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Research paper

Role of hydration on the functionality of a proteolytic enzyme α-chymotrypsin under crowded environment

Pramod Kumar Verma, Surajit Rakshit, Rajib Kumar Mitra**, Samir Kumar Pal*

Unit for Nano Science & Technology, Department of Chemical, Biological & Macromolecular Sciences, S.N. Bose National Centre for Basic Sciences, Block JD, Sector III, Salt Lake, Kolkata 700098, India

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ABSTRACT

Enzymes and other bio-macromolecules are not only sensitive to physical parameters such as pH, temperature and solute composition but also to water activity. A universally instructive way to vary water activity is the addition of osmotically active but otherwise inert solvents which also mimic the condition of an intercellular milieu. In the present contribution, the role of hydration on the functionality of a proteolytic enzyme α-chymotrypsin (CHT) is investigated by modulating the water activity with the addition of polyethylene glycols (PEG with an average molecular weight of 400). The addition of PEG increases the affinity of the enzyme to its substrate, however, followed by a decrease in the turnover number (k_{cat}). Energetic calculations show that entrance path for the substrate is favoured, whereas the exit channel is restricted with increasing concentration of the crowding agent. This decrease is attributed to the thinning of the hydration shell of the enzyme due to the loss of critical water residues from the hydration surface of the enzyme as evidenced from volumetric and compressibility measurements. The overall secondary and tertiary structures of CHT determined from far-UV and near-UV circular dichroism (CD) measurements show no considerable change in the studied osmotic stress range. From kinetic and equilibrium data, we calculate 115 ± 30 numbers of water molecules to be altered during the enzymatic catalysis of CHT. Spectroscopic observation of water relaxation and rotational dynamics of ANS–CHT complex at various concentrations of the osmoting agent also support the dehydration of the hydration layer. Such dehydration/hydration processes during turnover imply a significant contribution of solvation to the energetics of the conformational changes.

1. Introduction

Almost all biological macromolecules (proteins, enzymes, and DNA) are inactive in absence of water [1–4]. The hydration shell formed by water molecules in the close vicinity of a protein plays the most significant role in protein structure, conformational changes, in substrate binding followed by enzyme catalysis, and in molecular recognition. This role of hydration in enzyme catalysis is well known and has recently been reviewed in a number of publications [1,5–7]. Dehydration makes a protein more rigid, increases its denaturation temperature and alters its physiological functionality [1,8,9]. Extensive studies show the existence of a "hydration shell" around the protein surface that excludes many solutes [10,11], and extended networks of water have been observed within protein crystal structures [12]. Hydrostatic pressure-induced changes in protein association [13] and in enzyme catalysis [14] strongly implicate changes in protein solvation through the electrostriction of water, but they remain difficult to separate from other possible density changes [15]. Hydrostatic pressure methods would not identify water that moves without density change. The induced-fit mechanism of substrate specificity [16] implies that substrate binding changes the hydration of the protein. Protein solvation has, however, been difficult to measure directly and its contribution to the energetics of conformational change is difficult to estimate.

One direct and universally applicable strategy for probing water's role is to examine the effects of reducing the availability of

Abbreviations: CHT, α-chymotrypsin; PEG, polyethylene glycol; CD, circular dichrosim; OS, osmotic stress; AMC, Ala–Ala–Phe 7-amido-4-methyl coumarin; ANS, 1-anilinonaphthalene-8-sulfonate; ES, enzyme-substrate complex; a_w, water activity; φ_v, apparent specific volume; φ_k, partial apparent adiabatic compressibility; $\Delta\nu$, Stokes shift; TRES, time-resolved emission spectra.

* Corresponding author. Present address: Noyes Laboratory of Chemical Physics, California Institute of Technology, 1200 East California Boulevard, Pasadena, CA 91125, USA.

** Corresponding author. Tel.: +91 33 2335 5705/6/7/8; fax: +91 33 2335 3477.
E-mail addresses: rajib@bose.res.in (R.K. Mitra), skpal@bose.res.in (S.K. Pal).

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water in protein hydration layer by the addition of osmotically active but otherwise inert solvents. Experimental and theoretical works have demonstrated large effects of osmotic stress (OS) on the thermodynamics and kinetics of many biological in vitro processes, including protein binding, folding, and aggregation [17–20]. OS has the unique feature of detecting even extremely weakly perturbed water molecules near the membrane [21] and polymer [22,23] surfaces, perturbations which sum to give large interaction energies when many such water molecules are simultaneously moved. Polyethylene glycols (PEGs) are routinely used as osmotic stressing agents to alter the water activity in solutions leading to hydration change. These polymers are highly water soluble non-charged inert macromolecules that have been used to investigate the effects of macromolecular crowding [24] as it is not expected to bind directly to the biomolecules [25]. These polymers are preferentially excluded from the hydration shells around proteins or other biomolecules. This exclusion creates an OS, which draws water away from the protein surface or out of a polymer-inaccessible crevice. Osmotically stressed enzyme environment results in a change in catalytic activity as a result of the change in the number of bound water molecules in enzyme and substrate and the enzyme-substrate complex (change in K_M, Michaelis–Menten constant) or between the ground state and transition state of the complex (change in V_{max}, maximal reaction rate). In a classic example of regulation of enzyme activity by an osmolyte [26], the dissociation constant (K_d) for glucose binding to hexokinase has been reported to decrease with increasing the osmotic pressure (OP) of the assay medium, which can be tuned by the addition of PEG in the solution [25]. A complementary approach towards the understanding of the effect of water activity (in phenomenon like dehydration) is to move away from aqueous solution completely, and study systems where even the strongly bound waters can be removed. This requires solid enzymes to be dispersed in organic solvents which lead to some remarkable properties such as greatly increased thermal stability and strikingly different substrate specificity. Low bio-catalytic activity in non-aqueous media is supposed to emanate from the poor compatibility between the solvent and enzymatic transition state [27], reduced protein flexibility [28–30], ground-state stabilization of the substrate [31], water stripping [32,33] and partial denaturation of the enzyme [30,34]. All these studies furnish evidences of the essential role of hydration and flexibility or mobility of enzyme in bio-catalytic activity. However, the exact role of hydration and flexibility of enzyme in substrate–enzyme interactions in osmotically stressed condition are still poorly understood and a detailed understanding in this field is highly demanding due to the highly compact packing in vivo condition leading to a molecular crowding [35].

