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Interaction of S-Carboxymethylated Uteroglobin with Progesterone†

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ABSTRACT: S-Carboxymethylated uteroglobin, a small progesterone-binding globular protein, was studied by means of high-field proton magnetic resonance spectroscopy. Conformational changes induced by the steroid have been observed and indicate a well-defined rearrangement of the structure of the protein. The unusual stoichiometry of one progesterone to two uteroglobin dimers is confirmed by ^{1}H NMR. His-8 plays a central role in the mechanism of interaction of uteroglobin with progesterone. The pH dependence of affinity constant for the complexation of the steroid parallels the titration of this histidine. Although it is not a part of the active site, it influences a crucial conformational transition of the protein through the charge carried by its imidazole ring. Formation of a tetramer of uteroglobin subunits able to bind progesterone is critically compared to a kinetic scheme involving only dimers.

Uteroglobin is a small globular protein induced by progesterone and normally present in the rabbit uterus during the preimplantation phase of pregnancy. It is composed of two identical subunits of 70 amino acid residues each, held together by two disulfide bonds and other noncovalent interactions (Nieto et al., 1977). This protein, whose sequence has been recently established (Ponstingl et al., 1978), is able to bind progesterone and other progestins with high affinity and specificity (Beato & Baier, 1975). Steroid binding is dependent on the presence of reducing agents such as DTE[1] or DTT, i.e., on the reduction of the disulfide bonds that connect the two subunits of the protein (Beato, 1976; Beato et al., 1977). A recent ^{1}H NMR study of uteroglobin (Puigdoménech & Beato, 1977) has shown that reduction with DTT or DTE or carboxymethylation induces only some modifications of the tertiary (and/or secondary) structure of the protein but did not lead to denaturation (as it can be inferred from the persistence of some ring current shifted peaks).

The same preliminary ^{1}H NMR study shows that S-(carboxymethyl)uteroglobin (henceforth called SCU) is ideally suited for a study on the interactions with progestins. Indeed, SCU may be considered a good "model protein" for studies of ligand–protein interactions from many points of view. This protein has a low molecular weight (Nieto et al., 1977); unlike enzymes, it *does not* irreversibly modify the substrate upon binding (Fridlansky & Milgrom, 1976); it has very simple "windows" in its ^{1}H NMR spectrum (i.e., a clear aromatic region plus some other easily identifiable peaks) and a neat globular structure that is reflected by the presence of diagnostically valuable ring current shifted peaks.

The ^{1}H NMR study of the interaction of SCU with progesterone we present here, besides the general purpose of uncovering the main aspects of the mechanism of the interaction, has also the more "trivial" goal of performing an independent determination of the stoichiometry of the complex. Using two independent methods for measuring progesterone binding, we found an unusual stoichiometry of two uteroglobin molecules per molecule of progesterone, leading to complicated models for the binding reaction (Beato et al., 1977; Fridlansky & Milgrom, 1976).

Materials and Methods

Isolation. Uteroglobin was purified from the uterine flushes of rabbits treated sequentially with estradiol and progesterone, according to a previously described procedure (Nieto et al., 1977).

Reduction was achieved by incubation of 5 mg of protein in 1 mL of 10 mM DTT and 10 mM phosphate, pH 7.0, at 37 °C for 15 min. The sample was then dialyzed to achieve a final concentration of 1 mM DTT and 10 mM phosphate, pH 7.0. Carboxymethylation was carried out as described (Ponstingl et al., 1978; Nieto et al., 1977).

Sample Preparation. The lyophilized powder of the carboxymethylated protein was dissolved in 99.75% ^2H_2O, left at 37 °C for 1 h, and lyophilized again. Protein samples of 10 mg/mL were used in ^{1}H NMR experiments, dissolving 4 mg of protein in 0.4 mL of ^2H_2O, 10 mM KH_2PO_4, and 80 mM NaCl. The actual protein concentration was checked by means of UV spectroscopy, using a double-beam Cary 14 spectrometer and a molar extinction coefficient of 2800 at 280 nm. The progesterone solution used for protein titrations had a progesterone concentration of 4.96 mM in 99.5% $C^2H_3O^2H$. Aliquots of added progesterone were measured by weighing. Progesterone, DSS, 99% ^{2}HCl, 99% NaO^2H, 99.5% $C^2H_3O^2H$, and 99.75% ^2H_2O were purchased from Merck, Milano. NaCl and KH_2PO_4 were purchased from C. Erba, Milano. The 5-mm Royal Imperial tubes were purchased from Wilmad, New Jersey.

^{1}H NMR Spectra. ^{1}H NMR spectra were run on a Bruker WH-270 spectrometer, operating in the Fourier transform mode and equipped with a 32K 24 bit Aspect 2000 data system. The sample temperature was 29 ± 1 °C. For each spectrum ~2000 transients were accumulated by using 8K or 16K data points. Solvent suppression was achieved by means of gated decoupling.

