A Comparison of the Denaturation of Bovine β-Lactoglobulins A and B and Goat β-Lactoglobulin*

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ABSTRACT: This paper presents an experimental study of the urea denaturation of three β-lactoglobulins, bovine β-lactoglobulins A and B (βA and βB) and goat β-lactoglobulin (Gβ). βA differs from βB at only two amino acid residues, a Val/Ala substitution at position 68 and an Asp/Gly substitution at position 120–123. Gβ differs from βB at its amino- and carboxyl-terminal residues as well as at a minimum of three internal residues. The optical rotatory properties of the three proteins are similar at high urea concentrations where denaturation is complete and suggest that the proteins are randomly coiled under these conditions. In contrast, the optical rotatory properties of native Gβ differ significantly from those of βA and βB and suggest that Gβ may have a significantly different conformation in its native state. The midpoints of the isothermal urea denaturation curves at 25° and pH 3 occur at 5.30, 5.19, and 4.68 M urea for βA, βB, and Gβ, respectively. In each case denaturation is completely reversible. An analysis of these data shows that βA is 250 cal/mole more stable than βB and 950 cal/mole more stable than Gβ. It is shown that these differences in stability appear reasonable when model compound data are used to predict the effect of differences in amino acid sequence on stability. The dependence of the free energy of denaturation on urea concentration, d(ΔG)/d(urea), was found to be 1900, 2200, and 2200 cal per mole per M urea for βA, βB, and Gβ, respectively. The lower cooperativity observed for the denaturation of βA, along with other evidence, suggests that the denaturation of βA deviates from a two-state mechanism to a greater extent than the denaturation of βB and Gβ. The effect of temperature on denaturation is similar for the three proteins and indicates that for each the heat capacity of the denatured state is 2000 cal/(mole deg) greater than that of the native state.

The availability of a set of homologous proteins provides a ready-made system for investigating the effect of minor changes in structure on the properties of a protein molecule. One interesting question is what effect a small change in amino acid sequence has on the stability of a globular protein. This information can potentially be derived from a study of the denaturation of homologous proteins and may be useful in reaching a better understanding of protein denaturation and of the forces which contribute to the stability of globular proteins.

No detailed study of the denaturation of a set of homologous proteins has been published. It has been shown, however, that small changes in amino acid sequence affect the denaturation of hemoglobin (Molday and Steinhardt, 1969; Stamatoyannspoulos and Yoshida, 1969), TMV protein (Jockush *et al.*, 1969), and carboxypeptidase A (Petra and Neurath, 1969).

We report here a study of the denaturation of three β-lactoglobulins, bovine β-lactogolbulins A and B (βA and βB), and goat β-lactoglobulin (Gβ). βA differs from βB at only two positions, a βA-Val/βB-Ala substitution at position 68 and a βA-Asp/βB-Gly substitution at position 120–123 (Frank and Braunitzer, 1967). Numerous studies aimed at detecting differences in the physical and chemical properties of these two proteins have been reported (Townend *et al.*, 1969; McKenzie, 1967). Most of the properties of the proteins are very similar, suggesting that they have similar conformations, but a few interesting differences have been observed. For example, βB is five times more soluble than βA (Treece *et al.*, 1964). Gβ differs from βB at its amino- and carboxyl-terminal residues as well as at a minimum of three internal residues (Phillips and Jenness, 1965). Less is known about Gβ than βA and βB, but is the best characterized of the non-bovine β-lactoglobulins. There is evidence that the conformation of Gβ may differ significantly from the conformation of βA and βB (Townend *et al.*, 1969).

The fact that βA is one of the few proteins whose denaturation has been studied in reasonable detail (Pace and Tanford, 1968) was an important consideration in choosing the β-lactoglobulins for this study.

Experimental Section

Materials. Bovine β-lactoglobulins A and B were prepared from the milk of typed, homozygous cows by the procedure of Aschaffenburg and Drewry (1957). Goat β-lactoglobulin was prepared from the milk of two Saanen goats by the procedure of Townend and Basch (1968). All of the proteins were recrystallized at least twice before use.

