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Tyrosine Flourescence to Monitor the Denaturation of a Bacterial Protein

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TYROSINE FLUORESCENCE TO MONITOR THE DENATURATION OF A BACTERIAL PROTEIN

by

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ABSTRACT

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Tyrosine fluorescence was used to monitor the denaturation of a protein. The protein used as our model system was the regulatory subunit (RSU) of aspartate transcarbamoylase from *E. coli*. RSU is a dimer of two identical chains with one zinc ion bound per chain. It is also a tryptophan-deficient protein so tyrosine fluorescence was used as a probe to monitor changes during denaturation. The tyrosine fluorescence emission spectrum of RSU (λ_{max}=304 nm; I_{304}/I_{340} = 5.1 ± 0.3) is almost indistinguishable from that of pure tyrosine, indicating that the protein preparation was not contaminated with tryptophan containing protein. The quantum yield of RSU relative to that of free tyrosine is 0.69 ± .007. This is relatively high for a tryptophan-deficient protein and this may be due to the fact that two of the three tyrosine residues are exposed to solvent. Complete denaturation with guanidine-HCl or urea results in a decrease in fluorescence intensity to about 66 ± 3% of the original native protein. This is contrary to the increase in fluorescence which is observed for most tryptophan-deficient proteins. A cooperative denaturation was observed between 0.2 M and 0.7 M in guanidine and between 1.0 M and 2.0 M in urea. Assuming a single-step denaturation ($A_2 \underset{}{\overset{Ku}{\rightleftharpoons}} 2U$), the free energy of unfolding could be calculated and was found to be 12-14 kcal/mole. There was no significant difference when the denaturant or the concentration was changed. There was also no significant change in the denaturation profile when the zinc ion concentration in the buffer was increased from 0.0 to 2.2 mM. The Zn^{2+} was successfully removed from the RSU so future studies can be performed in order to gain information about the role of zinc in the protein stability.

Introduction:

Proteins are made up of specific sequences of amino acids, and it is this unique amino acid sequence which defines how the protein folds into its three dimensional structure. The "folded" three dimensional structure of a protein is stabilized by several types of forces: hydrogen bonding, hydrophobic interactions, Van der waals' forces, and electrostatic interactions. Experimentally induced disruption of the folded conformation of the protein chain is called denaturation (Creighton, 1984). Denaturation can be brought about in four different ways: variation of pH, increase in temperature or pressure, or the addition of a chemical denaturant. When a protein denatures it unfolds, and portions which are buried inside the folded structure are exposed to the aqueous solvent. Some small proteins have been shown to denature in a single concerted reaction; these proteins originate in their native state and denature to their unfolded or random coil state (Creighton, 1984). Many protein denaturation reactions, however, do not proceed this simply. These reactions proceed to completion through a series of partially unfolded intermediates.

One way to experimentally monitor protein denaturation is with fluorescence spectroscopy. After light is absorbed by a molecule, its electrons are excited to a higher state. Fluorescence is the emission of light resulting from the return of an excited electron to its ground state (Lakowicz, 1983). In order to monitor the fluorescence changes in proteins, one of the

three aromatic amino acids, tyrosine, tryptophan or phenylalanine must be present because these are the only fluorescent amino acids. Since both tyrosine and tryptophan are much more fluorescent than phenylalanine, the fluorescence effect of phenylalanine almost negligible in comparison (Figure 1). In proteins which contain both tyrosine and tryptophan, the fluorescence effect of the tryptophan tends to hide that of the tyrosine. Therefore, it is only generally possible to observe tyrosine fluorescence in proteins which contain no tryptophan residues. In this research project I used tyrosine fluorescence of a tryptophan-deficient protein to monitor the denaturation of the protein.

The particular protein which was chosen for this study was the regulatory subunit (RSU) of aspartate transcarbamoylase (ATCase) from *E.coli.* ATCase is a large oligomeric protein (MW: 300,000 daltons) with a very complex quaternary structure. Each ATCase molecule consists of 6 identical catalytic chains, grouped as two trimers. The trimer is referred to as the catalytic subunit (CSU). There are also 6 identical regulatory chains, grouped as three dimers; the dimer is referred to as the regulatory subunit (RSU). One trimer lies above and one lies below a belt of regulatory dimers (Figure 2). Each RSU chain (153 amino acids) contains one bound zinc ion. The zinc ions are tetrahedrally bound to four cysteine residues (positions 109, 114, 138, 141) on the regulatory chain. These interactions lie in the region of contact between the catalytic and the regulatory chains (Jefferson, 1990).

Since it is possible to separate the regulatory dimers from the catalytic trimers, we can use the isolated regulatory subunit (RSU) (MW: 34,000 daltons) as our model system. The x-ray crystalline structure of the two alpha

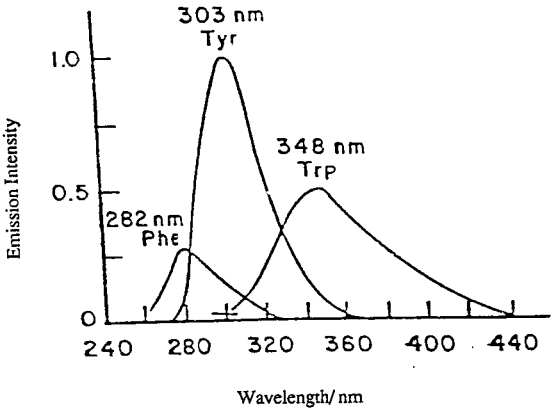


Figure 1: The fluorescence emission spectra of the aromatic amino acids. In folded proteins, the intensity of tryptophan is dominant over that of both tyrosine and phenylalanine because of the energy transfer between tyrosine and tryptophan.
Lakowicz, J.R. *Principles of Fluorescence Spectroscopy.* (1983)

