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Laboratory Exercises

Influence of Enzyme Conformational Changes on Catalytic Activity Investigated by Circular Dichroism Spectroscopy

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Carolina Fortes Rigos‡§, Hérica de Lima Santos‡¶, Geraldo Thedei, Jr.‖, Richard John Ward‡, and Pietro Ciancaglini‡**

From the ‡Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo (FFCLRP-USP), 14040-901 Ribeirão Preto, São Paulo, and the ‖Instituto de Ciências Biológicas e da Saúde, Universidade de Uberaba, Minas Gerais, Brazil

Enzyme activity is dependent on native conformational integrity. Here we present a simple laboratory exercise based on dichroism spectroscopy in which the change in enzyme structure induced by denaturation is correlated with the loss of catalytic activity. The results of circular dichroism spectra show that enzyme denaturation by either trifluoroethanol (enhancement of α-helix structure) or guanidinium chloride (reduction of α-helix and enhancement of random coil structure) leads to a concomitant reduction in enzyme activity, which demonstrates the relationship between structure and catalytic activity of the enzyme. This simple experimental approach demonstrates that only a single native protein (enzyme) conformation has the ability to catalyze substrate hydrolysis.

Keywords: Protein structure, α-helix, circular dichroism, enzyme activity, Na,K-ATPase.

Proteins are abundant biological macromolecules, and a single cell may contain proteins ranging in size from relatively small peptides to extended polymers of hundreds or even thousands of amino acid residues. These proteins mediate important activities such as catalysis, defense, transport, regulation, and movement [1]. Cells produce proteins with strikingly different properties and activities by the covalent linkage, via peptide bonds, of the same set of 20 amino acids in many different combinations giving rise to diverse amino acid sequences. Despite the complexity of these molecules, there are four generally recognized levels of protein structure. Primary structure refers to the amino acid sequence and the location of disulfide bounds, secondary structure is the defined spatial relationship of adjacent amino acids in localized stretches, tertiary structure describes the three-dimensional conformation of an entire polypeptide chain, and quaternary structure involves the spatial relationship of multiple polypeptide chains (subunits) in stable associations [1, 2].

Every protein has a unique three-dimensional structure that determines its function and which is stabilized by multiple weak interactions, which include hydrophobic interactions, hydrogen bonds, and ionic interactions (forces). Treatments that perturb or disrupt these interactions can destroy the three-dimensional structure of proteins with the concomitant loss of protein function, thereby demonstrating the relationship between native protein structure and function [1, 2].

The main objective of this laboratory exercise is to demonstrate and discuss the dependence of intact secondary structure on the biological activity of a protein (the example used is Na,K-ATPase) by correlating the results from circular dichroism spectroscopy with catalytic activity.

Background Theory and Pre-laboratory Preparation—Useful information concerning the structure of proteins in solution can be obtained from measurement of their optical activity. The inherent asymmetry of amino acid molecules is responsible for the large signals observed in proteins using the related methods of optical rotatory dispersion (ORD)[1] and circular dichroism (CD). ORD is the measurement of the ability of a molecule to rotate the plane of linearly polarized light; CD measures unequal absorption of right- and left-handed circularly polarized light by the molecule. Although all amino acids except glycine contain at least one asymmetric carbon atom either in the L or D configuration, most amino acids display only small ORD and CD signals [3, 4].

The protein main chain assumes several principal conformations in aqueous solution: α-helix, extended β structure (or β-sheet), turn, and so-called "random coil," which are non-regular structures. The α-helix and β-sheet conformations are characterized by optimal hydrogen bonding

§ Recipient of a Masters from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

¶ Recipient of a Ph.D. fellowship from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

** To whom correspondence should be addressed. E-mail: pietro@ffclrp.usp.br.

[1] The abbreviations used are: ORD, optical rotatory dispersion; CD, circular dichroism; TCA, trichloroacetic acid; PNPP, *p*-nitrophenyl phosphate di(Tris) salt; PNPPase, *p*-nitrophenyl phosphatase; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; TFE, trifluoroethanol; EDTA, ethylenediaminetetraacetic acid; MRW, mean of residue weight of the repeating unit.

between peptide groups in the polypeptide backbone and give rise to strong CD signals [3, 4].

