Chaperone-like effects of a scFv antibody on the folding of human muscle creatine kinase

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Molecular chaperones play an essential role in assisting the folding of a myriad of nascent peptides to form different biologically active proteins. Therefore, their low substrate specificity is important for the functions of these housekeeping proteins. However, discovering chaperones which assist the folding of a particular protein can shed new light on the folding pathway of the protein, offering an interesting approach for developing specific therapeutic agents to treat protein-misfolding diseases. Screening of antibodies with chaperone-like function represents a novel strategy to meet the challenges. In this study, some single-chain variable fragment (scFv) antibodies were selected from a high-capacity phage antibody library using human muscle creatine kinase (HCK) as antigen. A scFv antibody (scFv A4) was determined to inhibit aggregation and favor recovery of the native conformation of HCK during its refolding. This antibody also increased the stability of HCK during its heat-induced unfolding process. Our findings demonstrate that scFv A4 has dual-chaperone-like activities: assisting in correct protein folding as well as protecting the native protein from unfolding. A molecular mechanism by which scFv A4 exhibits chaperone-like effects on HCK was proposed. This study demonstrates that phage antibody libraries can lead to chaperone-like proteins, and the specificity of the resulting antibody toward its antigen could provide new molecular details regarding how the chaperone interacts with the protein’s unfolding and folding pathways.

Keywords: antibody library/chaperone-like effects/folding/human muscle creatine kinase/molecular chaperone

Introduction

The folding of polypeptide chains both in vitro and in vivo does not always proceed in a productive manner and may lead to the formation of misfolded conformations or even aggregations lacking their inherent biological activities (Dobson, 2004; Saunders and Bottomley, 2009; Wittung-Stafshede, 2011). A number of human diseases, such as Alzheimer’s, Huntington’s, Parkinson’s and Creutzfeldt-Jakob’s diseases, are associated with the misfolding and aggregation of proteins inside or outside cells (Horwich, 2002; Heller, 2010). Thus, the study of the causes and mechanisms of protein misfolding and aggregation as well as the search for techniques favoring productive protein folding in vivo are important tasks for both biophysics and biomedical research.

Protein folding is often assisted by two types of factors in vivo, foldases and molecular chaperones. Molecular chaperones have been shown to play an essential role in assisting the folding of nascent peptides to form biologically functional proteins by binding to the folding intermediate to avoid kinetic traps, suppressing aggregation of the substrate (Ellis and Hemmingsen, 1989; Ellis, 2006). Cellular molecular chaperones are effective in preventing the misfolding of different disease-causing proteins, reducing the severity of several neurodegenerative disorders and many other protein-misfolding diseases. Therefore, chaperones have been proposed to be useful therapeutics in the treatment of protein-misfolding diseases (Yokota et al., 2000; Cohen and Kelly, 2003; Neckers, 2007).

Chaperones interact with a myriad of nascent peptides to assist the folding of different proteins. Therefore, the promiscuity of their substrate recognition is important for the functions of these housekeeping proteins. On the other hand, discovering chaperones which can assist the folding of a particular protein can shed new light on the folding pathway of the protein and become an interesting approach for developing specific therapeutic agents to treat protein-misfolding diseases. Screening antibodies from phage antibody libraries is a potent method with high efficiency and accuracy. Phage display antibody library technology is also a good platform for the production of human antibodies. This technology is a product of the significant progress made in the area of antibody engineering and has been widely applied in biomedicine (Silacci et al., 2005; Kotlan and Glassy, 2009; Patel et al., 2011). In fact, a monoclonal antibody (mAb) specific for NBD1ΔPhe508 of CFTR (cystic fibrosis transmembrane conductance regulator), selected from a large synthetic human antibody phage display library, was reported to be able to inhibit the aggregation of NBD1 in vitro (Lovato et al., 2007).

