

Thermodynamics of the self-association of glucagon

(hydrophobic)

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ABSTRACT In water, glucagon exists in an equilibrium between a trimer in which more than half of the peptide groups are in an α -helical configuration and a monomer which has a random coil configuration with few α -helical residues. The thermodynamics of this self-association have been evaluated by studying the temperature- and concentration-dependence of the mean residue ellipticity at 220 nm. The enthalpy and entropy changes of association were negative at all temperatures between 5° and 50° and had large negative temperature dependencies. Usually an association that involves nonpolar groups is considered to be driven by a positive entropy term. Such an explanation is not tenable in the case of glucagon. However, if the effects of nonpolar groups on the coil-to-helix transition of a polypeptide are included into the thermodynamic considerations of hydrophobic interactions, then the negative parameters observed for glucagon association can be readily understood. The hydrophobic interaction is therefore not necessarily controlled by the entropy change because, if there are significant conformational changes, the reaction may be controlled by the enthalpy change. Consequently, the more important parameter characteristic of all hydrophobic reactions is the heat capacity change.

Glucagon is a 29-residue polypeptide hormone that is bound to target tissues and activates adenyl cyclase. Treatment of liver plasma membranes with reagents that modify membrane structure (i.e., digitonin or phospholipase A) inhibits both glucagon binding and stimulation of adenylate cyclase activity (1). It has been suggested from studies of the binding of glucagon fragments that the hydrophobic region at the carboxyl end of the molecule is important for binding and activation of adenylate cyclase (2). Recent x-ray diffraction studies of glucagon crystals revealed the formation of a trimer with very strong hydrophobic interactions between the glucagon chains which are largely α -helical (3). In order to understand these interactions, which may play an important role in glucagon binding to its membrane receptor (4), we have evaluated the thermodynamic parameters of the self-association of glucagon. It has been shown recently that the binding of insulin to its membrane receptor in leukocytes involves strong hydrophobic interactions as shown by a large decrease in heat capacity (M. Waelbroeck, E. Van Obberghen, and P. De Meyts, personal communication).

A recent conformational analysis based on the sequence of glucagon suggests that glucagon can readily fold into different conformations (5). In very dilute solutions, glucagon is largely unfolded with few stable intramolecular bonds (6–9). With increasing concentration, in dilute alkali glucagon forms trimers that are highly α -helical and in acid it forms fibrils whose folding is mainly of β -structure (6). In certain organic solvents (ethylene or propylene glycol/water mixtures), glucagon folds

into a α -helical structure but does not associate (10). Glucagon also becomes more α -helical when bound to lipids—i.e., cationic detergents (11), phospholipid micelles (12), or bilayers (13).

We have evaluated the thermodynamic parameters of glucagon association between 5° and 50°. These should be of interest in understanding not only the interactions between glucagon molecules but also other reactions that depend on hydrophobic forces. Typical examples are certain self-assembly systems such as microtubules (14, 15) and flagellin (16).

MATERIALS AND METHODS

Crystalline glucagon was obtained from Elanco Products Co. (Division of Eli Lilly and Co.) for us by M. Rodbell (National Institutes of Health). Without further purification, it was dissolved in 0.2 M K_2HPO_4 , pH 10.6, at room temperature. The solution was adjusted to pH 10.6 with KOH and centrifuged in order to clarify it completely. The concentration of glucagon was determined by absorbance measurements at 278 nm and pH 10.6, using the molar specific absorbance of 8260 (i.e., $E_{1cm}^{1\%} = 23.7$) which was measured at pH 10.2 (8). Because the isosbestic point of tyrosyl ionization in glucagon occurs at 278 nm, we made the absorption measurements at the pH value of the experimental solutions. A Radiometer pH meter (model 26) was used for the pH measurements. Glass-distilled water was used throughout, and all chemicals were reagent grade.

