Identification of Subunit Contact Sites on the α-Subunit of Lutropin†

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ABSTRACT: Peptides corresponding to the entire sequence of the α-subunit of the human glycoprotein hormones were synthesized by using standard solid-phase procedures. Purified peptides were incubated in the presence of α- and β-subunits of bovine lutropin, and subunit recombination was monitored by difference spectroscopy, reverse-phase high-pressure liquid chromatography, and gel filtration chromatography. Although the binding of α-peptides to either subunit could not be detected by these techniques, it was possible to demonstrate that some peptides could inhibit the recombination of α- and β-subunits. Specifically, α-peptide 33–58 allowed only 0–11% of subunit recombination in 24 h (38–56% after 48 h), while α-peptide 51–65 allowed 10–60% of subunits to recombine in 24 h (65–94% in 48 h). Peptides 1–15, 11–27, 22–39, 61–78, and 73–92 of the α-subunit could not inhibit subunit recombination at any time or at any concentration tested. The data suggest that at least a portion of the α-subunit contact site has been identified, and results are discussed in terms of protein structure assessment tools.

In attempts to design and synthesize proteins which will perform novel functions, it may be necessary to include the aspect of quaternary structure in order to achieve all of the desired functions. It is important, then, to understand the types of interactions between subunits and to be able to mimic them upon demand. An excellent model with which to study the interactions of subunits may be provided by the glycoprotein hormones which are composed of an α- and a β-subunit, noncovalently but very tightly associated. The primary structure of the α-subunits is common among all of the glycoprotein hormones (follitropin, lutropin, chorionic gonadotropin, and thyrotropin) within a species, and the sequence is highly conserved between species as well. The sequence of the β-subunits is unique to each hormone within a species, thus conferring hormonal specificity, but is also highly conserved among species. In addition, there is a substantial amount of information known about these hormones from chemical modification studies and other work, summarized in reviews (Ward, 1978; Pierce & Parsons, 1981; Saxena & Rathnam, 1978; Giudice & Pierce, 1978; Garnier, 1978; Ryan et al., 1987). Although Salesse et al. (1990) have begun an important series of studies, to date there has been no definitive determination of what portions of the individual subunits interact with the other subunit.

Chemical synthesis of peptides corresponding to various portions of the primary structure of a subunit provides a powerful tool with which to investigate subunit–subunit interactions. If a peptide can be identified which inhibits the association of subunits, it can be considered a likely candidate for a subunit contact site. Given the extensive amount of information available concerning the glycoprotein hormones, it may be possible, a priori, to select potential subunit contact sites. However, the individual subunits are not so large as to prohibit the synthesis of a complete set of peptides, and the need for control peptides would dictate that such a program be undertaken. In this paper, we have synthesized peptides corresponding to the sequence of the α-subunit of human glycoprotein hormones and show that two particular peptides are capable of inhibiting the association of the α-subunit and the β-subunit of lutropin.

Materials and Methods

Materials. Protected amino acids, resins, diisopropylethylamine, and hydroxybenzotriazole were obtained from Milligen/Biosearch (Burlington, MA). Diisopropylcarbodiimide, anisole, dimethyl sulfide, and cresol were obtained from Aldrich (Milwaukee, WI), and trifluoroacetic acid was from Kali-Chemie (Greenwich, CT). Dimethylformamide, methylene chloride, methanol, and acetonitrile were from Burdick and Jackson (Muskegon, MI).

Computerized Structural Assessment. Computer-assisted structural assessment tools (Krystek et al., 1985a,b) were used to examine the hydropathy, flexibility, and secondary structure of the α-subunit. Hydropathy assessment was a modification of the Kyte and Doolittle (1982) method, flexibility is the method of Karplus and Schultz (1985), and secondary structure is a modification of the Chou–Fasman (1976) method.

Peptide Synthesis. Peptides were assembled using solid-phase peptide synthesis methodology on a Biosearch 9500 synthesizer, employing the *t*-Boc strategy. The sequences were as follows: α1–15, A-P-D-V-Q-D-C-P-E-C-T-L-Q-E-N amide; α11–27, T-L-Q-E-N-P-F-F-S-Q-P-G-A-P-I-L-Q amide; α22–39, G-A-P-I-L-Q-C-M-G-C-C-F-S-R-A-Y-P-T amide; α33–58, F-S-R-A-Y-P-T-P-L-R-S-K-K-T-M-L-V-Q-K-N-V-T-S-E-S-T amide; α51–65, K-N-V-T-S-E-S-T-C-C-V-A-K-S-Y amide; α61–78, V-A-K-S-Y-N-R-V-T-V-M-G-G-F-K-V-E-N amide; α73–92, G-F-K-V-E-N-H-T-A-C-H-C-S-T-C-Y-Y-H-K-S acid. Peptides (except 73–92) were assembled on methylbenzhydrylamine resin, while the C-terminal peptide was synthesized on a Merrifield resin. Side chain protecting groups used were Asp(OBzl), Glu(OBzl), Cys(4-MeBzl), Lys(ClZ), His(DNP), Ser(Bzl), Thr(Bzl), and Tyr(BrZ). Completed peptides were removed from the resin by using the "low–high" HF cleavage method of Tam et al. (1983). Peptides were washed with ethyl acetate and ether, extracted into 1.0 M acetic acid, and lyophilized. Peptides 1–15, 22–39, 33–58, 61–78, and 73–92 were purified by ion-exchange

