Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1764 (2006) 190 – 198
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

Reversible inactivation of alkaline phosphatase from Atlantic cod (*Gadus morhua*) in urea

Bjarni Ásgeirsson *, Katrín Guðjónsdóttir

Department of Biochemistry, Science Institute, University of Iceland, Dunhaga 3, 107 Reykjavik, Iceland

Received 30 June 2005; received in revised form 9 December 2005; accepted 14 December 2005
Available online 10 January 2006

Abstract

Alkaline phosphatase (AP) from Atlantic cod (*Gadus morhua*) is a zinc and magnesium containing homodimer that requires the oligomeric state for activity. Its kinetic properties are indicative of cold-adaptation. Here, the effect of urea on the structural stability was studied in order to correlate the activity with metal content, the microenvironment around tryptophan residues, and events at the subunit interface. At the lowest concentrations of urea, the first detected alteration in properties was an increase in the activity of the enzyme. This was followed by inactivation, and the release of half of the zinc content when the amount of urea reached levels of 2 M. Intrinsic tryptophan fluorescence and circular dichroism ellipticity changed in the range 2.5 to 8 M urea, signaling dimer dissociation, followed by one major monomer unfolding transition at 6–8 M urea as indicated by ANS fluorescence and KI fluorescence quenching. Gibbs free energy was estimated by the linear extrapolation method using a three-state model as 8.6 kcal/mol for dimer stability and 11.6 kcal/mol for monomer unfolding giving a total of 31.8 kcal/mol. Dimer association had a very small ionic contribution. Dimers were stable in relatively high concentration of urea, whereas the immediate vicinity around the active site was vulnerable to low concentrations of urea. Thus, inactivation did not coincide with dimer dissociation, suggesting that the active site is the most dynamic part of the molecule and closest related to cold-adaptation of its enzymatic activity.

Keywords: Cold-adaptation; Psychrophilic; Dimer; Metalloenzyme; Folding; Stability

1. Introduction

Cold-adapted enzymes have evolved to function well at low temperatures, but at the same time, their temperature stability is lower than in homologous enzymes from warmer environments [1–3]. There has been some debate as to whether this global instability is necessary to ensure sufficient flexibility for catalytic movement by reducing weak non-covalent interactions in the structure, or due to lack of evolutionary pressure in the cold [4,5]. There is ample experimental evidence that enzyme activity requires some minimal mobility in active-sites, although it is not fully understood how the global dynamic motions of protein molecules contribute to their catalytic mechanisms [6]. Thus, the global stability of an enzyme is not necessarily an accurate indication of the flexibility at its active site, and inactivation of enzymes often precedes measurable global conformation changes for this reason. For the catalytic efficiencies of enzymes from psychrophilic organisms to be maintained at low temperatures, flexibility at the active site must be comparable to heat-adapted homologues [1–3]. Studies on cold-active enzymes have revealed that mutations can affect the mobility of specific parts that are involved in rate-determining catalytic conformational changes. Examples include lactate dehydrogenase [7], chitobiase from a psychrophilic Antarctic bacterium [8], and uracil DNA glycosylase [9].

Dimerization is the most common oligomeric form of proteins, bringing advantages in terms of stability and activity [10,11]. Alkaline phosphatase is a good example where monomers are inactive and the dimer intersubunit surface shapes the functional form of the catalytic site. Thus, dimerization may determine the catalytic efficiency of a particular enzyme through regulation of conformational

Abbreviations: AP, Alkaline phosphatase (EC 3.1.3.1); ANS, 8-Anilino-1-naphtalene sulfonic acid; GdmCl, Guanidine hydrochloride; PAR, 4-(2-pyridylazo)resorcinol

* Corresponding author. Tel.: +354 525 48 00; fax: +354 552 89 11.
E-mail address: bjarni@raunvis.hi.is (B. Ásgeirsson).

1570-9639/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbapap.2005.12.015

flexibility. This has been shown by model studies [12], or site-directed mutagenesis with several enzymes, including alkaline phosphatases (APs) [13,14]. The cold-adapted cod AP formed stable monomers upon treatment with guanidinium salt (GdmCl), in contrast to calf intestinal AP, which unfolded in a two-state process [15]. Thus, cod AP offers a unique opportunity to study the interface between subunits in a thermodynamic fashion and what role dimerization plays in structural and functional properties. Although, the majority of dimeric proteins are broadly characterized by two-state or three-state unfolding pathways, where the intimidate state is either folded monomers or the partially unfolded dimers, some dimeric proteins have been observed to form more intermediate states [16], and five intermediates were proposed for human placental AP [17].