A Cary model 60 spectropolarimeter, equipped with a temperature-controlled cell holder, was used to measure the ellipticities at 220 nm. Mean residue ellipticities were calculated by:

$$[\theta]_{\lambda} = MRW \theta_{obs} / 10lc \quad [1]$$

in which $[\theta]_{\lambda}$ is the mean residue ellipticity at a wavelength λ ; θ_{obs} is the observed ellipticity; MRW is the mean residue molecular weight (120 for glucagon); l is the optical path length in cm; and c is the concentration in g/ml.

Data analysis was accomplished with an on-line modeling program, MLAB (17, 18). This program utilizes the Marquardt–Levenberg algorithm to perform a least-squares fit of data to an arbitrary equation.

The ellipticity (at 220 nm) of glucagon was taken to be a weight average of the ellipticities of the monomeric and trimeric species. The molar association constants, at each temperature, and the ellipticities of the monomer and trimer were then evaluated by a simultaneous least-squares fit of the concentration- and temperature-dependence of the glucagon ellipticity. This involved the assumption that the total concentration of monomer could be expressed by a simple monomer \rightleftharpoons trimer equilibrium:

$$[Ct] = [Cm] + 3K_a[Cm]^3 \quad [2]$$

in which $[Ct]$ is the total concentration expressed in monomer units; $[Cm]$ is the free monomer concentration; and K_a is the molar trimerization constant. There have been conflicting in-

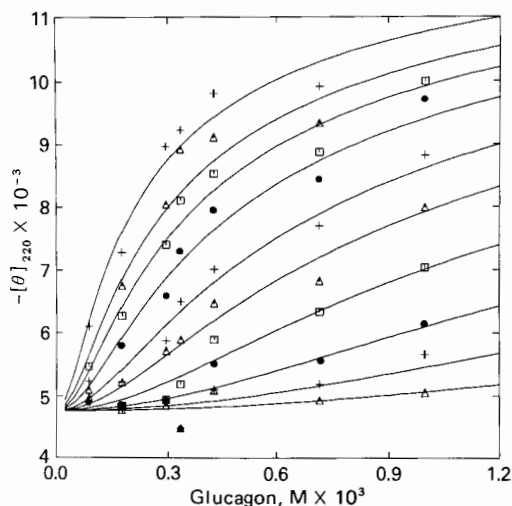


FIG. 1. The effect of glucagon concentration and temperature on the mean residue ellipticity of glucagon at 220 nm ($[\theta]_{220}$). +, Experimental data at 5°, 25°, and 45°; Δ , data at 10°, 30°, and 50°; \square , data at 15° and 35°; and \bullet , data at 20° and 40°. The solid lines were calculated from the "best fit" to a monomer \rightleftharpoons trimer equilibrium, Eq. 2, corresponding to temperatures of 5° (top), 50° (bottom), and intermediate values in 5° increments. See *text* for details of the "best fit".

interpretations of the polymeric species formed in glucagon solutions. Both a monomer \rightleftharpoons trimer and a monomer \rightleftharpoons dimer \rightleftharpoons hexamer association have been reported (8, 19). More recently, Gratzner *et al.* (20) found, by crosslinking experiments with dimethyl suberimidate, that the associated species is a trimer; no dimers or hexamers were found. Moreover, x-ray studies show that glucagon is a trimer in its crystalline state (3).

A second assumption used for the analysis of the experimental data was that the ellipticities of the monomer and trimer are independent of temperature. This assumption appears to be justified because Panijpan and Gratzner (9) have shown that the ellipticity (at 220 nm) of succinylated glucagon, which does not show the concentration-dependent association of glucagon, did not change between 5° and 90°. Moreover, they found that the ellipticity of a dilute solution of glucagon did not change between 50° and 90°.