Human muscle creatine kinase (HCK, adenosine triphosphate (ATP):creatine N-phosphotransferase, EC 2.7.3.2) is a member of the subclass of guanidino-kinases distributed in human muscle. It catalyzes the reversible phosphoryl transfer from phosphocreatine to adenine dinucleotide phosphate and plays an important role in cellular energy metabolism in human (Watts, 1973; Jacobus, 1985). HCK is an 86 kDa homodimer and is an excellent model for studying protein refolding (Li et al., 2001, 2006).

In this study, single-chain variable fragment (scFv) antibodies were screened from a high-capacity phage antibody library using HCK as the target ligand and their chaperone-like activities were studied. The scFv antibody A4 was observed to inhibit the aggregation of HCK during its refolding. Furthermore, scFv
A4 favored recovery of the native conformation of HCK and increased the stability of HCK during the unfolding process. These findings demonstrate that scFv A4 has chaperone-like activity. This novel chaperone-like antibody performs two functions: assisting in correct protein folding and protecting the native protein from unfolding. A possible molecular mechanism by which scFv A4 exhibits the chaperone-like effect on HCK was also proposed.

Materials and methods

Materials

The gene encoding HCK was cloned into the pET21b expression vector, expressed in *Escherichia coli* BL21(DE3) and purified as previously described (Ritter et al., 1981). The purified HCK was observed to be homogeneous by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). HCK concentration was determined by the Bradford assay using bovine serum albumin (BSA) as a protein standard. The human scFv-phage library (typically 10^{13} cfu) was constructed in the Centre of Clinical Laboratory in Navy Hospital of PLA, Beijing, P.R. China (Du et al., 2010). Guanidine chloride (GdmCl, ultra pure grade) and BSA were obtained from Sigma (St Louis, MO, USA). Creatine and ATP were purchased from Fluka (Milwaukee, WI, USA) and dithiothreitol (DTT) was obtained from Promega (Madison, WI, USA). All other chemicals were local products of analytical grade.

Selection of anti-HCK scFvs from phage display library

The selection of human scFvs specific for HCK was performed using four rounds of biopanning of the human scFv-phage library. Briefly, immunotubes coated with the antigen HCK were incubated with the phage library at 37 °C for 2 h. After washing with 0.05% Tween-phosphate buffered saline (PBS), the adherent phages were eluted using elution buffer (0.1 M HCl, 0.1% BSA, pH adjusted to 2.2 with solid glycine) and neutralized with 2 M Tris-buffer. Eluted phages were used to infect *E. coli* XL1-Blue cells, and the Helper phage VCS-M13 (10^{12} pfu) was subsequently added and incubated at 30 °C overnight. Phage preparation and screening was repeated four times. Individual ampicillin-resistant colonies (phage clones) were selected. Fifty-six positive anti-HCK phage clones were further screened by phage enzyme-linked immunosorbent assay (ELISA) in Microtiter plates (Nunc) coated with purified HCK as antigen. Further characterization by DNA fingerprinting and sequencing revealed that six clones with different scFv antibody sequences could be isolated. The selected clones were highly specific as analyzed by ELISA.

Expression and purification of soluble anti-HCK-scFvs

The six selected phage clones obtained from *E. coli* XL1-blue cells were used to infect the *E. coli* HB2151 non-suppressor bacterial strain to obtain solubile scFvs. After overnight induction with 1 mM isopropyl-β-D-1-thiogalactopyranoside at 30 °C, antibody fragments were harvested from the supernatant and periplasmic space. The harvested protein was precipitated with ammonium sulfate (60% saturation) and further purified on a HisTrap FF column (GE). Purified scFvs were dialyzed overnight against PBS, concentrated and stored at ~70 °C.

Unfolding and refolding of HCK

HCK was denatured at 25 °C for 1 h in 3 M guanidine chloride dissolved in buffer containing 10 mM Tris-HCl and 2 mM DTT (pH 8.0). In refolding studies, the denatured enzyme described above was diluted 30-fold into refolding buffer (10 mM Tris-HCl and 2 mM DTT, pH 8.0) containing different concentrations of scFvs. The final concentration of HCK dimer used for most experiments was 4 μM unless otherwise noted.

