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SHORT COMMUNICATION

A diketoreductase exhibits unique renaturation profile from thermal-induced protein unfolding

Meiling Lu · Xu Cao · Xin Yang · Heng Zheng · Nan Liu · Yun Jiang · Donghai Lin · Yijun Chen

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Abstract Proteins can refold from thermal-induced denaturation. Apo-diketoreductase exhibited a unique refolding profile, in which the degree of refolding from higher temperature was more complete. Partial aggregation and structural change may provide possible explanation on this phenomenon.

Keywords Diketoreductase · Refolding · Self-renaturation · Thermal induced · Unfolding

Introduction

Proteins are usually unfolded by chemical and physical treatments, which lead to alterations on their conformations (Dobson 2003; Fersht and Dagget 2002). One of the common methods of denaturing proteins is heating. Reports on conformational state of the denatured proteins have varied from apparently fully unfolded to substantial remaining structure (Goto et al. 1990). Unfolding caused by structural changes of proteins significantly influences their functional properties and results in pathological consequences in organisms (Selkoe 2003; Tsigelny et al. 2008; Lauren et al. 2009).

In certain cases, protein unfolding can be reversed through a refolding process, but the refolding typically requires the presence of other additives, such as glycerol, salt, metal ions (Mayor et al. 2000; Shortle and Ackerman 2001; Dyson and Wright 2005) or the dilution of denaturants, such as guanidine chloride, urea (Hagihara et al. 1993; Sandberg et al. 2002; Kohn et al. 2004). Usually, such reversibility of unfolding–refolding occurs for proteins or peptides with predominant α-helical structures (Tokunaga et al. 2004; Landfried et al. 2008).

We recently reported the cloning, expression and characterization of a novel diketoreductase (DKR) (GenBank accession no. EU273886) from *Acinetobacter baylyi* ATCC 33305. The recombinant DKR (rDKR) catalyzes double reduction of β, δ-diketo ester with extremely high stereoselectivity (Wu et al. 2008, 2009). In present work, we studied the thermal-induced unfolding and renaturation of this apo-DKR. Different from previous observations, the degree of refolding was more complete than that at lower temperatures from activity assays and CD analyses. An explanation of the unique renaturation profile of rDKR was attempted by UV scans and molecular dynamics of homolog modeling, indicating that partial aggregation and structural change may contribute to such unusual phenomenon.

M. Lu and X. Cao contributed equally to this work.

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M. Lu · X. Yang · N. Liu · Y. Jiang · Y. Chen (✉)
Laboratory of Chemical Biology, China Pharmaceutical University, 24 Tongjia Street, Nanjing 210009, Jiangsu, People's Republic of China
e-mail: yjchen@cpu.edu.cn

X. Cao · H. Zheng · D. Lin
School of Life Science and Technology, China Pharmaceutical University, 24 Tongjia Street, Nanjing 210009, Jiangsu, People's Republic of China

D. Lin
Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 555 Zuchongzhi Road, Shanghai 201203, People's Republic of China
e-mail: dhlin@mail.shcnc.ac.cn

Materials and methods

Protein expression and purification

Recombinant *Escherichia coli* cells were cultivated in LB media, and rDKR expression was induced by 0.1 mM IPTG. Crude enzyme was obtained by cell disruption and centrifugation. rDKR was purified sequentially by DEAE Sepharose FF column and Sephadex-100 column as previously described (Wu et al. 2008, 2009).The purity of enzyme preparations was examined to be greater than 99.9% on SDS-PAGE by Coomassie blue staining. Protein concentration was determined by standard BCA method.

Enzyme activity assay

Spectrophotometric method was used to determine the rDKR activity on a UV-1700 array spectrophotometer according to the literature (Wu et al. 2008, 2009). For all assays, enzyme activity was defined as one unit representing the oxidation of 1 μm of NADH per minute per milligram protein. Ethyl 3,5-diketo-6-benzyloxy hexanoate was used as the substrate to assay enzymatic activity (Wu et al. 2008, 2009).

CD spectroscopy

CD spectra in far UV region (190–250 nm) were recorded on a Jasco-810 spectropolarimeter equipped with a PTC-348 WI temperature controller and a Peltier cell holder. Thermal unfolding or refolding experiments were carried out by increasing or decreasing the temperature from 25 to 90°C or from 90 to 25°C at a rate of 1°C min^{-1} with protein concentration of 0.1 mg ml^{-1}.

UV scanning

UV spectra were scanned on UV-1700 with CPS-Controller (Shimadzu) in the temperature range of 25–60°C with wavelength of 600–200 nm.

Unfolding and renaturing of rDKR

Recombinant DKR with concentration of 0.1, 0.15 and 0.2 mg ml^{-1} in 10 mM potassium phosphate buffer (pH 7.0) were heat treated at 60, 70, 80 and 90°C for 5–30 min in Eppendorf tubes. After incubation, enzyme solutions were immediately cooled at 25°C for activity assays at various time points for renaturation. CD spectra and enzymatic activity were determined after both unfolding and refolding.