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chromatography on a 0.9 × 15 cm Dowex AG 50W-X4 column eluted with a linear gradient of 0–100% trifluoroacetic acid. Peptides 11–27 and 51–65 were purified by gel filtration chromatography on a 2.5 × 100 cm Sephadex G-10 column eluted with 20% acetic acid. For all peptides, the final purification step was reverse-phase HPLC using a DuPont Zorbax Protein Plus column (1.0 × 25 cm) with C_3 bonded groups, eluted with a linear gradient from 0 to 60% acetonitrile containing 0.1% trifluoroacetic acid. Quantitative amino acid analysis using the method of Bidlingmeyer et al. (1984) was used to confirm the composition of each purified peptide and to monitor the sequence of peptides during synthesis. For the latter purpose, aliquots of resin-bound peptide were removed from the reaction vessel and subjected to hydrolysis without cleavage from the resin.

Subunit Dissociation. Intact bovine lutropin was dissolved (10 mg/mL) in 0.1 M sodium phosphate, pH 6.8, containing 1 mM azide and 6 M guanidine hydrochloride. The solution was incubated for 16 h at room temperature and applied to a Vydac 218TP104 column (0.46 × 25 cm) eluted with a linear gradient of 0.1 M sodium phosphate, pH 6.8, to buffer containing 50% acetonitrile at a flow rate of 1 mL/min over 1 h. The α- and β-subunits eluted as described (Parsons et al., 1984) and were further identified by first-derivative spectroscopy. Subunits were lyophilized, dissolved in water, dialyzed against 1% ammonium bicarbonate, pH 8.0, and stored in the lyophilized state until needed.

Subunit Association. To quantitate subunit reassociation, difference spectroscopy, reverse-phase chromatography, and gel filtration chromatography were employed. Methods similar to those used by other workers were employed (Pierce & Parsons, 1981; Guidice & Pierce, 1978; Salesse et al., 1975; Ingham et al., 1976; Schmid, 1989; Herskovits, 1967), and similar results were obtained. For difference spectroscopy, 500 μg of each subunit in 1% ammonium bicarbonate, pH 8.0, was added in a final volume of 1 mL to a single compartment in the cuvette in the spectrophotometer's sample beam (the other compartment contained buffer). The reference beam cuvette contained the same amount of proteins in separate compartments. Difference spectra were measured between 340 and 240 nm in a Perkin-Elmer 320 spectrophotometer at various time points over 72 h. It has been shown (Ingham, 1976) that a difference spectrum between 280 and 290 nm is indicative of recombination. To monitor the effects on recombination of the various synthetic peptides corresponding to portions of the α-subunit, peptides (5-, 10-, or 20-fold molar excess over subunit) were first incubated with β-subunit for 24 h. No difference spectrum was observed for any α-peptide in the presence of β-subunit at any time (data not shown). Following this 24-h incubation, α-subunit was added and incubated for an additional 48 h. Peptides able to inhibit or delay generation of the difference spectrum attributable to subunit recombination were considered candidates for a subunit contact site.

Reverse-phase chromatography was used to monitor the appearance of intact (recombined) hormone in parallel with difference spectroscopy studies. Samples were removed from the cuvettes at the end of the incubation, and 500 μg of protein was loaded onto a Vydac 218TP104 column (0.46 × 25 cm). A linear gradient from 0.1 M sodium phosphate, pH 6.8 with 1 mM sodium azide, to buffer containing 50% acetonitrile at a flow rate of 1 mL/min, and developed over 1 h, was used to elute the subunits, α-peptides, and reassociated hormone. Samples from the reference cuvettes served as controls to identify α-peptide and re-formed hormone. First-derivative