Various methods of resolution enhancement were used, as appropriate, the two main techniques being those of Campbell et al. (1973) and of De Marco & Wüthrich (1976). pH measurements were performed on a Radiometer digital pH meter equipped with an Ingold combined microelectrode. No correction was made for deuterium isotopic effect in pH measurements. The pK of histidine was calculated by using the limiting chemical shifts at high and low pH values to

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[1] Abbreviations used: ^{1}H NMR, proton magnetic resonance; DTE, dithioerythritol; DTT, dithiothreitol; SCU, dimers of S-carboxymethylated uteroglobin (M_r ~15 000); DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; P, progesterone.

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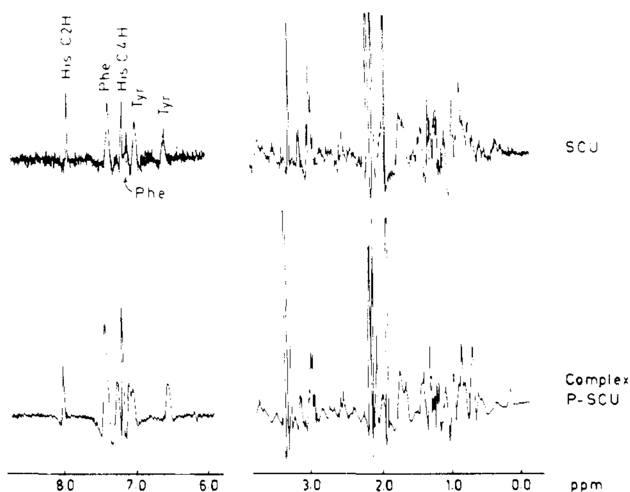


FIGURE 1: 270-MHz ^{1}H NMR spectra of S-carboxymethylated uteroglobin (SCU) and of the complex progesterone–SCU. Both samples were ^2H_2O solutions of 0.6 mM SCU, 10 mM KH_2PO_4, and 80 mM NaCl at pH 7.8. The chemical shift scale is in parts per million, referred to internal DSS (not shown). Both spectra were treated in the same way with techniques of resolution enhancement, so that relative apparent areas may not correspond to composition. The vertical scales of aromatic and aliphatic regions are different.

Table I: Amino Acid Sequence of the Subunit of Uteroglobin (Ponstingl et al., 1978)

Gly-Ile-Cys-Pro-Arg-Phe-Ala-His-Val-Ile-
Glu-Asn-Leu-Leu-Leu-Gly-Thr-Pro-Ser-Ser-
20
Tyr-Glu-Thr-Ser-Leu-Lys-Glu-Phe-Glu-Pro-
Asp-Asp-Thr-Met-Lys-Asp-Ala-Gly-Met-Gln-
40
Met-Lys-Lys-Val-Leu-Asp-Ser-Leu-Pro-Gln-
Thr-Thr-Arg-Glu-Asn-Ile-Met-Lys-Leu-Thr-
60
Gln-Lys-Ile-Val-Lys-Ser-Pro-Leu-Cys-Met
70

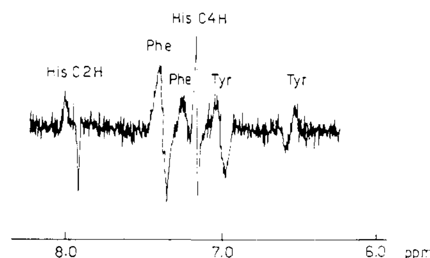


FIGURE 2: Difference spectrum between the complex progesterone–SCU and SCU at pH 7.8 in the aromatic region. Tentative assignments of the peaks are shown.

evaluate the fractional populations of discharged and charged forms, respectively (Handloser et al., 1973). DSS was used as the internal chemical shift reference. Integration of C2H histidine ^{1}H NMR peaks was made by weighing several replicas of the peaks cut from an enlarged section of the aromatic region; such a technique is more reliable than electronic integration for small closely spaced peaks.

Progesterone Binding. S-Carboxymethylated uteroglobin was dissolved in 10–20 mM sodium phosphate buffers of various pH values, containing 0.1 M NaCl. Apparent association constants and stoichiometries for progesterone binding were first calculated with the aid of Scatchard plots (Scatchard, 1949) from spectrophotometric data collected according to the procedure of Beato et al. (1977). Final values of the association constants were derived from linear least-squares adjustment of the data, based on the equation

$$\left(\frac{[P]_{bound}}{[P]_{free}}\right)^{1/2} = K^{1/2}([SCU]_{total} - 2[P]_{bound})$$

Gel Filtration. S-Carboxymethylated uteroglobin was dissolved in Tris–progesterone buffer (20 mM Tris, pH 7.5; 0.1 M NaCl; 5 mM progesterone) and incubated at 0 °C for 15 min. After addition of cytochrome *c*, an internal marker, the sample was applied to a column of Sephadex G-50 equilibrated and eluted with the same buffer. The position of carboxymethylated uteroglobin was determined by measuring the absorbance at 230 nm, and that of cytochrome *c* was determined by following the absorbance at 405 nm.

The progesterone-binding capacity was measured in 45 μL aliquots of each fraction of incubation at 0 °C for 60 min with 0.1 mM [^{3}H]progesterone (sp act. 20 Ci/mmol), followed by adsorption of the free steroid to charcoal (Beato, 1976).