Free energy changes were fitted to a series expansion in temperature, Eq. 3, and the remaining thermodynamic parameters were evaluated from the appropriate derivatives; ΔS^0 from Eq. 4, ΔH^0 from Eq. 5, and ΔC_p^0 from Eq. 6.

$$\Delta G^0 = A + BT + CT^2 + DT^3 \quad [3]$$

$$\Delta S^0 = -\partial(\Delta G^0)/\partial T = -B - 2CT - 3DT^2 \quad [4]$$

$$\Delta H^0 = \partial(\Delta G^0/T)/\partial(1/T) = A - CT^2 - 2DT^3 \quad [5]$$

$$\Delta C_p^0 = \partial(\Delta H^0)/\partial T = -2CT - 6DT^2 \quad [6]$$

RESULTS

The self-association of glucagon at pH 10.6 in water is rather weak, and therefore it is difficult to convert most of the glucagon into its associated state at reasonable concentrations. The situation can be improved somewhat by using phosphate to enhance the association (unpublished data).

The mean residue ellipticity of glucagon as a function of concentration and temperature is plotted in Fig. 1; theoretical curves based on a "best fit" to a monomer \rightleftharpoons trimer equilibrium are also shown. The equilibrium constants are given in Table

Table 1. Apparent association constants

Temperature, K	K_a , $\times 10^6 \text{ M}^{-2}$	$\pm \text{SEM}^*$, $\times 10^6 \text{ M}^{-2}$
278	12.96	2.58
283	6.406	1.119
288	4.127	0.733
293	2.330	0.393
298	1.064	0.173
303	0.561	0.094
308	0.241	0.046
313	0.093	0.024
318	0.037	0.016
323	0.014	0.012

These were evaluated from the ellipticity as a function of temperature and concentration; see *text* for details. Monomer ellipticity ($\pm \text{SEM}$) was -4736 ± 69 and for the trimer, $-12,953 \pm 318$.

* SEM are approximate because no rigorous theory exists for their evaluation when fitting to a nonlinear model.

1. The relative error increased substantially because the total change in ellipticity decreased with increasing temperature. The change in free energy as a function of temperature is shown in Fig. 2.

Gratzner and Beaven (8) have reported an association constant of 2.5×10^6 in 0.2 M phosphate, pH 10.2 at 23°. We have recalculated the gel filtration data of Swann and Hammes (19), assuming a monomer \rightleftharpoons trimer equilibrium (Eq. 2) instead of the monomer \rightleftharpoons dimer \rightleftharpoons hexamer that they assumed, and found a trimerization constant of $6.1 \times 10^6 \pm 2.5 \times 10^6$ in 0.2 M phosphate, pH 10.0 at 23–25°. Our value of $1.06 \times 10^6 \pm 0.17 \times 10^6$ in 0.2 M phosphate, pH 10.6 at 25°, is in accord with theirs because increasing the pH or the temperature decreases the association.

Gratzner and Beaven (8) discussed the linkage between structure formation and subunit association of glucagon. They suggested that an intermediate that either is structured (high helical content) monomer or unstructured (low helical content) trimer may be present in significant amounts. By assuming that the change in ellipticity (i.e., change in structure) can be expressed as a monomer \rightleftharpoons trimer association (i.e., Eq. 2), we have in effect assumed that at equilibrium only unstructured

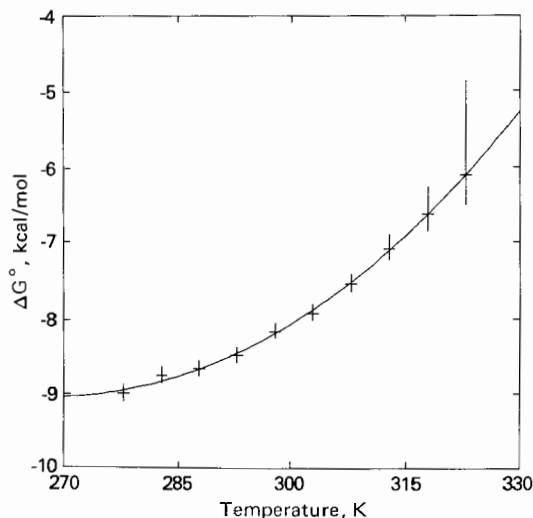


FIG. 2. Free energy changes of glucagon trimerization as a function of temperature. Data points correspond to the values listed in Table 1. Vertical lines are approximate SEM as described in Table 1. The solid curve is the "best fit" of this data to Eq. 3.

