Solvent Exposure of Aromatic Groups in Human Luteinizing Hormone and Its Subunits. Measurement by Solvent Perturbation Difference Spectroscopy†

William H. Bishop* and Robert J. Ryan

ABSTRACT: Recent observations with a variety of methods have suggested that, in the process of association of the gonadotropin subunits to form the native hormone, there is a change in the microenvironment of some aromatic side chains. It is, therefore, of interest to quantitate these net changes under well-defined equilibrium conditions. The split-beam, dual tandem (four cell), solvent perturbation spectroscopic method was applied to native human luteinizing hormone (hLH) and to each of its subunits, α and β. The perturbing solutions were 20% sucrose and 20% dimethyl sulfoxide. Difference absorption spectra for the proteins, taken in the 250–310-nm range, were compared with similar spectra for small model compounds containing the tyrosine and tryptophan chromophores. Under the usual assumption that model spectra represent complete exposure of chromophore to solvents, estimates were made of fractional exposure of aromatic groups in free subunits and native hormone. Spectra taken in dimethyl sulfoxide showed behavior suggestive of a differential solvation of the proteins by the solution components but were qualitatively completely consistent with the sucrose perturbation data. Based on the sucrose perturbations, all four tyrosines of hLH-α and both tyrosines of hLH-β are solvent exposed while the single tryptophan of hLH-β has 0.6 fractional exposure. The native hLH molecule has four of the six tyrosines and 0.6 of the tryptophan residue exposed. An approximate estimate of the entropy of transfer, at 25°, of two tyrosyl groups from an aqueous to a nonpolar environment (i.e., the favorable hydrophobic interaction entropy) is similar in magnitude and opposite in sign to an equally approximate estimate of the unfavorable entropy change when the relative motions of free subunits become restricted to those characteristic of subunits in the native hormone.

Since the initial demonstration that the gonadotropins, luteinizing hormone (LH),[1] follicle stimulating hormone (FSH), and human chorionic gonadotropin (hCG), as well as the thyrotropic hormone (TSH), are protein molecules having a quaternary structure of two nonidentical subunits, much chemical, immunochemical, and physicochemical work has been directed toward attempting to understand two additional important findings. These are: (1) neither of the subunits alone possess intrinsic biological activity in standard biologic assays; and (2) one of the subunits, approximately one-half the mass of the intact hormone, is identical, or nearly so, among the four hormones mentioned. Therefore, a problem of major interest in this work has been in defining the nature of the intersubunit and intrasubunit interactions which result in an association of the subunits to form the biologically active quaternary structure.

Specifically, and as only a part of this large general problem, several recent studies have emphasized the behavior of aromatic side chains located on both the α and β subunits of the gonadotropins. Chemical modification procedures (Sairam et al., 1972; Cheng and Pierce, 1972; Liu et al., 1974) and various spectroscopic methods (Mori and Hollands, 1971; Bewley et al., 1972; Combarnous and Maghuin-Rogister, 1974; Bishop and Ryan, 1974; Puett et al., 1974) have suggested that the character of the immediate environment of some of these aromatic side chains may be dependent on whether the subunits are associated or not. Additionally, comparisons of the amino acid sequences of these several hormones, particularly in the β subunits, suggest that these aromatic residues are in homologous positions.

The observation (Bishop and Ryan, 1974), using dual-wavelength mode spectroscopy, that the ultraviolet absorption spectrum of a mixture of α and β subunits of hLH (under conditions allowing reassociation) showed time-dependent red shifts, suggested that the techniques of split-beam mode difference spectroscopy might be used to quantitate the recombination process. In the present study the solvent perturbation methods of Herskovits and Laskowski (1962) were applied to the native hormone and to each subunit to estimate in the three cases the average numbers of tyrosine and tryptophan side chains exposed to the aqueous phase under conditions of neutral pH and constant temperature. The assumption has been made that the conformations of the three molecules are in states of thermodynamic equilibrium with the aqueous perturbant solutions and that these states of conformational equilibrium are identical or, more likely, very similar to those found in aqueous phases without perturbant. Based on these assumptions, the experimentally derived solvent exposure data were used to estimate certain relevant thermodynamic parameters for the subunit association process.

Experimental Section

Materials. The sucrose and dimethyl sulfoxide (DMSO) used were Schwarz/Mann products of Spectroscopic grade.