The moisture content of lyophilized, salt-free preparations of each of the proteins were determined by drying samples to constant weight at 107°. These preparations could then be used for preparing protein stock solutions of known concentration.

Calbiochem A grade urea was recrystallized from 80% ethanol at temperatures below 50°. Aqueous stock solutions were prepared by weight and their molar concentrations calculated using densities given by Kawahara and Tanford (1966). All other chemicals were reagent grade commercial preparations.

Methods. Optical rotation measurements were made at 365.4 nm on a Cary Model 60 spectropolarimeter using quartz cells with a 1-cm path length. The results are reported as the reduced specific rotation, $[\alpha']$, defined by $[\alpha'] = 3[\alpha]/(n^2 + 2)$, where $[\alpha]$ is the specific rotation and n is the refractive index of the solvent (Fasman, 1963).

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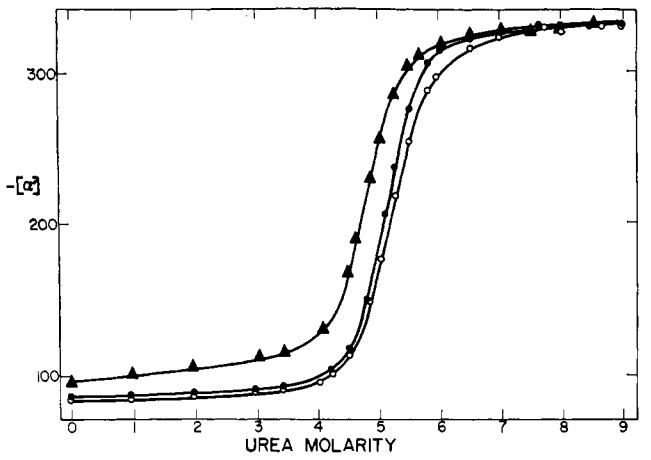

FIGURE 1: Typical urea denaturation curves for βA (○), βB (●), and Gβ (▲). Temperature 25°, pH 2.9, ionic strength 0.15 M KCl–HCl. The optical rotations were measured at 365.4 nm.

All pH measurements were made at room temperature using a Radiometer Model 26 pH meter.

In the isothermal urea denaturation experiments (Figure 1) each experimental point represents a freshly prepared solution. In the thermal denaturation experiments (Figure 5) a single solution was used to determine all experimental points. The temperature of the solutions was maintained to within ±0.05°.

Results

Typical isothermal denaturation curves for βA, βB, and Gβ are shown in Figure 1. For each of the proteins urea denaturation is completely reversible below pH 3.5. It is apparent that observable differences in the urea denaturation curves exist. The main objective of this paper will be to analyze these differences in an attempt to determine what they reveal about the proteins and their denaturation. For convenience, the differences in amino acid sequence are summarized in Figure 2. Note that the differences shown in parentheses for Gβ are minimal differences in sequence since they are based solely on differences in amino acid composition.[1]

Denatured States. Tanford and coworkers (Tanford *et al.*, 1967) have presented convincing evidence that proteins denatured by guanidine hydrochloride are randomly coiled. This conclusion was based in part on the finding that differences in the optical rotation of guanidine hydrochloride denatured proteins result solely from differences in amino acid composition. Using their estimates for the contribution of individual residues to the rotation, it can be shown that the values of $[\alpha']$ at 365.4 nm for βA and βB and Gβ should not differ by more than about 2% if the proteins are randomly coiled.

As Figure 1 shows, above 7 M urea the values of $[\alpha']$ for the three proteins are identical within experimental error. This is also true at other temperatures between 5 and 50°. These observations suggest that for each of these proteins urea denaturation leads to a randomly coiled product.

[1] Amino acid compositions for Gβ have been reported by three different groups (Phillips and Jenness, 1965; Bell *et al.*, 1968; Townend and Basch, 1968). At least two of the compositions agree on all of the differences shown in Figure 2, except for Leu which would differ in Gβ from βB by −1, −2, or −3 residues depending on which amino acid composition is used. We have assumed that Gβ has two less residues than βB.