Measurements of aggregation and enzyme activity

The aggregation of HCK during refolding was monitored by measuring the turbidity (absorbance at 450 nm) using a GBC UV spectrophotometer. To correct for the absorbance of buffer components (including scFv antibodies), measurements obtained for blank samples were subtracted from those of all experiments.

The aggregation of HCK was also detected by SDS–PAGE analysis. At the endpoint of HCK refolding in the absence or presence of scFv antibodies, samples were centrifuged at 15 000 g for 10 min at 4 °C to separate pellets and supernatants. Pellets were dissolved and diluted to the same final volume as the supernatants by water and gel loading buffer. Twenty micro-liters of each pellet and supernatant sample were then analyzed by SDS–PAGE.

Refolding samples of denatured HCK were assayed for enzyme activity at different time intervals after the refolding process began. Enzyme activity was determined as described by Yao et al., 1982. All enzyme activities were normalized against and expressed as a percentage of a 4 μM solution of native HCK in refolding buffer. Control experiments were performed to ensure that the scFv antibodies used had no influence on the assay. Enzyme activity was measured using a GBC UV spectrophotometer.

Aggregation and enzyme activity were all measured at 25 °C.

Fluorescence measurements

The fluorescence of HCK samples refolding for 2 h was monitored with a Fluoromax-2 fluorescence spectrophotometer. Intrinsic emission fluorescence spectra were recorded using a 1-cm path length cuvette with an excitation wavelength of 295 nm, which specifically excites the tryptophan residues of HCK. The excitation wavelength used for acquiring 8-anilino-1-naphthalene sulfonate (ANS)-binding fluorescence spectra was 380 nm. Each spectrum was corrected by subtracting the corresponding background spectrum of scFv antibodies with the same concentration.

Surface plasmon resonance analysis

The binding kinetics of scFv antibodies to HCK was measured by using surface plasmon resonance (SPR) analysis with BIACore 3000 (Biacore, Uppsala, Sweden) according to the manufacturer’s instructions. HCK was immobilized on a CM5 sensor chip by using an amine coupling kit with a contact time on/off of 4 s. The dissociation constant (K_{off}) and association (K_{on}) were obtained at five different scFv concentrations ranging from 10 to 160 nM. Measurements were carried out at a flow rate of 20 μl/min. The dissociation constant (K_{d}) was calculated from the ration of each rate constant (K_{off}/K_{on}). Sensorgrams were analyzed with the BIA evaluation 2.1 program.
Size-exclusion chromatography
Size-exclusion chromatography was performed on a GE Amersham Pharmacin ÄKTA FPLC system using a Superdex 200 HR 10/30 column. The refolding buffer was used to equilibrate and run the gel filtration column. Each collected fraction was then used for SDS–PAGE.

Thermally induced unfolding of HCK in the presence and absence of scFv antibodies
HCK was incubated with scFv antibodies of various concentrations for 1 h. Protected and unprotected HCK samples were then incubated in a water bath at 56°C. Samples were taken out at different time intervals, cooled on ice and residual enzyme activity was measured as described above. To measure aggregation, mixtures of HCK and antibody were incubated in the U/V spectrophotometer at 56°C and the turbidity (absorbance at 450 nm) of each sample was measured.

Results
Affinity and binding kinetics of scFv antibodies for HCK
In this experiment, six scFv antibodies (scFv A1–A6) with different amino acid sequences against HCK were selected using phage display technology. The amino acid sequences of scFv A1–A6 are provided in Supplementary Results. The six scFv antibodies have different complementarity determining regions (CDRs), while the similarity between CDRs of scFv A4 and A5 are larger than other scFv antibodies. The scFvs tagged with V5(GKPIPNPLLGLDST) and His6 epitopes were expressed in E.coli HB2151 and purified by immobilized metal affinity chromatography. The binding affinity of six scFv antibodies to HCK was examined by ELISA using the anti-V5 antibody and a secondary antibody. The ELISA results (Table I) show that all six scFv antibodies bind with relatively high affinity to HCK, whereas they bound weakly to other proteins (pepsin, BSA and ovalbumin), which were used as controls.

