FEBS Letters 583 (2009) 2054–2060

journal homepage: www.FEBSLetters.org

Reversible unfolding of dimeric phosphofructokinase-2 from *Escherichia coli* reveals a dominant role of inter-subunit contacts for stability

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ARTICLE INFO

Article history:
Received 6 March 2009
Revised 2 May 2009
Accepted 15 May 2009
Available online 22 May 2009

Edited by Judit Ovádi

Keywords:
Reversible unfolding
Subunit interaction
Folding intermediate
Dimer–monomer transition
Ribokinase superfamily
Bimolecular domain

ABSTRACT

Escherichia coli **phosphofructokinase-2 (Pfk-2) is a homodimer whose subunits consist of a large domain and an additional β-sheet that provides the interfacial contacts between the subunits, creating a β-barrel flattened-like structure with the adjacent subunit's β-sheet. To determine how the structural organization of Pfk-2 determines its stability, the reversible unfolding of the enzyme was characterized under equilibrium conditions by enzymatic activity, circular dichroism, fluorescence and hydrodynamic measurements. Pfk-2 undergoes a cooperative unfolding/dissociation process with the accumulation of an expanded and unstructured monomeric intermediate with a marginal stability and a large solvent accessibility with respect to the native dimer.**

1. Introduction

Phosphofructokinase-2 (Pfk-2) from *Escherichia coli* is a member of the ribokinase superfamily, a group of sugar and vitamins kinases formed by a common α/β/α domain [1]. In sugar and sugar phosphates kinases, such as ribokinase [2], tagatose-6-P kinase [3], 2-keto-3-deoxygluconate kinase [4] and Pfk-2 [5], there is an additional structure that forms a lid for the active site. This structure is a four stranded β-sheet inserted non-sequentially near the N-terminal end of the conserved α/β/α domain. On the other hand, vitamins and small molecules kinases are mainly formed by the conserved α/β/α domain since their structures lack the additional β-sheet structure [6]. Pyridoxal kinases correspond to such kind of kinases and have been characterized as active monomers [7], although a dimer can be observed in the protein crystal [8,9]. The guanidine hydrochloride (GdnHCl) induced unfolding of pyridoxal kinase from human and sheep brain, was reported as a two-state reversible equilibrium with calculated stability values of 1.2 kcal mol^{-1} [10] and 1.55 kcal mol^{-1} [11] respectively. These values indicate that in both cases the conserved α/β/α domain behaves as single cooperative unit exhibiting a marginal stability. However, in the case of sugar and sugar phosphate kinases, such as *E. coli* Pfk-2, is not known how the β-sheet insertion cooperates to bring about the stability of the native structure.

Light scattering and gel filtration analyses [12,13] have shown that *E. coli* Pfk-2 is primarily a dimer in solution, which quaternary structure is required for enzymatic activity [12,14]. As shown by its crystal structure (PDB ID code 3CQD), each subunit can be divided in two parts: the conserved α/β/α domain and the additional β-sheet structural element that protrudes from it. In Pfk-2 and in close family homologues, the additional β-sheet structure works as lid for the active site and also creates the interaction surface between the subunits, forming an intertwined interface denominated β-clasp [8]. Thus, Pfk-2 seems to be stabilized by two domains: the β-clasp interface and the conserved α/β/α domain that accounts for the mayor part of the intermolecular contacts of each subunit. In this kind of dimers both domains are separated by the active site, which is mainly self-contained in each subunit [8].

The GdnHCl unfolding of Pfk-2 was recently studied in order to obtain information regarding the structure of the separated subunits [14]. The dissociation of the enzyme originates a semi structured monomer with an expanded volume. However, a thermodynamic analysis of the unfolded transitions has not been explored in detail. In this work, we describe the reversible effect of GdnHCl on the dissociation and unfolding of Pfk-2 following

Abbreviations: Pfk-2, phosphofructokinase-2; GdnHCl, guanidine hydrochloride; ANS, 8-anilino-1-naphthalene sulfonic acid; DTT, dithiotreitol; SEC, size exclusion chromatography; CD, circular dichroism spectroscopy; DLS, dynamic light scattering; Cm, guanidine hydrochloride concentration at the middle of the observed property change; Rs, Stokes radius

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0014-5793/$36.00
doi:10.1016/j.febslet.2009.05.034

enzymatic activity, intrinsic tryptophan fluorescence and circular dichroism. Moreover, the hydrodynamic properties Pfk-2 were followed by size exclusion chromatography and dynamic light scattering measurements. The simplest model to describe the global unfolding of Pfk-2 is a three-state unfolding reaction $N_2 \leftrightarrow 2I \leftrightarrow 2U$, characterized by a semi-unstructured monomeric intermediate with an expanded volume. The energetic parameters extracted from the three-state unfolding model, indicate that the inter-subunit contact between the four-stranded β-sheets extra domain confers the global stability to Pfk-2 since the isolated subunits present a reduced stability with respect to the native dimer. Taken together the physical characteristics of the monomeric intermediate and its stability, is postulated that the conserved α/β/α domain and the β-sheet insertion are thermodynamically coupled in the dimeric structure of Pfk-2.

2. Materials and methods

2.1. Pfk-2 purification and storage

E. coli Pfk-2 was purified and stored as described by Cabrera et al. [15]. Previous to the unfolding or refolding experiments, the storage buffer was changed to the standard buffer (50 mM Tris pH 7.8, 5 mM $MgCl_2$ and 10 mM dithiotreitol (DTT)) by using a HiTrap desalting column (Amersham Biosciences, Uppsala, Sweden), before a 3 h dialysis in the same buffer. The enzyme was concentrated by using a centricon-60 concentrator (Amicon, Berverly, USA). Protein concentration was determined by the Bradford assay (Bio-Rad) and is expressed in terms of the monomer concentration.

