Effect of Single Amino Acid Substitutions on the Thermal Stability of the α Subunit of Tryptophan Synthase†

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ABSTRACT: The effects of single amino acid substitutions on the energetics of the reversible thermal unfolding of the α subunit of tryptophan synthase from *Escherichia coli* were studied by differential scanning calorimetry. The replacement of glycine at position 211 in the wild type protein with either arginine or glutamic acid in missense mutant proteins has little effect on the thermal stability of the α subunit. The midpoints of the thermal transitions for wild type and arginine and glutamic acid mutants are 57.8, 58.0, and 59.6 °C, respectively, at pH 7.8. In contrast to the small differences in free energy, large differences are observed in the enthalpy and entropy changes associated with the unfolding transition. The calorimetric enthalpy changes for both mutant proteins were 15–17 kcal mol^{-1} higher than the value for the wild type protein, 93.8 kcal mol^{-1}. As a consequence of the small changes in free energy and large increases in enthalpy, substantial increases in the entropy changes are found for the two mutant proteins. Structural considerations of the α subunit support the view that the replacement of the proton side chain of glycine at position 211 with either of the bulkier, charged side chains of glutamic acid or arginine alters numerous noncovalent interactions in the native conformation and thereby has a significant effect on the energetics of this protein.

The native conformation of proteins is maintained by numerous noncovalent interactions resulting from hydrogen bonds, salt bridges, and the hydrophobic effect. Although the stabilization energy contributed by any one of these interactions is small, it has long been known that they act in a cooperative fashion to stabilize a single three-dimensional structure for a given amino acid sequence (Sela et al., 1957; Anfinsen, 1973). The role of an individual amino acid in contributing to the overall stability can be determined, in principle, by altering the structure of that residue and studying the effect on thermal stability. Missense mutations are a particularly attractive mechanism for obtaining such alterations since the base change in the DNA and the resulting amino acid substitution occur at a single position. The process of thermal unfolding disrupts the noncovalent interactions involved in maintaining the native structure and thereby provides a means of detecting changes in this network due to an amino acid replacement.

An excellent candidate for this approach is the α subunit of trytophan synthase (*Escherichia coli*), in which over 2 doz missense mutations have been isolated and identified (Yanofsky, 1967; Yanofsky & Horn, 1972; Murgola & Yanofsky, 1974a,b). This protein contains a single polypeptide chain of molecular weight 28 800 and no prosthetic groups. We report here a differential scanning calorimetric (DSC)[1] study of the thermal unfolding of the wild type α subunit and two missense mutants.

Experimental Procedures

The wild type α subunit from *E. coli* was isolated from strain B8/F′B8, the Glu-211 mutant protein from strain A46, and the Arg-211 mutant protein from strain A23 by the methods of Kirschner et al. (1975). There is no possibility of heterogeneity in this protein since these bacterial strains have only a single copy of the tryptophan operon (except in the case of wild type protein where an F′ episome containing the identical operon has been introduced to increase the quantity of protein synthesized). The purity and homogeneity of the protein were ascertained by both $NaDodSO_4$ and native polyacrylamide gel electrophoresis where only a single band was apparent in the electrophoretograms. The activity of the various α subunits was determined by measuring their ability to enhance the activity of the β_2 subunit in the condensation of indole and serine to form tryptophan (Smith & Yanofsky, 1962). The specific activities of the proteins were the following: wild type α, 5000 ± 500 units/mg; Glu-211, 4000 ± 400 units/mg; Arg-211, 5000 ± 500 units/mg. The reported maximum specific activity of wild type protein under the conditions used in this assay is 5500 units/mg (Kirschner et al., 1975). The concentration of the three α subunits was determined by absorption spectroscopy using a specific absorbance value of 4.4 at 278 nm for a 1% solution of the protein at pH 7.8 (Adachi et al., 1974). This value has been measured previously for the wild type protein and was found in the present study to be identical for the two mutant proteins when the concentrations were checked by the procedure of Lowry et al. (1951).

