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Denaturation Studies on Aspartate Transcarbamoylase and its Catalytic Subunit

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DENATURATION STUDIES ON ASPARTATE TRANSCARBAMOYLASE AND ITS CATALYTIC SUBUNIT

by

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ABSTRACT

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Two separate but overlapping projects were studied. First, the tryptophan corrected fluorescence emission spectra of ATCase and CSU were characterized under a variety of denaturant conditions at excitation 295 nm. Because CSU is a portion of ATCase, the environments of the same two tryptophan residues were being probed in both systems. In the absence of denaturant ,native protein), the spectrum for ATCase (λ_{max} = 337.0 nm) was reproducibly blueshifted from that of CSU (λ_{max} = 339.0 nm). Therefore, association of CSU with RSU affects the environment of at least one of the two tryptophans in CSU. In the presence of 4.0 M guanidine HCl (completely denatured protein), both ATCase and CSU gave emission spectra with λ_{max} = 352 nm. This red shift relative to the native proteins reflects exposure of the tryptophan(s) to the polar aqueous environment. Treatment of CSU with 1.25 M NaSCN (dissociated but folded monomers) (*J. Biol. Chem.*, 257, 8638) caused only a 2.0 nm red shift relative to native protein, indicating that dissociation of the trimer has only a small effect on the environment of the tryptophans. The denaturation profile of CSU done previously by Ira Gurland was highly cooperative but displayed asymmetry, indicating more than one transition in denaturation of CSU with the second larger transition reflecting the unfolding of the equatorial domain where the two tryptophans are located. Comparison of the denaturation profiles of ATCase and CSU (Ira Gurland) showed that the difference in λ_{max} between the two proteins dissipated at guanidine HCl concentrations of 1.75 M or greater.This result is consistent with the dissociation of RSU from CSU at low (0-1.75 M) guanidine HCl concentrations. The second study involved the investigation when, if at all, zinc release from ATCase and CSU under denaturing conditions caused a change in A_{500} resulting from chelation to 100 uM PAR . There was no detectable change in A_{500} of PAR upon denaturation of ATCase. A large increase in A_{500} was observed, however, from the unfolded regulatory chain upon addition of PMPS. Therefore, zinc was still bound to unfolded ATCase. Due to this surprising result, the release of zinc from three other proteins were studied: alcohol dehyrogenase , carbonic anhydrase and thermolysin.

Introduction:

E. Coli aspartate transcarbamoylase (ATCase) is a well studied enzyme which catalyzes the first step in the biosynthesis of pyrimidines. ATCase is large (MW=300,000) and complex. Each ATCase molecule has twelve polypeptide chains: six identical catalytic chains and six identical regulatory chains. The six catalytic chains are grouped into two trimers (MW=100,000) called the catalytic subunit (CSU), while the six regulatory chains are grouped into three dimers (MW=33,000) called the regulatory subunit (RSU) (see Figure 1). Each catalytic chain is folds into two domains: the carboxy-terminal or "equatorial" domain (residues 7-132) and the N-terminal or "polar" domain (residues 155-283) joined by two α-helices. (Stevens, R.C.,1990).

There are four forces that determine the structure and stability of a protein. These are electrostatic interactions, van der Waals interactions, hydrogen bonding, and hydrophobic interactions. These forces that hold the protein together can be disrupted either chemically or by heat, causing the protein to unfold. This process is called denaturation. Denaturation of many small unidomain proteins have been shown to occur in one step (N <==> D) where N is the folded native protein and D is the denatured protein. However, many other proteins can denature in more than one step with intermediate step(s) $I_1....I_{1+n}$ (N <==> I_1 <==> I_{1+n} <==> D). Therefore, the single step process of unfolding might not apply if the concentration of the intermediates is significant enough. (Pace, C.N., 1975)

The denaturation of a protein can be monitored by various spectroscopic methods including fluorescence spectroscopy, ultraviolet

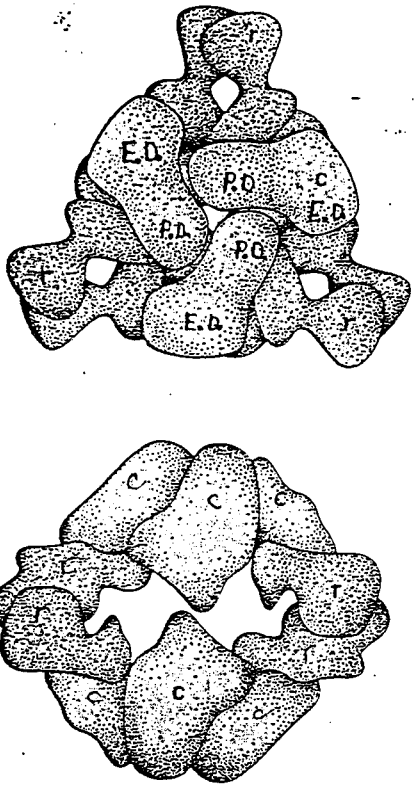


Figure 1 A space filling model of ATCase. In the upper diagram (top view), it is possible to see all 3 RSU dimers, denoted by r, while in the lower diagram (side view), it is possible to see both CSU trimers, denoted by c. The bidomain structure of CSU can be seen in the upper diagram with the equatorial domain denoted E.D. and the polar domain denoted P.D. From Stryer, 1988.

difference spectroscopy, and circular dichroism spe ..oscopy. In this thesis, fluorescence was used to monitor the denaturation of ATCase and its CSU. Fluorescence is the emission of light from a molecule as a result of being excited by light of a lower wavelength. In proteins, there are three amino acids that are fluorescent: phenylalanine, tryptophan, and tyrosine. These are the aromatic amino acids. Phenylalanine is only weakly fluorescent compared to tyrosine and tryptophan (see Figure 2). However, the fluorescence of proteins is dominated by tryptophan fluorescence because of energy transfer from tyrosine to tryptophan. (Lakowicz, J.R., 1983) Furthermore, it is possible to select an excitation wavelength of light that exclusively excites tryptophan, i.e. 295 nm because tyrosine does not absorb at that wavelength.

In ATCase, there are two tryptophan residues per catalytic chain (310 amino acids) while there are none in the RSU. The two tryptophans are both located in the equatorial domain of the catalytic chain at positions 209 and 284. Therefore, the same tryptophan residues are being monitored in CSU and the holoenzyme. There are tyrosines in both CSU and RSU, however, as stated earlier, the tryptophans can be studied exclusively.