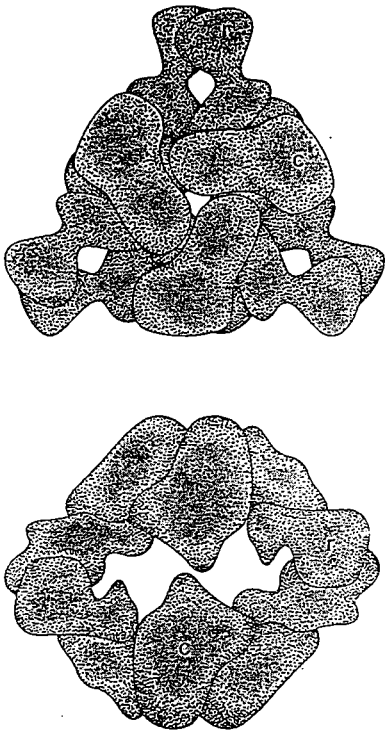


Figure 2: The three dimensional subunit arrangement in aspartate transcarbamoylase. There are three catalytic chains (c) and three regulatory chains (r). A side view of the molecule can be seen above and the perpendicular view can be seen below.
Stryer, L., *Biochemistry*. (1988)

carbon chains of one regulatory dimer allows the two domains, which comprise each chain, to be observed (Figure 3). Each chain folds into the "allosteric" amino terminal domain (residues 1-100) and the "zinc binding" carboxyl-terminal domain (residues 101-153), containing the single zinc ion. There are three tyrosine residues per chain at positions 77, 89, and 140. Two of the residues (positions 77 & 89) are located in the allosteric domain and the other (position 140) is located in the zinc binding domain. Since ATCase is a tryptophan-deficient protein, and since the contribution of phenylalanine to the fluorescence is negligible, tyrosine is the last remaining amino acid that contributes to the fluorescence. Therefore, tyrosine was used as a probe to monitor the confirmational changes during the denaturation of RSU.

There have been a limited number of investigations which use tyrosine fluorescence to monitor protein denaturation. Some research has been concerned with structural and environmental effects of quenching while others have dealt with quantum yield and denaturation studies. The two proteins in particular which have dealt with the environmental effects of quenching are neurophysin and calmodulin. Those dealing specifically with denaturation are histone H1, ribonuclease A and tropomyosin. Each of these proteins is tryptophan-deficient and, therefore, exhibits only fluorescence characteristics due to the tyrosine residues.

Neurophysin and calmodulin are proteins and each has a unique biological function. Neurophysins are carriers for oxytocin and vasopressin within the hypothalamo-neurophyseal tract. Calmodulin is a calcium binding protein which is important in the regulation of calcium ions in cells. Each neurophysin contains one tyrosine and three phenylalanine residues; it is the

Figure 3: The x-ray crystalline structure of the alpha carbon chain of the regulatory subunit (RSU) of ATCase. RSU folds into two domains, the "allosteric" amino terminal and the "zinc binding" C-terminal domains. Each domain contains three tyrosine residues and one zinc atom. The zinc atom is the red ball and the tyrosine residues are highlighted in pink. Nanovision - ACS Molecular Graphics Program

tyrosine which contributes to the fluorescence. Sur and Rabbani (1979) compared the fluorescence of tyrosine to that of neurophysin in order to use the tyrosine fluorescence to probe the non-duplicated binding region of the neurophysin. When neurophysin was bound to oxytocin and vasopressin, disrupting the tyrosine, the fluorescence intensity increased as the environment of the tyrosine became more polar with binding. In the neurophysin, quenching of the fluorescence was seen only when it interacted with peptides, carboxylates, and disulfides.

Similarly, calmodulin contains two tyrosine residues which are used as probes to monitor the fluorescence. One residue is situated so that it faces into a calcium binding site, whereas the other points into a hydrophobic pocket and is inaccessible. When calmodulin binds calcium, a new emission band is seen at 400 nm (Malencik, 1987). Malencik determined that conformational changes accompanied calcium binding and affected the reactivity of the protein. The new fluorescence band was a result of the formation of dityrosine from the intramolecular cross-linking of the two residues. The binding of calcium also causes a two-fold increase in the quantum yield (Malencik, 1987).

Further quantum yield and denaturation studies were investigated in three additional proteins. Histone H1 is an abundant chromosomal protein which is obtained from calf thymus. Each histone contains three tyrosines. Histone H1 is randomly coiled in dilute acidic solution and is induced to fold by increasing the pH or the ionic strength. The fluorescence intensity of H1 increases as the protein undergoes a conformational change from the random coil to the folded form (Giancotti, 1977). The buried tyrosines are supposedly quenched relative to the exposed ones but here H1, the buried,

unquenched tyrosine, shows enhanced fluorescence with folding. The quantum yield of the random coil is .30 whereas that of the folded protein is 1.3.

Similarly, the tyrosyl fluorescence of RNase A is effected by denaturation. RNase A is a small globular protein with six tyrosyl residues: two are adjacent to disulfide groups, and three are buried in the interior of the molecule (Cowgill, 1976). The buried proteins do not fluoresce because they are hydrogen-bonded to the carbonyl groups of the peptide backbone and/or to aspartic and glutamic acid residues. All of the fluorescence of the native protein resulted from the exposed tyrosine residues. Cowgill also saw an increase in the protein fluorescence intensity when it was denatured using urea and when the temperature was varied. The quantum yield of the denatured protein was .16, but with a reduction of the disulfides, the quantum yield increased to 0.28.

Likewise, Cowgill also studied the tyrosyl fluorescence of the muscle protein tropomyosin, a two-stranded tryptophan-deficient alpha helical rod. Its function in relaxed muscles is to prevent the actin from interacting with the myosin unit to generate force. He found that the helical conformation was lost in thermal denaturation, and this was accompanied by a decrease in the fluorescence emission of the protein. A typical quantum yield for the denatured protein was 0.25-0.35. Since a fluorescence decrease was not typical, he suggested certain factors which might have influenced it. First, the decrease in fluorescence was determined to be a result of quenching from dissociated carbonyl groups which were present in the random coil but not in the full helix. An increase in this fluorescence in an acidic solution suggested that the fluorescence at neutral pH's was quenched by a

collisional interaction with nearby ionic carboxylate groups.

Cowgill compiled data on the environmental effects of tyrosine fluorescence and systematically assigned eight number types to tryptophan-deficient proteins. This was formulated on the basis of the relative quantum yield and the probable effects of the environmental and structural conditions effecting the tyrosyl fluorescence (Figure 4). The eight types were based on the publications cited above as well as other research dealing with tryptophan-deficient proteins.