CD bands of proteins occur in two spectral regions. The far-UV or amide region (130–250 nm) is dominated by contributions of peptide bonds, and analysis of spectra in this region can give information about secondary structure. The CD bands in the near-UV region (250–300 nm) originate from asymmetry in the immediate environment around aromatic amino acids and yield information about protein tertiary structure [3–5].

The α-helix signal is denominated by negative CD bands at 222 and 208 nm and a positive CD band at around 190 nm. β-Sheet structures give rise to a negative CD band in the region of 210–220 nm, β-turn structures show a negative CD band between 180 and 190 nm, and the spectra of random coil has a characteristic negative CD band in region of 200 nm. CD data in the far-UV region can be used to determine the relative properties of the different secondary structural elements of protein [4, 7], and various computer programs are available on the Internet that facilitate the desconvolution of complex spectra. Detailed discussion of the electronic transitions of the peptide groups, involving $\eta \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions of the hydrogen-bonded peptide bond, as applied to CD spectroscopy has been presented previously [4–6].

These methods can also be used to measure enzyme interactions with substrates, inhibitors, or coenzyme and binding of metal ions and dyes to proteins and polypeptides [4]. These characteristics make CD and ORD spectroscopy excellent methods for measuring protein denaturation and helix-coil transitions of polypeptides in small volumes at low protein concentrations (~0.1 mg) [3].

General Note—In this class experiment, we describe a methodology adapted to the use of Na,K-ATPase (an enzyme available in our laboratory); however, other peptides, proteins, or enzymes can be used, for example alkaline phosphatase or phospholipase [8–11]. This class experiment does not use hazardous reagents; however, care should be used in handling trichloroacetic acid (TCA), which should be manipulated with gloves, and disposal should be made in accordance with local safety procedures.

Alternatively, if a spectrophotopolarimeter is not available, the conformational change on the tertiary structure of some proteins can be monitored by changes in the UV absorption spectra using a spectrophotometer and measuring the change in absorbance of aromatic amino acids caused by denaturing agents.

EXPERIMENTAL PROCEDURES

Materials—All solutions were prepared using Millipore Milli-Q® Ultrapure apyrogenic water. TCA, tris(hydroxymethyl)aminomethane (Tris), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), adenosine 5′-triphosphate Tris salt (ATP), *p*-nitrophenyl phosphate di(Tris) salt (PNPP), bovine serum albumin (BSA), trifluoroethanol (TFE), and octaethylene glycol dodecyl ether ($C_{12}E_8$) were from Sigma. Ethylenediaminetetraacetic acid (EDTA), potassium chloride, sodium chloride, guanidinium chloride, and magnesium chloride were from Merck. Analytical grade reagents were used without further purification.

Preparation of the $(\alpha\beta)_2$ Dimer of Solubilized Na,K-ATPase—Membrane-bound Na,K-ATPase was obtained from the dark red, outer kidney medulla of adult New Zealand white rabbits as described previously by Santos *et al.* [12]. Briefly, the solubilized enzyme was obtained at 4 °C after rapidly mixing 1 mg of Na,K-ATPase membrane fragments with 1 mg of $C_{12}E_8$/ml. The nonsolubilized residues were removed by centrifugation at 4 °C for 1.5 h at 100,000 × *g* in a Hitachi CP70-MX ultracentrifuge. The $C_{12}E_8$-solubilized enzyme was concentrated with a YM-10 Amicon filter, dialyzed overnight at 4 °C against 5 mM Tris·HCl buffer, pH 7.0, containing 1 mM EDTA, 150 mM KCl, and 0.005 mg/ml $C_{12}E_8$, and purified by gel filtration at 4 °C on a Sepharose 6FF column (26 × 200 cm) equilibrated and eluted in the same buffer using a flow rate of 1 ml/min in an AKTA system (Amersham Biosciences). Finally, 1.0-ml aliquots were frozen in liquid nitrogen and stored at −20 °C. This protocol yields purified $C_{12}E_8$-solubilized Na,K-ATPase that has an $(\alpha\beta)_2$ association. The enzyme is designated as solubilized Na,K-ATPase.