The column was calibrated in a parallel run with ovalbumin, chymotrypsinogen, and cytochrome *c*.

Results

High pH ^{1}H NMR Spectra. Exploratory spectra were run of both native and S-carboxymethylated uteroglobin with and without progesterone, under various experimental conditions.

Addition of progesterone to solutions of native uteroglobin, in its oxidized state, does not induce significant spectral changes. The ^{1}H NMR spectrum of native uteroglobin we observe is in fact identical with that already published by Puigdomênech & Beato (1977). On the other hand, the spectra of SCU complexed with progesterone show modifications of the protein resonances of potential diagnostic value.

Figure 1 shows a comparison between the ^{1}H NMR spectra of SCU and of the system obtained by saturation of SCU with progesterone, both at pH 7.8.

The binding of progesterone induces many spectral changes; they may be either direct for protons of residues involved in the active site or indirect from changes in the three-dimensional structure of the protein. The clearest indication of the existence of a definite tertiary structure in a protein is probably furnished by the presence of ring current shifted peaks in its ^{1}H NMR spectrum. Such is indeed the case for SCU, as already described in the quoted preliminary work (Puigdomênech & Beato, 1977).

A comparison of the upper high-field regions at pH 7.8 shows that saturation of the protein with progesterone causes a (nearly) complete disappearance of the peaks at 0.360 ppm and at 0.662 ppm; at the same time, however, new peaks appear at 0.147, 0.380, and 0.610 ppm, thus showing that the tertiary structure has been only modified. In fact, the appearance of ring current shifted peaks at an even higher field is sometimes regarded as an indication of a more stable globular structure. A more detailed interpretation of these changes rests necessarily on a possible (but difficult) assignment of the ring current shifted peaks to specific residues along the sequence of uteroglobin. On the other hand, most aromatic peaks can be assigned with some confidence to corresponding residues.

In particular, the presence of a single histidine residue per protein monomer (as illustrated by Table I) makes it trivial to assign the peak at 7.87 ppm, in the spectrum of free SCU, to the C2H proton of His-8.

Figure 2 shows the aromatic region of the difference spectrum between the systems SCU and progesterone-saturated SCU.

The assignments are consistent with the relative areas predictable from composition and with literature indications (Crane-Robinson et al., 1976).

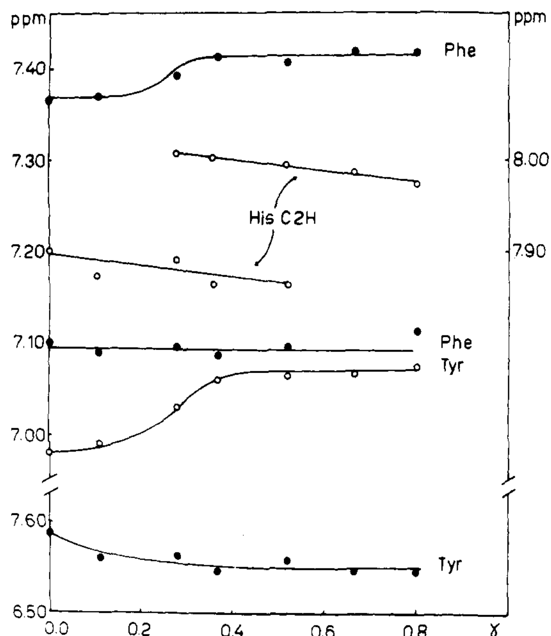


FIGURE 3: Variation of the chemical shifts of some aromatic peaks as a function of progesterone additions at pH 8.2. The amounts of progesterone are reported as fractions (γ) of the amount of SCU. The right-hand scale of the ordinates refers to the His C2H.

All peaks are somewhat shifted by the binding of progesterone, whereas in other regions of the spectrum the changes are more difficult to observe. Figure 2 also shows that the shifts of the aromatic resonances are both to high and low field, a behavior that can not be attributed to any conceivable bulk effect.

Chemical shift changes in the aromatic region were also monitored vs. small stepwise additions of progesterone with the aim of an accurate determination of the stoichiometry of the binding. These experiments were performed at a slightly higher pH value (namely, pH 8.2) in order to assure a safer pH interval with respect to the "low pH" experiments described later. Not all aromatic peaks are clearly identifiable at intermediate progesterone additions owing to superpositions and, occasionally, to signal to noise ratios not entirely satisfactory. Figure 3 shows the changes of the chemical shifts of five of these peaks as a function of progesterone concentration. The continuous curves can be used to yield the stoichiometry of the complex between progesterone and SCU.

All shifts reach an asymptotic value at a concentration of progesterone corresponding roughly to a ratio of 1 mol of progesterone to 2 mol of SCU. Further additions of progesterone are accompanied by the onset of a slight opalescence, as might be induced by incipient precipitation of the (sparingly soluble) progesterone.

At intermediate progesterone concentrations the ^{1}H NMR spectra present a striking phenomenon, i.e., the doubling of the His C2H resonance while other aromatic peaks only shift.