Molecular dynamics of homology modeling

Molecular modeling of DKR with human HAD (PBD:1f14) as a template was conducted according to reported methods (Sali and Blundell 1993; Shen and Sali 2006). Molecular dynamics simulation was performed according to the previous report (Schroder et al. 2005). The simulation temperature was from 298–363 K with a solvent model of GBSW.

Results and discussion

To investigate the thermal stability of the recombinant enzyme, apoprotein of rDKR solution without any cofactors and substrate was treated at different temperatures, and then enzymatic activity was determined by NADH oxidation in the presence of substrate. As expected, the enzymatic activity was reduced by the increase of temperature with a T_m of 60.1°C (Supplementary Fig. 1). Remarkably, within a narrow range of temperature (58–62°C), activity change was dramatic, showing complete inactivation at 61°C. CD analyses indicated that the secondary structure of rDKR was maintained as a stable conformation from 25 to 55°C, sharply changed at 60°C, and then attained another stable conformation from 65 to 90°C (Supplementary Fig. 2). The existence of any bound cofactors of NADH or NADPH was ruled out by UV spectroscopic analysis and activity determination without the addition of cofactors (data not shown). Furthermore, we recorded the thermal melting spectra of rDKR by CD. The onset, mid and end temperatures of melting were calculated to be 56, 60 and 65°C from the melting curve. Consistent with the enzymatic assay, rDKR lost its secondary structure sharply in the temperature range of 58–62°C (Supplementary Fig. 1).

As shown in Supplementary Fig. 1, the direct correlation between the changes in activity and secondary structure suggested a possible reverse of activity and structure from its thermal-induced unfolding state without assistance of any other additives. Therefore, we unfolded the enzyme at high temperatures, and then promptly cooled at 25°C. Indeed, the thermal-induced loss of enzymatic activity was regained after cooling at 25°C for a period of time. Because protein concentration plays important roles in the folding process (Lu and Liu 2008), different concentrations of rDKR were examined for its renaturation, and 0.1 mg ml^{-1} was identified as the best for the compromise of activity assay and CD analysis (Supplementary Table 1). Based on the T_m of rDKR, we chose temperatures ranging from 60 to 90°C, with a 10°C interval, to investigate the effects of unfolding temperature. Surprisingly, contrary to previous reports (Dill et al. 1989; Jennings and Wright 1993; Harald et al. 2003; Tokunaga et al. 2004; Landfried et al. 2008), the degree of renaturation from 90 and 80°C was more complete than that from 70 and 60°C, with a clear tendency of better recovery of enzymatic activity from higher unfolding temperatures, although the activity was not totally recovered in all cases (Fig. 1a). The results

indicated that rDKR showed a novel and unprecedented profile in the process of protein renaturation.

To confirm the findings of rDKR self-renaturation, CD spectra under different temperature conditions were measured and compared. After unfolding at 90°C for 30 min and then incubating at 25°C for 1 h, the CD signals were identical to that prior to heating, indicating that rDKR denatured at 90°C was almost recovered to its native state (Supplementary Fig. 3a). To clarify the relationship between heating temperature and refolding ratio of the secondary structure, we recorded CD spectra of rDKR at 60, 70 and 80°C with the same procedure as at 90°C. Recovery of secondary structural elements at 90°C was better than that at lower temperatures as well (Supplementary Fig. 3b–d). The structural changes based on the CD spectra were in excellent agreement with the results above of enzymatic activity. Because cooling rate typically plays a role in protein renaturation, we also controlled it at 1°C min^{-1}, same as the unfolding process. The refolding spectra of rDKR from 90°C exhibited gradual recovery of its secondary structure elements, and regain of the secondary structure was mostly seen when temperature reached 25°C (Supplementary Fig. 4). These results clearly indicated the difference from previous thermal-induced unfolding–refolding studies on globins, ribonucleases, β-lactamases, and others (Dill et al. 1989; Jennings and Wright 1993; Tokunaga et al. 2004; Landfried et al. 2008).

