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The low-temperature folding intermediate of hyperthermophilic D-glyceraldehyde-3-phosphate dehydrogenase from *Thermotoga maritima* shows a native-like cooperative unfolding transition

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Hyperthermophilic D-glyceraldehyde-3-phosphate dehydrogenase from *Thermotoga maritima*, after denaturation in 6 M guanidinium chloride and subsequent renaturation by dilution at 3°C, forms a 'low-temperature intermediate' with its native quaternary structure and most of its dichroic absorption restored, but with significant differences in its fluorescence properties compared to those of the native enzyme. Shifting the temperature beyond 10°C, the enzyme is reconstituted to high yields and to an overall structure indistinguishable from the initial native state [FEBS Lett. 290 (1991) 235-238]. These criteria suggest that the cold intermediate represents an 'assembled molten globule'. However, present equilibrium transition data prove the cold intermediate to be native-like, in that it exhibits a reversible highly cooperative conformational transition to the unfolded state which is incompatible with the typical characteristics of the molten globule state of globular proteins.

Protein folding; Molten globule state; D-glyceraldehyde-3-phosphate dehydrogenase; Cold intermediate; Thermophile; *Thermotoga maritima*

1. INTRODUCTION

The translation of the one-dimensional information at the DNA level into the three-dimensional structure of a protein is determined by the amino acid sequence and the cellular environment. So far, no systematic studies on the effects of physiologically relevant solvent parameters on the folding of globular proteins are available. Certain conclusions may be drawn from the expression of active recombinant proteins in cases where the optimal growth conditions of the host cell are different from the natural folding environment of the guest molecule. In asking how critical in vivo conditions are in the acquisition of the native state of a given protein, a remarkable temperature tolerance is observed, as seen by the fact that thermostable enzymes may be expressed in mesophilic hosts. In this context, it has recently been shown that hyperthermophilic D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) from *Thermotoga maritima* with a thermal denaturation transition at > 105°C [1] is expressed in active form in *Escherichia coli* (A. Tomschy, R. Glockshuber, R. Jaenicke, unpublished). In vitro reconstitution has been successfully performed at temperatures between 10 and 90°C [2]. However, at lower temperature, the enzyme is found to be trapped as an inactive 'cold intermediate', with its native quaternary structure and most of its dichroic absorption restored, but with significant differences in its fluorescence properties compared to those of the native enzyme [3]. Given these criteria, one may assume that the cold intermediate represents some kind of assembled molten globule. In the present work, this hypothesis has been investigated by studying the conformational stability of the reconstituting enzyme at low temperature. Spectroscopic evidence clearly shows that the tetrameric cold intermediate undergoes highly cooperative unfolding transitions incompatible with the fluctuating tertiary contacts characteristic for both the 'structured molten globule' and the 'collapsed unfolded polypeptide chain' [4–6].

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Abbreviations: CD, circular dichroism; F, fluorescence; GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; GdmCl, guanidinium chloride.

2. MATERIALS AND METHODS

2.1. *Chemicals*

NAD^+ and glyceraldehyde-3-phosphate (barium salt), were purchased from Boehringer Mannheim, cysteamine from Fluka (Buchs, Switzerland). Ultra-pure guanidinium-chloride and trypsin were products of Schwarz-Mann (Orangeburg, NY) and Sigma (Deisenhofen), respectively. All other chemicals were A-grade substances from Merck (Darmstadt). Quartz-bidistilled water was used throughout. Buffer solutions were filtered and carefully degassed.

2.2. *Cultivation of* Thermotoga maritima *and enzyme purification*

Cultivation of *Thermotoga maritima* (MSB 8, DSM strain 3109) and enzyme purification were performed as reported previously [1,7].