	1	68	121	162
βA	Leu....	Val.....	Asp....	Ile
βB	Leu....	Ala.....	Gly....	Ile
Gβ	Ile....	(+Gly, −Leu, −Ile)....		Val

FIGURE 2: Differences in the amino acid sequences of βA, βB, and Gβ. The differences shown for Gβ are relative to Bβ. The residues shown in parentheses for Gβ represent minimal differences in sequence since they are based on amino acid compositions. (See footnote 1.)

Native States. At pH 3 at 25° in the absence of urea βA exists in monomer–dimer equilibrium (Herskovits *et al.*, 1964). It has been shown that urea promotes the dissociation of βA and that dissociation is complete at urea concentration of 3 M and above (Pace and Tanford, 1968). Since the free energies of dimerization of βB (Townend and Timasheff, 1961) and Gβ (Townend and Basch, 1968) differ only slightly from the values for βA, βB, and Gβ are also likely to be dissociated into monomers before denaturation begins. Consequently, the native state which undergoes denaturation is probably a monomer for each of the three proteins.

An important question is whether the conformation of the polypeptide chain is similar in the native state of the three proteins or whether the differences in amino acid sequence lead to differences in conformation. In contrast to the results obtained for the denatured state, differences do exist in the optical rotatory properties of the proteins in their native state. In the absence of urea, the values of $[\alpha']$ do not differ significantly for βA and βB, but the value for Gβ is approximately 10° more negative. In terms of the Moffitt–Yang parameters it has been shown that this difference is related to a more negative value of a_0 for Gβ (Townend and Basch, 1968). In addition, the dependence of the Gβ rotation on urea concentration in the linear portion of the denaturation curve is about twice as large as it is for βA and βB.

Differences in the optical rotatory properties of globular proteins are difficult to interpret. One obvious explanation for the differences noted above is that the conformation of native Gβ differs from that of βA and βB. This has been suggested to be the case on the basis of differences in sedimentation properties and titration behavior (Townend and Basch, 1968) as well as differences in the reactivity of the tyrosine residues (Townend *et al.*, 1969). Thus, while it appears that native βA and βB have very similar conformations, the conformation of Gβ may be significantly different.

Isothermal Urea Denaturation. In order to analyze the data shown in Figure 1 and similar data at other pH values, we have assumed that denaturation is a two-state process in which all molecules are in either the native state (N) or denatured state (D) at any point in the transition. (This assumption will be discussed below.) For a two-state process the equilibrium constant can be calculated using

$$K = \frac{[\alpha'] - [\alpha']\mathrm{N}}{[\alpha']\mathrm{D} - [\alpha']} \qquad (1)$$

where $[\alpha']$ is the observed rotation, and $[\alpha']$N and $[\alpha']$D represent the rotations which the native and denatured states would have under the same conditions. The values of $[\alpha']$N and $[\alpha']$D are determined by extrapolation of the linear portions of the denaturation curves, from 0 to 3 M and from 7 to 9 M urea, into the transition region.

The data from Figure 1 have been analyzed in this way and

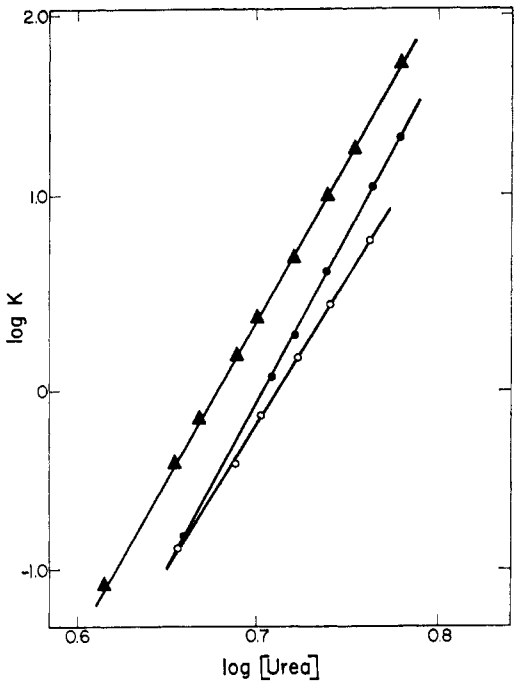

FIGURE 3: Effect of urea concentration on the equilibrium constant for βA (○), βB (●), and Gβ (▲). Calculated from the data shown in Figure 1.