† From the Department of Molecular Medicine, Mayo Clinic and Mayo Foundation, Rochester, Minnesota 55901. *Received March 13, 1975.* Supported by funds from the National Institutes of Health (HD 03726 and HD 09140) and the Mayo Foundation.

[1] Abbreviations used are: hLH, human luteinizing hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone; hCG, human chorionic gonadotropin; TSH, thyrotropic hormone.

The spectroscopic standards employed were *N*-acetyl-L-tyrosine ethyl ester, a product of Schwarz/Mann, and *N*-acetyl-L-tryptophanamide, a Sigma product. Electrolytes and buffer salts were ACS Certified Fisher Chemicals. The water used was deionized and glass distilled.

Hormone and Subunits. Acetone-dried human pituitary glands were supplied by the National Pituitary Agency. Native luteinizing hormone was prepared from this starting material by the methods of Hartree (1966) and Ryan (1968) and had a biologic potency of 3 NIH-LH-S1 U/mg. The subunits of the hormone were separated and purified by the procedures of Ward et al. (1973). As a final step, immediately before weighing, all preparations were lyophilized from 0.10 *M* ammonium bicarbonate solution, then relyophilized twice from deionized glass-distilled water. Samples of the dried proteins, in the range 3–6 mg, were weighed to the nearest hundredth of a milligram on an EMB-1 (RIIC, London) electric microbalance, then dissolved in a known small volume of buffer. The quantitation of solvent-perturbed groups per protein molecule was made on the basis of this dry weight and the molecular weights given by Bishop and Ryan (1973). The appropriate dilutions from this stock solution were then made for both the base line setting runs and the difference spectral runs described below.

Methods. Spectroscopy. The general methods of split-beam solvent perturbation difference spectroscopy described by Herskovits (1967) were adapted for use with an American Instrument Company DW-2 spectrophotometer. A special cell housing for the dual tandem (four cell) arrangement was designed and constructed locally for use in combination with a Haake constant temperature circulation bath which was maintained at 25 ± 0.1° for all experiments.

The estimates of protein side chain exposure were based on perturbation spectra for the model compounds acetyltyrosine ethyl ester and acetyltryptophanamide where it was assumed that "full exposure" was observed. The description which follows pertains to both the protein solutions and the model standard solutions.

For both the base line setting procedure and the experimental runs the dual tandem cell arrangement, employing four matched quartz cells (Savant product), was used. Due to the small volumes used and possible systematic dilution errors, the same set of micropipets was employed for preparing solutions in both the reference (unperturbed) beam and the sample (perturbed) beam. For each experiment a stable, flat base line was established, using a bank of 31 trimpots, in the wavelength range 250–310 nm. Since the absolute spectral changes were small, the 0–0.1 full scale expansion was used for base-line runs as well as experimental runs. The base-line setting was accomplished with protein (or standard), at a particular concentration in buffer, placed in both sample and reference beams while the blank cell in each beam was filled with perturbant at the proper concentration. To produce the perturbation difference spectrum, the perturbant in the sample beam was moved into the protein (or standard)-containing cell while keeping the total concentration of all components in both beams constant at their values during the base-line setting. During the transfer, dilution, and mixing manipulations, between base-line run and perturbation run, care was taken not to disturb the alignment of cells and housing with respect to the incident beams.

Though these procedures are essentially nondestructive, in the interest of conserving the valuable supplies of hormone and subunits, small quartz cells were used having a path length 1.0 cm, and width of 4 mm and in which the sample volume was 0.9 ml. Under these conditions, it was found necessary to attenuate the incident beams by the use of masks. The masks consisted of 4-mm wide slits cut in small, black Bakelite plastic plates which were placed on the light source end of the cell housing directly adjacent to, and properly aligned with, the cells. If the masks were not used, small amounts of light passing through the quartz sides (but not through chromophore solution) reached the photocell and deviations from Beer's law were found. The masking procedure reduced the total intensity reaching the photocell and, if the instrument was operated with the dynode voltage selector in the "Auto" mode, the reduction in intensity was compensated by an automatic gain in dynode voltage. This in turn had the effect of magnifying noise to troublesome levels if too narrow a spectral band width was employed. Since the wishes to conserve material, reduce noise, and achieve maximum resolution were not completely compatible aims, the compromise arrangement of masked 4-mm wide cells, dynode selector on "Auto" mode, and 30-Å spectral band width was adopted. With these choices, it was found that, within the absorbance and wavelength ranges for these experiments, Beer's law was obeyed in both direct and difference modes, i.e., these parameters were linear functions of chromophore concentration. This 30-Å band width was larger than that recommended by Herskovits and Sorensen (1968a), for their Cary 14 spectrophotometer, and it is possible that we have sacrificed at least some spectral resolution in using it. However, the resolution obtained for our estimated exposed Tyr/Trp ratios compares quite favorably with that found for protein mixtures with similar known exposed Tyr/Trp ratios (Herskovits and Sorensen, 1968b).