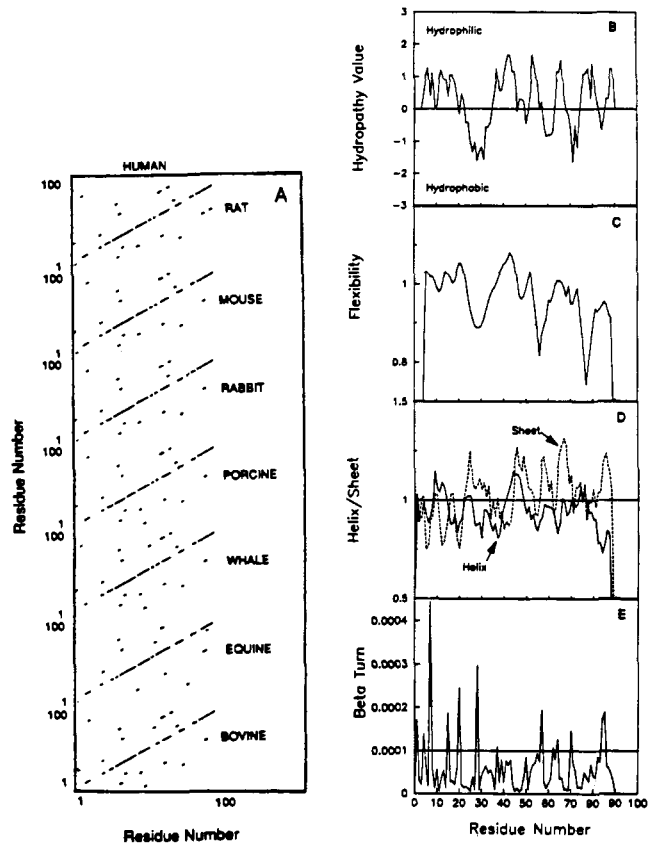

FIGURE 1: Computer-assisted analysis of the structure of the α-subunit. (Panel A) Matrix of the homology between the human α-subunit and α-subunits from various species. The numbering scheme utilized is that of bovine subunits. Consecutive adjacent dots represent sequence homology. (Panel B) Hydropathy profile (Krystek et al., 1985b; Kyte & Doolittle, 1982). (Panel C) Flexibility profile (Karplus & Schultz, 1985). (Panel D) Helix (solid line) or sheet (dashed line) prediction. (Panel E) β-Turn prediction.

spectroscopy of the eluted peaks further confirmed the identity of each polypeptide as either α-subunit, β-subunit, or re-formed hormone. Similar approaches have been used by others, notably Garnier (1978).

Gel filtration chromatography was also used to quantitate the effects of α-peptides on lutropin subunit recombination. An HPLC system consisting of one Bio-Sil TSK-125 column (0.46 × 25 cm) and one Bio-Gel TSK-30 column (0.46 × 25 cm) connected in series was used to separate free subunits from reassociated hormone, similar to the method employed by others (Parsons, 1984). The eluent was 0.2 M ammonium acetate, pH 6.0, with 30% acetonitrile at a flow rate of 1.0 mL/min. α- and β-subunits (100 μg) were incubated in 200 μL of 1% ammonium bicarbonate at room temperature in the presence of either a 5-, 10-, or 20-fold molar excess of α-peptide. Subunits could not be resolved from each other, but could be resolved from recombined hormone and from synthetic peptide.

Results

Peptide Synthesis. Amino acid analysis performed on peptide–resin samples taken at multiple points during synthesis suggests that each peptide is comprised of the desired sequence. Reverse-phase HPLC analysis of each peptide resulted in a single major peak in each case, the composition of which reflected that of the desired peptide (data not shown).

Structural Assessment. Figure 1 shows the results of the computerized structural assessments of the α-subunit of the glycoprotein hormones. A dot matrix homology assessment

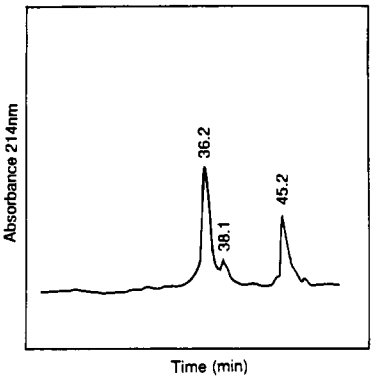

FIGURE 2: Reverse-phase HPLC separation of bovine lutropin subunits. Bovine lutropin was incubated for 20 h in 6 M guanidine hydrochloride at a concentration of 10 mg/mL. Successive aliquots were applied to a Vydac 218TP104 column (0.46 × 25 cm) and eluted with a linear gradient (60 min) of 0–50% acetonitrile with 0.1 M sodium phosphate, pH 6.8, plus 1 mM sodium azide. α-Subunit peak eluted at 36.2 min, β-subunit peak eluted at 45.2 min, and intact hormone eluted at 38.1 min. The identity of the peaks was confirmed by first-derivative spectroscopy and comparison to published data (Parsons et al., 1984).

for α-subunits of different species shows regions which are most highly conserved, and presumably important for function. The hydropathy analysis (panel B) suggests that the amino terminus is hydrophilic, while residues 22–33 are contained in a strongly hydrophobic region. Residues 33–60 are strongly hydrophilic, while the remainder of the molecule alternates short stretches of hydrophilic and hydrophobic areas. Panel C is a flexibility analysis which suggests that segments 33–50 and 1–22 are the most flexible, while the C-terminus is much less flexible. Panels D and E are secondary structure prediction methods, showing little α-helix and substantial numbers of turns and amounts of β-structure. This assessment is in keeping with circular dichroism assessments of the glycoprotein hormones (Giudice & Pierce, 1978; Puett et al., 1976).