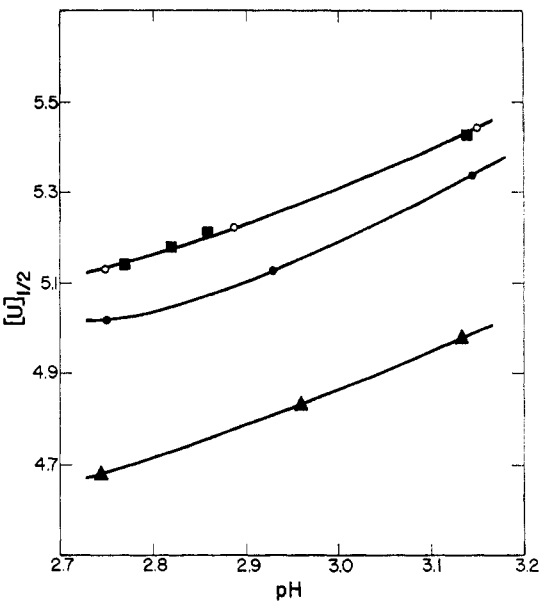

FIGURE 4: Effect of pH on $[U]_{1/2}$ for βA (○), Bβ (●), and Gβ (▲). Data from a previous study (Pace and Tanford, 1968) of the denaturation of βA (■).

the results are shown in Figure 3 where log K is plotted as a function of the logarithm of the urea concentration. As has been found in previous studies of this kind, the plots are linear between ±1 in log K. Thus the data in the transition region can be tabulated using the slope of these plots, which we designate n, and the urea concentration at which log K = 0, which we designate $[U]_{1/2}$. Values for n and $[U]_{1/2}$ for the denaturation of βA, βB, and Gβ at various pH values at 25° are given in Table I. The pH values given are the values at the midpoint of the transition. There is a small variation in pH over the course of the transition but corrections have been applied to the values of K to correct all data for a given denaturation curve to the pH at the midpoint.

The values of $[U]_{1/2}$ for the three proteins are plotted as a function of pH in Figure 4. The filled squares on the βA curve are data from a previous study of the denaturation (Pace and Tanford, 1968). Over the limited pH range investigated the pH dependence of $[U]_{1/2}$ is similar for the three proteins. At each pH it can be seen that the values of $[U]_{1/2}$ increase in the order βA > βB > Gβ.

It is evident from Figure 4 that although the denaturation curves for βA and βB lie quite close to one another in Figure 1, the $[U]_{1/2}$ values for the two proteins differ significantly. The sensitivity of these urea denaturation curves is worth noting. Differences of less than 0.05 M in the $[U]_{1/2}$ values can be readily detected. As will be seen below, this corresponds to a free-energy difference of less than 100 cal/mole.

The parameter n is a measure of the steepness of the transition. The values of n do not vary significantly as a function of pH and in the calculations below we will use the average of the three values of n for each protein. The average values are 16.9, 19.8, and 18.2 for βA, βB, and Gβ, respectively.