The DSC measurements were made with a calorimeter designed by Privalov that has previously been described in detail (Privalov et al., 1975); the heating rate was 1 K min^{-1}. The protein concentration varied from 0.70 to 1.86 mg mL^{-1} in a solution containing 1 mM sodium phosphate, pH 7.8, 0.1 mM dithioerythritol, and 0.2 mM EDTA. This concentration of buffer was found to be sufficient to maintain the pH constant to within 0.1 pH unit over the temperature range studied but did not cause precipitation of the protein at high tem-

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[1] Abbreviations used: DSC, differential scanning calorimetry; Glu-211, mutant α subunit with glutamic acid at position 211; Arg-211, mutant α subunit with arginine at position 211; EDTA, ethylenediaminetetraacetic acid; DTE, dithioerythritol; T_d, temperature at the midpoint of the thermal transition; ΔH_{Cal}, calorimetric enthalpy change; ΔH_{VH}, van't Hoff enthalpy change; ΔC_p, difference in heat capacities between native and unfolded forms; ΔC_p^d, difference in heat capacities between native and unfolded forms at T_d; $NaDodSO_4$, sodium dodecyl sulfate.

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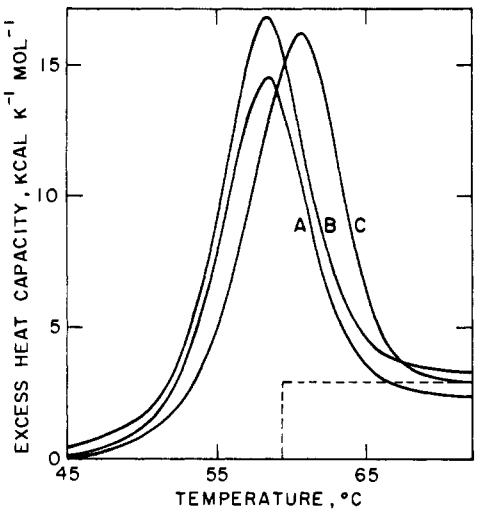

FIGURE 1: The excess specific heat of the wild type (A), Arg-211 (B), and Glu-211 (C) α subunits as a function of temperature in 1 mM sodium phosphate, pH 7.8, 0.1 mM DTE, and 0.2 mM EDTA. Protein concentrations are all normalized to the same arbitrary value in order to show the difference in the excess specific heats. As an example, the heat adsorbed in the thermal transition for Glu-211 was calculated by measuring the area bounded by the absorption peak and the dashed lines with a planimeter. More details on the analysis of the data can be found in Velicelebi & Sturtevant (1979).

perature. To achieve reversibility for the thermal unfolding, it was necessary to degas rigorously the samples by aspiration and thereafter to maintain an argon atmosphere over the protein solutions. All chemicals were reagent grade.

Results

Representative DSC scans for the wild type protein, which has a glycine at position 211, and the Arg-211 and Glu-211 mutant proteins are shown in Figure 1. The denaturation profiles of all three proteins display single major heat absorption peaks centered around 60 °C at pH 7.8. The thermal unfolding transitions for these proteins begin in the range of 45–50 °C and are complete by 70 °C. A scan for the wild type protein from 10 to 90 °C revealed no additional heat absorption processes. The positive slopes of the base lines in the pre- and posttransition regions indicate that the temperature coefficients of the heat capacities of the native and unfolded forms are positive with values typical for many proteins (0.001 cal K^{-2} g^{-1}). The reversibility of the unfolding was ascertained by rescanning samples that had been removed from the calorimeter at the conclusion of a heating cycle (~70 °C) and rapidly cooled on ice to 0 °C. This procedure was required to reduce irreversible inactivation that occurs with prolonged exposure to high temperatures. Although the nature of this inactivation process was not ascertained, the requirement for the exclusion of oxygen to achieve reversibility suggests that the oxidation of one or more of the three cysteine residues is involved.