The binding kinetics of six scFv antibodies to HCK was determined by using SPR analysis. The association and dissociation rate constants ($k_{on}$ and $k_{off}$, respectively) were measured and the dissociation equilibrium constant ($K_d$) was calculated as $k_{off}/k_{on}$ (Table II). The results show that among the six scFv antibodies, the $K_d$ value of scFv A1 is the largest and that of scFv A4 is the smallest.

Effects of scFv antibodies on HCK aggregation during refolding
The aggregation of HCK has been reported to occur during refolding when enzyme concentration is high (Li et al., 2006). In this experiment, we studied the aggregation of HCK with high concentrations refolding in the absence or presence of the six scFv antibodies (scFv A1–A6) using turbidity measurements. The results are shown in Fig. 1A. Of the six scFv antibodies, scFv A1, A2, A3 and A6 had no apparent effect on the aggregation of HCK, whereas scFv A4 and A5 effectively prevented the aggregation of HCK during the refolding process. The inhibitory effect of scFv A4 was observed to be stronger than scFv A5.

The concentration-dependent inhibitory effect of scFv A4 on the aggregation of HCK is shown in Fig. 1B. The extent of aggregation decreased with increasing scFv A4 concentrations, indicating that the molar ratio of antibody to HCK influences the aggregation. The aggregation could almost be suppressed as the molar ratio of scFv A4 to HCK monomer reached 1:1.

The aggregation suppression effect of HCK by scFv antibodies was further examined by SDS–PAGE analysis of pellet and supernatant (Fig. 1C and D). In good agreement with the turbidity assay, these results show that among the six scFv antibodies, scFv A4 has yielded substantially increased amount of soluble protein and decreased amount of insoluble protein. Moreover, scFv A4 prevents aggregation of the target protein in a concentration-dependent manner.

Effects of scFv A4 on the conformational changes of HCK during refolding
The intrinsic tryptophan fluorescence of HCK refolded for 1 h in the presence of different concentrations of scFv A4 was measured to detect tertiary structural changes, and the results are shown in Fig. 2A. The fluorescence of HCK significantly changed as the protein unfolded, shifting the emission maximum from 334 to 353 nm. The emission maximum of spontaneously refolded HCK was ~339 nm, indicating partial renaturation of the unfolded HCK. In the presence of increased concentrations of scFv A4, the emission maximum blue-shifted toward that of native HCK. The emission maximum of refolded sample without antibody, indicating that scFv A4 can favor recovery of the native conformation and suppress the formation of misfolded species.

We studied the ANS fluorescence of refolded HCK in the absence and presence of scFv A4. The fluorescence emission of ANS dye increases when it binds to hydrophobic regions of proteins. The result in Fig. 2B shows that the self-refolded HCK in the absence of scFv A4 has a significantly higher level of ANS-binding fluorescence than native HCK. This observation indicates that the folding of HCK in the absence of any chaperone could produce misfolded proteins with large exposed hydrophobic surface. HCK folded in the presence of scFv A4 shows weaker ANS fluorescence compared with the refolded sample without antibody, indicating that scFv A4 could assist in the correct folding of HCK and result in less misfolded protein.