Cowgill supported his classification by explaining the fluorescence effects of certain environmental conditions as well as the effects of each amino acid residue. Environmentally, he determined that oxygen did not quench the tyrosyl fluorescence but an increase in the temperature did increase the fluorescence yield of the sample. Cowgill also found that many amino acids (arg, his, lys, met, phe) have no effect on the tyrosyl fluorescence. There were, however, a few which did contribute substantially. When the carboxylate groups of asp and glu were negatively charged, they were shown to quench the tyrosyl fluorescence through a collisional process. On the other hand, the disulfides were shown to quench fluorescence, while free sulfhydrals had no effect. The remaining aliphatic residues (gly, leu, ala, ile, val, phe) alone have no fluorescence effects, although, as a group, they form a hydrophobic region; the resulting quenching properties are not fully understood.

In this study I used tyrosine fluorescence to monitor the denaturation of the regulatory subunit of aspartate transcarbamoylase. The basic features of the tyrosine fluorescence of RSU were first characterized. The fluorescence intensity of RSU was then measured, and this led to the further study of the

Classification of Tyrosyl Residues

Type I:	Exposed[a] and nonquenched[b] (R_{Tyr} approx 1.0)
Type II:	Exposed and quenched by hydrated peptide carbonyl groups (R_{Tyr} = 0.25 to 0.35)
Type III:	Quenched by disulfide groups[c]
Type IV:	Quenched by ionized Tyr residues[c]
Type V:	Buried in a hydrophobic environment and hydrogen bonded to peptide carbonyl groups (R_{Tyr} = 0.00)
Type VI:	Quenched by collision with ionized carboxylate groups[c]
Type VII:	Quenched by Tyr residues of Types V and VI acting as energy sinks[c]
Type VIII:	Quenched by Trp residues[c]

[a] Buried Tyr that have been studied are all of Types V and VII.

[b] Quenching by the peptide carbonyl group being abolished by a helical conformation or location of the peptide bond in a nonpolar environment.

[c] Degree of quenching depends upon spatial location of the interacting groups.

Figure 4: Cowgill's table for classification of tyrosyl residues on the basis of the relative quantum yield and the probable effects of environmental and structural conditions.
Cowgill, R. "Tyrosyl Fluorescence in Proteins and Model Peptides". (1968)

denaturation profiles using guanidine-HCl and urea as the denaturants. These results were then analyzed in terms of the known location of the tyrosine residues in RSU. The profiles were also studied using various concentrations of zinc ion in the buffer. Finally, the apo-protein (no zinc present) was prepared, and preliminary fluorescence and zinc analyses were performed.

Materials and Methods:

Chemicals. Ultrapure guanidine hydrochloride and urea were purchased from Bethesda Research Labs. Neohydrin was synthesized by the method of Rouland and Perry (1950). PMPS (p-hydroxymercuriphenylsulfonic acid) was purchased from Sigma Chemical. The trizma base which was used in all of the tris buffers was purchased from Sigma. The phosphate buffers were made from K_2HPO_4 and KH_2PO_4 (Sigma) .

Proteins and Reagents. ATCase was purified based on published procedures (Gerhart and Holoubek, 1967) from the *E. coli* strain EK1104/pER1 which uses the concept of genetically engineered overexpression in order to maximize the production of ATCase. A single colony of EK1104/ pER1 was inoculated into 2 ml of an enriched "LB medium and was grown at 37^{o} for 24 hrs. with concurrent shaking at 200 rpm. Then

0.60 ml of the solution was inoculated into 60 ml of minimal derepressing media (42 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 19 mM NH_4Cl, pH 7.4), supplemented by 2.0 mM $MgSO_4$, 0.10 mM $CaCl_2$, .20% glucose, .50% casamino acids, 0.001% B1, 0.023 mM ZnOAc, 50 ug/ml ampicillin and 0.003% uracil and was grown overnight. This 60 ml was inoculated into 6 L of the same minimal buffer, having a lower concentration of uracil; the culture was again allowed to grow for 24 hours.

Preparation of Extract. The cells were then collected by centrifugation (4^o, at 8000 rpm, for 25 min.) from the 6L culture and then resuspended in 130 ml of 0.1 M Tris-Cl pH 8.5, containing 24 mM magnesium acetate. Next, the cells were incubated in a water bath for 30 min. at 37^o in order for the lysozyme (90 mg) to break open the cell walls. This suspension of cells was then frozen in a dry ice-ethanol bath (about -65^o) and then thawed in a 37^o water bath to complete the disruption of the cell walls; this sequence was repeated twice. Once the cell walls were completely destroyed, DNase and RNase (3 mg each) were added to break down their respective nucleic acids, resulting in a less viscous solution. These cells were again incubated at 37^o for 30 min. Finally centrifugation (10,000 rpm, 4^o, for 1 hr.) was used to separate out the insoluble debris so that the supernatant, containing the soluble ATCase, could be collected.

Heat Step. For every 100 mls of supernatant collected, 35 mls of $(NH_4)_2SO_4$ and 4 mls of 1 M Tris-HCl, pH 8.9 was added. This solution was then

incubated in a preheated, 65°, water bath with gentle, constant swirling until the protein reached 60°. It was left at this temperature for 5 min. and was then cooled to 10° in an ice bath. Since ATCase is a relatively heat stable protein, the heat step conditions cause it to remain in solution where many other proteins denature and coagulate. Centrifugation (8000 rpm for 30 min.) was again used to separate the supernatant containing the ATCase from the coagulated debris. In order to concentrate the protein, the supernatant solution was then dialyzed against 3.6 M $(NH_4)_2SO_4$, pH 7.0 with 5 mM β-mercaptoethanol.