Tryptophan fluorescence is sensitive to the polarity of the tryptophan's environment. When the protein is in its native state, the tryptophan residues are buried in a nonpolar hydrophobic pocket. However, as the protein denatures, the tryptophan residues are exposed to water and "move" to a polar environment. This change from a nonpolar to a polar environment of the tryptophans during denaturation causes the peak emission wavelength of the tryptophans to shift to a higher wavelength, known as a red shift. This is what allows us to monitor the denaturation of a protein using fluorescence

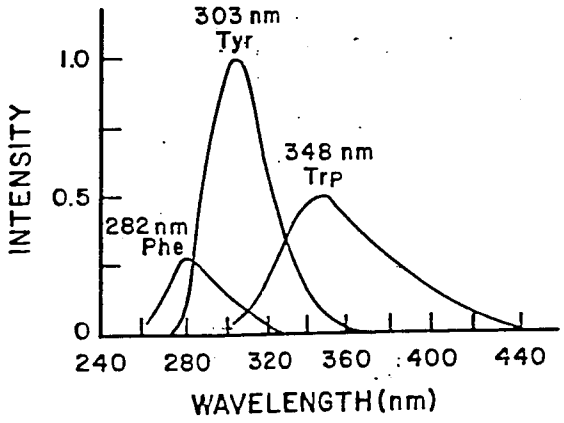
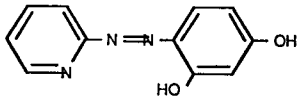


Figure 2 The fluorescence emission spectra of tryptophan, tyrosine, and phenylalanine. From Lakowicz, J.R., 1983.

spectroscopy. (Lakowicz, J.R., 1983) In this study, the denaturants guanidine hydrochloride and sodium thiocyanate (NaSCN) are used.

One thing that may contribute to the stability of some proteins is a metal atom which is bound to amino acid ligands. In ATCase, there is one zinc bound to each regulatory chain (or two zincs per RSU dimer), and therefore, six zinc ions per ATCase enzyme. Each zinc is bound tetrahedrally to four cysteines, Cys-109, -114, -137, and -140. The polypeptide chain in the region forms two loops, which the zinc atom holds together. Stabilization of these loops, which form part of the RSU/CSU interface, is thought to be responsible for stabilizing the quaternary structure. (Vallee, B.L., 1990)

In this project we investigated when, if at all, the zinc ion is released during the denaturation of ATCase by guanidine HCl. To perform these studies we took advantage of an indicator which binds zinc strongly : 4-(2-pyridylazo)resorcinol (PAR), which is pictured below:



Upon binding zinc, PAR undergoes a dramatic increase in absorbance at 500 nm due to the increase in molar extinction coefficient when the zinc and PAR complex. As the zinc is being released from the protein, the PAR indicator turns from yellow to orange, so the zinc release can be visualized. This indicator has been previously used to detect the displacement of zinc by 4-(2-pyridylazo)resorcinol in ATCase. (Hunt, J.B., 1985)

Other zinc containing proteins are also examined for their release of zinc during denaturation. These were alcohol dehydrogenase from *yeast*,

carbonic anhydrase from *bovine erythrocytes*, and thermolysin from *Bacillus thermoproteolyticus*. Alcohol dehydrogenase (YADH) has its catalytic zinc bound to two cysteine ligands (Cys-46 and -174), a histidine ligand (His-67), and a water molecule. The peptide backbones of all three ligands are firmly anchored in secondary structural elements, not in a flexible loop region. Cys-46 is the first residue of a short α-helix and the last residue of a β-strand; His-67 is the first residue of a β-strand; and Cys-174 is in the middle of an α-helix. The geometry about the zinc is a distorted tetrahedron. (Vallee, B.L., 1990) Carbonic anhydrase has three histidine residues (His-94, -96, -119) and a water molecule as ligands to zinc. A β-strand encompassing residues 88-108 supplies His-94 and His-96, while another β-sheet extending from residues 113 to 126 contributes His-119. The geometry about the zinc is a distorted tetrahedron. (Vallee, B.L., 1990) Thermolysin has one catalytic zinc that is bound to His-142, His-146, Glu-166, and a water molecule. These residues are located around the zinc in a distorted tetrahedron.The two histidines are part of an α-helical domain that extends behind the zinc and traverses the entire center of the molecule. The glutamic acid residue comes from another long internal helix. (Vallee, B.L., 1990)

This thesis describes work concerning two distinct but overlapping projects. The first project describes fluorescence emission spectra of ATCase and CSU using guanidine HCl and NaSCN as denaturants. The second project describes monitoring when, if at all, the zinc ion is released during the denaturation of ATCase using the indicator PAR.

Materials and Methods:

Chemicals. Ultrapure guanidine hydrochloride was purchased from Bethesda Research Labs. 4-(2-pyridylazo)resorcinol (PAR) was purchased from Aldrich Chemical Co. *p*-(hydroxymercuri)phenylsulfonic acid (PMPS) was purchased from Sigma Chemical Co.

Proteins (other than ATCase). Alcohol dehydrogenase from *yeast*, carbonic anhydrase from *Bovine erythrocytes*, and thermolysin from *Bacillus thermoproteolyticus* were purchased from Sigma Chemical Company.

Buffers. Phosphate buffer was made from K_2HPO_4 and KH_2PO_4 (Sigma). Tris buffer and Hepes buffer were purchased from Sigma Chemical Co. The buffer used for the tryptophan fluorescence experiments was 40.0 mM Tris-OAc, pH 7.5. The buffer used for the zinc release absorption experiments was 40.0 mM Hepes-KOH, pH 7.0 which was first passed through a column (5 cm x 20 cm) of Chelex-100 resin purchased from Bio-Rad.

Proteins and Reagents. ATCase was purified by a procedure based on published methods (Gerhart and Holoubek, 1967) from strain EK1104/PER1. The phenomenon of overexpression is utilized by this method. Overexpression is the process that causes a cell to synthesize very large amounts of a particular protein. The catalysis of ATCase is the first step in the bisynthesis of pyrimidines. If the cells are deprived of the pyrimidines in the medium, the cells will produce large amounts of enzymes, mainly ATCase, involved in the synthesis of pyrimidines. Low concentrations of uracil is provided in the media, which is used up quickly by the dividing cells.

After using up the uracil in the media, the cells transcribe large amounts of mRNA to code for ATCase off an inserted plasmid. The ATCase can then be isolated from the cells.