That is, addition of progesterone apparently causes the His residue to be "trapped" in a local conformation of long residence time while all other resonances of aromatic residues exchange rapidly between the limiting positions characteristic of SCU and of the progesterone–SCU complex. A lower limit of $\sim 5 \times 10^{-3}$ s for the residence time of His can be easily estimated from the separation of the two peaks ($\sim$35 Hz). Figure 4 shows this resonance for different amounts of progesterone added to the SCU solution. There is no change in position but only a decrease in area of the peak at 7.87 ppm, paralleled by an equivalent increase in the area of a new peak appeared at 8.00 ppm. That is, the two limiting conditions

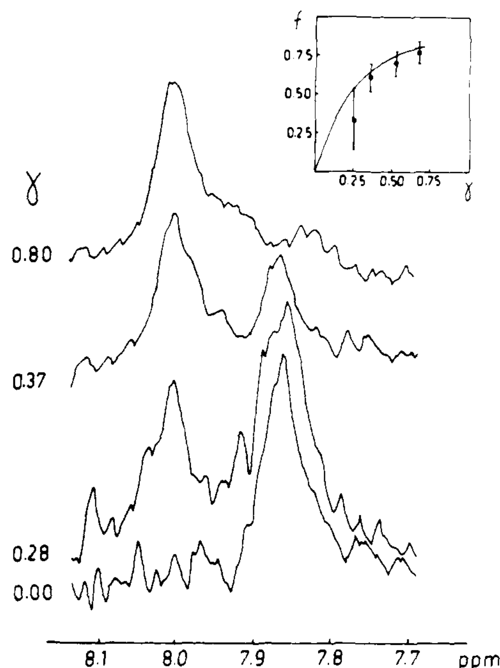


FIGURE 4: Line-shape changes of the C2H resonance of histidine at pH 8.2 as a function of addded progesterone amounts. The inset shows the fraction of complexed protein (f) as a function of the fractional amount of progesterone (γ), referred to the total SCU concentration. The continuous curve was calculated by using an association constant $K = 1 \times 10^8$ M^{-2}.

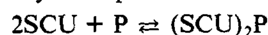
of free SCU and of progesterone-binding SCU are seen simultaneously by means of this resonance, whereas all other aromatic peaks reflect an averaged situation. In fact, when the progesterone added corresponds to a ratio (to SCU) much greater than 1:2, the area of the C2H resonance is entirely attributable to the peak at 8.00 ppm.

This circumstance, in turn, lends itself to another possible measurement of the stoichiometry of progesterone binding. The areas of the two peaks can be regarded as strictly proportional to the populations of protein molecules, with or without progesterone. The results of the integrations, performed by weighing, as described under Materials and Methods, are reported in the inset of Figure 4.

The ordinates (f) represent the fraction of complexed uteroglobin, calculated from the ratio of the area of the 8.00-ppm resonance to the total area of the C2H resonance. The abscissas are the concentrations of the added progesterone, reported directly as fractions (γ) of the total SCU concentration.

The data of Figure 4 are not compatible with a 1:1 stoichiometry, since even quantitative reaction between SCU and P would lead to fractions of complexed protein (f) consistently lower than any of the experimental points, well beyond experimental errors.

The fairly large errors connected with the integration of small ^{1}H NMR peaks and the limited number of experimental points prevent an accurate evaluation of the association constant. The fit obtained with a constant $K_{assoc} = 1 \times 10^8$ M^{-2} is nonetheless satisfactory as shown by the continuous line of the inset of Figure 4 whose points were calculated on the assumption that only the equilibrium



$$2SCU + P \rightleftarrows (SCU)_2P$$

exists in solution.

Histidine Titration. Histidine resonances are well suited for the determination of local pK values in proteins. We performed a titration of the His-8 residue of SCU in solutions

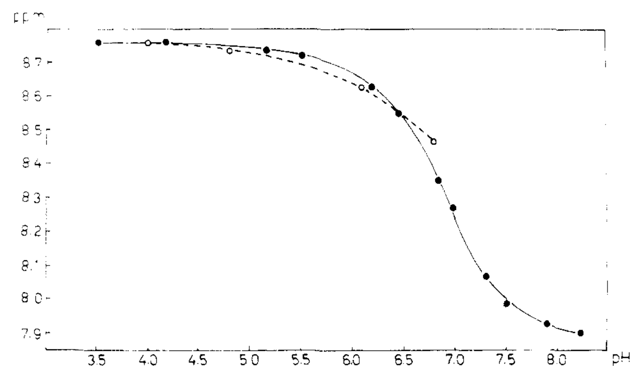


FIGURE 5: pH dependence of the chemical shifts of the histidine C2H resonance of free (●) and complexed (○) SCU. The continuous line was calculated from the equation $pK = pH + \log([His^+]/[His])$ by using the value of 6.95 for the pK and the limiting values of 8.75 and 7.90 ppm, respectively, for the chemical shifts of His^+ and of His (Handloser et al., 1973).

of both free and complexed protein. Figure 5 shows the variation of the chemical shift of the C2H resonance of free SCU as a function of pH. The curve is consistent with a pK_b of 6.95, a value similar to those found in many other proteins (Markley, 1974). Both this average value and the regular shape of the curve of Figure 5 are consistent with a histidine site open to the solvent. On the other hand, attempts to measure a pK_b in complexed SCU failed since the points in the alkaline region were not reproducible; more precisely, the C2H chemical shifts of complexed SCU show a marked dependence on previous history of the sample at high pH values. In fact, in the acidic range the chemical shifts of complexed SCU fall on the curve of Figure 5, but in the basic range they may be different in different experiments, depending on whether the actual pH was reached starting from a higher or lower value.

pH Influence on Binding. The behavior of the histidine resonance implies a pH dependence of the binding of progesterone to SCU. It was investigated by means of the spectrophotometric technique previously described (Beato et al., 1977).