Table I. ELISA results for the binding of six scFv antibodies to HCK and other proteins

<table>
<thead>
<tr>
<th>Antibody name</th>
<th>HCK</th>
<th>Pepsin</th>
<th>BSA</th>
<th>Ovalbumin</th>
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<tbody>
<tr>
<td>scFv A1</td>
<td>0.840 ± 0.032</td>
<td>0.006 ± 0.001</td>
<td>0.015 ± 0.002</td>
<td>0.014 ± 0.002</td>
</tr>
<tr>
<td>scFv A2</td>
<td>0.929 ± 0.045</td>
<td>0.008 ± 0.001</td>
<td>0.019 ± 0.001</td>
<td>0.034 ± 0.002</td>
</tr>
<tr>
<td>scFv A3</td>
<td>1.081 ± 0.036</td>
<td>0.011 ± 0.002</td>
<td>0.018 ± 0.002</td>
<td>0.059 ± 0.003</td>
</tr>
<tr>
<td>scFv A4</td>
<td>1.027 ± 0.041</td>
<td>0.012 ± 0.002</td>
<td>0.019 ± 0.002</td>
<td>0.034 ± 0.002</td>
</tr>
<tr>
<td>scFv A5</td>
<td>1.264 ± 0.042</td>
<td>0.044 ± 0.004</td>
<td>0.022 ± 0.003</td>
<td>0.068 ± 0.004</td>
</tr>
<tr>
<td>scFv A6</td>
<td>1.096 ± 0.045</td>
<td>0.064 ± 0.003</td>
<td>0.032 ± 0.004</td>
<td>0.042 ± 0.003</td>
</tr>
</tbody>
</table>
Interactions between scFv A4 and HCK

We studied the interactions of scFv A4 with HCK using size-exclusion chromatography. After guanidine-denatured HCK was diluted into renaturation buffer containing different concentrations of scFv A4, the mixed solution was subjected to size-exclusion chromatography (Fig. 3A). We also performed size-exclusion chromatogram analysis of native HCK dimer (86 kDa), HCK monomer (43 kDa) refolded in the presence of 20 μM ZnCl₂ (Li et al., 2002) and native scFv A4 (31 kDa) as control (Fig. 3B). By comparing the results with standard size-exclusion chromatography curves of native HCK and scFv A4, we determined that the protein in peak I was native HCK dimer and the protein in peak III was scFv A4. A new component peak II appeared in the presence of scFv A4. Because the molecular weight of this component is less than that of the native HCK dimer and higher than those of HCK monomer and scFv A4, the component is likely composed of one HCK monomer and one scFv A4 molecule. Samples in peaks I, II and III were then analyzed using SDS–PAGE (Fig. 3C). The results of SDS–PAGE proved that peak I contained only HCK and peak III contained only scFv A4. Moreover, the component in peak II can be separated into HCK and scFv A4 by SDS treatment, which indicates that this component is the complex of HCK and scFv A4.

Effects of scFv A4 on reactivation of HCK

When unfolded HCK was diluted 30-fold into refolding buffer containing different concentrations of scFv A4, the reactivation rate of HCK decreased with increasing antibody concentrations dramatically (Fig. 4). The RA₅₀ time (reactivation of
The experiments reported in this paper were designed to screen human scFv antibodies for HCK from a large human scFv-phage library and study their chaperone-like activity. All six selected scFv antibodies (A1–A6) have shown relatively high affinity to folded HCK dimer (Table II), although it is difficult to assess their binding to the folding intermediates. One antibody (scFv A4) effectively prevented the aggregation of HCK during the refolding process. In addition, scFv A4 promoted recovery of the native conformation of HCK. Furthermore, this antibody was observed to increase the thermal stability of HCK and inhibit its aggregation during heat-induced unfolding.