2.3. *Preparation of the cold intermediate*

Holo-GAPDH from *Thermotoga maritima* was denatured over a

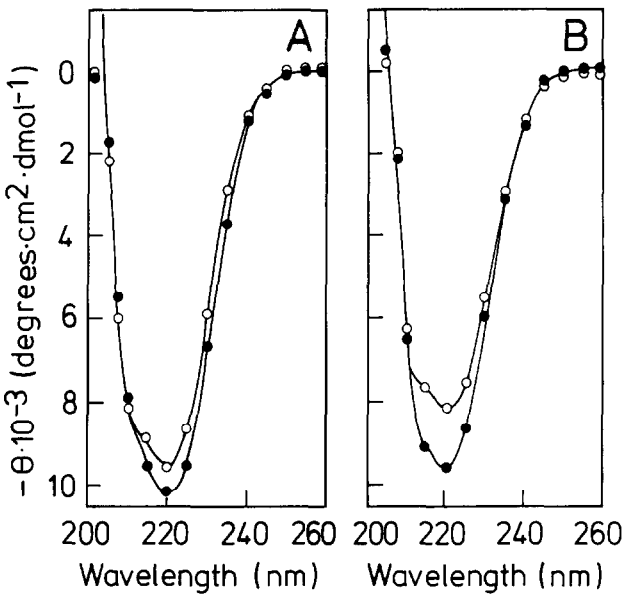

Fig. 1. Far-UV CD spectra of native GAPDH from *Thermotoga maritima* and its cold intermediate. 50 mM sodium phosphate buffer pH 7.0, 5 mM EDTA, 5 mM cysteamine at an enzyme concentration of 31 μg/ml. (●) Native GAPDH, (○) cold intermediate. (A) Far-UV CD spectra at 3°C. (B) Far-UV CD spectra after shifting the temperature to 50°C.

period of ≥ 120 min at 40°C in 50 mM sodium phosphate pH 8.0, 5 mM EDTA, 5 mM cysteamine in the presence of 6 M GdmCl. After cooling down to 3°C, renaturation was started by dilution with GdmCl-free buffer, keeping the temperature constant at 3°C. The residual concentration of GdmCl was 103 mM. The reactivation mixture was incubated in the cold for at least 10 days.

2.4. *Enzyme assays*

Enzyme assays made use of the oxidation of glyceraldehyde-3-phosphate, monitoring the increase of NADH absorbance at 366 nm and 40°C in an Eppendorf spectrophotometer. The cuvettes were thermostatted, and the actual temperature in the cuvettes was monitored by a thermistor unit mounted in a reference cuvette. In order to exclude reactivation during the standard assay, trypsin was added [8,9]. Enzyme assay mixtures contained 3 mM NAD^+, 10 mM disodium hydrogen arsenate, 5.2 mM glyceraldehyde-3-phosphate and trypsin at a concentration of 20 μg/ml in 50 mM Tris-HCl pH 8, 5 mM EDTA, 5 mM cysteamine.

2.5. *Spectral analysis*

Fluorescence emission spectra were measured in the wavelength range 300–400 nm (λ_{exc} = 280 nm) using a Perkin-Elmer luminescence spectrometer LS 5B and 1 cm cuvettes. GdmCl-induced unfolding transitions were measured at 3°C and a constant wavelength of 320 nm. Circular dichroism spectra were monitored at 3°C in a Jasco J500A CD spectropolarimeter using a mean residue weight of 109.4 [7]. Far-UV CD spectra were measured at 200–260 nm in thermostatted 0.5 cm quartz cuvettes; denaturant-induced unfolding transitions were monitored at 222 nm. For near-UV CD spectra at 250–350 nm, thermostatted 1 cm quartz cuvettes were used.

3. RESULTS

3.1. *Spectral analysis*

In order to determine the secondary structure of the native enzyme and the cold intermediate (after denaturation/renaturation), far-UV CD spectra were recorded at 3°C. As shown in Fig. 1, the spectra of both the native enzyme and its cold intermediate show the characteristics of α-helical proteins. After correcting the spectrum of the cold intermediate for incomplete reconstitution (82% yield), the amplitudes at 222 nm are indistinguishable. Thus, we may conclude that the cold intermediate on the folding pathway at ≤ 3°C is trapped in a native-like conformation. Raising the temperature to 50°C leads to the typical temperature-dependent decrease in amplitude reported previously [1]. The dichroic absorption in the near-UV (which reflects the tertiary contacts in the local environment of aromatic residues) shows that the cold intermediate differs from the native enzyme. As depicted in Fig. 2, the spectrum of the intermediate at 3°C, in the presence of 0.1 M residual GdmCl can be mimicked by applying the same conditions to the native enzyme. On the other hand, at room temperature, the near-UV spectrum of the native enzyme remains practically unchanged in the absence and in the presence of the denaturant; this holds also if one compares the native enzyme at 3°C and at 25°C. Thus, one may exclude that spectral differences between the native enzyme and the cold intermediate