In APs, conformational changes are dependent on the metal-ion content of the enzyme, and significant conformational changes take place upon dimerization which enhance thermal stability, metal binding, and catalysis [13,14]. Most APs require zinc and magnesium, or rarely other metals such as cobalt, for activity and stability. The three-dimensional structure of three APs from very different sources have been solved to date, namely, from *E. coli* [18], North Atlantic shrimp [19], and human placenta [20]. Given the high level of sequence identity amongst known bacterial and vertebrate APs, and similarity in tertiary structure, it can be assumed that all APs have same reaction mechanism [21]. However, subtle differences do exist as borne out by different kinetic properties of bacterial, mammalian, and psychrophilic AP variants from fish or bacteria [22–26]. We have previously characterized the cold-adaptation of the homodimeric AP from Atlantic cod [24]. The catalytic efficiency (k_{cat}/K_m) was over twice higher for the cod AP compared with calf intestinal AP at 5 °C. Heat stability of cod AP was clearly much reduced compared with the calf AP, and the cod AP also had reduced affinity for inorganic phosphate [24]. Dissociation of the reaction product from APs is the overall rate-limiting step at alkaline pH, and the dynamic properties of the protein structure may play a key role in facilitating this step of the catalytic function. Other psychrophilic APs have been isolated for study [23,25–28], but information about the stability of these proteins in terms of Gibbs free energies, and a description of structural changes accompanying unfolding, is lacking.

In this paper, we examine the dissociation and unfolding of the cod AP dimer in the presence of urea. We have previously found that the stability of cod AP in the presence of GdmCl is to a large extent dependent on dimerization [15]. The fact that dimer association in cod AP is weaker than in vertebrate APs suggested that movement at the dimer interface may accompany the catalytic cycle. It may be noted that AP monomers are inactive, presumably because of slight malformation of the active site. Using an uncharged modulator like urea, instead of the previously used GdmCl [29], may be expected to give a fuller picture of stabilizing interaction in terms of Gibbs free energies, the possible existence of intermediary steps on the unfolding pathway and the role of the metal ions, as well as allowing determination of the relative contribution of hydrophobic forces versus non-covalent ionic bonds in forming the stable enzyme.

2. Materials and methods

Cod alkaline phosphatase (AP) was purified as previously described [24]. Calf intestinal AP was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Substrates, ANS, PAR, and buffer salts were purchased from Sigma Chemical Company (St. Louis, MO). Urea was supplied by Acros Organics (New Jersey, USA).

2.1. Protein assays

Enzyme activity was measured with 5 mM *p*-nitrophenyl phosphate in 1.0 M diethanolamine buffer at pH 9.8 containing 1.0 mM magnesium chloride, and protein concentration was estimated by Coomassie Blue G250, or by using a calculated extinction coefficient of 52000 M^{-1} cm^{-1} [15].

2.2. Fluorescence and circular dichroism measurements

Intrinsic fluorescence was measured with a Spex Fluoromax instrument and analyzed with Datamax software (Jobin Yvon). Measurements were carried out in 1 ml cuvettes and either in 25 mM Mops, or 20 mM disodium tetraborate 10-hydrate (borax), with 1 mM $MgSO_4$ at pH 7.5 and 25 °C. The excitation wavelength was 295 nm and emission was monitored at 310–400 nm (5 nm bandwidths). An average of three scans was taken. The different concentrations of urea were obtained by dilution from a 9-M stock solution, and the enzyme pre-incubated for 4 or 24 h prior to measurements. The concentration of urea was determined by refractometry [30]. The final enzyme concentration was 0.012 mg/ml with either cod AP or calf intestinal AP. Background spectra were determined at each urea concentration and subtracted. General data handling followed well-established procedures [30] as previously described [15].

Tryptophan exposure was further monitored by collision quenching experiments using KI at 25 °C. Enzyme was added to the desired concentration of urea in 25 mM Mops buffer at pH 7.5, and KI then added from a 5 M stock solution containing 0.01 M Na_2SO_3 to a concentration in the range 0–0.3 M. Excitation was at 295 nm and data were collected as the integral emission intensity in the range 310 to 400 nm by averaging three scans. The Stern–Volmer equation ($F_0/F = 1 + K_{SV}$ [KI]) was used for analyzing the data and to determine K_{SV}, the Stern–Volmer quenching constant [31]. K_{SV} gives the approximate degree of exposure of tryptophan residues to the solvent.

ANS fluorescence was determined in 25 mM Mops containing 1 mM $MgSO_4$ at pH 7.5. Protein concentration was 0.05–0.06 mg/ml and final concentration of ANS 0.02 mM from a 2 mM stock solution. The excitation wavelength was 380 nm and spectra collected over the range 420–620 nm. Circular dichroism spectra were collected on a Jasco 810 instrument using 2 mm or 5 mm cells at 25 °C. The buffer was 20 mM borate containing 10 mM $MgSO_4$ at pH 7.5.

2.3. Size-exclusion chromatography

Size-exclusion chromatography was performed on a Pharmacia FPLC apparatus equipped with a Superose 12 column. All solution were filtrated through 0.45 μm membranes (Millipore). The column was pre-equilibrated with three column volumes of the same denaturant solution as used for incubating the samples in each case. The buffer was 25 mM HEPES, 10 mM $MgSO_4$ (pH 7.5) containing varying amounts of urea. The final active enzyme concentration was about 0.06 mg/ml. Analysis of the data was performed with the computer program IgorPro (WaveMetrics, Inc., Oregon, USA).

2.4. Refolding experiments

Protein samples were incubated in various concentrations of urea buffered with 25 mM Mops buffer containing 1 mM $MgSO_4$ at pH 7.5, and then usually diluted 15-fold with 20 mM borate at pH 7.8 containing 10 mM $MgSO_4$ and 50% (v/v) glycerol.