The energetics of the thermal unfolding process was determined by evaluating the data as described in detail by Privalov & Khechinashvili (1974) and Velicelebi & Sturtevant (1979). The thermodynamic parameters, T_d, ΔH_{Cal}, ΔH_{VH}, and ΔC_p^d, obtained from this analysis are shown in Table I. The van't Hoff enthalpy, ΔH_{VH}, was calculated according to the equation $\Delta H_{VH} = 4RT_d^2C_{excess}/\Delta q_{Cal}$, where R is the gas constant, T_d is the temperature at half-transition, C_{excess} is the observed excess specific heat at T_d, and Δq_{Cal} is the observed total heat absorbed in the unfolding (Velicelebi & Sturtevant, 1979). Within experimental error, the wild type protein with glycine at position 211 and the Arg-211 mutant have the same

Table I: Thermodynamic Parameters for the Unfolding of Wild Type and Mutant α Subunits at pH 7.8

protein	T_d (°C)	ΔH_{Cal} (kcal mol^{-1})	ΔH_{VH} (kcal mol^{-1})	$\Delta H_{VH}/\Delta H_{Cal}$	ΔC_p^d (kcal mol^{-1} K^{-1})
wild type	57.8 ± 0.5	93.8 ± 5.9	109 ± 11	1.16 ± 0.14	2.1 ± 0.4
Arg-211	58.0 ± 0.2	111 ± 4	113 ± 11	1.02 ± 0.11	2.8 ± 0.9
Glu-211	59.6 ± 0.6	109 ± 6	107 ± 11	0.98 ± 0.11	2.7 ± 0.7

Table II: Free Energy and Entropy Changes for the Unfolding of Wild Type and Mutant α Subunits at pH 7.8, 57.8 °C

protein	ΔG (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})
wild type	0.0	283 ± 18
Arg-211	0.1 ± 0.1	337 ± 12
Glu-211	0.6 ± 0.2	342 ± 19

temperature at the midpoint of the transition (T_d); the value for wild type is 57.8 °C and that for Arg-211 is 58.0 °C. The Glu-211 mutant, with a T_d of 59.6 °C, is more stable with respect to thermal unfolding than the other two proteins.

In contrast to the similarities in the values of T_d, the calorimetric enthalpies for these proteins show large differences. The substitution of the large, cationic guanidinium moiety of arginine for the proton of glycine at position 211 results in an increase in ΔH_{Cal} of 17 kcal mol^{-1}, and the insertion of the aliphatic carboxylic acid moiety of glutamic acid results in an increase of 15 kcal/mol^{-1}. These differences represent an increase of 18 and 16%, respectively, for the enthalpy change accompanying the unfolding. The ratio of the calorimetric and van't Hoff enthalpies for each protein is unity, to within experimental uncertainty, showing that the thermal unfolding in each case is best described as a two-state process. The difference in heat capacity between the native and unfolded forms, ΔC_p^d, is similar for all three proteins, within experimental error.

Other pertinent thermodynamic parameters for the thermal unfolding can be calculated from the values of ΔH_{Cal}, ΔC_p^d, and T_d by using the formalism described by Privalov & Khechinashvili (1974) and assuming that ΔC_p is not a function of temperature. The absence of a temperature dependence for ΔC_p for other proteins has been observed previously (Privalov & Khechinashvili, 1974). The values for ΔG and ΔS at 57.8 °C, the T_d of wild type protein, are shown in Table II. As expected for proteins with nearly equal transition temperatures, the differences in the free energy changes near the melting points are small. The Glu-211 mutant is ~0.6 kcal/mol^{-1} more stable with respect to its unfolded form at 57.8 °C than either the wild type or Arg-211 mutant is to its unfolded form at this temperature. This difference is significant since the extrapolations of the free energy changes for the Arg-211 and Glu-211 mutants to this temperature are less than 2 °C.