Acid Precipitation of ATCase. The sample was centrifuged (8000 rpm, for 30 min.) in order to sediment the precipitated protein, and the supernatant was discarded. The protein was then resuspended in a minimal amount of standard phosphate buffer (40 mM potassium phosphate, pH 7.0, 2 mM EDTA and 5 mM β-mercaptoethanol). This solution was dialyzed against 1L of 10 mM KH_2PO_4, pH 5.9, and 5 mM β-mercaptoethanol for 24 hrs. At pH's less than 6.0, ATCase reversibly self aggregates causing it to precipitate out of solution as a nearly pure white precipitate. The dialyzed sample was then recentrifuged. The supernatant was dialyzed against 1L of the same phosphate buffer (pH 5.9) while the pellet was resuspended again in the standard phosphate buffer, pH 7.0. This solution was centrifuged (10,000 rpm for 30 min.) in order to remove the insoluble aggregate; this was the first batch of ATCase. The supernatant in dialysis was also worked up in the

same fashion two additional times in order to obtain three batches of ATCase.

In order to separate the monomeric ATCase from the aggregate and the trace amounts of other protein, the ATCase was run through a Sephadex G-200 chromatography column. The column was equilibrated with the same standardized phosphate buffer, which had been degassed. The column flow rate was 15 ml/hr (80% of 1x on pump), and fractions were collected at 20 min. intervals. This column separates by weight into two main peaks; the ATCase aggregate is eluted first and then the monomeric ATCase (Figure 5). The ATCase fractions were checked for purity with polyacrylamide gel electrophoresis and absorbances at 280 nm; the purest fractions were pooled and stored as an $(NH_4)_2SO_4$ precipitate.

Polyacrylamide Gel Electrophoresis. In order to check the purity of the separations, a 7.5% native gel was made from 6 ml of distilled water and 3 ml of 1.5 M Tris-HCl, pH 8.9. To this solution, 3 ml of 30% Acryl / 8% Bis solution, along with 200 ul of a "fresh" 10% ammonium persulfate solution was added. Finally, 20 ul of temed (N,N,N-N'-tetramethyelthylenediamine) was added, causing the solution to polymerize. This solution was rapidly applied between two gel plates which had first been sealed at their base with a 1% agarose solution. Each gel plate contained 10 wells, and the samples were placed within these wells. A sample was prepared using 3 ul of bromphenol blue and 10 ul of the protein fraction. The gel buffer was a .15 M Tris-HCl, pH 8.9 solution and the gels were run at 20 mA and 50V for 1-2 hrs. When the gels were complete they were stained with a 12.5% trichloro

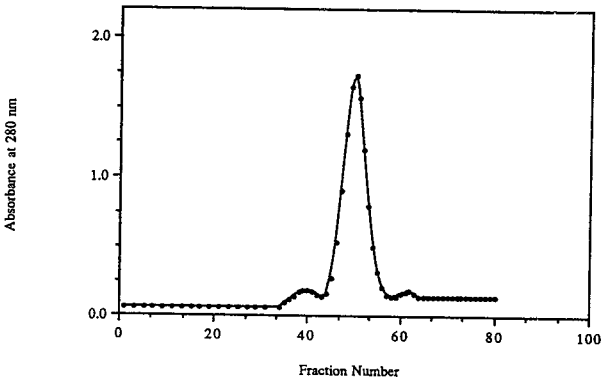


Figure 5: The elution profile of aspartate transcarbamoylase (ATCase) from a Sephadex G-200 chromatography column. The column separates by molecular weight into two main peaks; the ATCase aggregate is eluted first (fractions 33-43) and then the monomeric ATCase (fractions 48-54). The column is 2.6 cm x 101 cm.

acetic acid solution with commassie blue G for 3-4 hrs. They were then destained with a 45% methanol, 45% water and 10% acetic acid solution for another 2 hrs. Finally the gels were dried in a Hoeffer Dry Gel apparatus attached to a Cerco Pressovac vacuum pump.

Separation of Subunits. ATCase was separated into its regulatory (RSU) and catalytic (CSU) subunits by published procedures (Yang et. al., 1978). The ATCase precipitate was centrifuged and the resulting pellet was dissolved in a minimal volume of .01 M Tris-HCl buffer, pH 8.7 with .1 M KCl (no β-mercaptoethanol) while the supernatant was discarded. This protein solution was then dialyzed against the same buffer overnight. The resulting solution was treated with neohydrin, a mercurial compound which separates ATCase into its subunits. Neohydrin was added in a ratio of 8 mg of neohydrin to 100 mg of ATCase, and the reaction was allowed to proceed for 1.5 hrs. Immediately thereafter, the protein was applied to a 30 ml Whatman DE-52 anion exchange column. This column was equilibrated with the same .01 M Tris-HCl/.1M KCl, pH 8.7 buffer. Fractions were collected on a dropwise basis (150 drops/fraction), and the RSU was detected at an absorbance of 280 nm (OD^{280}). After the RSU fractions were pooled, 2 mM ZnOAc and 10 mM β-mercaptoethanol were added and the solution was dialyzed versus 3.6 M $(NH_4)_2SO_4$. In order to elute the CSU from the column, the salt concentration of the buffer was increased to .5 M KCl; this allowed the resin to exchange the bound CSU for the excess chloride ions in the salt. Fractions containing CSU were also evaluated by OD^{280} and 2mM β-mercaptoethanol was added to the pooled fractions which were also

stored as an $(NH_4)_2SO_4$ precipitate.

Apo-Protein Preparation. After the ATCase had been separated into its subunits and the RSU had been pooled, three additional columns were employed to remove the bound zinc ligand, according to published procedures (Jefferson et. al., 1990). All columns and resins were purchased from BioRad. The RSU (1.32 mg/ml to 3.08 mg/ml, either obtained from ammonium sulfate storage or freshly prepared from ATCase) was first passed through a P-10 (0.75 cm x 24 cm) desalting resin column which removed the protein from the salts in which it was sitting. This column was equilibrated with a 20 mM Hepes KOH/100 mM KCl, pH 7.2 buffer solution. Fractions were collected at 50 drop intervals and the RSU was located and pooled using absorbances of 280 nm. To the resulting RSU, .28 mg PMPS (p-hydroxymercuriphenylsulfonic acid) per mg of RSU was added to displace the bound Zn^{2+} with Hg. This solution was applied to the third column. This was a 30 ml Chelex 100 column, and it was used in order to remove the excess PMPS and the zinc, which had been removed from the protein. These substances adhere to the column, and the rest of the protein is flushed through. This column was equilibrated with the same Hepes/KOH buffer, and fractions were again collected at the same intervals; absorbances were then taken at 280 nm. Finally, β-mercaptoethanol was added to the pooled protein to remove the mercury from it; it was then applied to the final P-10 column. This column was again used for desalting purposesin order to separate the apo-protein from the removed mercury. The column was equilibrated with the same Hepes/KOH buffer with the addition of 5 mM β-

mercaptoethanol. This column buffer had also been run through a Chelex 100 column before being applied to the P-10 column in order to minimize the concentration of metal ions. The concentration of the RSU which resulted was checked at OD^{280}.