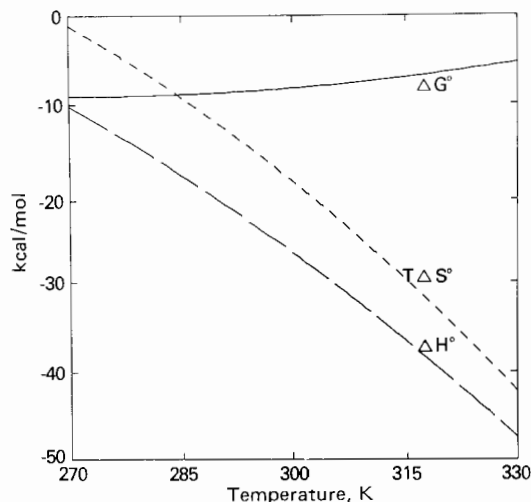


FIG. 3. Thermodynamic parameters for glucagon trimerization. The parameters were derived from the data in Table 1 and Fig. 2 by fitting to Eq. 3 and then evaluating Eqs. 3, 4, and 5 as described in the text. The constants in Eq. 3 were found to be: $A = 23.344$ kcal/mol; $B = -0.12796$ kcal/mol per degree; $C = -4.0123 \times 10^{-4}$ kcal/mol per degree²; and $D = 1.59482 \times 10^{-6}$ kcal/mol per degree³.

monomers and structured trimers exist in significant amounts. If structured monomers or unstructured trimers are present, measurements by gel filtration and ellipticity would be expected to yield different results because they weight the various species differently. The close agreement between association constants measured by these two methods indicates that the concentration of structured monomers and unstructured trimers, is not significant. This applies only to equilibrium conditions and does not rule out kinetic intermediates.

The temperature dependence of the ellipticities of the monomer and trimer species was tested by analyzing the concentration-dependence data for the monomer and trimer ellipticity values at each temperature. At 5°, 15°, and 25° the ellipticities of the monomer and of the trimer did not vary (within their standard errors). At higher temperatures the conversion to trimer was too small for meaningful extrapolation. However, from inspection of Fig. 1 it is obvious that the monomer ellipticity is constant to at least 50°.

ΔH° , $T\Delta S^\circ$, and ΔG° of glucagon trimerization are shown in Fig. 3 as a function of temperature. In contrast to the free energy change, the enthalpy and entropy changes were strongly dependent on temperature.

Our measurements were made at pH 10.6 which is in the region of the tyrosyl ionization of glucagon. The thermodynamic parameters reported in Fig. 3 include a contribution from any tyrosyl groups if their ionization changes with polymerization. Gratzer and Beaven (8) have measured the tyrosyl ionization curves of glucagon at 0.106 and 4.5 mg/ml. There are several ways of treating their data but the most direct is to take the difference in α at pH 10.6 from figure 4 of their paper (i.e., $\Delta\alpha = -0.06$). This value of $\Delta\alpha$ must be corrected for the change in degree of association at their glucagon concentrations (i.e., 53%) and the six tyrosyl groups per trimer which could change their ionization. Consequently, the total change in tyrosyl ionization is -0.66 per trimer.