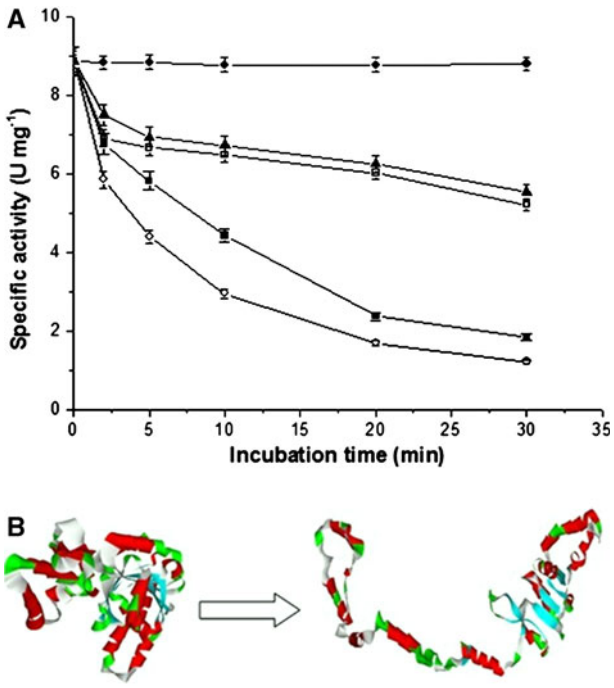

Fig. 1 Renaturation of rDKR from different temperatures and modeled and simulated structures of rDKR. **a** Renaturation of rDKR from different temperatures rDKR samples were incubated (denaturing) at 25, 60, 70, 80 and 90°C for different periods of time, and then promptly cooled at 25°C (renaturing). The data are averages of three independent experiments, *solid circles* 25°C, *open circles* 60°C, *solid squares* 70°C; *open squares* 80°C, *solid triangles* 90°C. **b** Modeled and simulated structures of rDKR, *left* molecular model of rDKR, *right* simulated structure of rDKR at 90°C, α-helix *red* cylinder, β-sheet *light blue arrow*, turn *green line*, random coil *white line*

According to Greenfield and Fasman (1969), the secondary structural elements of rDKR were calculated as shown in Supplementary Table 2. The native state of rDKR was α-helical dominant (45.7%). The α-helical content of rDKR gradually reduced during the process of temperature increase. However, after heating at 60, 70, 80 and 90°C, the α-helical elements decreased to 27.3, 22.9, 20.3 and 19.0%, respectively, and random coil fractions increased correspondingly. On the other hand, after heat treatment and then being promptly cooled at 25°C, the α-helical elements were progressively regained. The changes of all secondary structural elements during heating at 90°C were mostly recovered, even attaining values close to those of rDKR's native state.

To examine the possibility of protein aggregation during the heating, UV spectra of rDKR at different temperatures were scanned, and they showed aggregation characteristics with dramatic increase of absorbance at 280 nm when scanned at 60°C, the highest temperature of the equipment allowed (Supplementary Fig. 5). Even though we were unable to observe the aggregation states at higher temperatures, the aggregation behavior of rDKR at intermediate temperatures, such as 60°C suggested that its relatively lower renaturation rates at this temperature range are possibly caused by partial aggregation.

Although the less renaturation at intermediate temperatures could be explained by partial aggregation, more complete renaturation at higher temperatures was not adequately addressed. Therefore, to find out additional factors influencing the renaturation profile, we took homolog modeling approach to build a tertiary structure for rDKR based on the crystal structure of a homologous apoprotein, 3-hydroxyacyl CoA dehydrogenase (PDB code: 1f14, 52% amino acid homology), and utilized molecular dynamics to simulate structural change at 90°C (Qin and Marttila 1999; Schroder et al., 2005; Ylianttila and Pursiainen 2006). As a result, rDKR consists of two domains with a short linker in its native state. The C-terminal domain is smaller and folds into a globular structure full of α-helix, and the N-terminal domain contains a 5-stranded β-sheet surrounded by α-helices (Fig. 1b, left). After unfolding, the protein at 90°C, the results of molecular dynamics showed that these two compact domains became loose, and some secondary structures were lost (Fig. 1b, right). Thus, we suggest that, at molecular level, thermal-induced unfolding of rDKR probably results from tertiary structural collapse by the loss of α-helical elements. In addition, the faster folding of the C-terminal domain as

an α-protein (Gianni and Guydosh 2003) and the short linker between the two domains may have facilitated the folding independently to prevent inter-domain misfoldings (Batey et al. 2005; Robertsson et al. 2005).

The mechanism of protein folding has not been completely understood, despite many studies devoted to this subject (Creighton 1992). Combined the results of UV spectra and molecular simulation in the present study, the thermal-induced unfolding–refolding could be explained by a collective contribution of partial aggregation and structural change. However, the reasons on the difference between higher (90°C) and intermediate (60°C) temperatures still remain as a topic of further investigation. Thermal stability of proteins is directly connected to their folding pathways, in which the mechanism of thermal-induced protein unfolding–refolding has been relatively less studied due mainly to the difficulty of capturing intermediate states when compared with chemical treatments (Dobson 2003; Fersht and Dagget 2002). Different from previous studies focused on globular proteins, the present study may provide a new model to investigate temperature related protein folding processes, which will result in new insights into the mechanism of protein thermostability.

Conclusion

In this report, apoprotein of DKR was able to refold after thermal-induced unfolding. During the process of its self-renaturation, the degree of refolding from denaturing at higher temperature was more complete than that at lower temperature, which was different from any previous observations on protein folding. UV spectra at different temperatures and molecular dynamics simulation indicated that partial aggregation and structural change may play collective roles in such unusual renaturation profile. The present study may offer a new model for the study of novel pathways in protein folding.

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