Independently of the pH of the incubation, the number of "steroid binding sites" calculated from the Scatchard plots yields a stoichiometry of one molecule of progesterone per two uteroglobin dimers (M_r 15 000 each). These results confirm previous reports obtained by using Tris-HCl buffer or other binding assays (Beato et al., 1977; Fridlansky & Milgrom, 1976).

However, the Scatchard plots can only give a rough stoichiometry and "apparent association constants" in systems where the protein undergoes oligomerization in the formation of the complex (Tanford, 1963). That is, if the number of *independent* binding sites on the protein is less than 1, the association constants derived from the Scatchard plots, although useful for comparisons among different solutions, have little physical meaning. Accordingly, we calculated the association constants from plots of $([P]_{bound}/[P]_{free})^{1/2}$ vs. $[P]_{bound}$, i.e., under the assumption of an exact 1:2 stoichiometry. Figure 6 shows the pH dependence of the association constant of the binding of progesterone to SCU.

Optimal binding of progesterone was observed at pH 7.5; however, even at relatively extreme pH values (6.0 and 8.5) binding of lower affinity takes place.

Low pH ^1H NMR Spectra. The main features of the ^1H NMR spectra of free and complexed SCU at pH 7.1 are similar to those at pH 8.2, but for the histidine resonance.

Figure 7 shows the variation of the C2H resonance of His

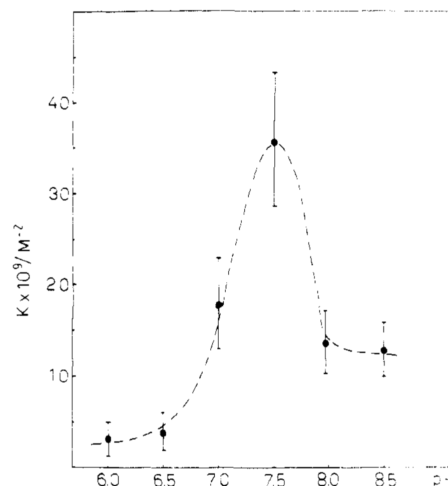


FIGURE 6: Influence of pH on progesterone binding to S-carboxymethylated uteroglobin. Solutions of SCU (0.66 mg/mL) were prepared in 10–20 mM phosphate buffers of different pH values, all containing 0.1 M NaCl. 1-μL aliquots of a concentrated solution of progesterone in ethanol were sequentially added, and the proportion of free and protein-bound steroid was determined from the quenching of the progesterone absorbance at 260 nm (Beato et al., 1977). The values of K, the association constant, were determined by means of a linear least-squares treatment of the data using the equation $([P]_{bound}/[P]_{free})^{1/2} = K^{1/2}([SCU]_{total} - 2[P]_{bound})$; they are plotted vs. pH values determined at the end of the incubation. All values represent the average of two series of experiments, carried out in duplicate. In one series, a 10 mM phosphate buffer was used and incubation was performed at 10 °C. In the second series, 20 mM phosphate buffer and 20 °C were employed. Separate experiments had previously shown that these changes in temperature and ionic strength do not significantly influence progesterone binding.

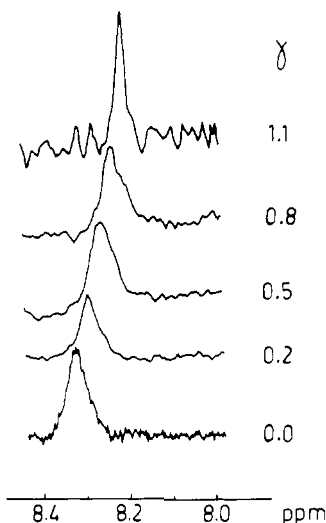


FIGURE 7: Line-shape changes of the histidine C2H resonance at pH 7.1 as a function of added progesterone expressed as fractions (γ) of total SCU. No doubling is observed, but the broadening observed around $\gamma = 0.5$ might be indicative of some chemical exchange contribution.

as a function of added progesterone at pH 7.1. It is seen that the peak remains single throughout all explored ranged *and* moves to high field. Thus, we can deduce that at lower pH values the environment of His in the system progesterone–SCU is probably different from that observed at high pH, and, furthermore, it exchanges rapidly with the corresponding site of free SCU. The stoichiometry of the protein–steroid complex at this pH can only be inferred from curves of chemical shift changes as a function of added progesterone.