Estimating the Differences in Stability. It has been shown (Tanford, 1964) that for a two-state transition the dependence of the free energy of denaturation, ΔG, on urea concentration is related to the values of n and $[U]_{1/2}$ by

$$\left(\frac{d(\Delta G)}{d(\text{urea})}\right)_{[U]=[U]_{1/2}} = \frac{RTn}{[U]_{1/2}}$$

Using the values of n given above and the values of $[U]_{1/2}$ at pH 3, values of $d(\Delta G)/d(\text{urea})$ of 1900, 2200, and 2200 cal per mole per M urea are obtained for βA, βB, and Gβ, respectively. Similar results are obtained when all of the data for a given protein are adjusted to a common pH and the slope of a plot of ΔG *vs.* urea concentration is determined. Multiplying these values of $d(\Delta G)/d(\text{urea})$ by the differences in the values of $[U]_{1/2}$ for the proteins yields an estimate of the differences in stability. We find that βA is 250 ± 50 cal/mole more stable than βB and 950 ± 100 cal/mole more stable than Gβ. These are the differences in stability near 5 M urea, not in aqueous solution, and they depend on the validity of the assumed two-state mechanism. As will be seen below, these values may underestimate the magnitude of the differences in stability but probably not by much.

TABLE I: Values of $[U]_{1/2}$ and n at 25°.[a]

	pH	$[U]_{1/2}$ (M)	n
βA	2.75	5.12	17.3
	2.89	5.22	16.7
	3.15	5.43	16.7
βB	2.75	5.01	20.0
	2.93	5.13	19.5
	3.14	5.34	20.0
Gβ	2.75	4.67	17.8
	2.92	4.82	18.2
	3.14	4.98	18.6

[a] Summary of all data from plots of the type shown in Figure 3. $[U]_{1/2}$ is the urea molarity at which log K = 0 and n is the slope of the plot.

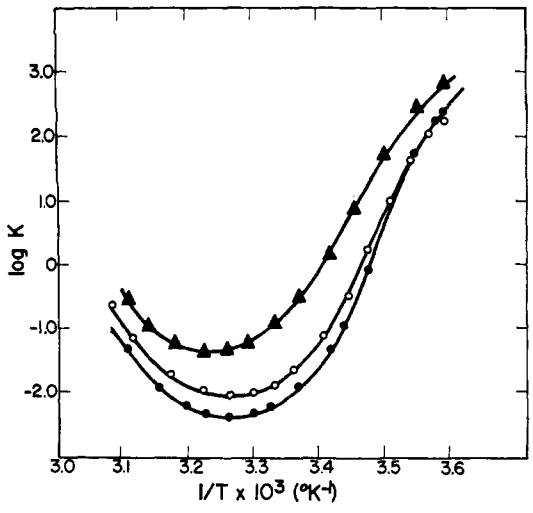

FIGURE 5: Van't Hoff plots for βA (○), βB (●), and Gβ (▲). Urea = 4.63 M; ionic strength = 0.15 M KCl–HCl; pH 2.78 (βA), 2.89 (βB), and 2.90 (Gβ).

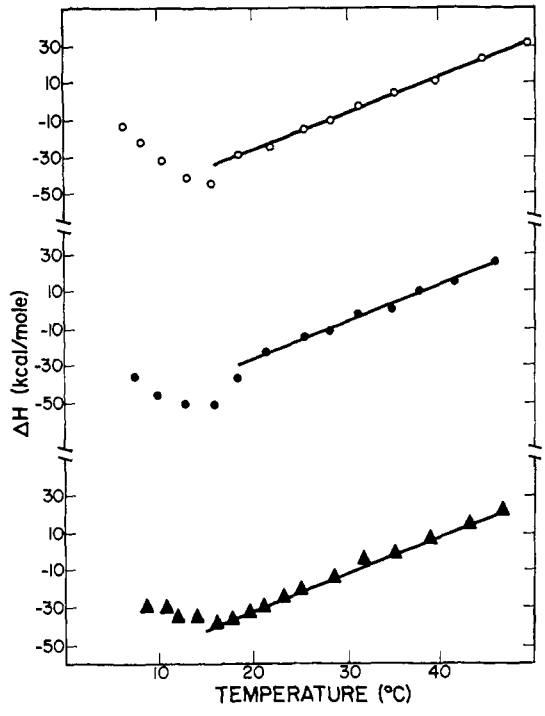

FIGURE 6: Temperature dependence of ΔH for βA (○), βB (●), and Gβ (▲). These results were obtained from the data shown in Figure 5.