Sufficient thermodynamic data are available for hydrogen-bonded and non-hydrogen bonded salicylic acid that it can be used as a model for the thermodynamics of tyrosine ionization in proteins. Hermans *et al.* (21) measured the enthalpies of phenolic ionization of *ortho* and *para* salicylic acid (and ethyl salicylate). In the *ortho* compounds, the ΔH_i° are 10.5 and 7.1

kcal/mol for the acid and the ester, respectively. In the *para* compounds, ΔH_i° are 3.4 and 1.5 kcal/mol for the acid and the ester, respectively. The average total change therefore in the former (hydrogen-bonded cases) is $\frac{1}{2}(10.5 + 7.1)(-0.66) = -6.0$ kcal/mol and in the latter (non-hydrogen-bonded cases) is $\frac{1}{2}(3.4 + 1.5)(-0.66) = -1.6$ kcal/mol. Sasaki *et al.* (3) did not report whether the tyrosyl residues are hydrogen-bonded in the trimer. Consequently, -6.0 and -1.6 constitute upper and lower limits of the enthalpy change at 25° due to the ionization of tyrosine. Most of the measurements reported by Hermans *et al.* (21) were performed between 1° and 45°, and no dependence of ΔH_i° on temperature was observed for any of their compounds. Consequently, the limits cited above should be independent of temperature. The entropy changes reported by Hermans *et al.* (21) varied between -22 and -33 kcal/mol per degree at 25°. Thus, the $T\Delta S^\circ$ change due to tyrosyl ionization in glucagon is between 4.3 and 6.5 kcal/mol at 25°. It thus appears that the thermodynamics of tyrosyl ionization in glucagon modify somewhat the magnitudes of the parameters of glucagon association but have no effect on the heat capacity change and only a minor effect on the temperature dependence of $T\Delta S^\circ$.

DISCUSSION

In studies of glucagon self-association, it has been assumed from the red shift in absorption and increase in optical activity of the tyrosyl and tryptophanyl chromophores that these hydrophobic residues become sequestered (19, 22). X-ray results show the participation of tryptophan-25, tyrosine-10 and -13, phenylalanine-6 and -22, and leucine-26 in interchain interactions (3). It has become clear since the original description by Kendrew (23) of the nonpolar interactions in myoglobin and the thermodynamic analyses by Kauzmann (24) and Scheraga (25) that hydrophobic interactions could account for most of the favorable free energy change involved in protein folding. However, it still remains to be demonstrated that the free energy change for the association of glucagon is derived principally from hydrophobic interactions.

Glucagon affords an interesting model for protein folding as well as association because there is a significant acquisition in secondary and tertiary structure concomitant with association. It is a tenet of protein solution and crystal studies that proteins undergo very little or no intramolecular unfolding upon going into solution from their crystalline forms (26). For larger proteins, this point of view appears to hold. For smaller proteins, this assumption may not be true because glucagon reveals important structural changes when it dissolves. Another system displaying a strong dependence of secondary and tertiary structure on quaternary structure is the reduced alkylated chain of the apoprotein from human serum lipoprotein (Cm apoA-II) containing 77 residues (27). Size is clearly only one consideration because the pancreatic inhibitor of trypsin contains only 58 residues but remains highly organized in solution and apparently retains the same structure as in the crystal (28).

The thermodynamic parameters of glucagon association, ΔH° and ΔS° , are strongly temperature dependent and decrease with increasing temperature. This dependence on temperature can be considered as characteristic of a reaction controlled by hydrophobic interactions between the nonpolar groups (29, 30) because hydrogen bonding, the second most prevalent interaction in proteins, appears to have little or no temperature dependence (31, 32). The few available studies on hydrogen bonding in water indicate that there is very little difference in enthalpy or free energy if polar side chains or