Figure 8 shows these curves for several aromatic resonances. Only the curve corresponding to one of the Phe resonances has

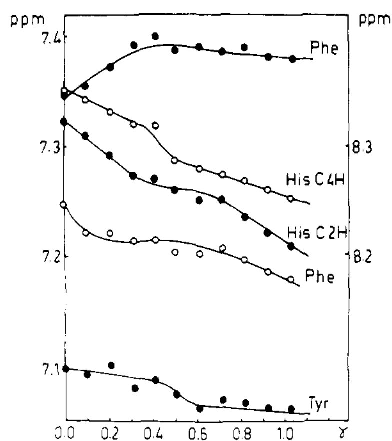


FIGURE 8: Chemical shift changes of the aromatic resonances vs. progesterone additions at pH 7.1. The amounts of progesterone are reported as fractions (γ) of total SCU. The chemical shift scale of the C2H resonance is on the right.

a behavior similar to that observed at pH 8.2 (see Figure 3); that is, it goes to low field until the ratio of progesterone to SCU reaches the value of 1:2 and remains more or less constant for higher ratios. All other aromatic chemical shifts reproduced in Figure 8 go to high field even for progesterone additions larger than that corresponding to the 1:2 ratio, yet they all show some sort of discontinuity (or even a clear inflection point) at a percentage corresponding to the 1:2 stoichiometry.

Another important difference at this pH is that precipitation of progesterone apparently only begins when the ratio to protein dimers reaches the value of 1:1.

These data suggest that at low pH values we have the coexistence of different progesterone-protein complexes in solution.

Gel Filtration. In previous reports we have shown that the complex of native uteroglobin and [^{3}H]progesterone elutes from Sephadex columns in a position corresponding to a molecular weight of 15 000, suggesting that the uteroglobin dimer is the steroid-binding species (Beato & Baier, 1975; Beato et al., 1977).

Since the stoichiometry of the binding (see above) is one molecule of steroid per two uteroglobin dimers, we repeated the gel filtration experiments under conditions that would facilitate the detection of an even unstable tetrameric complex. For this purpose, a column of Sephadex G-50 was equilibrated with Tris-HCl buffer containing 5 μM progesterone, prior to the chromatography of a sample of S-carboxymethylated uteroglobin equilibrated with progesterone. An analysis of the eluate from this column demonstrates that both the absorbance of the protein and the progesterone binding activity elute ahead of the internal marker cytochrome *c* in the position of the uteroglobin dimer (Figure 9). This experiment does not provide any direct indication for dissociation of the protein into the monomer (M_r 7500) nor for association into a tetramer (M_r 30 000).

However, it will be shown under Discussion that it is not in contradiction with any stoichiometry determination.

Discussion

The many spectral changes observed upon varying the pH or adding the ligand emphasize the suitability of SCU as the "model protein" for nuclear magnetic resonance studies of protein-steroid interactions. These changes include ring current shifted peaks which are the best probe for tertiary structure variations. Also quite noteworthy are the changes

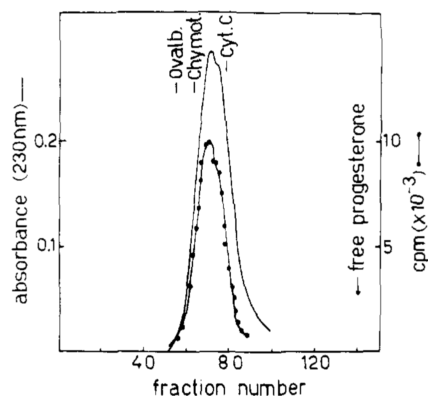


FIGURE 9: Column chromatography of SCU on Sephadex G-50 in the presence of progesterone. SCU (0.8 mg) was dissolved in 0.5 mL of Tris-saline buffer (20 mM Tris-HCl, pH 7.5; 0.1 M NaCl) containing 5 μM progesterone. After incubation at 0 °C for 15 min, 200 μg of cytochrome *c* was added, and the sample was applied to a Sephadex G-50 column (1.5 × 70 cm) equilibrated with Tris-saline buffer containing the same concentration of progesterone. Elution was carried out with the same buffer. The positions of the protein and the progesterone binding activity were determined as described under Materials and Methods. The position of the internal marker cytochrome *c* is shown, along with those of ovalbumin and of chymotrypsinogen and free progesterone, as determined in a parallel run.

in the aromatic region where *all* the aromatic resonances are influenced by progesterone complexation.

It is appropriate to note that all aromatic residues are located in a relatively small portion (between residues 6 and 28) of the sequence of uteroglobin, at the beginning of the N-terminal end of each subunit (Table I).

Such a circumstance may furnish in principle useful structural indications, also considering the very specific spectral changes induced by progesterone.

These considerations are liable to be further strengthened as soon as the three-dimensional structure in the solid state will be available, but a simple comment can already be made on the basis of the ^{1}H NMR spectra alone. For instance, the widening of the Tyr multiplet at pH 7.8 (Figure 2) is compatible with a well-defined structural change and not with an overall unspecific rearrangement. Even more interesting is the fact that the two Phe resonances shift toward opposite directions at pH 7.1 since it may be a strong indication that the two Phe residues are located in different environments. In particular, it seems fair to speculate that the resonance that has a similar behavior upon addition of progesterone at different pH values is the one further away from histidine along the sequence, i.e., Phe-28.