Protein Concentration. All protein concentrations were quantitated by measuring absorptions at 280 nm. ATCase has an extinction coefficient of .59 $(mg/ml)^{-1}$ cm^{-1} and for RSU it is .30 $(mg/ml)^{-1}$ cm^{-1}. The instrument used for these measurements was a Perkin Elmer Lambda 3B UV/Vis Spectrophotometer, along with a Perkin Elmer R-100 A chart recorder.

Fluorescence Measurements. Fluorescence data was collected on a Perkin Elmer LS-5B Luminescence Spectrometer, in conjunction with a Perkin Elmer R-100 A chart recorder. All measurements were obtained at 21^{o} using a circulating water bath. Instrument conditions were: slit widths of 3 nm(excit)/10 nm (emm), a fixed scale of .60 and a response time of "3". Samples were excited at 268 nm and emission spectra were collected between 268-400 nm. For the denaturation experiments, after mixing the protein and denaturant, emission spectra were recorded until no further changes were observed.

Results & Discussion:

This research dealt with the characterization of several properties of the regulatory subunit of aspartate transcarbamoylase. RSU is a unique protein since it is tryptophan-deficient, and its fluorescence is due solely to its tyrosine residues. My research had four parts: first, the fluorescence characteristics of RSU were compared to those of free tyrosine. Second, denaturation studies were performed using tyrosine fluorescence as a probe of denaturation. The analysis of this data gave insight into possible structural effects involving the environment of the tyrosine residues during denaturation. Third, denaturation profiles at different concentrations of RSU gave insight into possible mechanisms of denaturation. Lastly, studies aimed at elucidating the role of zinc in the denaturation were initiated.

Protein Purification of ATCase.

The final process in the purification of ATCase from E. coli is the passage of the protein through a Sephadex G-200 chromatography column. The column separates into two peaks by molecular weight (Figure 5). The ATCase aggregate is eluted first (fractions 33-43), and then the monomeric ATCase (fractions 48-54) follows. The purity of the fractions was assessed by polyacrylamide gel electrophoresis. The ATCase bands traveled much further down the gel than the aggregate bands which remained near the top; this allowed for the proper separation of the components. A clean separation was obtained because the initial aggregate peak was relatively small in comparison

to that of the pure ATCase.

Fluorescence Studies.

Characterization of RSU Spectra and Relative Quantum Yield.

It was first necessary to characterize the tyrosine fluorescence of RSU. In order to accomplish this the fluorescence characteristics of RSU were compared to those of free tyrosine at an excitation wavelength of 268 nm (Figure 6). The emission spectra are very similar, and both the RSU and the tyrosine have their maximum wavelengths occurring at 304 nm. These similarities were an indication of a pure RSU preparation because the RSU curve would have been much broader and found at a higher wavelength if there had been any tryptophan-containing protein contaminants. A quantum yield value for the RSU fluorescence relative to that of free tyrosine was then calculated using data such as that shown in figure 6.

The quantum yield is a measure of the proportion of absorbed light which is emitted as fluorescence. In order to obtain a quantitative value the emission spectra of RSU and tyrosine were measured at an excitation wavelength of 268 nm and in the same buffer at nearly identical absorbances. An average quantum yield of 0.69 ± .007 was obtained. This was determined using the following equation:

$$R_{tyr} = \frac{\text{integrated area RSU}}{\text{integrated area tyr}} \cdot \frac{\text{absorbance of tyr}}{\text{absorbance of RSU}}$$

This equation takes into account the actual absorbances of the samples and the integrated areas under both the RSU and the tyrosine fluorescence emission

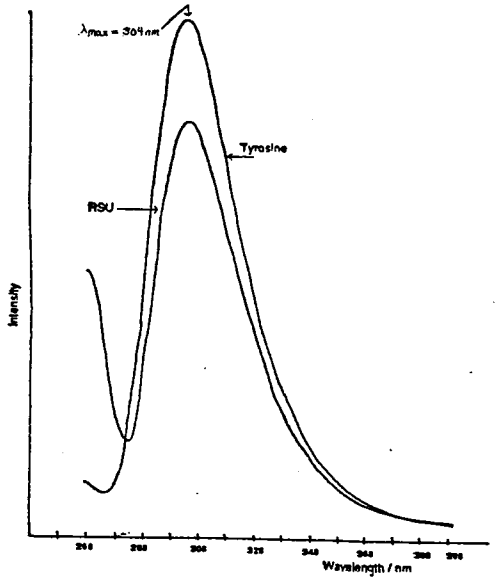


Figure 6: Fluorescence emission spectra of RSU versus that of free tyrosine.

curves. The value of 0.69 is relatively high for a tryptophan-deficient protein; for example, the quantum yield for RNase A is 0.16 and that for ovomucoid is 0.12. I hope to eventually explain this difference in terms of the environment of the tyrosine residues in RSU.

The two tyrosine residues at positions 77 and 89 in the allosteric domain are highly exposed (hydrophilic environment) whereas the tyrosine at position 140, near the zinc atom, is buried by the rest of the chain (Figure 7). It has been shown that exposed tyrosine residues have higher quantum yields than buried residues due to the quenching effects of various groups within the protein (see discussion below). Perhaps the relatively high quantum yield of the folded RSU is due to the exposure to solvent of two out of its three tyrosines in the native state.

Denaturation Studies of RSU.

Once the quantum yield data had been obtained, the protein was then denatured using guanidine hydrochloride and urea as the denaturants. In other fluorescence studies of tryptophan-deficient proteins it has been found, that they increase in fluorescence as they denature. Therefore an increase was anticipated. It was actually found that when comparing the 0.0 M concentration of denaturant to the fully denatured version, there was a decrease in the fluorescence intensity to about 66 $\pm$ 3% of the original native protein (Figure 8). Since a significant difference was observed, this difference was used to monitor the unfolding of the RSU as a function of denaturant concentration.