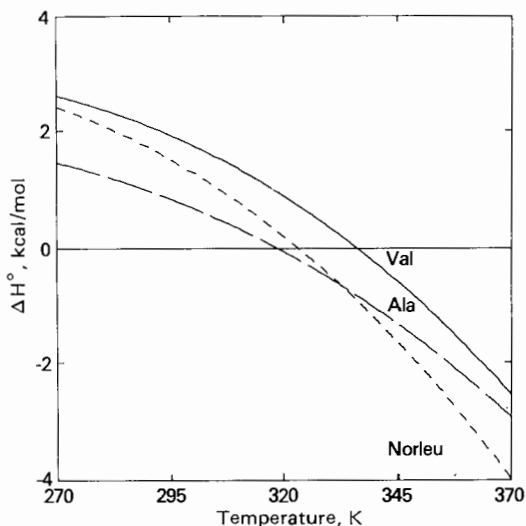


FIG. 4. Enthalpy changes for transfer of the side chains of alanine, norleucine, and valine from water to 95% ethanol. These were calculated by using Eqs. 3 and 5 and the data presented by Brandts (35, 36).

peptide groups are hydrogen bonded to water molecules in the solvent or to each other in the interior of the native protein (31, 33).

The enthalpy change is negative between 5° and 50° and controls glucagon association because the entropy change is also negative. Similar thermodynamic changes are found in most protein renaturation reactions involving large changes in secondary and tertiary structure (34). There is no apparent means of explaining enthalpy-controlled reactions by the thermodynamics of a simple transfer model because these show negative heat capacity changes and positive enthalpy changes (entropy-driven reactions) at low temperatures. This can be seen from the positive enthalpy changes below 320 K for the transfer of the amino acid side chains of alanine, norleucine, and valine from water to 95% ethyl alcohol, as shown in Fig. 4 (35, 36). The enthalpy changes for the vaporization of the hydrocarbon gases, CH₄ to C₄H₁₀, are also positive at low temperature and only become negative at temperatures similar to those at which the enthalpy changes reverse for the three amino acid side chains (29, 37). Recent calorimetric measurements of the heats of solution and partial molal heat capacities at 25° and 30° show that other amino acids side chains also have positive enthalpy (and heat capacity) changes at room temperature (see Table 2 and ref. 38).

Table 2. Side chain enthalpies (kcal/mol)

Side chain	Transfer from water		Coil→helix transition	
	Calor.* 25°	v. Hoff† 25°	Scheraga‡ 20°	Fig. 5 20°
Ala	1.29	0.79	-0.24	-0.19
Val		1.86	0.64	0.66
Norleu		1.42		
Leu			0.10	0.08
Phenyl	1.54		-0.17	
Threo	1.07			
Pro	4.05			
Glu			-1.07	
Glu ⁻			-0.19	
Tyr			-0.93	

* From Prasad and Ahluwalia (38).

† Calculated from data of Brandts, as in Fig. 4.

‡ From Scheraga and coworkers (39-43, 46).