The final solvent conditions were 0.05 *M* phosphate (total), 0.10 *M* NaCl, and pH 6.95. Final perturbant concentrations were 20% w/v sucrose and 20% v/v DMSO. The initial scans were taken 5 min after diluting, mixing, and capping the cells. Repeated scans over 30 min were taken and the difference spectra were found to be stable. The protein solutions with buffer and with buffer plus perturbant remained clear.

Results

We used *N*-acetyl-L-tyrosine ethyl ester as spectral standard for the tyrosyl chromophore. This compound was also used by Herskovits and Sorensen (1968a,b). We found good agreement with their data regarding the essential spectral parameters (the difference extinction coefficients and the wavelengths of difference maxima) with these parameters, of course, being functions of the particular perturbant. *N*-Acetyl-L-tryptophanamide was employed as standard for the tryptophyl chromophore in the present work while Herskovits and Sorensen (1968a,b) used the ethyl ester. Only minor qualitative and quantitative differences were found between the difference spectra for the two compounds so that the same fractional exposure for the chromophore in the proteins would be estimated using either small molecule as the model for complete tryptophyl exposure.

Figures 1 and 2 show the perturbation difference spectra using, respectively, sucrose and DMSO as perturbants. These figures are reproductions of tracings of the spectra taken directly from the instrument chart paper and are, therefore, presented as experimentally observed difference

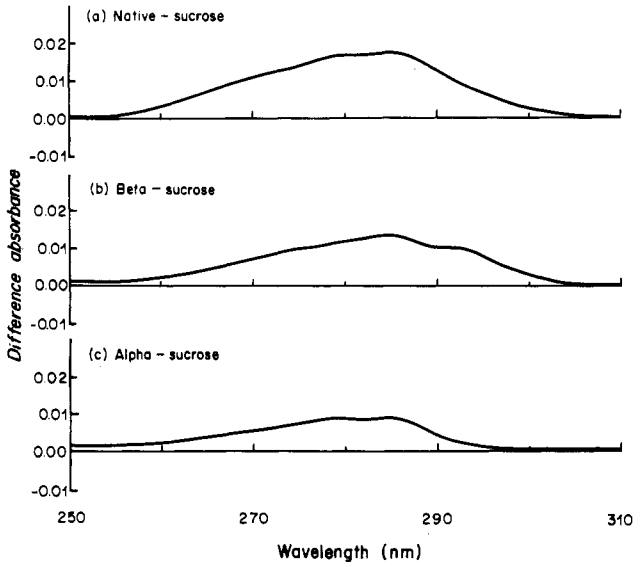

FIGURE 1: Perturbation difference spectra of human luteinizing hormone and its subunits in 20% (w/v) sucrose. Protein concentrations were: native, 2.07 mg/ml; β subunit, 1.19 mg/ml; α subunit, 1.04 mg/ml. The solvent was 0.10 *M* NaCl-0.05 *M* phosphate (pH 6.95) and the temperature was 25 ± 0.1°.

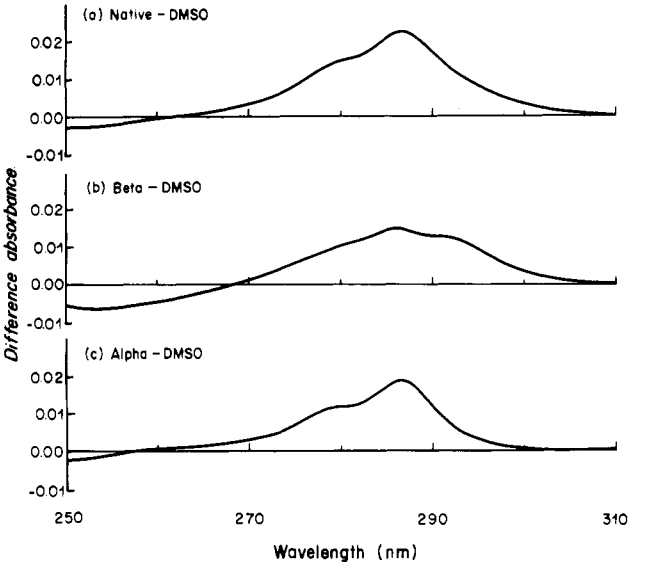

FIGURE 2: Perturbation difference spectra of human luteinizing hormone and its subunits in 20% (v/v) dimethyl sulfoxide (DMSO). Protein concentrations were: native, 0.98 mg/ml; β subunit, 0.57 mg/ml; α subunit, 0.54 mg/ml. The solvent was 0.10 *M* NaCl-0.05 *M* phosphate (pH 6.95) and the temperature was 25 ± 0.1°.