The discovery of chaperones is one of the greatest advances in the field of protein folding. Chaperones are defined as proteins that assist in the non-covalent folding and assembly of other macromolecular structures. One major function of chaperones is to prevent both newly synthesized polypeptide chains and assembled subunits from aggregating into non-functional structures. Thus, many chaperones are also heat shock proteins because the tendency to aggregate increases as proteins are denatured by stress. Jakob and Buchner (1994) suggested that the following four key properties can be used to characterize a protein as a molecular chaperone: (i) suppression of aggregation during protein folding; (ii) suppression of aggregation during protein unfolding; (iii) influence on the yield and kinetics of folding; and (iv) effects exerted at near stoichiometric levels. The effects of the scFv A4 on HCK during its refolding from the guanidine-denatured state and thermal unfolding from the native state observed in this study appear to satisfy all of these four criteria. The properties of scFv A4 in preventing the aggregation and promoting the refolding of unfolded HCK upon dilution are similar to those of well-studied molecular chaperones, such as Hsp90 and Hsp60. The function to inhibit aggregation and assist in folding of denatured HCK in vitro reflects the general chaperone-like activity of scFv A4. By analyzing the time course of aggregation and reactivation for HCK during refolding in the presence of different

50% of HCK activity) in the absence of scFv A4 was ~1 h, whereas in the presence of 1 equivalent or higher concentrations of scFv A4, the RA50 time extended to more than 40 h. Most interestingly, in the absence of antibody, the HCK started to lose activity after 12 h, while the chaperone-like effect of the antibody resulted in slower but higher recovery of HCK’s activity. Furthermore, this experiment has also revealed that a 1:1 ratio of scFv A4 to HCK monomer provides the greatest reactivation of HCK’s activity.

**Effects of scFv A4 on the thermal stability of HCK**

Samples of HCK were incubated with different concentrations of scFv A4 for 1 h. The mixtures were then incubated at 56°C for various times up to 40 min, and the samples were subsequently chilled on ice. After the last sample was withdrawn, all samples were assayed for enzymatic activity. The results in Fig. 5A show that untreated HCK was readily inactivated, losing almost all activity after 25 min of incubation at 56°C. In contrast, antibody-treated enzyme was relatively stable. The time at which the residual activity of HCK is 50% of native CK (T1/2) increased from 6.5 to 16.5 min as the molar ratio of scFv A4 to HCK monomer increased from zero to 1:1. At a molar ratio of 1:1, the HCK sample still retained ~7% of its original activity after heating at 56°C for 40 min.

HCK is prone to temperature-dependent aggregation during heat-induced unfolding (Tong et al., 2000). We measured the turbidity of HCK-antibody mixtures at 56°C. Figure 5B shows that the presence of scFv A4 could suppress the aggregation of HCK during thermal unfolding. This aggregation-inhibitory effect of scFv A4 is dependent on its concentration.

**Discussion**

The experiments reported in this paper were designed to screen human scFv antibodies for HCK from a large human...
Fig. 3. Analysis of binding between scFv A4 and HCK. (A) Size-exclusion chromatogram of HCK refolded in renaturation buffer containing different concentrations of scFv A4. The HCK dimer concentration was 4 μM. The scFv A4 concentrations used were 0, 4, 8 and 12 μM for curves 1–4, respectively. (B) Size-exclusion chromatogram of native HCK dimer (curve 5), HCK monomer refolded in the presence of 20 μM ZnCl2 (curve 6) and native scFv A4 (curve 7). (C) SDS-PAGE analysis of samples in each peak of curves 1–4. Samples were molecular weight marker, native HCK, samples in peak I of curve 1, samples in peak I of curve 2, samples in peak II of curve 2, samples in peak II of curve 3, samples in peak III of curve 4 and native scFv A4 from left to right (lanes 1–8, respectively).

Fig. 4. Effects of different concentrations of scFv A4 on reactivation of HCK during folding. The refolding conditions used were the same as those listed in Fig. 1. The HCK dimer concentration was 4 μM. The following scFv A4 concentrations were used: 0 (filled circle), 2 (empty triangle), 4 (filled inverted triangle), 8 (filled rhombus) and 16 (empty square) μM.
concentrations of scFv A4, we observed that one scFv antibody could potentially bind to one HCK subunit. We further examined the binding profiles of scFv A4 with HCK by size-exclusion chromatography and SDS–PAGE, and the results indicate that one molecule of scFv A4 could capture one HCK monomer during its refolding process. The stoichiometry of scFv A4’s chaperone-like action is very similar to that of the known chaperones (Buchner et al., 1998).