Subunit Separation and Purification. Reverse-phase HPLC separation of bovine lutropin subunits is shown in Figure 2. The α-subunit eluted at 36.2 min and the β-subunit at 45.2 min, with a small amount of material eluting at 38.1 min corresponding to intact (heterodimeric) lutropin. Greater than 90% of intact lutropin could be dissociated under these conditions, and rechromatography of any of the three peaks shown in Figure 2 yielded a single peak (data not shown).

Effect of Peptides on Subunit Recombination. Subunit recombination was monitored by difference spectroscopy, reverse-phase HPLC, and gel filtration chromatography. Figure 3 shows the results from difference spectroscopy. In panel A, α-subunits and β-subunits were allowed to interact in the absence of peptides. Initially, the spectrum showed almost no difference [between the sample beam (α-subunit and β-subunit together) and the reference beam (α-subunit in one compartment and β-subunit in the other)]. After 24 h, a pronounced difference spectrum was seen which was slightly greater after 48 h of incubation. This difference is indicative of subunit recombination (Ingham et al., 1976). Panel B shows the results of a similar experiment in which α-peptide 1–15 was included in the subunit compartment of the sample beam and in the β-subunit compartment of the reference beam. After 24 h, a difference spectrum is apparent, and after 48 h, the profile resembles that of the control recombination. Thus, α-peptide 1–15 had no discernible effect on (i.e., did not diminish) subunit recombination. Figure 3C shows the results of subunit recombination measured by difference spectroscopy in the presence of α-peptide 33–58. After 24-h incubation, there is no increase over base line in the spectrum,

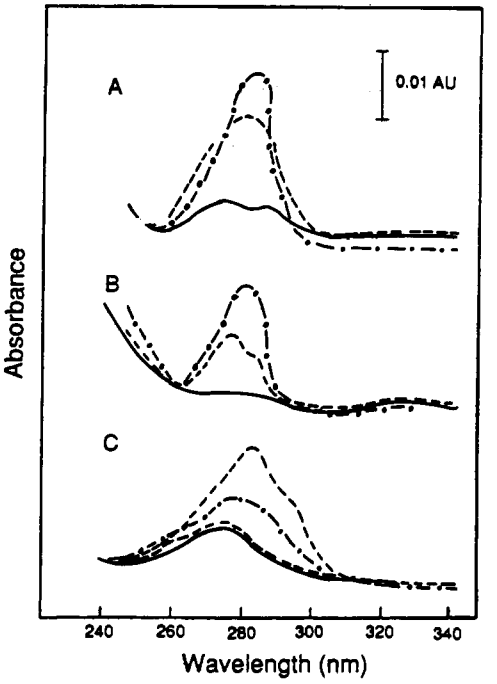

FIGURE 3: Recombination of lutropin subunits measured by difference spectroscopy. Recombination was measured at 0 h (solid line), 24 h (dashed line), 48 h (dash/dot line), and 72 h (dotted line). (A) Recombination of lutropin subunits in the absence of a peptides; (B) recombination of lutropin subunits in the presence of α1–15; (C) recombination of lutropin subunits in the presence of α33–58. The shoulder seen at 290 nm is unique to this peptide, but is otherwise unexplained.

Table 1: Bovine Lutropin Subunit Recombination

peptide	time (h)	difference spectroscopy (%)	rpHPLC (%)	gel filtration (%)
none	24	100	100	100
1–15	24	82	82	86
11–27	24	91	76	75
22–39	24	92	84	80
33–58	24	0	11	0
	48	39	38	56
	72	76	67	ND[a]
51–65	24	61	29	10
	48	94	65	87
61–78	24	90	71	63
	48	92	74	90
73–92	24	88	86	88

[a] ND = not determined.

suggesting that this peptide can inhibit the recombination of lutropin subunits. After 48 h, a difference spectrum is apparent. The data in Table I indicate that 76% recombination of α- and β-subunits had occurred after 72 h as estimated by difference spectroscopy. In similar experiments, α-peptide 51–65 decreased subunit recombination after 24 h, but all other peptides had no effect on subunit recombination at any time point (Table I).

The reaction was also monitored by reverse-phase HPLC. After various time points, aliquots were removed from the spectroscopy experiments and applied to a reverse-phase column as described under Materials and Methods. Figure 4A shows the elution profile for subunits in the absence of peptides. Although not all of the subunits recombined, substantial heterodimer is apparent, and its identity was further confirmed by derivative spectroscopy (data not shown). This amount of heterodimer (i.e., that formed in the absence of any peptide) is defined as 100% recombination in each experiment.