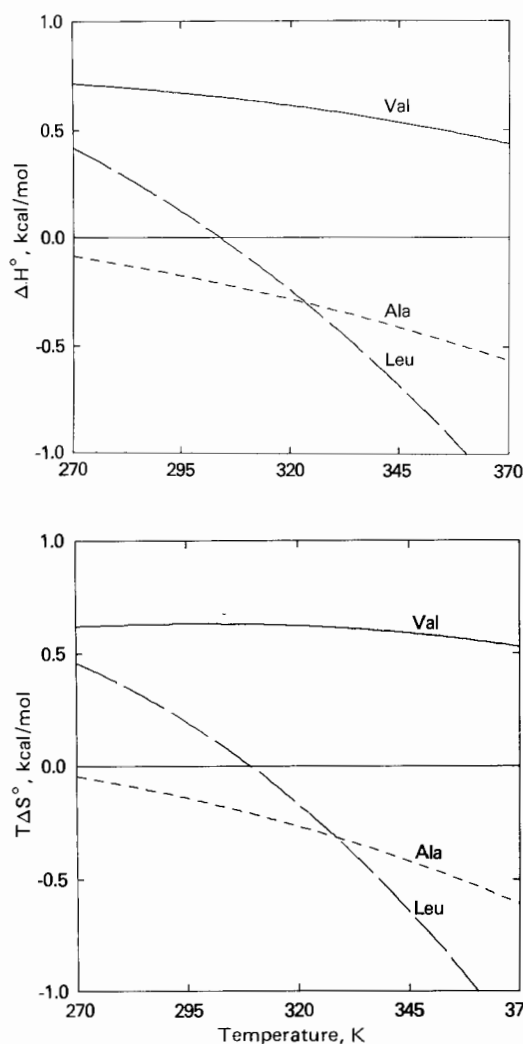


FIG. 5. Enthalpy change (*Upper*) and entropy change (*Lower*) for the coil-helix transition of valine, alanine, and leucine. (*Upper*) Calculated from the host-guest data of Scheraga and coworkers (39-43) by applying Eqs. 3 and 5, as described in the text. (*Lower*) Calculated as in upper, by using Eqs. 3 and 4.

The above thermodynamic values were obtained from solubility data or calorimetry on dilute solutions of various nonpolar solutes. Because glucagon association and many protein reactions involve conformational changes, the simple transfer or heat capacity measurements do not take into account more complex protein reactions. The effects of nonpolar residues on the thermodynamics of the helix ⇌ coil equilibrium have been measured for numerous side chains by Scheraga and colleagues in an extensive series of studies using a host-guest procedure (39-43). They estimated the Zimm-Bragg σ and s parameters of the guest residue from its effect on the coil→helix transition of the host—i.e., either hydroxybutylglutamine or hydroxypropylglutamine. Scheraga and coworkers fitted their data to a linear dependence of $RT \ln s$ versus T to obtain temperature-independent values of ΔH^0 and ΔS^0 . We have reanalyzed their experimental data for the alanyl, leucyl, and valyl side chains by using Eq. 3 and calculated their thermodynamic parameters as a function of temperature (Fig. 5) in order to compare the parameters for helix propagation with those for transfer (see Table 2). The limitations in the analysis of Scheraga and coworkers for evaluating σ and s values have been emphasized in their articles. Consequently, only the signs and the

trends of the thermodynamic parameters we have calculated should be considered and not the exact values.

An interesting and important difference emerges in the contributions of these nonpolar residues. The enthalpy changes for transfer (from water) of the three side chain groups are quite similar at a given temperature whereas those for helix propagation have very different values and temperature dependencies. The enthalpy of helix propagation is always positive for valine, is always negative for alanine, and is positive at low temperature but negative at high temperatures (>313 K) for leucine. It should be noted that the variation in s for phenylalanine, another nonpolar side chain, resembles that for alanine and it also has negative enthalpy change at all temperatures (44). The enthalpies reported by Maxfield *et al.* (45) for ionized and un-ionized glutamic acid are strongly and weakly negative, respectively, at 293 K. These negative enthalpy values for the coil→helix transition must predominate in the contributions of the nonpolar residues to the thermodynamics of glucagon self-association. This is a reasonable assumption because important changes in α -helical structure accompany the reaction.

There are not enough thermodynamic data available on the behavior of the remaining nonpolar side chains of proteins, either for transfer or for the coil→helix transition to permit a more complete analysis of the effects of all the side chains of glucagon. Nevertheless, the thermodynamic data for transfer of the above typical nonpolar side chains do not reveal any negative enthalpy changes at low temperatures and cannot account for glucagon association. Hydrophobic interactions are therefore characterized by a large temperature dependence of their thermodynamic constants (i.e., enthalpy and entropy) and not necessarily by a large entropy change. It is evident that the same nonpolar groups can contribute either positive or negative enthalpy changes during protein refolding or association, depending on whether the number of peptide groups in α -helical segments change in the process. The most typical parameter that describes hydrophobic controlled reactions, therefore, is the heat capacity change and not the enthalpy or entropy because both of these may be positive or negative at any temperature.

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