No resonances of the substrate could be identified with certainty, although, at the steroid concentration corresponding to full saturation, we expected to be able to spot the peaks of the three methyl groups.

The reasons for this may be a marked broadening of the progesterone resonances and/or a complete concealment under protein resonances. It is important to note that exchange broadening would imply a facile rearrangement of the steroid inside the protein. However, it is clear that sound information on the state of bound progesterone can only be gained through the use of labeled compounds. Work along this line is currently in progress in our laboratory.

Most of the information derived from the ^{1}H NMR study presented here stems from the behavior of His-8, the single histidine residue present in each SCU subunit.

Addition of progesterone (at alkaline pH values) causes a splitting of the C2H resonance of histidine into two peaks that can be assigned to free and complexed SCU. Besides giving

one of the various stoichiometry measurements, the peculiar doubling of the His C2H resonance (while other aromatic peaks only shift) provides a deep insight into the mechanism of progesterone binding. The doubling phenomenon is not entirely new; actually, it is reminiscent of the behavior of His-46 of staphylococcal nuclease (Markley et al., 1970) or of the His residues of neurophysin II (Cohen et al., 1972). In these cases titration of the protein is accompanied by splitting of the C2H signal, whereas we observe splitting at constant pH, on addition of the substrate.

The pH range in which we observe the rapid rise of 1:2 complexation coincides with the usual range of His titration in most peptides and proteins (Markley, 1974).

These circumstances concur to assign a central role in progesterone complexation to the single His residue of each SCU subunit. It would seem only reasonable to postulate the presence of His-8 in the active site of SCU.

This hypothesis, however, is not consistent with the long residence time of histidine. In fact, we observe a single C2H resonance for all four histidines of the 1:2 complex, a circumstance that it is nearly impossible to reconcile with the fact that the progesterone molecule ought to spend as much as 5×10^{-3} s in only one of the four subunits. This would render one of the four His residues markedly different from the other three, thus leading to three distinct peaks for the C2H resonance. Rather, it is more likely that the His be involved in a conformational transition.

It seems reasonable to think that both the long residence time of the His residue and the limited accessibility to solvent reflect its "trapping" in a rigid enclave. If this enclave is hydrophobic and/or very large, the His residue can occupy it only when the imidazole ring is not charged. Conversely, a charged His residue would prevent the entire subunit from assuming a conformation compatible with steroid binding.

Bearing in mind that the stoichiometry requires an identical behavior of the four peptide chains, it is admissible to correlate the sudden rise of activity between pH 6.5 and pH 7.5 with the simultaneous discharge of the imidazole rings of four His residues. While this paper was being written, A. Saavedra and M. Beato (unpublished experiments) have shown that histidine modification with diethyl pyrocarbonate prevents progesterone binding; on the other hand, SCU complexed to progesterone can still be modified at His-8. These findings show conclusively that His-8 is not part of the active site but is essential for the stability of the complex.

The gel filtration experiment shows that bound progesterone elutes with an apparent molecular weight corresponding to that of a uteroglobin dimer (M_r 15 000). This is in apparent contradiction with the 1:2 stoichiometry measured by ^{1}H NMR and by other techniques (Fridlansky & Milgrom, 1976; Beato et al., 1977).

It is only fair to say that the results of this experiment and of the several independent stoichiometry determinations are not mutually exclusive. The simplest, albeit trivial, rationale is that the conditions of the gel filtration experiment cause a shift of the multiple equilibria of our system toward the 1:1 complex. Let us imagine that it is possible to restrict the description of the system to the two mentioned 1:1 and 1:2 complexes. Their association constants will be defined by the relationships

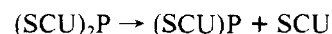
$$K_1 = \frac{[(\mathrm{SCU})\mathrm{P}]}{[(\mathrm{SCU})][\mathrm{P}]}$$

$$K_2 = \frac{[(\mathrm{SCU})_2\mathrm{P}]}{[(\mathrm{SCU})\mathrm{P}][(\mathrm{SCU})]} = \frac{[(\mathrm{SCU})_2\mathrm{P}]}{[(\mathrm{SCU})]^2[\mathrm{P}]}$$

The ratio between dimer and tetramer concentrations will depend both on the constant ratio and on the free protein concentration:

$$\frac{[(\mathrm{SCU})\mathrm{P}]}{[(\mathrm{SCU})_2\mathrm{P}]} = \frac{K_1}{K_2}[\mathrm{SCU}]$$

By the very nature of the gel filtration, SCU and (SCU)P molecules will be separated from the tetramers $(\mathrm{SCU})_2\mathrm{P}$. Thus, provided the kinetics of the dissociation process



$$(\mathrm{SCU})_2\mathrm{P} \rightarrow (\mathrm{SCU})\mathrm{P} + \mathrm{SCU}$$

is not extremely slow, the equilibrium (SCU) concentration will soon be so low as to favor quantitative dissociation even if K_1 is much smaller than K_2, as is probably the case at pH 7.5.

In fact, what the gel filtration experiment is telling us is that the 1:1 complex although thermodynamically less stable than the 1:2 complex is kinetically very stable with respect to the subunits of SCU (M_r 7500).