Table I: Summary of Group Exposure Data for hLH, hLH-α, and hLH-β.[a]

Molecule	20% (w/v) Sucrose: Apparent No. of Groups Perturbed per Protein Molecule	Total No. of Groups per Protein Molecule	20% (v/v) Dimethyl Sulfoxide: Apparent No. of Groups Perturbed per Protein Molecule
Native	4.10 Tyr	6 Tyr	2.60 Tyr
	0.57 Trp	1 Trp	0.55 Trp
β	2.00 Tyr	2 Tyr	1.35 Tyr
	0.55 Trp	1 Trp	0.55 Trp
α	3.70 Tyr	4 Tyr	2.20 Tyr
	0.00 Trp	0 Trp	0.00 Trp

[a] Apparent numbers of tryptophyls and tyrosyls perturbed are the coefficients *a* and *b*, respectively, in eq 1 and 2.

Table II: Comparison of Observed and Calculated Perturbation Difference Absorbance, ΔA, of hLH-β in 20% Sucrose.

λ (nm)	ΔA(obsd)[a] − ΔA(calcd)[b]
262	0.0018
266	0.0020
270	0.0017
274	0.0010
278	<0.0003
282	<0.0003
284	<0.0003
286	0.0000
290	0.0010
292	0.0000
296	<0.0003
300	0.0000
304	0.0000

[a] Figure 1b. [b] Computed on basis of numbers of exposed Tyr and Trp groups from Table I, molar concentration of protein in Figure 1b, and molar difference extinction coefficients of model chromophores discussed in text.

absorbance instead of as molar difference extinction coefficients (which are calculated quantities). The total absorbance, to be compared to the difference absorbances observed, was kept in the approximate range 0.5–1.0.

The group exposure data shown in Table I were derived with the use of the equations discussed by Herskovits (1967).

$$\Delta\epsilon_{292\text{-}294}(\text{protein}) = a\Delta\epsilon_{292\text{-}294}(\text{Trp}) + b\Delta\epsilon_{292\text{-}294}(\text{Tyr}) \quad (1)$$

$$\Delta\epsilon_{286\text{-}288}(\text{protein}) = a\Delta\epsilon_{286\text{-}288}(\text{Trp}) + b\Delta\epsilon_{286\text{-}288}(\text{Tyr}) \quad (2)$$

In these equations the $\Delta\epsilon_\lambda$ are the molar difference extinction coefficients, at the wavelengths designated in the subscript, for the model standards or for the protein of interest. The coefficients *a* and *b* are the apparent numbers, per protein molecule, of tryptophyl and tyrosyl chromophores, respectively, which are exposed to the solvent. These coefficients are derived by use of the iterative methods described by Herskovits and Sorenson (1968b).

An implicit assumption in these calculations is that only tyrosine and tryptophan contribute to the perturbation spectrum of the protein near the chosen wavelengths. This assumption was examined by calculating difference spectra for the proteins from (1) the derived *a* and *b* of Table I and from (2) the $\Delta\epsilon_\lambda$, over a larger wavelength range, taken from the model compound spectral runs. A representative result, reported in Table II as the difference between observed (Figure 1b) and calculated difference absorbance, supports the original assumption that the protein difference spectra in the wavelength range 275–305 nm are due to tyrosine and tryptophan. Below 275 nm it seems clear that perturbation effects of other protein chromophores are also being observed.

The immediate and important observation from Table I is that the sum of the numbers of exposed tyrosyl groups in the separated subunits is greater than the number exposed in the native molecule. Further, the fractional exposure, about 0.6, of the single Trp side chain of the β subunit does

not change (by the criteria of perturbation spectroscopy) when the subunits associate.