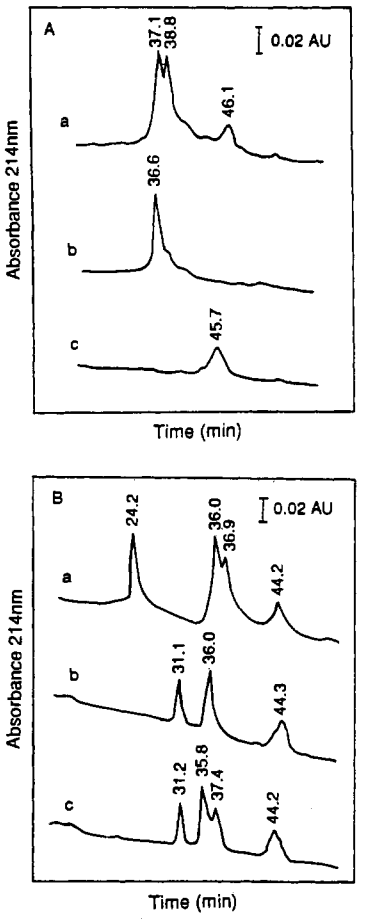

FIGURE 4: Reverse-phase HPLC measurement of lutropin subunit recombination. (A) The contents of the sample and reference cuvette compartments from difference spectroscopy were applied to a Vydac 218TP04 column and eluted with a linear gradient of 0–50% acetonitrile with 0.1 M sodium phosphate, pH 6.8, plus 1 mM sodium azide. (a) Aliquots of the sample cuvette which contained reassociated subunits were injected and eluted as follows: α-subunit eluted at 37.1 min, β-subunit eluted at 46.1 min, and reassociated dimer eluted at 38.8 min. (b) Aliquots of the reference cuvette compartment which contained α-subunits were injected and eluted at 36.6 min. (c) Aliquots of the reference cuvette compartment which contained β-subunit were injected and eluted at 45.7 min. The identity of the peaks was confirmed by first-derivative spectroscopy and comparison to literature (Parsons et al., 1984). (B) (a) 24-h incubation of α1–15 with lutropin subunits eluted as follows: α1–15, 24.2 min; α-subunit, 36.0 min; reassociated dimer, 36.9 min; and β-subunit, 44.2 min. (b) 24-h incubation of α33–58 with lutropin subunits eluted as follows: α33–58, 31.1 min; α-subunit, 36.0 min; β-subunit, 44.3 min. (c) 48-h incubation of α33–58 with lutropin subunits eluted as follows: α33–58, 31.2 min; α-subunit, 35.8 min; reassociated dimer, 37.4 min; and β-subunit, 44.2 min.

Figure 4B, trace a, shows the result obtained in the presence of α-peptide 1–15, which by itself elutes at 24.2 min. Again, substantial amounts of heterodimer are evident (82%, Table I) after 24-h incubation, indicating that this peptide did not diminish subunit recombination. In contrast, Figure 4B, traces b and c, shows the results obtained with α-peptide 33–58 after 24 and 48 h, respectively. The peptide alone elutes at 31.1 min, but after 24 h, little or no heterodimer is formed (trace b). After 48 or 72 h, heterodimer can be detected (trace c) and is quantitated in Table I. As estimated by reverse-phase HPLC, only peptides 33–58 and 51–65 exhibited the ability to inhibit subunit recombination.

Gel filtration was employed to monitor the recombination reaction using two HPLC size-exclusion columns in series in order to resolve the subunits from heterodimeric lutropin (though it was not possible to resolve α- and β-subunits from

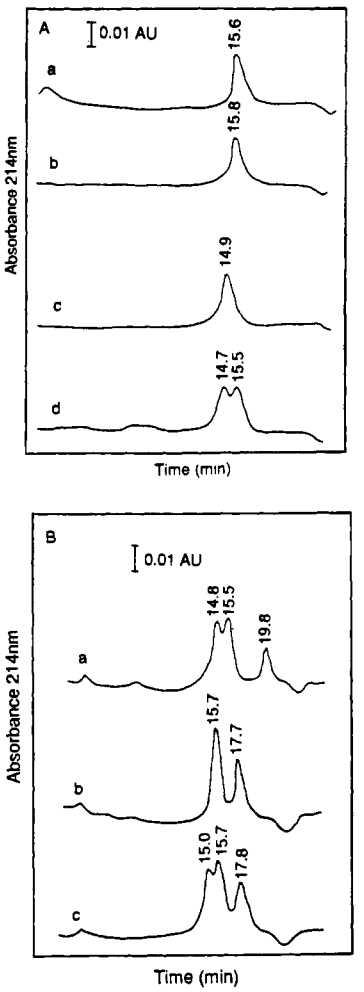

FIGURE 5: (A) Gel filtration measurement of bovine lutropin subunit recombination. (a) α-Subunit eluted at 15.6 min. (b) β-subunit eluted at 15.8 min. (c) Native hormone eluted at 14.9 min. (d) Subunit recombination after 24-h incubation of α- and β-subunits. Subunits eluted at 15.5 min while the reassociated dimer eluted at 14.7 min. Gel filtration was performed with a BioSil TSK-125 column and a BioGel TSK-30 column in series. Samples were eluted with 0.2 M ammonium acetate, pH 6.0, with acetonitrile. (B) Gel filtration measurement of bovine lutropin subunit recombination in the presence of α-peptides. (a) Subunit recombination in the presence of α1–15. α1–15 eluted at 19.8 min, subunits eluted at 15.5 min, and reassociated dimer eluted at 14.8 min. (b) Subunit recombination in the presence of α33–58 after 24-h incubation. α33–58 eluted at 17.7 min, and subunits eluted at 15.7 min. (c) Subunit recombination in the presence of α33–58 after 48-h incubation. α33–58 eluted at 17.8 min, free subunits eluted at 15.7 min, and recombined dimer eluted at 15.0 min.