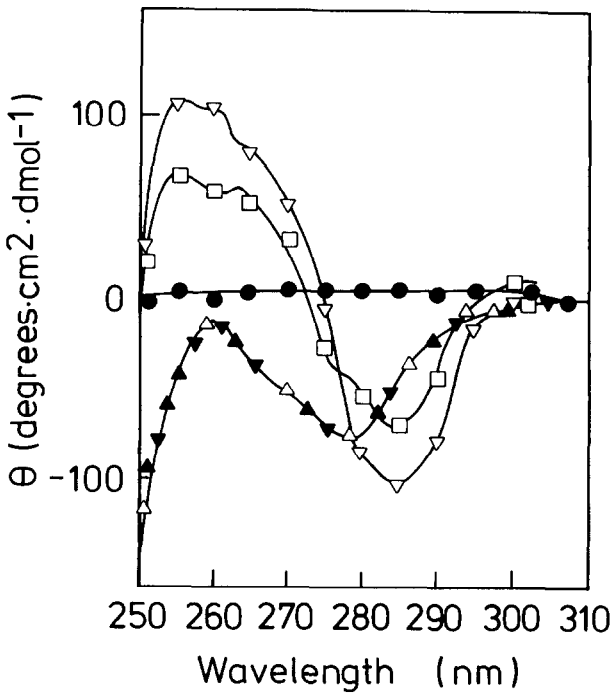

Fig. 2. Near-UV CD spectra of the cold intermediate from GAPDH from *Thermotoga maritima* and native and denatured controls. 50 mM sodium phosphate pH 7.0, 5 mM EDTA, 5 mM cysteamine at an enzyme concentration of 31 μg/ml. Cold intermediate at 3°C, in the presence of 103 mM residual GdmCl concentration (□). Controls using 31 μg/ml GAPDH not subjected to denaturation/renaturation refer to: 25°C, 6 M GdmCl (●); 3°C, 103 mM GdmCl (▽); 25°C, 103 mM GdmCl (▼); 3°C, without GdmCl (△) and 25°C, without GdmCl (▲); the latter three coincide.

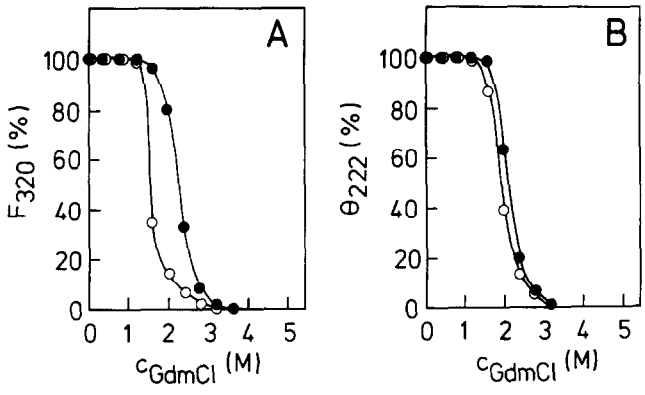

Fig. 3. GdmCl-induced unfolding transitions of native GAPDH from *Thermotoga maritima* and its cold intermediate at 3°C, as monitored in fluorescence emission and far-UV circular dichroism. (●) native GAPDH, (○) cold intermediate. Prior to the measurements, samples were incubated in 50 mM sodium phosphate pH 7.0, 5 mM EDTA, 5 mM cysteamine at the given concentrations of GdmCl and 3°C for 4 d. (A) Equilibrium transitions, monitored by fluorescence emission at 320 nm; enzyme concentration was 10 μg/ml. (B) Equilibrium transitions, as monitored by circular dichroism at 222 nm; enzyme concentration was 31 μg/ml.

after denaturation/renaturation are caused by cold denaturation [10]. Clearly, low denaturant concentrations favor cold denaturation of the enzyme [2,3]. After denaturation and subsequent renaturation at low temperature, the residual GdmCl concentration keeps the enzyme in its partially unfolded state. Shifting the temperature beyond 10°C restores the native state. In 6M GdmCl, both far-UV and near-UV CD prove the enzyme to be fully unfolded.