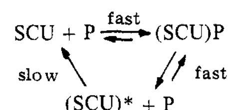
It is important to emphasize the complementary nature of the ^{1}H NMR with respect to spectrophotometric data. In fact, we are using two independent "windows" in the two experiments; i.e., when we measure the C2H resonance areas we are looking directly at concentrations of free and complexed protein, whereas the spectrophotometric data are related to concentrations of free and bound progesterone. In view of such a circumstance, the ^{1}H NMR result should be regarded as something more than a simple confirmation; rather, it is akin to a real "proof" of the 1:2 stoichiometry.

The only other alternative to explain the apparent discrepancy between the stoichiometry and the gel filtration experiment would be to consider the 1:2 stoichiometry as an artifact induced by the kinetics of the reaction.

The long correlation time of the C2H resonance is indeed compatible with the measurement of a relative amount of complexed protein higher than that required by the stoichiometry.

That is, when we measure the concentration of the complex from the area of the C2H resonance at lower field, we may be measuring also the concentration of protein molecules without progesterone but still in the same conformation of the complex (at least in that part of the molecule where the histidine residue is located).

According to the scheme



we would be measuring both (SCU)P *and* (SCU)* concentrations in the ^{1}H NMR experiments. On the other hand, the measurement of the same 1:2 apparent ratio from ligand concentrations requires that (SCU)* molecules react very slowly (or not at all) with progesterone to give (SCU)P.

In fact, when we monitor complexation by measuring free and bound ligand concentrations, the conditions required for simulating a stoichiometry higher than the true one are opposite than those required when measuring protein concentrations. That is, we measure an apparent higher stoichiometry from ligand concentrations only if part of the protein molecules are nonreactive, whereas we measure the same apparent stoichiometry from protein concentrations only if a fraction of protein molecules devoid of ligand appear as complexed.

Since the only direct measurement of the molecular weight of the complex (i.e., gel filtration) can be easily reconciled with the 1:2 stoichiometry, it seems reasonable to consider this

stoichiometry as a true one at the moment.

The rather complicated kinetic scheme required to simulate the 1:2 ratio with a true 1:1 stoichiometry will be fully justified only if we fail to observe the tetramer with any direct measurement in equilibrium conditions.

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Characterization of the Slowly Dissociable Human Growth Hormone Binding Component of Isolated Rat Hepatocytes†

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ABSTRACT: Human growth hormone (hGH) bound to specific sites on rat hepatocytes. The time course of hGH dissociation was comprised of more than one component. Dissociation was resolved into rapid ($t_{1/2}$ = 10.5 min) and slow ($t_{1/2}$ = 6.4 h) fractions. The amount of slowly dissociable hormone increased for the first 75 min during which time cells and [^{125}I]hGH associated. Subsequently, the amount of slowly dissociable hGH was constant. The time courses of hGH receptor binding and subsequent retention of slowly dissociable label were similar. The capacity of hepatocytes to accumulate slowly dissociable label was saturated by hGH over the same concentration range as the high-affinity binding site ($K_D \approx 2$ nM). This suggested that a receptor-mediated process was responsible for the accumulation of slowly dissociable hGH. Rapidly dissociable label was intact [^{125}I]hGH and fragments resulting from growth hormone degradation. Slowly dissociable hGH recovered from hepatocytes by acid extraction was intact and immunocompetent. There was a large increase in the extent of [^{125}I]hGH degradation between 23 and 37 °C. Over this temperature range, the proportion of hGH not in rapid equilibrium with the medium decreased. High concentrations of hGH decreased the amount of slowly dissociable [^{125}I]hGH retained by hepatocytes by competing for high-affinity sites. The interaction of [^{125}I]hGH with low-affinity degradative systems was favored by the presence of hGH. The temperature and concentration dependencies of hGH retention and degradation distinguished these processes.

Mechanistic descriptions of peptide hormone–receptor binding and subsequent cellular response have assumed that bound hormone was free to dissociate rapidly to the medium (Cuatrecasas, 1974; Kahn, 1976). Isolated rat hepatocytes accumulate a slowly dissociable human growth hormone (hGH)[1] binding fraction with increasing incubation time (Donner et al., 1978a). Some characteristics of the accumulation and retention of insulin (unpublished experiments) and glucagon (Martin et al., 1978a,b) are similar to those of hGH. This suggested that rapidly reversible equilibrium binding may not entirely describe peptide hormone–receptor interactions.

Retention of intact, receptor-bound epidermal growth factor (Schecter et al., 1978) and thyrotropin (De Rubertis et al., 1975) was required for persistent cellular response to hormone. Therefore, a first step toward evaluating the ultimate significance of cellularly retained hGH was to determine whether this fraction of bound label was intact and receptor bound. The slowly dissociable hGH binding fraction on hepatocytes

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[1] Abbreviations used: hGH, human growth hormone; [^{125}I]hGH, iodine-125-labeled human growth hormone; HBSS, Hank's balanced salt solution; BSA, bovine serum albumin, fraction V; Cl_3AcOH, trichloroacetic acid.

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