A colony of EK1104/pER1 *E. Coli* was inoculated into 2.0 ml of rich LB media and grown for 24 hours at 37°C, while shaking at 200 rpm at 37°C. Then, 0.6 ml of the culture was inoculated into 60 ml of the minimal buffer (42 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, and 18 mM NH_4Cl, pH 7.4) supplemented with 2.0 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.2% glucose, 0.5% casamino acids, 0.001% B1, 0.023 mM ZnOAc, 15 ug/ml uracil, and 50 ug/ml ampicillin. After 20 hours of growth, 60 ml (6 x 10 ml) of culture was inoculated into 6 L (6 x 1L) of the same minimal buffer with the same supplements, except only 5 ug/ml of uracil was present, for maximal overexpression of ATCase.

Preparation of Extract. The cells were harvested from the 6 L culture by centrifugation and were resuspended in 150 ml of 0.1 M Tris-Cl, pH 8.5 with 14 mM magnesium acetate. 90 mg of lysozyme was added to the suspension and it was incubated at 37°C for 30 minutes. The cells were then frozen in a dry ice/ ethanol bath (-70°C) until frozen and then thawed in a 37°C water bath. The freeze/thaw sequence was repeated once more. 3.0 mg DNase and 3.0 mg RNase were added and then incubated at 37°C for 30 minutes. The cell debris was centrifuged (8000 rpm x 1.0 hour) and the supernatant was collected. (Saved 0.5 ml for a gel).

Heat Step. For every 100 ml of supernatant, 35 ml of 3.6 M $AmSO_4$ and 4 ml of 1.0 M Tris-HCl, pH 8.9 was added. The mixture was then incubated in a pre-heated water bath (65°C), and swirled gently, until the protein mixture

reached 60°C. After 5 minutes, the protein was cooled in an ice water bath immediately. The precipitate was centrifuged (8000 rpm x 30 minutes) and the supernatent was dialyzed against 2 L of 3.6 M $AmSO_4$ pH 7.0 buffer, with 5.0 mM β-mercaptoethanol. The protein precipitated out of solution.

Acid Precipitation. The sample was centrifuged (8000 rpm x 30 minutes) to sediment the precipitated protein. The precipitant was resuspended in a minimal volume (20 ml) of standard phosphate buffer (40 mM potassium phosphate, 0.2 mM EDTA, 5 mM β-mercaptoethanol, pH 7.0), and then was centrifuged (8000 rpm x 30 minutes). The solution was dialyzed against 1 L of 10 mM KH_2PO_4, 5mM β-mercaptoethanol, pH 5.9 for 24 hours. The sample was centrifuged (8000 rpm x 30 minutes). The supernatant was dialyzed against 1 L of pH 5.9 buffer for 24 hours. The precipitate was resuspended in 12 ml of standard phosphate buffer and then centrifuged (10,000 rpm x 30 minutes) to remove insoluble aggregated protein. The following day, the dialyzed sample was worked up the same way as this batch. The supernatent was redialyzed and worked up for a third batch.

G-200 Chromatography. A G-200 Sephadex (5.3 cm^2 x 100 cm) chromatography column was used to separate monomeric ATCase from higher aggregates and other trace amounts of other proteins. The G-200 column was equilibrated with standard phosphate buffer (40 mM phosphate, 0.2 mM, 5 mM β-mercaptoethanol pH 7.0. All buffer running through the column was previously degassed. The sample was loaded onto the column (13 ml maximum). The flow rate was about 15 ml an hour (80% of "1x" on the pump), and 20 minute fractions were collected. The fractions were analyzed by absorbance at 280 nm and the ATCase fractions were pooled

and dialyzed against 3.6 M $AmSO_4$, 15 mM β-mercaptoethanol. The individual fractions were also analyzed on a polyacrylamide gel to determine which fractions contained pure, monomeric ATCase. The protein was stored as a precipitate.

Separation of Subunits. ATCase was separated into CSU and RSU according to a published procedure (Yang et. al., 1978). The ATCase was dialyzed against 0.01 M Tris-Cl, 0.1 M KCl, pH 8.7 buffer (no β-mercaptoethanol). Neohydrin was then dissolved into 1 ml of 0.1M Tris-Cl, pH 8.7 buffer. This was added to the protein (7 mg/ 100 mg ATCase) and left to react for 15 minutes. Afterwards, the solution was applied to a 30 ml DEAE-cellulose column equilibrated in a buffer of 0.01 M Tris-Cl, 0.1 M KCl, pH 8.7. This buffer was then applied to the column to elute the RSU, which was detected by absorbance at 280 nm. The RSU fractions were pooled, and 2 mM ZnOAc and 10 mM β-mercaptoethanol were then added. The fractions were then dialyzed against 3.6 M $AmSO_4$, 5 mM β-mercaptoethanol and stored as a precipitate. The CSU was eluded afterwards by using the same buffer supplemented with 0.4 M KCl. The fractions were then analyzed by absorbance at 280 nm for protein content. The fractions for CSU were also pooled and β-mercaptoethanol was added to a final concentration of 5 mM. CSU was dialyzed against 3.6 M $AmSO_4$, 5 mM β-mercaptoethanol, and stored as a precipitate.

Polyacrylamide Gel Electrophoresis Analysis. To prepare the gel, 3 ml of 30% acrylamide/ 0.8% bis (N,N-Methylene, bis acrylamide) solution was added to 3 ml of 1.5 M Tris, pH 8.9 buffer. Then 200 ul of fresh 10% ammonium persulfate solution was added. 20 ul of TEMED (N,N,N-N'-

tetramethylethylenediamine) was added immediately before the gel was poured into the plates and placed into the Hoeffer minigel apparatus due to polymerization. All of the above reagents were purchased from Biorad. The samples being analyzed were made from 10 ul of the protein samples and 2 ul bromophenol blue, glycerol solution. The gels were run in 0.15 M Tris, pH 9.0 buffer at 500 V and 200 mA for 2 hours. The gels were then stained with 12.5% trichloro acetic acid solution and Commassie blue for 1 hour, and then destained with a solution of 45% methanol, 45% water, and 10% acetic acid. The gels were dried with a Hoeffer Dry Gel apparatus in conjunction with a Cenco vacuum pump.

Protein Concentrations. For absorption at 280 nm the extinction coefficient was 0.59 $(mg/ml)^{-1}$ cm^{-1} for ATCase and 0.73 $(mg/ml)^{-1}$ cm^{-1} for CSU. The instrument used to measure protein absorbance was a Perkin Elmer Lambda 3B UV/Visible spectrophotometer with a Perkin Elmer R100A chart recorder. The extinction coefficient for YADH is 1.46 $(mg/ml)^{-1}$ cm^{-1} (Fasman, 1989), CBA is 1.68 $(mg/ml)^{-1}$ cm^{-1} (Fasman, 1989), and thermolysin is 1.76 $(mg/ml)^{-1}$ cm^{-1} (Fasman, 1989).