As a consequence of the large differences in ΔH and the small differences in ΔG between the wild type and mutant α subunits, large differences are observed in ΔS. The ΔS values for the two mutant proteins are 50–60 cal K^{-1} mol^{-1} higher than that for the wild type protein at 57.8 °C.

Discussion

Calorimetric experiments provide a more detailed description of the effect of single amino acid replacements on the

stability of a protein to unfolding than is available from other methods, e.g., urea or guanidine hydrochloride induced unfolding where only the value of ΔG can be readily obtained (Pace, 1975). The importance of measuring the enthalpy changes is emphasized in the study of the α subunit of tryptophan synthase reported here. The substitution of a glycine by glutamic acid at position 211 changed T_d by only 2 °C, and the substitution by arginine produced no significant change. Thus, all three proteins have very similar values for the free energy change in the vicinity of 60 °C (see Table II). What is quite remarkable, in contrast, is the effect that a single change in the primary sequence can have on the enthalpy and entropy changes for the unfolding process. The magnitudes of changes in the thermodynamic parameters, 15–17 kcal mol^{-1} for ΔH and 50–60 cal K^{-1} mol^{-1} for ΔS at 57.8 °C, suggest that the replacement of a single amino acid can have a substantial effect on the energetics of the protein.

The observation of large but opposing changes in ΔH and ΔS and small changes in ΔG that occur as a result of single amino acid substitutions is similar to that for the enthalpy, entropy, and free energy changes for the unfolding of other proteins (Lumry & Biltonen, 1969). The basis for the compensating effect in the mutant α subunits may reside in a particular feature of this protein. The selection procedure for the mutant α subunits requires that the α subunit be able to form a complex with the β_2 subunit and enhance the activity of the β_2 subunit in the condensation of indole and serine to form tryptophan (Smith & Yanofsky, 1962). It is possible that this requirement constrains the type of mutants isolated to those which do not drastically increase the free energy and, thereby, the stability of the protein. Obviously, an amino acid substitution which significantly decreases the stability of the protein would prevent it from spontaneously folding to the native form and complexing with the β_2 subunit. Thus, the only missense mutant α subuits which can be isolated are those in which small changes in the free energy of folding occur. A survey of these thermodynamic parameters for other mutant α subunits is required to test the validity of this hypothesis.

The small differences that are observed in the melting temperatures of the three proteins under consideration here suggest that the native conformations must be quite similar. Evidence from two independent probes of the three-dimensional structure supports this conclusion. Each of the two mutant α subunits examined in this study is capable of binding to the β_2 subunit and enhancing the activity of the β_2 subunit (Murphy & Mills, 1968). In addition, both mutants reacted with antisera to wild type protein exactly like wild type protein (Murphy & Mills, 1968). Thus, the native conformations of the three proteins are similar, with any tertiary changes that occur being rather limited. This observation is important, since comparative studies of the stabilities of wild type and missense mutant proteins are based upon the presumption that the native conformations are closely similar; the amino acid substitutions should not cause catastrophic perturbations which completely alter the structure.

In an approach similar to that described in the present work, Elwell & Schellman (1977) have compared the thermal stability of the wild type and two mutant T4 phage lysozymes. By monitoring the circular dichroism at 223 nm as a function of temperature at a series of pH values, estimates of the enthalpy and free energy changes involved were made by using a van't Hoff analysis. It was found that the replacement of tryptophan at position 138 with tyrosine decreased the enthalpy change by ~20 kcal mol^{-1} and the free energy by 1 to 2 kcal mol^{-1} at pH 2.0, 25 °C. Replacement of the tryptophans at positions 128 and 158 with tyrosines had little effect on the energetics. As in the case of the α subunit, the relatively large change in enthalpy is compensated for by a large opposing change in entropy and a small free energy change results.