This drives us in the present study in which we systemically study the role of water molecules on the substrate binding, functionality and hydration of a very commonly studied enzyme α-chymotrypsin (CHT) by modulating the solution water activity by the addition of PEG 400 (average molecular weight of 400). CHT is a proteolytic enzyme associated with the hydrolysis of peptide bonds in the mammalian digestive system. The structure of CHT [36,37], the specificity of substrates [38], the mechanism of hydrolysis, and temperature effects on catalysis and binding [39] are well-documented in the literature. In the present study the enzymatic activity of CHT on the substrate AMC (Ala–Ala–Phe 7-amido-4-methyl coumarin) is found to decrease with increasing PEG concentrations in the solution. Such a change in the activity of the enzyme with the addition of the crowding agent offers a unique opportunity to correlating its functionality with dynamics of the associated hydration layer, as the global structure and the binding site for recognition remain unchanged even at the highest concentration of PEG used, as confirmed by the far-and near-UV circular dichroism (CD) study. Densimetric and compressibility measurements of the protein in the presence of PEG provide information on the hydration state of the protein. To follow the environmental change at a particular site of the CHT upon addition of PEG, we study the fluorescence behaviour of proflavin. The overall environmental change around CHT as a function of the concentration of PEG is followed from the steady-state and time-resolved fluorescence spectroscopy of ANS (1-anilinonaphthalene-8-sulfonate), which binds rigidly at a single site on the surface of the enzyme near the Cys-1-122 disulfide bond. We also investigated the dynamical evolution at the active site of CHT as well as the hydration shell surrounding the enzyme using picosecond-resolved fluorescence anisotropy of a substrate mimic (inhibitor) proflavin and ANS.

2. Material and methods

2.1. Materials and sample preparation

Lyophilized α-chymotrypsin (CHT) powder was purchased from Sigma. ANS (1-anilinonaphthalene-8-sulfonate) and Ala-Ala-Phe 7-amido-4-methyl coumarin (AMC) were obtained from Sigma Aldrich. PEG 400 (Poly(ethylene glycol), average molecular weight of 400) was purchased from Sigma. The chemicals and the protein were used as received. All aqueous solutions were prepared in a phosphate buffer (10 mM). The PEG concentration was varied from 5% to 30% in terms of weight i.e., g PEG/(g PEG + g water).

2.2. UV–visible absorption spectroscopy

Concentration of the enzyme in the buffer was determined using the extinction coefficient (ε) value of 51 $mM^{-1}\,cm^{-1}$ at 280 nm [40]. Activity measurements were performed using AMC as the substrate. Concentration of the substrate in aqueous solution was estimated by taking the extinction coefficient value to be 15.9 $mM^{-1}\,cm^{-1}$ at 325 nm [40]. The enzyme cleaves the substrate and produces a free coumarin derivative. The absorbance of this product (coumarin derivative) was monitored in a Shimadzu Model UV-2450 spectrophotometer. The increase in the absorption at 370 nm due to the release of 7-amido-4-methyl-coumarin ($\varepsilon_{370} = 7.9\ mM^{-1}\,cm^{-1}$) was followed as a function of time. It is to be noted here that the substrate does not absorb at this monitoring wavelength. Initial rates of catalysis reactions were measured in the regime where the absorbance varies linearly with time. The reaction follows Michaelis–Menten kinetics [41] and the apparent K_M and k_{cat} values were derived by least squares fitting of the double reciprocal Lineweaver–Burk plot (Fig. 1 A). Steady-state absorption spectra were measured with a Shimadzu UV-2450 spectrophotometer.

2.3. Circular dichroism spectroscopy

Far-and near-UV circular dichroism (CD) measurements were performed on a JASCO 815 spectrometer at room temperature. Far-UV CD spectra were measured between 190 and 260 nm wavelength in 0.1 cm path length cell. Near-UV CD measurements were done in the region of 250–350 nm in 1.0 cm path length cell.

2.4. Steady-state and time-resolved fluorescence spectroscopy

Steady-state emission spectra were measured with a Jobin Yvon Fluoromax-3 fluorimeter. Fluorescence transients were measured using commercially available spectrophotometer (LifeSpec-ps) from Edinburgh Instrument, U.K. (excitation wavelength 409 nm and 375 nm, 80 ps instrument response function (IRF)) and fitted