each other). Figure 5A shows elution positions for the α-subunit (15.6 min, trace a), the β-subunit (15.8 min, trace b), heterodimer (14.9 min, trace c), and the recombination mixture after 24 h (trace d). Figure 5B shows the elution profile of the recombination mixture (trace a) in the presence of α-peptide 1–15, which by itself elutes at 19.8 min. Heterodimer is apparent and is quantitated in Table I. Trace b is the result of recombination in the presence of α-peptide 33–58 (which, by itself, elutes at 17.7 min) after 24-h incubation, and no heterodimer is evident. After 48 h, however, substantial recombination was detected (trace c, Table I).

Discussion

Theoretical Methods for Assessing Protein Structure. Since the glycoprotein hormones within a species have the same α-subunit, and β-subunits from one species can bind to α-subunits from different species (Bousfield et al., 1985; Roser et al., 1986), it was hypothesized that highly homologous

regions of the α-subunit should be involved in subunit–subunit interactions. Examination of homology plots for glycoprotein hormone α-subunits (Figure 1) shows that the most homologous region in the α-subunit is comprised of residues 25–48. Regions of the α-subunit that are least homologous are residues 1–24 and 65–75.

Surface profiles were used to identify regions of the α-subunit which should be accessible for protein–protein interactions. Figure 1 shows the Kyte and Doolittle scale, but correlation of five hydropathy scales revealed essentially equivalent results. Further, the surface profile for human α-subunit (Figure 1) is similar to profiles for α-subunits from several other species (not shown) in that regions of hydrophilicity and hydrophobicity are similar throughout their sequences. Residues 35–48 are predicted to be located in a hydrophilic portion of the α-subunit, and the beginning and end of this region are predicted to be hydrophobic. Since residues 35–48 are predicted to be hydrophilic, are surrounded by hydrophobic residues at each termini, and correspond to the homologous regions of the α-subunit, these residues (35–48) may comprise a portion of the α-subunit contact site. Other hydrophilic regions of the α-subunit are residues 10–20 and 50–60. However, residues 10–20 are contained in an area of the α-subunit shown to have little homology (Figure 1A). Therefore, this region of the subunit was not predicted to be an α-subunit contact site. Residues 50–60 comprise a hydrophilic region bordered by hydrophobic regions at both ends. Since residues 50–60 are predicted to be hydrophilic and are bordered by hydrophobic regions, this region may contain sites of protein–protein interaction, but further information is required before a prediction can be made. There are, of course, several limitations of the hydropathy analysis. The analysis does not take into account the carbohydrate chains located on proteins, and disulfide bonds are not adequately analyzed by these methods.

Correlation of flexibility plots with homology plots and surface profiles may be helpful in identifying specific regions of protein structure. The most flexible regions of a protein reportedly are associated with protein binding sites (Richardson, 1981; Van Regenmortel, 1987), antigenic determinants (Van Regenmortel, 1987; Westhof et al., 1984; Tainer et al., 1985), and enzyme catalytic sites (Tainer et al., 1985; Richardson, 1981). Flexibility plots ostensibly identify the most flexible regions of a polypeptide but do not take into account disulfide bonding (Spinella et al., 1989). Disulfides are known to stabilize protein structures (Richardson, 1981), and the flexibility of a molecule that is highly disulfide bonded is probably overestimated by this technique. Since the α- and β-subunits of the glycoprotein hormones contain a high number of disulfides, this limitation should be kept in mind. Regions of the α-subunit predicted to be most flexible are 5–20 and 30–55, but residues 5–20 contain little homology. Residues 30–55 overlap with a homologous region of the α-subunit and are predicted to be in the most accessible portion of the subunit. Residues 50–60 are also predicted to be fairly flexible.

The secondary structure of intact follitropin, as determined by circular dichroism, shows little α-helix (0–10%), 25–40% β-structure, and a large portion of structure classified as unordered (50%) (Pierce & Parsons, 1981; Giudice & Pierce, 1978). The method of Chou and Fasman (Figure 1) predicted that the α-subunit contains a large amount (28%) of unordered structure and significant amounts (38%) of β-structure, and suggests that α35–48 contains unordered structure and β-sheet, and follows a β-turn located at residues 28–31. The region α50–60 is predicted to contain β-sheet structure and a β-turn. This analysis is similar to a more thorough discussion by Erikson et al. (1990).