Fluorescence Spectroscopy

The instrument used was a Perkin Elmer LS-5B luminescence spectrophotometer interfaced with a Perkin Elmer 3600 data station and a Perkin Elmer R100A chart recorder. The protein sample was taken from an 3.6 M $AmSO_4$ precipitated stock solution, centrifuged (10,000 rpm x 10 minutes) to remove the $AmSO_4$ supernatent and dialyzed against the Tris-OAc buffer for 24 hours. The sample was then centrifuged and the protein's

concentration was found by A_{280} and diluted to a useful concentration with the dialysis buffer. The protein was added to a mixture of guanidine HCl and buffer or NaSCN and buffer that would lead to an appropriate concentration of both buffer and guanidine HCl or both buffer and NaSCN. The final protein concentration was 100 ug/ml for CSU and 150 ug/ml for ATCase. The slit width was 5 nm excitation and 5 nm emission, and the instrument was programmed to record corrected emission spectra. The experiments were done at an excitation wavelength of 295 nm and over an emission wavelength range of 310 nm to 420 nm. The scan rate was 60 nm/sec. Software was supplied from Perkin Elmer that allowed the computer to determine both the peak emission wavelengths (to 0.5 nm) and intensity at any wavelength.

Zinc Release Studies

All absorbance measurements used were made on a Perkin Elmer Lambda 3B UV/Visible spectrophotometer in conjunction with a Perkin Elmer R100A chart recorder. The ATCase protein sample was taken from the 3.6 M $AmSO_4$ solution, centrifuged to remove the $AmSO_4$ supernatant, suspended in 1.0 ml of buffer, and dialyzed against 40 mM Hepes, KOH pH 7.0 buffer. The other protein samples purchased from Sigma, YADH, CBA, and thermolysin, were dissolved and dialyzed against the same Hepes buffer. The samples were centrifuged and the concentration was found by A_{280}. The protein samples were then diluted to a useful concentration with dialysis buffer. The protein was added to a mixture of PAR (100 uM) and buffer or PAR (100 uM) and guanidine HCl (4.0 M) The final concentrations of protein were 3.17 uM ATCase and 19.0 uM for the other three proteins (YADH, CBA, thermolysin) since ATCase has 6 times the zinc of the other three proteins.

Absorption spectra were taken over a range of 250 to 600 nm. Absorption spectra were taken again 30 minutes later to see if any changes had occurred. The chart speed was 60 nm/min. Afterwards, aliquots of PMPS were added to the samples to monitor zinc release (at A_{500}) from samples that showed no initial release of zinc. Those results were graphed on Cricket Graph.

Results and Discussion:

Purification of ATCase from *E Coli*

Figure 3 shows the elution profile from the G-200 column in the final step of purification of ATCase. Fractions 39-46 represented aggregate ATCase while fractions 49-55 represented monomeric ATCase. The fractions containing monomeric ATCase were pooled and used for experimentation. The fractions from the column were analyzed by SDS polyacrylamide gel electrophoresis. This technique was used to confirm what exactly is in each representative fraction. The fractions that correspond to monomeric ATCase (49-55) clearly show migration down the lane to a point equal to that of reference ATCase. The earlier fractions show slower migration bands which indicates the presence of aggregated ATCase. These results verify the elution profile (figure 3).

Molecular Graphics Studies

We purchased a molecular graphics program from the American Chemical Society called *Nanovision* and structure coordinates from the Protein Data Bank at Brookhaven National Laboratory to assess the environment of the two types of tryptophans in ATCase. What was found was that tryptophan-209 had 5 atoms of its indole ring exposed to the polar aqueous environment but the remainder of its atoms were buried in a hydrophobic pocket. Tryptophan-284 had 2 atoms of its indole ring exposed to the polar environment while the remainder of its atoms were buried in a hydrophobic pocket. Since Trp-284 was more buried in a hydrophobic pocket than Trp-209, there is a possibility that the fluorescence changes upon

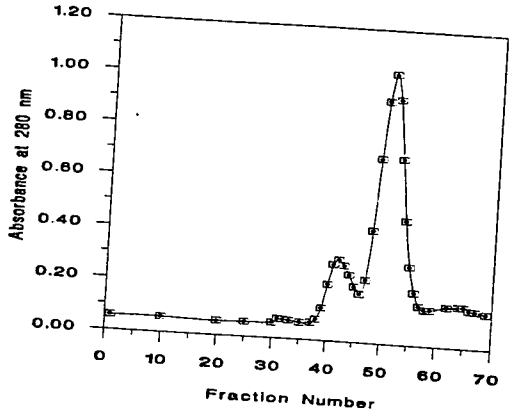


Figure 3 The elution profile of ATCase from the Sephadex G-200 column. Fractions 39-46 (first peak) represent aggregated ATCase while fractions 49-55 (second peak) represent monomeric ATCase.

unfolding are from mostly Trp-284. It was also found that the two tryptophan residues were far from the interchain interface with another catalytic chain (C1-C2 interface).

Tryptophan Fluorescence Studies

Tryptophan fluorescence is sensitive to the environment of the tryptophans in ATCase. When tryptophan is in its native state, the tryptophans are in a nonpolar hydrophobic pocket. When ATCase denatures, the tryptophans are exposed to polar aqueous environment. This change from a nonpolar to a polar environment causes a red shift in peak emission wavelength. Also, if any other changes in the environment of the tryptophans occurs, there is an effect on fluorescence measurements. This information can tell about the structure of ATCase and/or CSU. All fluorescent studies were done at an excitation wavelength of 295 nm, 5 nm slit widths, and the spectra were "corrected."