2.2. Pfk-2 unfolding and refolding

For the refolding experiments, the enzyme was first exposed to 3 M GdnHCl (Pierce, molecular biology grade) for at least 5 h at 20 °C. Under this condition Pfk-2 was completely unfolded, as indicated by its catalytic activity, circular dichroism spectroscopy (CD) and intrinsic fluorescence measurements. The refolding curve was obtained diluting the unfolded enzyme to several GdnHCl concentrations in the standard buffer. Under these conditions equilibrium was achieved after 5 h of incubation at 20 °C. The unfolding curves were constructed by diluting native Pfk-2 to several GdnHCl concentrations. In this case equilibrium was obtained after 48 h at 20 °C. GdnHCl concentrations were prepared as described by Pace [16].

2.3. Enzyme assays

Phosphofructokinase enzymatic activity was measured spectrophotometrically by a coupled assay as described by Babul [17]. The activity assay began by dilution of a 1 μl aliquot containing the enzyme and GdnHCl into 700 μl of assay mixture. Since this procedure implies the dilution of GdnHCl, renaturation should be expected. To determine the renaturation yield of Pfk-2 during the enzymatic assay, the enzyme was unfolded with GdnHCl and refolded into the assay mixture to bring about a protein concentrations of 0.004 μM (the protein concentration used in the coupled assay). Under these conditions, substrates were added at several times after refolding was initiated and the enzymatic activity was followed. The activity was only about 3% with respect to that measured with samples that were not previously denatured.

2.4. Intrinsic and ANS fluorescence

Measurements were done in a Perkin–Elmer LS 50 spectrofluorimeter. Protein samples at several GdnHCl concentrations were excited at 295 nm to limit the fluorescence to the single tryptophan per monomer of Pfk-2 (Trp-88). The emission spectra were recorded from 300 to 480 nm using emission and excitation slits of 5 nm. Stock 8-anilino-1-naphthalene sulfonic acid (ANS) solutions (Molecular Probes, Eugene, USA) were prepared in methanol and its concentration was determined using a ε of 7800 M^{-1} cm^{-1} at 372 nm. The Pfk-2 samples refolded and unfolded at different GdnHCl concentrations contained 80 μM ANS. The mixture was incubated for 48 h in the dark. Samples prepared in this way were excited at 380 nm and the emission recorded from 400 to 580 nm.

2.5. Circular dichroism spectroscopy

Far UV CD spectra were acquired in a Jasco J600 dichrograph, employing 1 mm cell. Each spectrum resulted from the accumulation of three scans (bandwidth 1 mm, scan rate 20 nm min^{-1}) between 210 and 260 nm (the high absorbance of 5–10 mM DTT did not allow to record spectra below 210 nm).

2.6. Size exclusion chromatography

Size exclusion chromatography (SEC) equilibrium experiments were performed using a Water Breeze HPLC system equipped with a Bio-Rad exclusion column (Bio-Sil SEC 250 gel filtration, 300 × 7.8 mm). The column was equilibrated with 60 ml of the mobile phase containing 0.2 M KCl in standard buffer with the same GdnHCl concentration of that of the sample to be injected. Calibration was performed by using the proteins provided by the manufacturer of the column (Vitamin B-12, 1.35 kDa, 8.5 Å Stokes radius (Rs); horse myoglobin, 17 kDa, 19 Å Rs; chicken ovalbumin, 44 kDa, 30.5 Å Rs; bovine gamma globulin, 158 kDa, 41.8 Å Rs; and bovine thyroglobulin 670 kDa, 85 Å Rs). Protein elution volumes were converted to Rs values using the linear relationship obtained with the molecular-mass markers). The column temperature was adjusted with a water jacket at 20 °C. Protein elution was followed at 220 and 280 nm. The concentration of the injected protein ranged between 2 and 20 μM.

2.7. Dynamic light scattering experiments

The Stokes radius (Rs) of Pfk-2 in 50 mM Tris buffer pH 8, 5 mM $MgCl_2$, 10 mM DTT incubated at different GdnHCl concentrations for 24 h at 20 °C, was determined by dynamic light scattering (DLS) using a DynaPro MSTC014 (Protein Solutions, Lakewood, NJ, USA) at a protein concentration of 14 μM. All solutions were centrifuged at 13 600×*g* for 30 min prior to data collection. The protein concentration of the samples was measured before and after these treatments and no significant loss of sample was observed. Data were acquired by accumulation of 18 readings of 5 s with detector sensitivity set to 80%. The particle size distribution was calculated by using the 'regularization' method provided with the DYNAMICS software, supplied with the instrument. The residual scattering intensity (intensity scattered by the protein without solvent contribution) was also determined.

2.8. Data analysis

CD unfolding curves were analyzed according to a three-sate model:

$$N_2 \overset{K_1}{\Longleftrightarrow} 2I \overset{K_2}{\Longleftrightarrow} 2U$$

where N_2 represents the native state dimer, I is a monomeric intermediate and U, is the unfolded polypeptide.

The changes in the Gibbs energy expressed in the unfolding sense of the reaction for the intermediate unfolding (ΔG_2) and the native dimer unfolding (ΔG_1) are defined as $\Delta G_2 = -RT \ln K_2$