The differences, for Tyr, between the numbers derived from sucrose perturbation and from DMSO perturbation seem systematic in that, for all three protein molecules, the ratio of groups exposed in sucrose to groups exposed in DMSO has nearly the same value. The observed behavior suggests the presence of differential solvation effects for the proteins in DMSO, a phenomenon discussed by Donovan (1969). That is, the local composition of the perturbing solution near the protein surface is not the same as its macroscopic composition. It is not clear why this effect should be so dramatic for the tyrosyl residues and only barely suggested for the tryptophyl residue.

Systematic experiments to obtain difference absorption as a linear function of perturbant concentration were not performed. These data would help ensure that the differing tyrosyl exposures using sucrose and DMSO, respectively, were not related to conformational changes induced by perturbant (Herskovits, 1967). However, earlier work (Bishop and Ryan, 1973), in which hLH and its subunits were centrifuged in 5–27% sucrose gradients, gave no indication of such behavior. Further, if some of the tyrosyls were located in protein surface crevices, perturbants of different mean diameters could yield differing degrees of exposure (Herskovits, 1967). Sucrose and DMSO have mean diameters of 9.4 and 4.0 Å, respectively. Therefore, the observed differences in exposure estimates are in a direction opposite to that expected if some tyrosyl residues were in surface crevices.

Discussion

For what follows, it may be helpful to review the numbers and locations of the tyrosyl residues in hLH, as well as hCG and ovine (o), bovine (b), and porcine (p) LH. hLH-α and hCG-α have four Tyr while hLH-β has two and hCG-β has three; o, b, and p-LH-α have five Tyr while o, b, and p-LH-β have two.

Several reports on chemical modification of ovine or bovine LH and their subunits are relevant to the present work (Sairam et al., 1972; Cheng and Pierce, 1972; Liu et al., 1974). While there is lack of detailed agreement as to the numbers and locations of tyrosines modified by tetranitromethane in LH and its subunits, there does seem to be agreement that the total number of tyrosine residues reacting with the reagent when the subunits are separated is about two more than when they are associated in the native hormone. Consistent with these observations, Yang and Ward (1972) found that iodination of native o-LH occurred only on the α subunit while both subunits reacted when separated, with the iodine introduced in rough proportion to the numbers of tyrosines on each subunit.

The hydrogen ion titration data for hCG (Mori and Hollands, 1971) and for porcine and bovine LH (Combarnous and Maghuin-Rogister, 1974) also demonstrate a reduced accessibility of some tyrosine side chains in the native molecule. Four of the seven tyrosyl groups of native hCG are reversibly titratable while all become available in concentrated guanidine hydrochloride solution. Five of the seven tyrosyl groups in native porcine or bovine LH titrate normally while all seven can be titrated in the isolated subunits. The solvent perturbation studies on hCG by Mori (1972) are consistent with the titrations in that four tyrosines in the native molecule can be solvent perturbed, two more become solvent exposed in concentrated urea solution, and the last becomes available in urea-mercaptoethanol solution.

If a macromolecule has a collection of closely related conformational states and these states are involved in a facile equilibrium among themselves and there is then introduced a kinetically irreversible step (chemical modification) or a stoichiometric conversion of an uncharged form to a charged form (titration) or a strongly solvating denaturant (urea), problems with estimating the degree of exposure of a group can potentially arise. This is especially true if the point of ultimate interest is whether, and how much, a change in average microenvironment of a particular group during some biochemical process contributes to the energetics of that process. The studies of Tulinsky et al. (1973) and Vandlen and Tulinsky (1973) on the tertiary structural variability of α-chymotrypsin under various conditions emphasize the potential mobility of surface or near surface groups. However, the results reported herein using dimethyl sulfoxide as a spectral perturbant indicate that, even in a situation where there is no reason to suspect significant conformational shifts (as with urea or guanidine), preferential solvation problems can complicate the interpretation (Donovan, 1969).

From these considerations it seems clear that neither the chemical methods, which *depend* on some net conversion, nor the perturbation spectroscopic methods, which must *assume* minimal disturbance of equilibria, are free from possible difficulties in interpretation. In spite of these problems, in the case of the gonadotropins, there is apparently rather good agreement between the two approaches in estimates of solvent exposure.

Because most of the data necessary for a systematic discussion of subunit–subunit interaction in the gonadotropins are not yet available, any attempt at a detailed interpretation is premature. However, in the interest of trying to place the present results in a more general perspective relative to other work, certain qualitative and rough quantitative points can be suggested. The following analysis is neither unique, in terms of interpretation, nor all-inclusive, in terms of completeness. It is, for the present purpose, only a device with which to examine certain aspects of gonadotropin structure and behavior.