In order to observe the intermediate steps in the denaturation, various concentrations of the denaturant were used. In the guanidine experiment a cooperative denaturation was observed between 0.20 M and 0.70 M guanidine;

Figure 7: A space filling model of the x-ray crystalline structure of the RSU dimer. Tyrosine 77 and tyrosine 89 are in somewhat of a hydrophilic environment but tyrosine 140, near the zinc atom, is essentially buried in the molecule. The tyrosine residues are highlighted in pink.
Nanovision - ACS Molecular Graphics Program

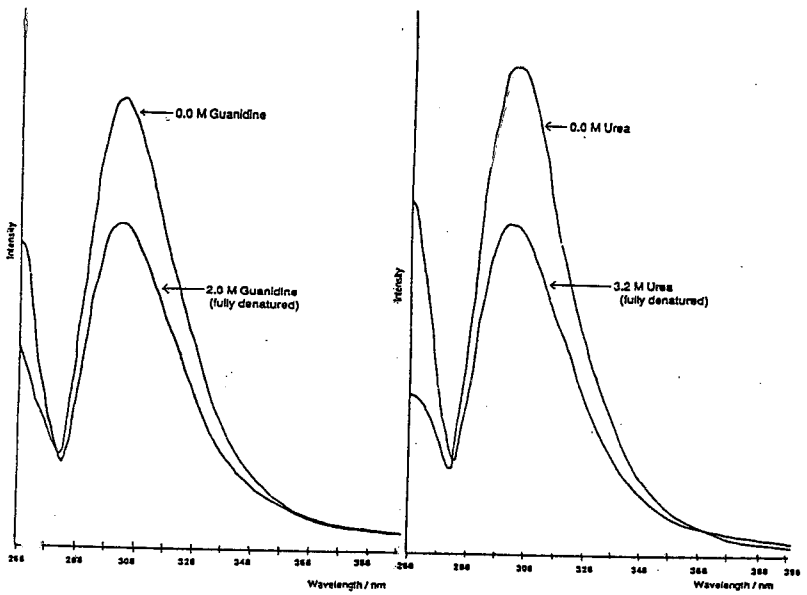


Figure 8: Denaturation spectra of RSU versus guanidine-HCl and urea. Comparison of the 0.0M concentration to that of the fully denatured concentration.

the largest percent decrease occurred between 0.50 M and 0.60 M guanidine (Figure 9). The denaturation profile of urea was also cooperative; the denaturation occurred between 1.0 M and 2.0 M urea, with the largest percent decrease occurring between 1.6 M and 1.8 M (Figure 10). It took more urea to unfold and denature the RSU since urea is not as strong a denaturant as guanidine. Both of these curves were very smooth and neither exhibited more than one transition. This is consistent with a single step denaturation process.

It was possible to take these denaturation conclusions one step further. Bowie and Sauer (1989), in their research with the Arc repressor dimer, considered two possible mechanisms of protein denaturation. In the first mechanism the dimer dissociated before unfolding ($A_2 \underset{}{\overset{K1}{\rightleftharpoons}} 2A \overset{K2}{\rightleftharpoons} 2U$; two-step denaturation). This model would predict that the denaturation profile would be independent of protein concentration. However in the second mechanism, the dissociation occurred concomitantly with unfolding, ($A_2 \overset{Ku}{\rightleftharpoons} 2U$), and was described as a concerted reaction. This model would imply that an increase in protein concentration would shift the denaturation curve to the right. This is because with increased protein concentration, the rate for the reverse reaction would increase more than the rate for the forward because of the squared term in U ($rate_{for} = k_{for}[A_2]$; $rate_{rev} = k_{rev}[U]^2$). The second mechanism is in agreement with the previously obtained single-step denaturation profile for RSU.

Assuming that the concerted mechanism applied to RSU, the equilibrium constant for the unfolding process of RSU was determined from the following equation: $K_u = 2P_t [f_u / (1-f_u)]$. The fraction that unfolded (f_u) was calculated from the denaturation profiles, and the total protein concentration (P_t) was

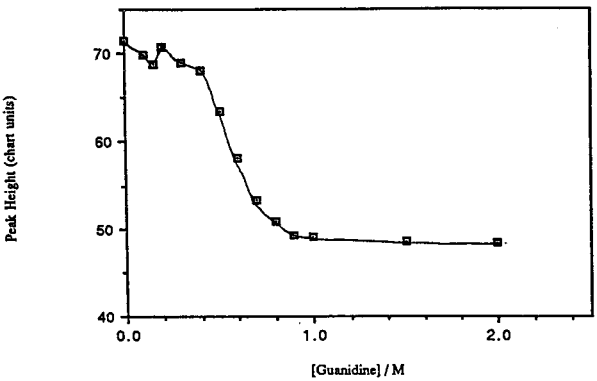


Figure 9: The denaturation profile of RSU using guanidine-HCl. The denaturation was cooperative between 0.20 M- 0.70 M guanidine-HCl.

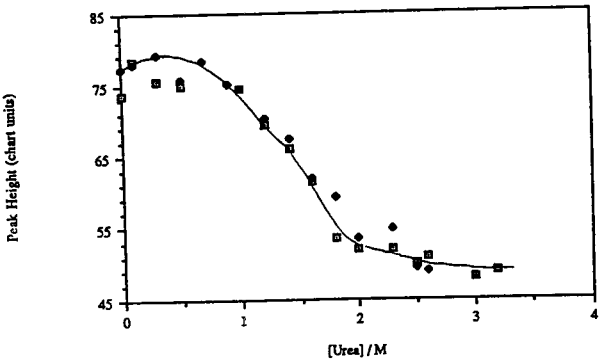


Figure 10: The denaturation profile of RSU using urea. There was a cooperative denaturation between 1.0 M- 2.0 M urea. The two sets of points represent two runs of this experiment on different days.

previously determined. The free energy of unfolding (ΔG_u°) was then determined from K_u. This method was applied to two experiments. In the first experiment 95 ug/ml of RSU was denatured using either guanidine-HCl or urea as the denaturants. The free energy values for these curves should have intersected the y axis at the same point because the free energy should not change with a change of denaturant. The intersection points were slightly different (Figure 11). The ΔG_u° for guanidine was 14.4 kcal/mole and that of urea was 12.9 kcal/mole. At this point, we are not sure why these values are not the same. One possibility is that each denaturant unfolds the RSU into a different state.