Chemical modification studies in the literature indicate that lysine residues in the α-subunit might be involved with subunit–subunit interactions (Pierce & Parsons, 1981). Specifically, lutropin α Lys-49 was cross-linked with lutropin β Asp-109. Since these residues were close enough to cross-link, forming an amide bond, it suggested that these residues were at or near one site of subunit contact. α Tyr-41 could not be nitrated in the intact hormone but could be nitrated in the free α-subunit (Combarnous & Maghuin-Rogister, 1974). The region near α Tyr-41 is predicted to be hydrophilic. Therefore, these observations are consistent with the idea that α Tyr-41 is protected from modification by the β-subunit. Thus, this residue is probably a portion of an α-subunit contact site. These data correlate well with our structural analyses in that one region predicted to contain the subunit contact sites encompasses α35–48. Therefore, both chemical modification studies and theoretical structural analyses suggest residues 35–48 as a potential subunit contact site.

Design of Peptides. On the basis of information from the α-subunit, peptides were designed to mimic the predicted subunit contact sites. The first site predicted, α35–48, was modified to α33–58 based upon the following reasoning. In order to ensure the entire predicted region be contained in a single peptide, we extended both the amino terminus and the carboxyl terminus. Residues 31 and 32 are half-cystines, probably paired with half-cystines at positions 7 and 10 (Pierce & Parsons, 1981; Ryan et al., 1987). We modified the peptide to begin at residue 33 in order to avoid these residues. Amino acids 59 and 60 are half-cystines involved in disulfide bonds elsewhere in the molecule (Pierce & Parsons, 1981; Ryan et al., 1987), so it was logical to end the peptide at residue 58. This peptide would now contain the predicted α-subunit contact site 35–48 and most of the second predicted α-subunit contact site 50–60. A second peptide was included, α51–65, to isolate this second site. Control peptides for this study were designed to correspond to the entire sequence of the α-subunit, and consisted of overlapping peptides (α-peptide 1–15, 11–27, 22–39, 61–78, and 73–92).

Experimental Assessment of Peptides. The ability of α-peptides to inhibit subunit reassociation was measured by using three techniques. Many spectral methods have been used to measure conformational changes in proteins and have been correlated with subunit recombination (Herskovits, 1967; Schmid, 1989). Specifically, difference spectroscopy has been used to monitor the unfolding and refolding of the glycoprotein hormones (Salesse et al., 1975; Ingham et al., 1976) as well as to monitor the dissociation and association of the subunits (Salesse et al., 1975; Ingham et al., 1976; Schmid, 1989). Spectral techniques are among the least perturbing of methods available (compared to chromatography, binding analyses, or other approaches), and we therefore attempted to use difference spectroscopy to monitor the binding of α-peptides to the β-subunit and induce a conformational change similar to the change seen for α-subunit binding to the β-subunit. While other means of obtaining a difference spectrum are possible (i.e., a polarity change), the bulk of the evidence strongly supports the existence of a conformational change (Garnier, 1978). No α-subunit peptide could produce a difference spectrum following incubation with β-subunit. This suggests that there is insufficient information in the peptide to cause the β-subunit to change conformation and that the peptide is incapable of yielding the difference spectrum. Since α-subunit would be incubated in the presence of α-peptide during dif-

ference spectroscopy measurements, it was necessary to determine if α-peptides could bind to α-subunit and cause changes in absorbance. Coincubation of α-peptides with α-subunit resulted in no change in the difference spectra for any α-peptide.

Difference spectroscopy measurements were made on the α-peptide–α-subunit–β-subunit mixtures in order to determine which of the α-subunit peptides could prevent or delay the development of a difference spectrum due to α–β interaction. When α-subunit was added to the peptide–β-subunit mixture and allowed to recombine with β-subunit for 24 h, a change in the difference spectrum was seen in the presence of the peptide α1–15. The difference was near 285 nm as expected, and suggested that α1–15 did not prevent the reassociation of lutropin subunits. Human α-peptides 1–15, 11–27, 22–39, 61–78, and 73–92 gave similar results. They did not prevent development of a difference spectrum (82–92% of the difference spectrum was obtained by 24-h incubation). These data suggest that α-peptides 1–15, 11–27, 22–39, 61–78, and 73–92 did not contain α-subunit contact sites for bovine lutropin.

However, a similar protocol using human α33–58 showed no change in the difference spectrum 24 h after α-subunit addition, suggesting α33–58 contained a subunit contact site. After 72 h, there was a significant change in the difference spectrum, suggesting that α33–58 could not prevent subunit recombination completely. By 72 h, 76% of the subunits had recombined. In addition, α51–65 was capable of decreasing subunit recombination: at 24 h, 61% of subunits had recombined in the presence of α51–65, and by 48 h, 90% of the subunits had recombined. The data may suggest that neither peptide contains the entire subunit contact surface and the correct conformation that would prevent recombination completely. However, these data suggest that α33–58 contains at least a portion of the α-subunit contact site and that additional binding determinants or structure were required to prevent subunit reassociation completely. Although these data are insufficient to provide a detailed kinetic analysis of the recombination process, they are consistent with the very high affinity of the subunits for each other. Peptide is in 5–20-fold molar excess and probably binds rapidly to β-subunit, but only weakly. When peptide does dissociate and α-subunit binds to β-subunit, this interaction becomes very strong and, for purposes of this experiment, irreversible.