Titration studies have shown that the net charge on βA and βB is identical at pH values below 5 (Nozaki and Tanford, 1959). Gβ, on the other hand, is more negatively charged than βA and βB in this pH range (Townend and Basch, 1968). Since a more negative charge on Gβ may contribute to its lower stability, we have also estimated the difference in stability between βA and Gβ when the net charge on the molecules is approximately equal. Under these conditions βA is found to be only 250 cal/mole more stable than Gβ.

Validity of the Two-State Mechanism. We have assumed that only two macroscopic states of the protein exist at significant concentration in the transition region. If, for example, the mechanism were actually $N \rightleftharpoons X \rightleftharpoons D$, the observed values of K and n would be different from those expected for a two-state transition. In our case, if the value of $[\alpha']$ for X is intermediate between $[\alpha']$N and $[\alpha']$D, the probable situation, the values of K and n will be less than if the transition were a two-state process (Tanford, 1968).

Recent kinetic studies of the guanidine hydrochloride denaturation of βA indicate that it is not a two-state process under all conditions (C. Tanford, personal communication). Other data suggest that this may also be the case for urea denaturation (Pace, 1966). The value of $d(\Delta G)/d(\text{urea})$ for βA is substantially lower than for βB and Gβ. To get an equivalent value for βA would require an n value of 20.2. The most likely explanation for this difference is that the denaturation of βA deviates from a two-state mechanism to a greater extent than does the denaturation of either βB or Gβ. This would indicate that the small difference in amino acid sequence between βA and the other two proteins specifically stabilizes an additional state (or states) of the molecule.

A theory has been developed which allows for the calculation of $d(\Delta G)/d(\text{urea})$ using data derived from solubility studies on model compounds (Tanford, 1964, 1970). When this theory is applied to βB and Gβ a theoretical value of $d(\Delta G)/d(\text{urea})$ in agreement with the observed experimental value is obtained when it is assumed that 45% of the hydrophobic and peptide groups are exposed to solvent in D and not in N. This is considerably larger than the change in the degree of exposure required to fit similar data for the denaturation of both lysozyme or ribonuclease (Tanford, 1970). Since the denaturation of both lysozyme (Aune and Tanford, 1969a,b, 1970) and ribonuclease (Salahuddin and Tanford, 1970; Brandts and Hunt, 1967) are thought to be two-state transitions, we feel this is good evidence that the denaturation of both βB and Gβ must closely approach a two-state mechanism.

Effect of Temperature. The denatured states of the β-lactoglobulins are insoluble in dilute salt solution; consequently, the effect of temperature on denaturation was investigated in the presence of urea. We have measured $[\alpha']$ as a function of temperature on solutions of the proteins containing 4.63 M urea. The variation is similar to that observed previously for βA (Pace and Tanford, 1968). The values of $[\alpha']$ are maximal at about 35° and decrease at both higher and lower temperatures. Values of $[\alpha']$N and $[\alpha']$D have been estimated under the same conditions using the extrapolation procedure described above. Using eq 1 these data were then used to calculate the equilibrium constant as a function of temperature. The results are shown in the form of a Van't Hoff plot in Figure 5 where the logarithm of the equilibrium constant is plotted *vs.* the reciprocal of the absolute temperature.

These data show that the proteins are maximally stable near 35° and either raising or lowering the temperature decreases the stability. Note that the curve for βA lies above the curve for βB. If the pH of the βA solution were the same as that of the βB solution, the curve for βA would lie slightly below that of βB. It was shown previously (Pace and Tanford, 1968) that small differences in pH and urea concentration do not significantly effect the thermodynamic data derived from these plots.