In the second experiment the protein concentration was varied. The concentration was increased from 95 ug/ml to 400 ug/ml of RSU and these samples were denatured using guanidine-HCl. Clearly, when the concentration of RSU was increased, it took more guanidine to denature the RSU, and the curve shifted to the right (Figu. 12). Although the curve was shifted to the right, this had no effect on the value of ΔG_u° as would be expected. In figure 13 both of the free energy curves had similar ΔG_u°'s with an average value of 14.4 kcal/mole. This result was independent of protein concentration and it was also consistent with the $A_2 \underset{}{\overset{Ku}{\rightleftharpoons}} 2U$ single-step mechanism.

The denaturation of RSU was accompanied by a decrease in fluorescence which is only observed in a few tryptophan-deficient proteins. This decrease may be better understood by investigating the environments surrounding each tyrosine residue. It has been shown that many amino acids (arg, his, met, lys, phe) have no effect on the tyrosyl fluorescence (Cowgill, 1976). There are, however, amino acids which do affect tyrosine fluorescence.

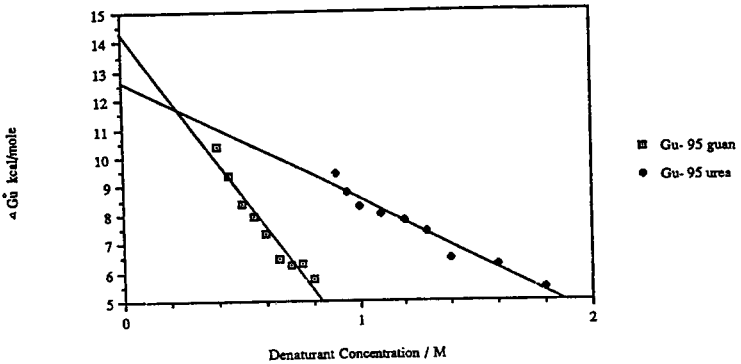


Figure 11: Free energy of unfolding (ΔG_u^e) of 95 ug/ml of RSU, using both guanidine-HCl and urea as the denaturants. The free energy of unfolding for guanidine was 14.3 kcal/mole and that of urea was 12.6 kcal/mole.

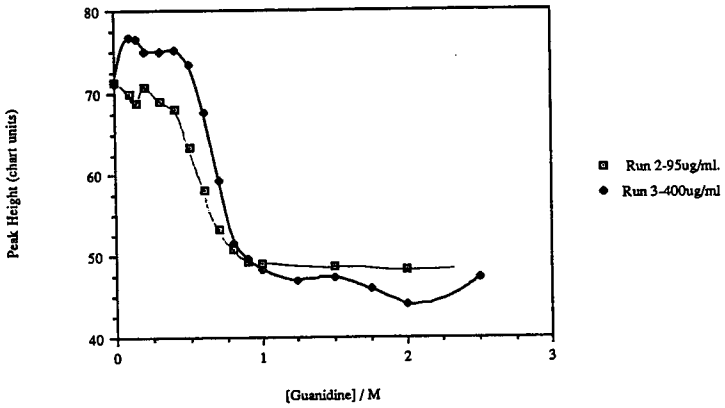


Figure 12: Denaturation profiles of RSU at 95 ug/ml and at 400 ug/ml using guanidine-HCl.

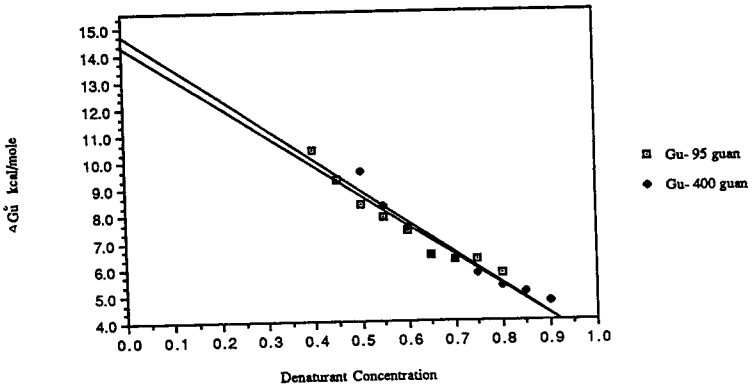


Figure 13: Free energy of unfolding (ΔG_u^o) for guanidine at two different concentrations of RSU. Two different concentrations of guanidine were used: 95 ug/ml and 400 ug/ml. The average ΔG_u^o was 14.45 kcal/mole.

The dissociated carboxyl groups of asp and glu have been shown to quench tyrosyl fluorescence through a collisional process (Cowgill 1976). Carbonyl groups have also been shown to quench fluorescence. In addition, disulfide bonds formed from cys residues quench tyrosine fluorescence as there is an increase in the fluorescence when the disulfide bonds are reduced. The remaining aliphatic residues (gly, leu, ala,ile, val) alone do not effect the fluorescence, yet as a group they form a hydrophobic region where the quenching properties are not fully understood.

The environments surrounding each tyrosyl residues in RSU were examined within a 3.35 A radius. Tyrosine 77 is surrounded mostly by aliphatic groups such as gly, pro, ala, and leu. These groups have been shown not to have any significant effect on the fluorescence. This residue is also the only tyrosine which is situated on an alpha helix. According to Cowgill it has the potential of being quenched by carbonyl groups, yet there are none present. Likewise, tyrosine 89, in the same region, is surrounded by some aliphatic residues (ala, gly, val) and also by one glu and one asp residue. Each of these residues (glu, asp) has a dissociated carboxyl group which quenches the tyrosyl fluorescence by a collisional process. This process occurs because these residues are in close proximity to the tyrosine. Finally tyrosine 140, located near the zinc atom, is surrounded by two ser, two asn and one cys residue. The ser and the asn residues do not affect the fluorescence. The cys residue would have the potential to quench the fluorescence if it were involved in disulfide bonding. Since there are no disulfide bonds, there is no fluorescence effect. Although this tyrosine is adjacent to the zinc atom, the zinc has not been found to contribute to the tyrosyl fluorescence (Chen, 1976). Although these environmental studies have aided in evaluating the quenchers

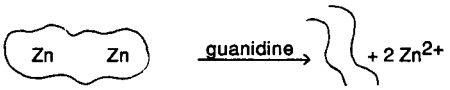
of tyrosyl fluorescence, there is still some doubt as to the actual cause of the decrease in intensity from the native state.