Effects of various denaturants on the tryptophan fluorescence emission of ATCase and CSU. CSU in buffer vs. CSU in 4.0 M guanidine HCl. Figure 4 shows examples of corrected fluorescence emission spectra of CSU (100 ug/ml) in buffer and CSU (100 ug/ml) in 4.0 M guanidine HCl. For CSU in buffer (native conformation), a wavelength emission maximum of 339.0±0.5 nm is observed. However, when the native protein is treated with 4.0 M guanidine HCl which totally denatures the protein, a red shift of 13 nm occurs in peak emission wavelength to 352.0±0.5 nm. This is a typical trend for a tryptophan containing protein and is indicative of the change in environment of one or more of the tryptophans as they are going from the relatively

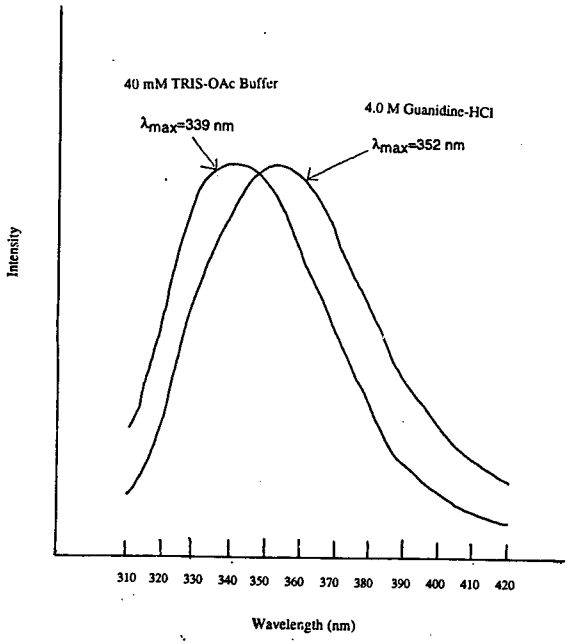


Figure 4 Comparison of corrected fluorescence emission spectra of CSU in buffer and CSU in 4.0 M guanidine HCl at excitation 295 nm. A red shift of 13 nm in peak wavelength is clearly indicated when CSU is denatured with 4.0 M guanidine HCl.

hydrophobic interior of a protein to being exposed to polar water solvent as the protein denatures (Lakowicz, J.R., 1983). For both naive CSU and denatured CSU, there was no significant change in intensities of the emission wavelength peaks. Therefore, the concentration of CSU was nearly identical (100 ug/ml) for both samples and in fact only CSU tryptophan fluorescence was being measured for both native and denatured CSU.

CSU in buffer vs. CSU in 1.25 M NaSCN. Insight into structural events which may be effecting the 13 nm red shift were obtained by using a mild denaturant sodium thiocyanate (NaSCN). It has been shown that a NaSCN concentration of 1.0 to 1.25 M dissociates the catalytic trimer but does not unfold it (Burns, D.L., 1982). Figure 5 shows the corrected fluorescence emission spectra of CSU (100 ug/ml) in buffer and CSU (100 ug/ml) in 1.25 M NaSCN. When CSU is in its native state, it has a peak emission wavelength of 339.0±0.5 nm, as before. However, when the native CSU is treated with 1.25 M NaSCN, there is a peak emission wavelength red shift of 2 nm to 341.0±0.5 nm. Therefore, it appears that the dissociation of the trimer into folded monomers has only a small effect on the environment of the tryptophan residues. This result is consistent with the fact that the tryptophan residues are far from the C1-C2 interface. Therefore, the observed 13 nm red shift in emission wavelength seen upon complete denaturation is due mostly to the unfolding of the individual chains, not the dissociation of the trimer. Furthermore, there appears to be little change in conformation of the chain as a result of dissociation.

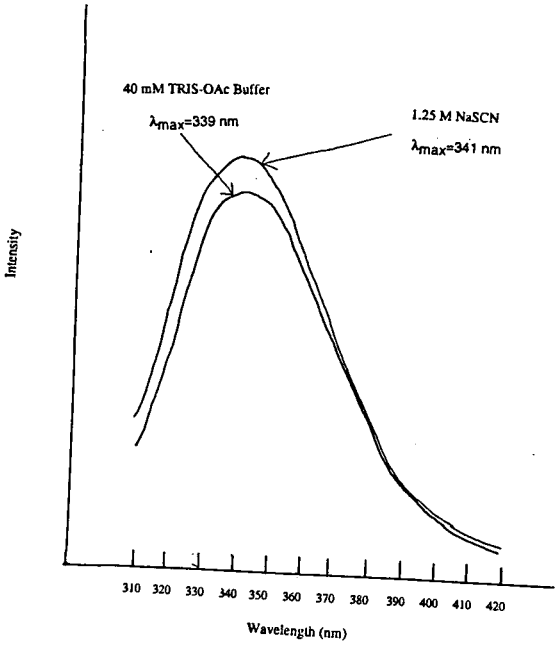


Figure 5 Comparison of corrected fluorescence emission spectra of CSU in buffer and CSU in 1.25 M NaSCN at excitation 295 nm. A red shift of 2 nm in peak wavelength is clearly indicated when CSU is dissociated with 1.25 M NaSCN.

ATCase in buffer vs. ATCase in 4.0 M guanidine HCl. Figure 6 shows the corrected fluorescence emission spectra of the holoenzyme, ATCase (150 ug/ml), in buffer and ATCase (150 ug/ml) in 4.0 M guanidine HCl. There are two points to be made from these spectra. The first point is when the holoenzyme is denatured, there is a red shift in peak emission wavelength of 15 nm from 337.0±0.5 nm to 352.0±0.5 nm. This latter value is the same peak emission wavelength as when CSU is fully denatured in 4.0 M guanidine HCl. This shows that the same tryptophans are being measured in CSU and the holoenzyme. Also, this is consistent with the fact that CSU and ATCase are completely dissociated and denatured since nothing is effecting the environment of the tryptophans other than the aqueous solution.

ATCase in buffer vs. CSU in buffer. The second point to be made is that in their native states, both CSU and ATCase had different peak emission wavelengths. This is shown more clearly in Figure 7 where ATCase's peak emission wavelength (337.0±0.5 nm) was reproducibly blue shifted 2 nm from that of CSU (339.0±0.5 nm). This implies that the association of CSU with RSU is slightly effecting the environment of at least one of the tryptophans in ATCase. Table 1 summarizes the results found in this study of tryptophan fluorescence.