With this intention, the standard free energy change, $\Delta F°$, for the process ($\alpha + \beta \rightarrow \alpha\beta$) will be separated into various components. It is to be emphasized, for this process, that the conditions of major interest would be an aqueous phase of dilute electrolyte near neutral pH and near room temperature and protein standard states of unit molarity:

$$\Delta F° = \Delta f_{es} + \Delta f_{conf} + \Delta f_{h\phi} - T\Delta s_{int} \qquad (3)$$

The contributions in this equation are the following. (1) The electrostatic component Δf_{es} which, as used here, is the free energy associated with changes in electrostatic (ionic and polar) interaction between the two subunits and between the subunits and all solvent species during the association process. (2) Δf_{conf} includes the changes, during association, in intrasubunit electrostatic, van der Waals, hydrophobic, and chain folding free energy. Δf_{conf} could, in the event of changes in peptide main chain or side chain conformation during the association process, contain significant intrasubunit entropic terms. (3) $\Delta f_{h\phi}$ is the free energy associated with changes in the degree of interaction between subunit surface nonpolar groups and the solvent water molecules at the regions of contact between the subunits. These hydrophobic contributions, as with those of a similar nature in Δf_{conf}, tend to be dominated by solvent entropy changes.

(4) Δs_{int} is the negative (unfavorable) intrinsic entropy change associated with reducing the relative translational and rotational mobility of the separated subunits to the relative mobility characteristic of subunits in the associated state.

Though at present quite fragmentary, there is experimental evidence to demonstrate that each of these free energy terms could contribute to the resultant affinity of subunit association. Gonadotropins tend to dissociate at reduced pH, presumably at least partly due to general electrostatic effects but possibly also involving titration of specific crucial groups. In at least one case (Aloj et al., 1973) the rate of subunit association is influenced by electrolyte concentration. These facts indicate that Δf_{es}, while pH dependent, may be significant. The results from several circular dichroism studies clearly show that changes in intrasubunit secondary and tertiary structural levels may accompany association (Jirgensons and Ward, 1970; Pernollet and Garnier, 1971; Bewley et al., 1972; Rathnam and Saxena, 1972; Puett et al., 1974). Though these laboratories are not all in agreement as to detailed interpretation and significance of these structural changes, the work demonstrates the necessity to consider the term Δf_{conf}.[2]

The data in Table I, combined with the earlier observations (Bishop and Ryan, 1974), indicate that, as a result of subunit association, about two neutral tyrosyl side chains get transferred from an aqueous to a nonpolar environment. This is a thermodynamically favorable process due principally to a solvent-related positive entropy change (Nemethy and Scheraga, 1962) and a rough estimate of +13 eu might be given as the entropy change, at 25°, for transferring the two tyrosyl groups. For the present, it is not clear whether this favorable hydrophobic free energy belongs in $\Delta f_{h\phi}$ or Δf_{conf}, however, the thermodynamic estimates would be about the same in either case.

The concepts developed by Steinberg and Scheraga (1963) may be used to calculate the quantity Δs_{int} approximately. If the more conservative limits of the intersubunit motions discussed by these authors are employed, then for molecules of the size of gonadotropin subunits, a value of −16 eu is found at 25°. It is emphasized that this estimated value is rather insensitive to the particular limits of motility used. The important point is that allowing even a modest range of relative subunit motion yields a Δs_{int} which, for subunit association, is much less thermodynamically unfavorable than that resulting from the formation of a completely rigid two-subunit quaternary structure.[3] Further, the favorable entropy yield discussed above, resulting from transferring the two tyrosyl groups, seems capable in approximate terms of almost compensating the unfavorable Δs_{int}. It is stressed, however, that such effects on the free energy of subunit association should be considered as acting in combination with the other contributions summarized in eq 3.

[2] Recent immunochemical work from this laboratory (Prentice and Ryan, 1975) also suggests conformational changes within the subunits resulting from their association. Antibody raised against isolated α subunit interacts with isolated α subunit with an affinity which differs from its affinity for α subunit in native LH. This result refers to data normalized to the same total mass of α subunit present in each reaction. Further, the affinity of this antibody for α subunit in native FSH and native TSH is different from that for native LH.

[3] Polarization of fluorescence measurements on hLH and its subunits, obtained recently in this laboratory (Bishop and Ryan, 1975), indicate that the subunits, when associated in the native hormone, are capable of a significant degree of relative motility.

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