Protein Concentration Analysis—Protein concentrations were estimated in the presence of 2% (w/w) SDS as described previously [13]. Bovine serum albumin was used to construct the reference curve.

Measurements of Enzymatic Activity—ATPase and *p*-nitrophenyl phosphatase (PNPPase) activities were determined for solubilized Na,K-ATPase as described previously [12, 14, 15]. Briefly, ATPase activity was assayed discontinuously at 37 °C by quantifying phosphate release in 1.0 ml of the assay medium. The reaction was initiated by addition of the enzyme and stopped with 0.5 ml of cold 30% TCA at appropriate time intervals. The assay medium was centrifuged at 4,000 × *g* immediately prior to phosphate measurement. Alternatively, PNPPase activity was assayed discontinuously at 37 °C in a Genesys 2 spectrophotometer by monitoring the release of *p*-nitrophenolate ion ($\epsilon_{1M,pH13}$ = 17,600 M^{-1} cm^{-1}) at 410 nm. The reaction was initiated by addition of the enzyme and stopped with 1.0 ml of 1 M NaOH. If PNPP is used as chromogenic substrate, the determination of the activity is more rapid and uses less enzyme.

The standard conditions for ATPase activity were 50 mM HEPES buffer, pH 7.5, containing 3 mM ATP, 10 mM KCl, 5 mM $MgCl_2$, and 50 mM NaCl in a final volume of 1.0 ml. PNPPase activity measurements were made in 50 mM HEPES buffer, pH 7.5, containing 10 mM PNPP, 15 mM KCl, and 10 mM $MgCl_2$ in a final volume of 1.0 ml. Measurements of catalytic activity were performed in triplicate; the initial velocities were constant for at least 30 min, provided that less than 5% of the substrate was hydrolyzed. Controls without added enzyme were included in each experiment to quantify non-enzymatic substrate hydrolysis. One enzyme unit is defined as the amount of enzyme hydrolyzing 1.0 nmol of substrate/min at 37 °C.

Circular Dichroism Spectroscopy—CD spectra were recorded with a Jasco 810 spectrophotopolarimeter equipped with a Peltier thermostatic system under constant nitrogen flux at 25 °C and with a 0.1-cm quartz cuvette. The protein concentration was in the range of 0.05–0.45 mg/ml, and spectra were measured in the absence or presence of the denaturing agent, 7 M guanidinium chloride or 20% (v/v) TFE, in 5 mM Tris·HCl buffer, pH 7.0, containing 0.005 mg/ml $C_{12}E_8$, 1 mM EDTA, and 150 mM KCl.

The CD spectrum of each sample was measured three times at a scan rate of 50 nm·min^{-1}. The average of these scans gave the raw protein spectrum, from which a baseline spectrum was substrated that was measured using the same cell and appropriate buffer minus the protein component.

Student Pitfalls—This experiment is generally straightforward; however, some doubts can arise in calculating (i) the enzymatic activity from absorbance data and (ii) a conversion to ellipticity units from CD data. Therefore it is recommended that the students review Lambert-Beer's law to understand the conversion of absorbance to micromoles of inorganic phosphate liberated in the enzyme assay. Modern CD spectropolarimeters use a high frequency photoelectric modulator to generate alternate left and right circular polarized light. This generates an alternating current contribution in the photomultiplier signal that is proportional to