More recently, Grütter et al. (1979) isolated a temperature-sensitive mutant of the T4 phage lysozyme and obtained an X-ray structure of the mutant protein at 2.4-Å resolution. Compared to the wild type protein, there are no detectable changes in the three-dimensional structure of the mutant in which arginine at position 96 is replaced with histidine, except for the change in the side chain at position 96. However, the melting temperature for the mutant protein in the pH range from 2.1 to 3.2 is lowered by 14 °C. Although the mutant protein may contain an additional hydrogen bond, any other structural changes induced by this substitution, perhaps at numerous locations throughout the molecule, appear to be too subtle to be observed at this resolution.

Yutani et al. (1977, 1979) have examined the urea and guanidine hydrochloride induced unfolding of the wild type α subunit of tryptophan synthase and several missense mutants at position 49. These workers also found that single amino acid substitutions affected the stability of the protein; however, the enthalpy and entropy changes were not examined. For these replacements, the changes in stability were attributed to different free energies of transfer from a hydrophobic to a hydrophilic environment for the side chain at position 49.

The interpretation of the observed changes in thermodynamic parameters in terms of changes in molecular interactions requires knowledge of the structure of the α subunit in the vicinity of position 211. Although a high-resolution X-ray structure of this protein does not exist, the nature of the amino acid replacements at this site provides information on this topic. The fact that 14 different amino acids can be substituted for the glycine in the wild type protein (Murgola & Yanofsky, 1974a) argues that this side chain must be on the surface of the protein in the native conformation. Among these replacement amino acids are tryptophan, arginine, lysine, aspartic acid, and glutamic acid. Thus, a change in size from the proton of glycine to the indole moiety of tryptophan as well as a change in charge from neutral to both positive and negative can be accommodated at this position without loss of binding capacity for the β_2 subunit. It is difficult to understand how the observed substitutions could be made in the interior of the protein without significantly altering its conformation. Further information on the solvent exposure of the amino acid side chain at position 211 is provided by the observation that the fluorescence emission maximum of the Trp-211 mutant occurs at 345 nm (Murgola & Yanofsky, 1974a). This value is quite close to that expected for the free amino acid, 350 nm. Since the wild type protein contains no tryptophan residues, it can be concluded that position 211 has a high degree of exposure to solvent.

Further consideration of the local environment at position 211 suggests that it is unlikely that there are charged groups nearby since arginine and glutamic acid do not cause opposing effects on the stability of the α subunit. The absence of nearby charged groups is also supported by the results of second-site reversion studies. Positions 175 and 211 are thought to be in close proximity in the native conformation since an amino acid replacement at position 175 restores activity to an α subunit which has been inactivated by a substitution at 211 (Yanofsky & Horn, 1972). Similarly, the mutant protein with the noninactivating amino acid serine at position 211 is inactivated by the replacement of phenylalanine at position 22 with leucine. Examination of the amino acid sequences out to four

residues away from 22, 175, and 211 (Yanofsky et al., 1967; Li & Yanofsky, 1972) reveals the absence of charged residues. Thus, it appears that the amino acid side chain at position 211 is located on the surface of the protein and is not involved in any significant electrostatic interactions.

The above picture, while consistent with the available data, does not provide any simple explanation for the large changes in the enthalpy of unfolding for the mutant proteins. Placement of the side chain at position 211 on the surface of the protein decreases the importance of a contribution to ΔG from the transfer of that side chain from a hydrophobic to a hydrophilic environment upon unfolding. Also, the net effect on the enthalpy change by hydrogen bonds that could be formed between the side chains of glutamic acid or arginine and other appropriate nearby groups would be expected to be small since these bonds would presumably be made to water in the unfolded form. Since the formation or disruption of a single noncovalent interaction involving the side chain at position 211 cannot explain the observed results, the most reasonable explanation is that the replacement of the glycine at this position by larger, charged amino acids causes small changes in numerous noncovalent interactions in the native conformation. Although the results of Grütter et al. (1979) on the wild type and mutant T4 phage lysozymes, where no detectable changes were observed at 2.4 Å, are consistent with this hypothesis, high-resolution X-ray structures for the wild type and mutant α subunits are required to prove its validity.

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