Zinc Analysis of Apo-protein Buffer Concentration Studies.

Once a reliable method to monitor the denaturation of RSU had been established, it was used to study the role of zinc in the denaturation of RSU. Since RSU is a zinc-binding protein, the role which zinc plays in the denaturation must be understood in order to gain insight into the the contribution of zinc to the overall stability of the protein. The study of the role of zinc was initiated by observing various concentrations of zinc ion in the buffer. For some metal binding proteins, when the metal ion concentration in the buffer is increased, an increase in their T_m (temperature at which the thermal induced denaturation occurs) is observed. This can be understood in terms of the following equilibrium:

$$\text{Folded protein} \cdot M \underset{\text{heat}}{\rightleftharpoons} \text{Unfolded protein} + M^+$$

Le Chatelier's principle tells us that if the concentration of the metal ion in the buffer is increased, this will push the equilibrium to the left and an increase in T_m should be observed. This model applied to RSU can be represented as follows:



In order to test this model various concentrations of zinc ion in the buffer were used to observe their effects on the profile. In each experiment 3.5 uM RSU was used, and the concentration of zinc in the buffer was varied between 0.0 to 2.2 mM. In every case RSU binding sites were saturated with zinc. At 0.0 M the intensities were very similar, but when the RSU was completely

denatured, small fluorescence differences were observed in the opposite direction predicted by the model (Figure 14).

The data in figure 14 was used to determine the concentration of guanidine required for half the complete denaturation to occur. It took slightly more guanidine to denature the RSU with no additional zinc added. However, these small differences may be due only to the differences in fluorescence of the unfolded state of the protein. In any case, evidence does not support our model, and this may indicate that a simple model with concomitant unfolding and zinc release is not appropriate for RSU denaturation. Further studies are necessary to confirm whether this observation was due to the fact that chemical denaturants were used rather than heat.

It is also of interest to compare the denaturation profiles of the RSU with the bound zinc to the apo-protein, the protein with the Zn^{2+} removed. These studies would give a direct measure of the role of zinc in the stability. Through an extensive process, the apo-protein was formed and the two zinc ions were removed. From the preliminary data (Figure 15), it appeared that the zinc was removed by this process because there was one tenth the amount of zinc left at the end of the process (Table 1). It was, however, difficult to determine whether or not the removal of the zinc had an effect on the profile.

In the future it would be interesting to perform the ΔG_U° studies with additional concentrations of RSU. It may also be appropriate to check with differential scanning calorimetry if the dismissal of concomitant unfolding and zinc release was due to the fact that a chemical denaturant, rather than heat, was used. Finally, additional studies should be carried out with the apo-protein in order to obtain a reliable denaturation profile and information

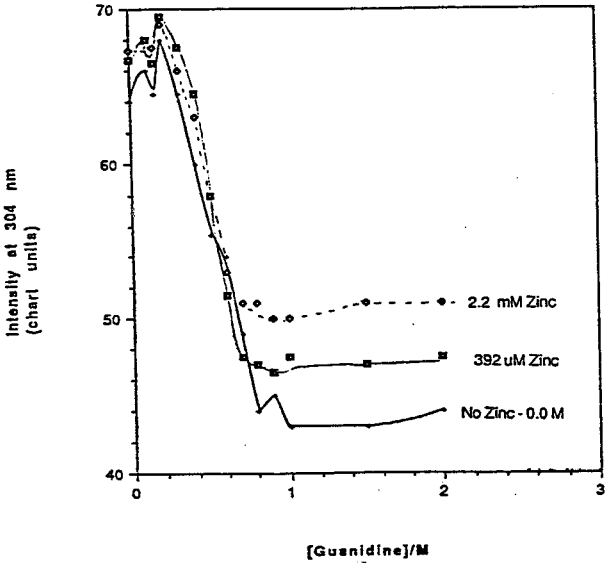


Figure 14: Zinc analysis of RSU using a constant protein concentration of 3.5 uM, while varying the concentration of zinc ion in the buffer from 0.0 to 2.2 mM.

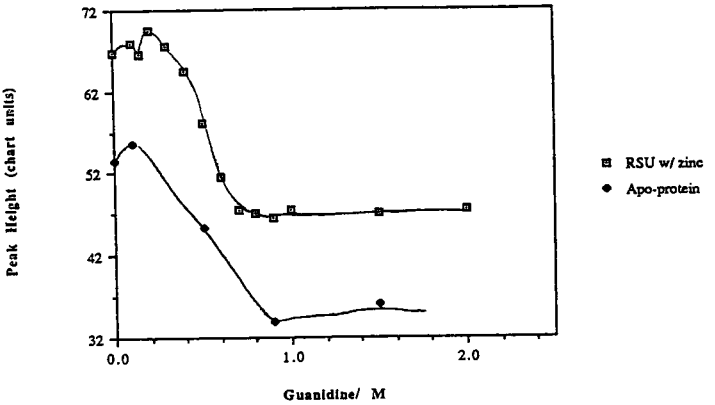


Figure 15: Preliminary apo-protein data showing the effects of the removal of the zinc ion on it's denaturation profile.

Sample	Initial Solution Concentration of RSU ug/ml	Final Zinc Concentration /ppb	Mole : Mole Ratio
RSU with Zinc	40.2	131.2 (theoretical=154 ppb)	0.85
Apo-Protein	40.0	13.1	0.085

Table 1: Preliminary apo-protein data , showing a ten fold decrease in the amount of zinc in the apo-protein from that of pure RSU.

about the role of zinc in the protein stability.

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