Fluorescence of CSU and ATCase as a function of guanidine HCl concentration. The red shift of 13-15 nm displayed by CSU and ATCase when they are denatured has previously been used as a tool to monitor the denaturation of CSU and ATCase. In his thesis, Ira Gurland measured peak emission wavelength as a function of guanidine HCl concentration for both

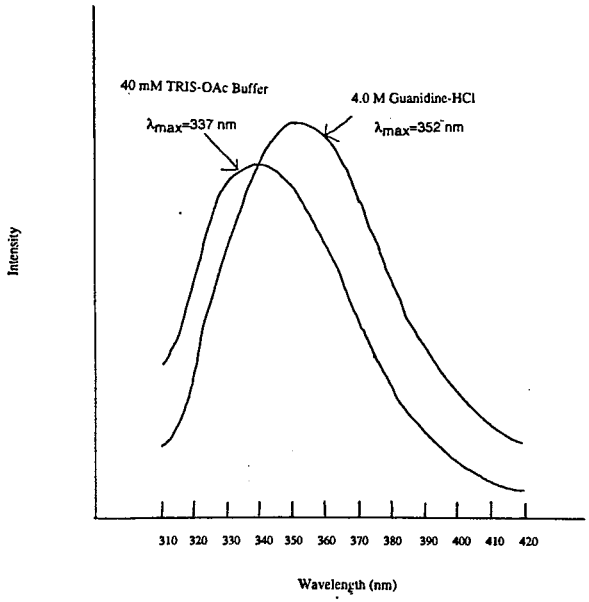


Figure 6 Comparison of corrected fluorescence emission spectra of ATCase in buffer and ATCase in 4.0 M guanidine HCl at excitation 295 nm. A red shift of 15 nm in peak wavelength is clearly indicated when ATCase is denatured with 4.0 M guanidine HCl.

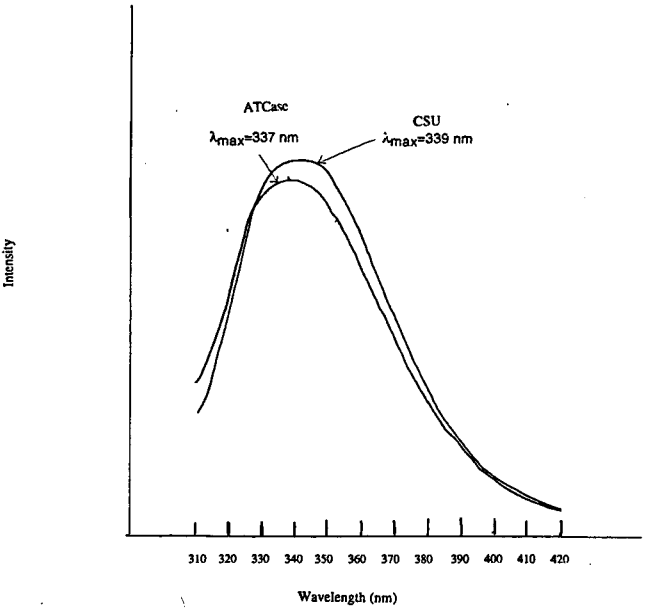


Figure 7 Comparison of corrected fluorescence emission spectra of CSU in buffer and ATCase in buffer at excitation 295 nm. A blue shift of 2 nm is clearly indicated for ATCase relative to CSU.

Table I:
Peak Emission Wavelengths (λ_{ex}=295nm)

Conditions	CSU λmax (+/-0.5nm)	ATCase λmax(+/-0.5 nm)
Buffer (native)	339.0	337.0
4.0 M Guanidine-HCl (unfolded & dissociated)	352.0	352.0
1.25 M NaSCN (dissociated only)	341.0	-------

ATCase and CSU. He used slightly different spectroscopic conditions than we used here. In particular he used 10 nm slit widths and uncorrected spectra. Figure 8 shows the denaturation profile of CSU in various concentrations of guanidine HCl. The sigmoidal curve is highly cooperative but displays asymmetry. Ira Gurland concluded that there is more than one transition in the denaturation of CSU. Most likely, the second larger transition may reflect the unfolding of the equatorial domain where both of the tryptophans are located, while the first smaller transition may be due to the unfolding of the polar domain. This behavior can be displayed by proteins with more than one domain.

Figure 9 shows the denaturation profiles of CSU and ATCase superimposed on each other. Reiterating the results showed earlier, ATCase is blue shifted 2 nm relative to CSU in 0 M guanidine HCl (native state) while upon complete denaturation, both ATCase and CSU converge to the same peak emission wavelength. However, what happens in the meantime? The differences in peak emission wavelength between CSU and ATCase dissipated after 1.75 M guanidine HCl. What is thought to be happening is that RSU dissociates from CSU at low concentrations of guanidine HCl. Therefore, at higher concentrations of guanidine HCl (latter part of the profile), the denaturation of CSU is being measured in both profiles.

Comparing our results to Ira Gurland's results at 0 M and 4.0 M guanidine HCl (figures 8 and 9), Ira found ATCase to be 2 nm blue shifted to CSU in 0 M guanidine HCl just as we did. In 4.0 M guanidine HCl, Ira also found that the peak emission wavelengths of both CSU and ATCase were in fact the same. However, Ira found different peak emission wavelengths. His were consistently 2 nm red shifted from our values for both ATCase and CSU

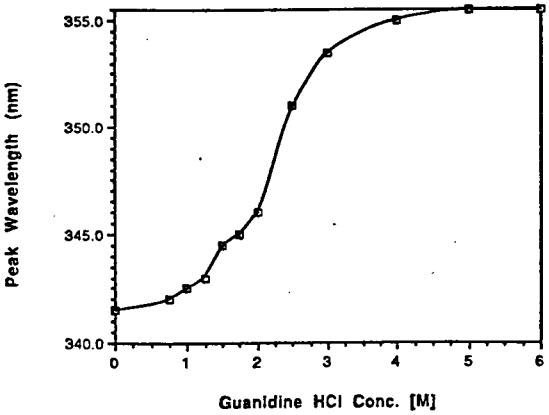


Figure 8 Peak Wavelength as a function of guanidine HCl concentration for CSU at excitation 295 nm. A sigmoidal curve of increasing wavelength with increasing guanidine HCl concentration is observed. A deviation from symmetry in the curve at 1.75 M guanidine HCl is noted. Figure done by Ira Gurland.

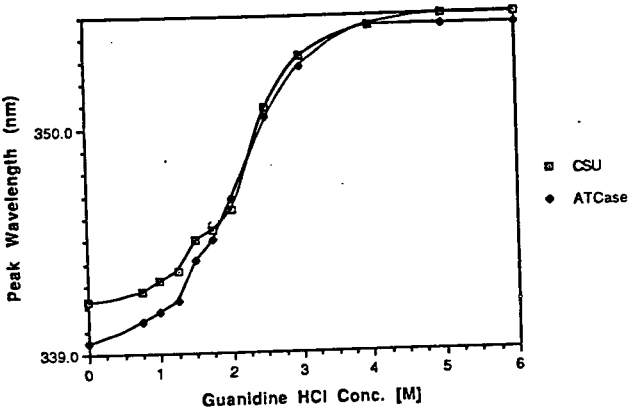
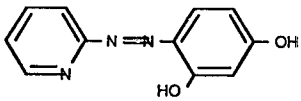


Figure 9 Comparison of peak wavelength as a function of guanidine HCl concentration for ATCase and CSU at excitation 295 nm. From 0 to 1.75 M guanidine HCl, ATCase is initially blue shifted 2 nm from CSU by 2 nm. From 2.0 to 6.0 M guanidine HCl, the curves are almost identical. Figure done by Ira Gurland.

in both 0 and 4.0 M guanidine HCl. This is probably due to the fact that Ira used uncorrected spectra and 10 nm slit widths since our shifts in peak emission wavelengths were identical consistently. In the future, the denaturation profiles of both ATCase and CSU will have to be examined using corrected fluorescence emission spectra and 5 nm slit widths.