3.2. *Equilibrium unfolding transition*

As indicated by the spectral data, the cold intermediate has recovered its native secondary structure, but its tertiary structure is still perturbed. Both criteria suggest the cold intermediate on the folding pathway to represent a 'molten globule' state [4,6,11]. On the other hand, previous ultracentrifugal analysis has shown that the cold intermediate is trapped in a state close to the tetramer: at 0°C, the non-linear lnc vs. r^2 dependence yields a weight-average molecular mass of 110 kDa, with the 36 kDa monomer as the smallest component. Increasing the temperature to 25°C (within the same experiment), shifts the dissociation equilibrium back to the homogeneous tetramer (144 kDa, correlation factor 0.9994) [2].

In order to further characterize the intermediate, the stability and possible cooperative equilibrium unfolding transitions of the cold intermediate were investigated. Since denaturation of the enzyme is accompanied by a shift in fluorescence emission from λ_{max} = 327 nm (native) to λ_{max} = 350 nm (6 M GdmCl) and a decrease in dichroic absorption at 222 nm, measurements made use of $\Delta F_{320\,nm}$ and $\Delta\Theta_{222nm}$, respectively. Deactivation cannot be applied because the cold intermediate shows no measurable catalytic function. As illustrated in Fig. 3, the cold intermediate undergoes a reversible unfolding transition with characteristics close to those observed for the native enzyme at room temperature. Monitoring fluorescence emission and circular dichroism, the denaturation profiles do not coincide: full exposure of the fluorophores (ΔF_{320nm}) precedes the cooperative helix-coil transition (ΔP_{222nm}). As one would predict, the GdmCl 'half-concentration', $c_{1/2}$, (where the residual structure shows 50% of its original value) for the native enzyme exceeds the value for the cold intermediate. Comparing $c_{1/2}$ for 3 and 25°C, the shift amounts to 0.7 M: 2.3 M vs. 1.6 M GdmCl for ΔF_{320nm}, and 2.6 M vs. 1.9 M GdmCl for $\Delta\Theta_{222nm}$. Thus, the cold intermediate is only slightly less stable than native GAPDH; also, there is no drastic decrease in cooperativity, in contrast to molten globule–coil transitions reported in the literature [12].

4. DISCUSSION

The 'molten globule state' has been widely accepted as an early intermediate on the folding pathway of globular proteins [4–6,12]. It is characterized by native-like secondary structure, fluctuating tertiary contacts (leading to the exposure of hydrophobic residues and dye binding), and no cooperativity in thermally induced unfolding transitions. The present study shows, that the cold intermediate on the folding pathway of hyperthermophilic GAPDH from *Thermotoga maritima* represents a native-like structure with a highly cooperative unfolding transition, similar to the one observed for the enzyme at temperatures above 3°C.

When Thermotoga GAPDH is subjected to a denaturation/renaturation cycle at ≤ 3°C, the residual GdmCl concentration of ca. 0.1 M, together with the low temperature, keep the enzyme in a cold-denatured state with the native secondary and quaternary structure practically restored. The characteristic spectral changes are also observed for the native enzyme, indicating that under moderately destabilizing conditions cold denaturation is detectable also for the enzyme that has not been subjected to the denaturation/renaturation cycle. Previous temperature shift experiments have shown, that the full recovery of the native state occurs in a fast reaction [3]. As taken from (i) ultracentrifugal analysis, (ii) the cooperativity of the ΔF_{320nm} and ΔH_{222nm} vs. c_{GdmCl} unfolding transitions, and (iii) the relatively high $c_{1/2}$(GdmCl) values, the cold intermediate must be stabilized by most of the secondary, tertiary and quaternary contacts present in the native enzyme. In conclusion, considering the complete consecutive uni-bimolecular reconstitution reaction of the enzyme, the cold-intermediate of GAPDH from *Thermotoga maritima* must be a well-defined stable intermediate, distinct from a 'molten globule', but close to the native state of the enzyme.

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