequence has not been proven; it is possible that NH₂-terminal or COOH-terminal residues are absent either as a consequence of pepsin digestion or even as a consequence of differences in crude TSH preparations. For the same reasons, the terminal residues of the α subunit fragment are only deduced and are not proven. Nevertheless, the fortunate circumstance of the amino acid and carbohydrate distributions make this a most likely "working" structure pending sequence studies.

Present work by Pierce localizing the disulfide links of the TSH α subunit indicate that a disulfide bond would have to be broken between cystine residues 32 and 64 in order for pepsin digestion to result in the release of the COOH-terminal fragment of the α subunit missing in this exophthalmogenic derivative. This result would be compatible with the data which show higher yields of the exophthalmogenic derivative after reductive tritiation and with our previous finding that different electrophoretic species of the TSH molecule form the exophthalmogenic derivative at different rates when exposed to pepsin, i.e. that disulfide rearrangements might contribute to the formation of the exophthalmogenic derivative of the TSH molecule described in this report. An alternate explanation is that pepsin digestion was able to excise the disulfide bond between residues 32 and 64 by digestion of peptide bonds linking amino acid residues adjacent to the half-cystine residue at position 32. This idea would be compatible with the amino acid data of Table II.
which shows that a methionine residue is missing beyond those predicted by the sequence detailed in Fig. 4. NH₂-
terminal and COOH-terminal residue analyses are presently being performed to evaluate these possibilities.

In its action on proteins, pepsin has been found to catalyze the hydrolysis of peptide bonds formed by all amino acids except proline and isoleucine. It is of interest in this regard that the NH₂-terminal and COOH-terminal halves of the α-TSH subunit are proline rich and poor, respectively, and can be viewed as two parallel peptide loops linked by the one disulfide bond between half-cystine residues 32 and 64 (3, 32). The proline-rich NH₂-terminal half of the α-TSH subunit should be relatively unaffected by pepsin digestion and remain united to the β-TSH subunit; in contrast, the proline-poor COOH-terminal portion of the α-TSH subunit should be more susceptible to pepsin digestion and the resultant small peptide fragments could easily drop off the β-TSH subunit.

Cleavage between valine 53 and proline 54 or proline 54 and lysine 55 of the α-TSH subunit (Fig. 4) by pepsin is unlikely (33). This probability, together with the experimental findings that the one carbohydrate moiety of the α subunit is attached to asparagine 56 and that all of the carbohydrate is missing in the Peak II component, suggest that pepsin cleavage is either between methionine 51 and leucine 52 or at some residue before methionine 51. Other amino acid differences (lysine, threonine, methionine, and leucine) in Table II suggest that the cleavage is between lysine residues 48 and 49 and that the 0.8 mol of valine is an artifically or experimentally high value. The presence of the α(Tm) 2 peptide (Fig. 2) would most certainly place the cleavage after the alanine 45 residue given the proline sequence of this peptide. Thus the most likely pepsin cleavage point would be between alanine 45 and valine 53 and heterogeneity of this end is entirely possible.

As noted above, the structure of Fig. 4 rests heavily on the presumption that TSH preparations used to sequence TSH and those used in the present report are essentially the same. In support of this presumption are the amino acid and carbohydrate analyses (Tables I and II). These are effectively the same with the exception of galactose values, and galactose differences appear to represent a methodologic problem as noted in the legend to Table I. In addition, the chromatographic patterns of the tryptic peptides of the maleylated α subunit isolated from TSH preparations used in this report (Fig. 3) and from TSH preparations used by Liao and Pierce (3) are effectively the same. Further support rests on the same tryptic peptide pattern when S-carboxymethyl TSH is hydrolyzed with trypsin and chromatographed on Dowex 50 (34). In the last analysis, however, the validity of the primary sequence suggested in Fig. 4 will be derived from sequence studies themselves.

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