The data from Figure 5 were used to calculate the enthalpy of the transition, $\Delta H = -R\,d(\ln K)/d(1/T)$, by measuring the slope between adjacent points on the curve. In Figure 6 ΔH is shown plotted as a function of temperature. For each of the proteins ΔH increases linearly with temperature between 20 and 50°, but there is a break in the curves between 10 and 20°. It is possible that the discontinuity observed at lower temperatures results because the two-state mechanism is no

TABLE II: Values of ΔH and ΔC_p for Denaturation in 4.63 M Urea.[a]

	pH	ΔH at 25° (kcal/mole)	ΔC_p (cal/(deg mole))
βA	2.78	−18	2030
βB	2.89	−18	2010
βC	2.90	−22.5	2020

[a] From the experiments shown in Figure 6.

longer applicable, *i.e.*, the concentration of one or more intermediate states becomes significant at lower temperatures.

The linear portions of the curves in Figure 6 have been used to calculate ΔC_p ($=d(\Delta H)/d(T)$), the difference in heat capacity between the denatured and native states. The values of ΔC_p along with the values of ΔH at 25° are listed in Table II.

Discussion

Although three-dimensional structures are not available for the β-lactoglobulins, it is interesting to consider the sign and magnitude of the differences in stability which might result from the differences in amino acid sequence. To do so obviously requires some assumptions. Note in Figure 2 that except for an Asp which is present in βA but not βB or Gβ, the substitutions all involve nonpolar aliphatic residues. There is good evidence that the Asp/Gly substitution in βA–βB occurs at a residue on the surface of the proteins (Townend *et al.*, 1969). Since we can compare the proteins under conditions where the net charge is the same, we will assume that this substitution has a negligible effect on stability. We will make the following assumptions for the substitution of the nonpolar aliphatic residues. (1) Substitutions at residues where the side chain is exposed to solvent will have no effect on the stability. Side chains which are exposed to solvent in the native state will also be exposed to solvent in the denatured state. Consequently, if the free energy of the native state is increased, the free energy of the denatured state should be increased to the same extent and the stability will not be altered. (2) Substitutions at residues in the interior of the molecule will exert only a "hydrophobic" bonding effect. Side chains in the interior of the protein will be in a nonpolar environment in the native state but exposed to solvent, in our case about 5 M urea, in the denatured state. We assume that the difference in free energy between two proteins which differ by one internal amino acid substitution will be equal to the difference in the free-energy changes accompanying the transfer of the two different side chains from a nonpolar environment to 5 M urea. The free energies of transfer for the side chains of Ala, Val, Leu, and Ile from ethanol—representing the interior of the protein—to 5 M urea were taken from Tanford (1962, 1970).

Proceeding under these assumptions we estimate that βA will be from 0 to 800 cal per mole more stable than βB and from 0 to 5000 cal per mole more stable than Gβ. It is encouraging to find that this analysis predicts that the stabilities will vary in the order $\beta A \geq \beta B \geq G\beta$, as observed experimentally, and that the experimental values fall in the predicted range. The greater stability predicted for βA by this analysis results because βA has larger, more hydrophobic residues than βB and Gβ. The fact that agreement is obtained may suggest that any unfavorable steric effects associated with fitting a larger residue into the interior of the protein are more than compensated by the increased contribution from hydrophobic bonding. Dickerson *et al.* (1971) have suggested that the polypeptide chain in globular proteins may indeed be elastic enough to allow larger groups to be inserted in the interior of the molecule.

Substitutions like the Val/Ala substitution in βA–βB are often referred to as conservative replacements (Smith and Margoliash, 1964). It should be noted that even in the absence of steric effects many conservative replacements are capable of altering the stability of a protein by over 1 kcal/mole. Under physiological conditions the native, globular state of proteins is only from 5 to 15 kcal per mole more stable than unfolded states for the six proteins where information is available (Tanford, 1970; Brandts, 1969; Lumry and Biltonen, 1969; Hermans and Acampora, 1967). Obviously a change of 1 kcal/mole in their stability would be significant. It could, for example, lead to a sizeable change in the *in vivo* steady-state concentration of the enzyme by altering its turnover rate (Shimke and Doyle, 1970).