Zinc Release from ATCase

We would like to know when, if at all, zinc is released from RSU of ATCase under various denaturing conditions. This might tell us some information regarding when RSU is denaturing on the denaturation profile of ATCase at low concentrations of guanidine HCl (0-1.75 M) (figure 9). One way to determine when zinc is being released from RSU is to use an indicator that binds zinc strongly and undergoes a change in absorbance upon chelation. The indicator chosen was 4-(2-pyridylazo)resorcinol (PAR) (Hunt, J.B.,1985):



We believe that zinc binds to the pyridyl nitrogen of PAR. PAR undergoes a change in absorbance at 500 nm upon chelation to zinc. When zinc is released from RSU, the PAR solution turns from yellow to orange. (Hunt, J.B., 1985).

Zinc release from ATCase using guanidine HCl. First, a previous experiment was repeated (Hunt, J.B., 1985) in which the absorption spectra of 3.17 uM

ATCase (951 ug/ml) with 100 uM PAR and 3.17 uM ATCase with 100 uM PAR and 100 uM PMPS were taken. My results were identical to those from the literature (figure 10). *p*-(hydroxymercuri)phenylsulfonate (PMPS) is an oxymercurial reagent that has been shown to displace zinc quickly and stoichiometrically from ATCase because mercury binds specifically to the cysteine sulfhydryls that hold zinc in ATCase . 100 uM PMPS was enough to release all of the zinc, which was seen by the change in absorbance at 500nm (figure 10). Afterwards, 4.0 M guanidine HCl was added to 3.17 uM ATCase (951 ug/ml) with 100 uM PAR and an absorption spectrum was taken. A surprising result occurred. Figure 11 shows that the addition of 4.0 M guanidine HCl (which presumably fully denatures ATCase) had no detectable effect on the absorbance at 500 nm. This suggests that zinc is still bound to the regulatory chain of ATCase after the protein is denatured with 4.0 M guanidine HCl. It was assumed that zinc would be released from ATCase as the protein is denatured. To conclusively confirm that zinc was still bound to the protein, 5 ml aliquots of PMPS (4000 uM stock) were added to the 3.17 uM ATCase/4.0 M guanidine HCl solution. Figure 12 shows that zinc was released stoichiometrically upon addition of PMPS and thus zinc was still bound to the denatured protein. This result is further confirmed with figure 13, the stoichiometric release of zinc from native ATCase. The graphs of the stoichiometric release of zinc from the native protein (figure 13) and that of the denatured protein (figure 12) are superimposable. PMPS displaced zinc from the denatured protein exactly as it did from the native protein again confirming that zinc was still bound to denatured ATCase. These results were within a few percent of quantitative results (Hunt, J.B. 1985).

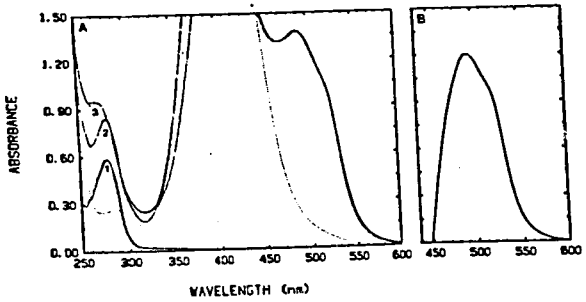


Figure 10 Previously published UV-visible absorption spectra of PAR experiment with ATCase. (A) Curve 1 shows the absorption spectrum of 3.17 uM ATCase. Curve 2 shows the absorption spectrum of 3.17 uM ATCase in the presence of 100 uM PAR. Curve 3 shows the absorption spectrum of 3.17 uM ATCase and 100 uM PAR after the addition of 100 uM PMPS. (B) The calculated visible difference spectrum by adding 100 uM PMPS to 3.17 uM ATCase and 100 uM PAR. From Hunt, J.B., 1984.

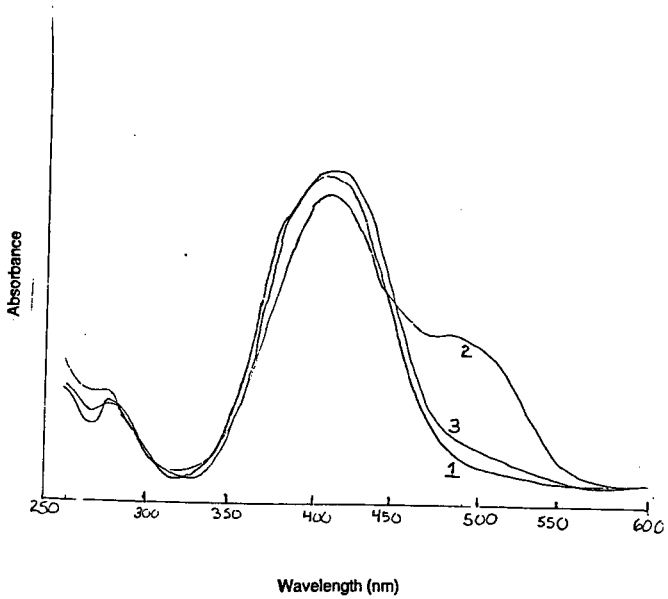


Figure 11 Experimental UV-visible absorption spectra of PAR experiment with ATCase. Curve 1 is the absorption spectrum of 3.17 uM ATCase and 100 uM PAR. Curve 2 is the absorption spectrum of 3.17 uM ATCase and 100 uM PAR in the presence of 100 uM PMPS. Note the change in A_{500}. Curve 3 is the absorption spectrum of 3.17 uM ATCase and 100 uM PAR in the presence of 4.0 M guanidine HCl. Note there is no change in A_{500}.