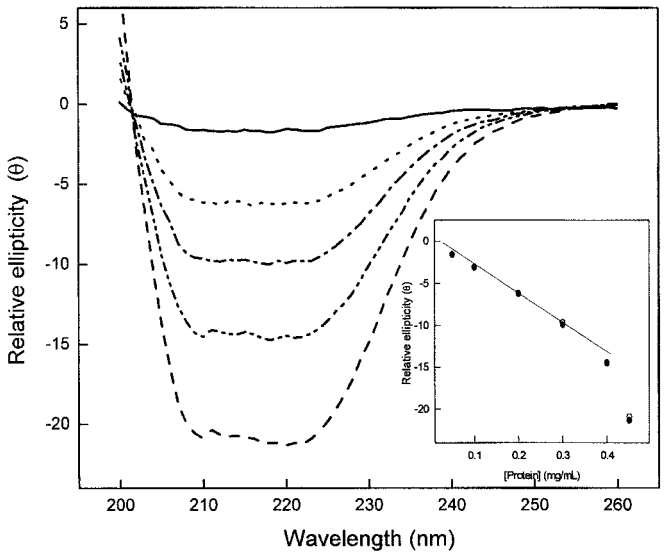

FIG. 1. **Circular dichroism spectra with increasing concentrations (0.05–0.45 mg/ml) of solubilized Na,K-ATPase: 0.05 mg/ml (——), 0.2 mg/ml (- - - -), 0.3 mg/ml (— - — - —), 0.4 mg/ml (— - - —), and 0.45 mg/ml (— — — —).** The spectra were obtained in buffer 5 mM Tris·HCl, pH 7.0, containing 1 mM EDTA, 150 mM KCl, and 0.005 mg/ml $C_{12}E_8$ at 25 °C. The CD spectra were recorded at 200–250 nm in a Jasco 810 spectropolarimeter using a 0.1-cm quartz cuvette. *Inset*, ellipticity change at 208 nm (○) and 222 nm (●) of CD spectra obtained at different protein concentrations.

the CD of the sample. The signal is generally recorded as the difference in absorbance (ΔA)

$$\Delta A = A \text{ of left-polarized light} - A \text{ of right-polarized light} \quad \text{(Eq. 1)}$$

or as molar ellipticity or residue ellipticity $[\theta]$ (degrees), which is defined in Equation 2

$$[\theta]_\lambda = \theta_{obs} \times MW \text{ (or} \times MRW)/10 \times d \times C \quad \text{(Eq. 2)}$$

where λ = wavelength, θ_{obs} = observed ellipticity in degrees, MW = molecular weight, MRW = mean of residue weight of the repeating unit (the MRW used for amino acids in proteins is ~115), d = path length in cm, and C = protein concentration in g/ml [4].

RESULTS AND DISCUSSION

Enzyme catalytic activity is dependent on native conformational integrity, and the effects of structural changes on the activity of enzyme have been studied for many proteins. It has been suggested that the active sites of the enzyme are usually situated in a region of the enzyme that is more susceptible to denaturants than the protein as a whole [9, 16]. This article studies this dependence using circular dichroism spectroscopy to gather data and analyze structural change in relation to the catalytic activity.

Fig. 1 shows the far-UV CD spectra of Na,K-ATPase at protein concentrations between 0.05 and 0.45 mg/ml. Note that the CD spectra are characteristic of α-helical secondary structure as distinguished by the two negative CD bands at 222 and 208 nm. A plot of ellipticity either at 208 or 222 nm as a function of protein concentration revealed linear behavior between 0.05 and 0.4 mg/ml of protein (*inset* of Fig. 1). Similar results were obtained from human placenta Na,K-ATPase [17].

Because of the complexity of the Na,K-ATPase structure in high concentrations of protein (up to 0.4 mg/ml) and low detergent concentrations (*inset* of Fig. 1), a protein-protein

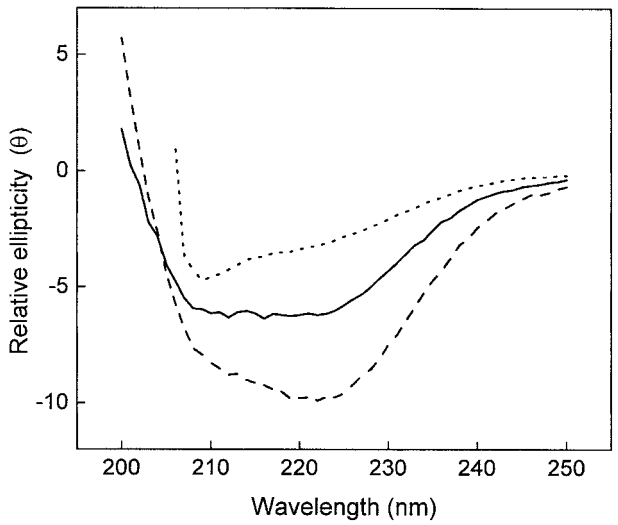

FIG. 2. **Circular dichroism spectra of the solubilized Na,K-ATPase (0.2 mg/ml): control (——), in the presence of 7 M guanidinium chloride (- - - -), or in the presence of 20% TFE (— — —).** The CD spectra were recorded at 200–250 nm in a Jasco 810 spectropolarimeter using a 0.1-cm quartz cuvette.