Reverse-phase HPLC is an excellent method for measuring subunit recombination because of its ability to resolve proteins based upon their hydrophobicities. The net hydropathy value (NHV) is a measure of the hydropathy for an entire molecule based upon various hydropathy scales (Krystek et al., 1985a). The NHV for lutropin β-subunit is 12 and for lutropin α-subunit 153. The NHV would suggest, for lutropin, that the α-subunit is more hydrophilic than the β-subunit and that the α-subunit should elute from a reverse-phase system well before the β-subunit. This is the case, as demonstrated in Figure 2.

Table I displays a summary of the data obtained using reverse-phase HPLC to measure subunit recombination. α-Peptides 1–15, 11–27, 22–39, 61–78, and 73–92 did not prevent subunit recombination, in keeping with the results obtained from difference spectroscopy. α33–58 was capable of preventing subunit recombination at 24 h, but by 72 h, 67% of the subunits had recombined as assessed by HPLC, corresponding to 76% as assessed by difference spectroscopy. α51–65 prevented subunit recombination to some extent at 24 h, allowing 29% of the subunits to recombine, but by 48 h, 65% of the subunits had reassociated. This compares to 94% recombination seen by difference spectroscopy. Difference spectroscopy and reverse-phase HPLC confirm that α33–58 can prevent the initial stages of subunit recombination but, in time, significant recombination occurs. These data suggest that α51–65 may be capable of decreasing subunit recombination through interaction at a single contact site (or only a few such sites), while α33–58 may interact at multiple contact sites.

The data representing the recombination of lutropin subunits as measured by gel filtration chromatography are also presented in Table I. The α-peptides 1–15, 11–27, 22–39, and 73–92 did not prevent or decrease subunit recombination of lutropin subunits. α61–78 appeared to decrease subunit recombination slightly at 24 h, but significant recombination was achieved by 48 h. The decrease may be significant, but the first two methods used to measure lutropin subunit recombination did not detect any decrease in subunit recombination due to α61–78. However, results for follitropin subunit recombination (unpublished experiments) suggested that this peptide was capable of decreasing subunit recombination, so it is possible that α61–78 may contain a portion of the subunit contact site. α33–58 prevented lutropin subunit recombination at 24 h, but significant recombination was attained by 48 h. α51–65 also prevented subunit recombination, but by 48 h, full recombination was achieved. Therefore, all three methods used to measure subunit recombination suggest that α33–58 (which was predicted to contain two subunit contact sites) and α51–65 (which was predicted to contain one subunit contact site) were capable of preventing lutropin α- and β-subunit reassociation.

A comparison of the results for subunit recombination measured by difference spectroscopy, reverse-phase chromatography, and gel filtration chromatography shows that the two chromatographic methods report lower values of recombination. The lower values obtained by the chromatographic methods may be explained by the dissociation of the α–β complex, which may have occurred during chromatography. Chromatography of the intact hormone using acetonitrile gradients for reverse-phase HPLC can cause significant dissociation of the α–β complex (Parsons et al., 1984). Similarily, the use of 30% acetonitrile for the gel filtration measurements may cause some subunit dissociation.

A recent report by Salesse et al. (1990) suggested that α5–16 and α52–72 may contain contact sites. These authors used chorionic gonadotropin and monitored recombination using a binding assay in the presence of peptides. Salesse's assessment of α52–72 as a putative contact site is similar to our assessment of α51–65. Their identification of α5–16 as a subunit contact site is not in keeping with our assessment of α1–15, nor is our assessment of α33–58 (which we identify as a major contact site in lutropin) in keeping with their assessment of α33–59. Their discussion of the chemical modification information available on this region, and a proposed conformational change, seems to strengthen the argument that one might expect this peptide to be a contact site. In Salesse's study, recombination was not measured by direct, nonperturbing physical techniques, but by an indirect assay which measured inhibition of radiolabeled chorionic gonadotropin binding to receptor. In the present study, direct measurement of recombination by three physical techniques (at least one of which employs minimal perturbation of the system) yielded similar results. Agreement with anti-peptide topographic analysis was observed for the major putative contact site. Utilizing specific anti-peptide antisera, we recently reported on the topographical determinants of human follitropin

(Weiner et al., 1990). Antisera raised against α33–58 bound to α-subunit to a much greater extent than to heterodimeric follitropin. However, antisera raised against α1–15 bound tightly to α-subunit and weakly to intact follitropin. Further analysis will be required to elucidate why α1–15, which appears to be a subunit contact site by other criteria, did not inhibit subunit association under the conditions employed here.

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