The marked temperature dependence of ΔH which is observed for the three proteins results from the large ΔC_p for the reaction. ΔC_p values of similar magnitude have been observed for the denaturation of several proteins (Jackson and Brandts, 1970; Tanford and Aune, 1970; Salahuddin and Tanford, 1970) and result predominately from the exposure of nonpolar groups to solvent on denaturation (Tanford, 1970). Model compound data indicate that each CH_2 group which is transferred from a nonpolar environment to water should contribute about 16 ± 3 cal/(mole deg) to ΔC_p (Tanford, 1970). Since βA has only two more CH_2 equivalents than βB and eight more than Gβ, changes in ΔC_p due to these extra CH_2 groups should be less than our experimental error. Thus the similarity in the ΔC_p values for the three proteins is reasonable.

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Isolation of Trypsins by Affinity Chromatography*

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ABSTRACT: Bovine α- and β-trypsin were separated by affinity chromatography on a column of chicken ovomucoid covalently bound to Sepharose. The two active fractions were selectively eluted by a pH gradient. The procedure was also applied to the purification of porcine and dogfish trypsins and to the isolation of trypsin from activated bovine pancreatic juice. Neither trypsinogen nor α-chymotrypsin was retarded by this column.

Affinity chromatography has recently been introduced as a method of enzyme purification (Cuatrecasas *et al.*, 1968). The method depends on the affinity of an enzyme toward specific substrate analogs or inhibitors covalently coupled to an insoluble matrix. In contrast to other chromatographic techniques which separate proteins on the basis of their molecular size, charge distribution, or both, affinity chromatography separates enzymes on the basis of their specificity and can be directly applied to the isolation of analogous enzymes from different species. The present investigation was undertaken to design a procedure for the large-scale purification of trypsins by affinity chromatography. After an examination of several protein inhibitors, chicken ovomucoid (CHOM)[1] covalently bound to Sepharose was selected because this protein inhibitor is highly specific in binding trypsin and can be easily prepared in large quantities. The method was successfully applied to the isolation of bovine trypsin, porcine trypsin, and trypsin from pancreatic extracts of the spiny Pacific dogfish. During the course of this investigation Feinstein (1970) reported the purification of bovine trypsin on a similar ovomucoid–Sepharose column. The present method extends the usefulness of this approach by demonstrating its applicability to other species of trypsin. Furthermore the method described herein is also capable of separating α- and β-trypsin, the two predominant species found in conventional preparations of bovine trypsin (Schroeder and Shaw, 1968).

Experimental Section

Materials. The whites of fresh chicken eggs were obtained from Gaffney Suppliers, Inc., Puyallup, Washington. Crystalline bovine trypsin, trypsinogen, and α-chymotrypsin were obtained from Worthington Biochemical Corp. Bovine α- and β-trypsin were prepared according to the method of Schroeder and Shaw (1968). Porcine trypsin was a product of Novo Industri, Copenhagen. Dogfish trypsinogen and trypsin were prepared as described by Tye (1971). Bovine pancreatic juice was collected as described by Keller *et al.* (1958) and was found to have undergone spontaneous activation. After adjustment to pH 3 with HCl, the insoluble material was removed by centrifugation and discarded. SE-Sephadex C-50, DEAE-Sephadex A-50, and Sepharose 4B were all purchased

* From the Department of Biochemistry, University of Washington, Seattle, Washington 98105. *Received March 11, 1971.* This work was supported by research grants from the National Institutes of Health (GM 15731) and the American Cancer Society (P-79).

† Taken from dissertations submitted by N. C. R. and R. W. T. to the Graduate School of the University of Washington in partial fulfillment of the requirements for the degree of Doctor of Philosophy. N. C. R. and R. W. T. were supported by a training grant from the National Institutes of Health (GM 00052-14) to the Department of Biochemistry.

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[1] The following abbreviations are used: BAEE, α-*N*-benzoyl-L-arginine ethyl ester; NPGB, *p*-nitrophenyl-*p'*-guanidinobenzoic acid; CHOM, chicken ovomucoid; MES, 2-(*N*-morpholino)ethanesulfonic acid; TAME, tosyl-L-arginine methyl ester.