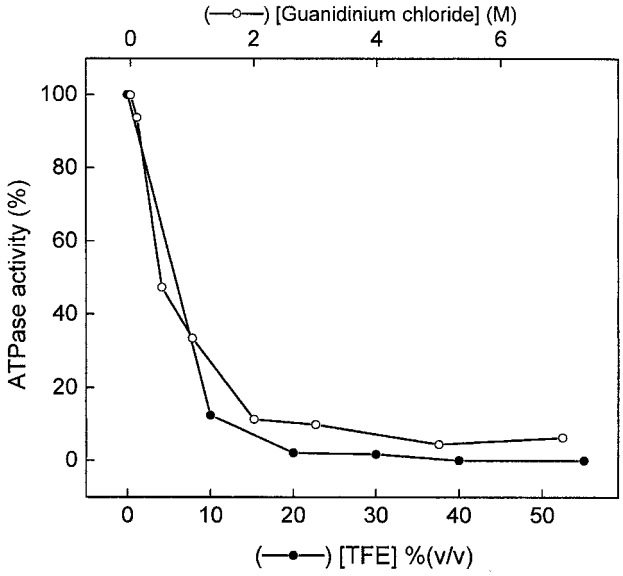

FIG. 3. **Residual ATPase activity of the solubilized Na,K-ATPase in the presence of different concentrations of guanidinium chloride (○) or TFE (●).** The ATPase activity was determined in 50 mM HEPES, pH 7.5, with 3 mM ATP, 10 mM KCl, 5 mM $MgCl_2$, and 50 mM NaCl. 100% ATPase activity corresponds to solubilized enzyme without addition of denaturing agent determined in the same conditions.

aggregation process could be occurring [12, 14, 15]. For peptides or soluble proteins, the linear behavior between relative ellipticity and protein concentration could be used as a method for protein quantification [18].

For the other experiment we used a protein concentration of 0.2 mg/ml, which present a relatively good CD signal. Addition of guanidinium chloride (7 M) caused significant changes in CD spectra of Na,K-ATPase; the two negative bands at 208 and 222 nm disappear, and a single negative band at 210 nm is present (Fig. 2), suggesting changes in the secondary structure of the protein (α-helix to random coil). Moreover, Fig. 3 shows a progressive

reduction of the ATPase activity of the enzyme in the presence of increasing concentrations of guanidinium chloride. These data confirm the dependence of the activity on the conservation of the native enzyme structure.

Addition of 20% (v/v) TFE resulted in CD spectra with more negative ellipticity at 208 and 222 nm (Fig. 2). TFE is known to enhance helical structure in polypeptides, and although the mechanism involved in this process is unknown [19], the ATPase activity of the enzyme was reduced by TFE denaturation (Fig. 3).

In conclusion, the changes in the CD spectra show that denaturation by guanidinium chloride (reduction of α-helix and enhancement of random coil structure) or trifluoroethanol (enhancement of α-helix structure) leads to a concomitant reduction in enzyme activity and demonstrates the relationship between structure and catalytic activity of the enzyme. These simple experimental approaches show the existence of a single protein (enzyme) conformational structure, which has an optimal ability to catalyze substrate hydrolysis.

Laboratory Time—Our 1-year experience with different classes of post-graduate students shows that two periods of 4 h are adequate to carry out the experiment, in which one period is used to obtain data from CD spectroscopy and the other period is used for the kinetic assay. More laboratory time is necessary to study the effect on binding of substrate, ions, inhibitors, or other modulators in the Na,K-ATPase structure using this experimental approach.

Study Questions

1. What is primary, secondary, tertiary, and quaternary protein structure?
2. How is this structure related to native protein conformation?
3. Why should loss of structure be associated with loss of activity?
4. CD is a "global" technique; what does this mean?
5. What other techniques could be used to monitor structure change in protein?

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