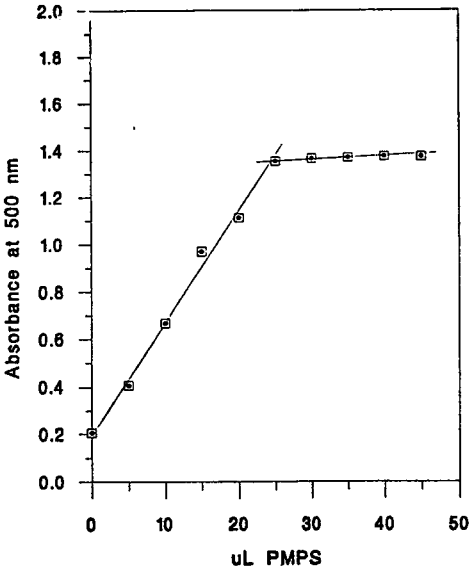


Figure 12 The stoichiometric release of zinc from denatured 3.17 uM ATCase during titration with PMPS (4000 uM stock). Note the superimposability of this plot with that of figure 13, the release of zinc from native ATCase.

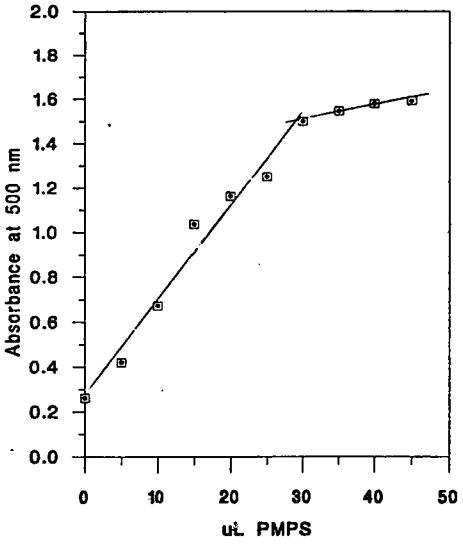


Figure 13 The stoichiometric release of zinc from native 3.17 uM ATCase during titration with PMPS (4000 uM stock). Note the superimposability of this plot with that of figure 12, the release of zinc from denatured ATCase.

Zinc release from other proteins using guanidine HCl. Due to the surprising result that zinc was not released from denatured ATCase, we decided to study the release of zinc from three other proteins, each with different zinc binding sites: alcohol dehyrogenase (YADH), carbonic anhydrase (CBA), and thermolysin.

Alcohol dehydrogenase. When 100 uM PAR was added to 19.0 uM YADH in buffer, A_{500}=1.296. After 30 minutes, A_{500}=1.302. Therefore, the zinc was released completely and immediately from the native protein. Aliquots of PMPS were added but showed no further zinc release, which is not surprising since zinc was already released. 4.0 M guanidine HCl was then added to the 19.0 uM YADH/ 100 uM PAR solution, which showed a change in A_{500} from 1.374 to only 1.395 over a 30 minute period. Again, this showed that zinc was already released from YADH, which confirmed the earlier result. Therefore, the off rate of zinc from YADH is fast even in its native state. Figure 14 shows the zinc binding sites in YADH.

Carbonic anhydrase. When 100 uM PAR was added to a solution of native CBA (19.0 uM), the solution remained yellow for 30 minutes, with A_{500} changing from 0.200 to 0.228. This could be due to a slow zinc off rate. When PMPS was added, there was still no appreciable change in A_{500} and the solution remained yellow. This implies that PMPS does not release zinc from CBA, which is not surprising because zinc is bound to CBA by three histidine residues (figure 14), and therefore, the mercury in PMPS is not going to want to bind to the histidine nitrogens due to their low polarizability.

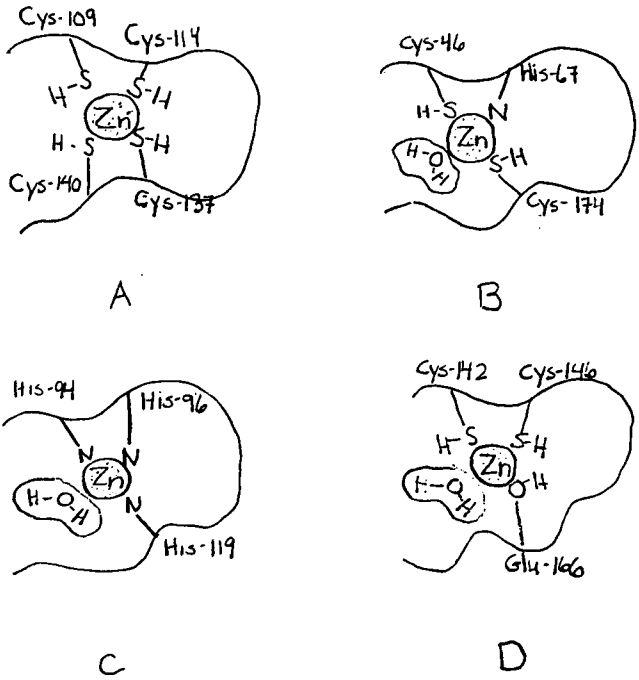


Figure 14 The zinc binding sites of (A) ATCase, (B) alcohol dehydrogenase, (C) carbonic anhydrase, and (D) thermolysin.

However, when 100 uM PAR was added to a solution of denatured CBA (4.0 M guanidine HCl), the solution was orange, with A_{500}=1.349. After 30 minutes, there was little change in A_{500} (to 1.375). This implies that zinc is released quickly upon unfolding of carbonic anhydrase (in 4.0 M guanidine HCl). However, as seen earlier, there is no zinc release from CBA in the protein's native state.

Thermolysin. When 100 uM PAR was added to native thermolysin (19.0 uM), there was no change in the color of the solution; it remained yellow (A_{500}=0.285). After 30 minutes, there was still no color change (A_{500}=0.324). The slight change in absorbance can be due to a very slow release of zinc. When aliquots of PMPS were added, again, there was no zinc release despite the fact that two of the three zinc ligands contributed by the protein are cysteine sulfhydryls (figure 14). However, when 4.0 M guanidine HCl was added to the 19.0 uM thermolysin/100 uM PAR solution, there was a very slight orange color with A_{500}=0.285. After 30 minutes, A_{500}=0.576 indicating that the rate of zinc release was increased compared to that of the native protein. Then, aliquots of PMPS were added to the solution, and there was no change in solution color or A_{500}. Therefore, there was a slow but detectable zinc release from native thermolysin, and the rate of zinc release is significantly higher with thermolysin in 4.0 M guanidine HCl.

Further study of the release of zinc from RSU in ATCase is needed due to the surprising result obtained for the release of zinc from denatured ATCase. To obtain this goal, further study of the release of zinc from YADH, CBA, and/or thermolysin might be needed.

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