For the chaperone-like effect of scFv A4 on the refolding of HCK, we propose a possible mechanism combining all the results we obtained in this study. The model for refolding of HCK is shown in Fig. 6, where $U$ is an unfolded HCK subunit, $I$ is a monomeric folding intermediate, $N'_0$ is a partly refolded HCK monomer which has a conformation similar to native HCK but has no activity and $N_2$ is an active HCK dimer. It can be seen that the total refolding process of HCK can be divided into stages and several intermediates are present in this process. Misfolding and aggregation process competes favorably with the proper folding process. ScFv A4 binds to the monomeric folding intermediate $I$ by recognizing the epitope already exposed on the surface of this intermediate. Binding of scFv A4 steers the folding process from $A4/I$ complex to $A4/N'_0$ complex by lowering the activation barrier through the stabilization of the folding transition state, and prevents aggregation by inhibiting misfolding and binding between the monomeric folding intermediate $I$. However, more experiments are required to confirm this hypothesis.

In this study, scFv A4 was determined to block the recovery of HCK enzyme activity during the early refolding process (within the first 90 min). One possible explanation is that scFv A4 inhibits dimerization of $N'_0$ due to steric hindrance caused by antibody binding, thus brings about the slow attainment of enzymatic activity. A similar phenomenon has been found in studies on the effects of molecular chaperone GroEL and peptidyl prolyl cis/trans isomerase Cyp18 on the refolding of rabbit muscle creatine kinase (Li et al., 2001; Ou et al., 2001).

Fig. 5. Effects of scFv A4 on inactivation and aggregation of HCK during thermal-induced unfolding. (A) Inactivation of HCK in the absence and presence of different concentrations of scFv A4. The HCK dimer concentration was 2 μM. The following scFv A4 concentrations were used: 0 (filled circle), 1 (filled rhombus), 2 (filled triangle), 3 (filled square), 4 (filled inverted triangle) and 8 (plus symbol) μM. (B) Aggregation of HCK in the absence and presence of different concentrations of scFv A4. Turbidity (OD at 450 nm) was measured for 8 min. The HCK dimer concentration was 2 μM. The following scFv A4 concentrations were used: 0 (1), 1 (2), 2 (3), 3 (4), 4 (5) and 8 (6) μM.

Fig. 6. Chaperone-like effects of scFv A4 on folding and unfolding of HCK. U: unfolded HCK subunit; I: monomeric folding intermediate; $N'_0$: refolded HCK monomer which has a conformation similar to native HCK but has no activity; $N_2$: active HCK dimer; O-minus: inhibition; O-plus: stimulation.
The high affinity of scFv A4 to HCK during folding process leads us to speculate that scFv A4 works as a ‘holdase’ rather than a ‘foldase’ (Bohen et al., 1995). ‘Holdases’ like the ATP-independent small Hsps bind stably to refolding target proteins and suppress otherwise lethal aggregation. Chaperones that stably bind target proteins may also serve as holdases for the subsequent action of proteases (Sherman and Goldberg, 1996). Remarkably after long-term incubation (up to 4 days), the HCK bound to scFv A4 could be released slowly. The released HCK monomers do not aggregate and can form active homodimers, leading to the increase of reactivation yield.

The results of our thermal denaturation experiments demonstrate that scFv A4 stabilizes the native HCK and prevents the aggregation of HCK induced by heat, which is an important characteristic of chaperones with holdase activity (Oh et al., 1997; Jakob et al., 1999). Thus, we conclude that scFv A4 could be referred to as a chaperone-like antibody with dual function: assisting in correct protein folding as well as protecting the native protein from unfolding. The chaperone-like effects of scFv A4 on folding and unfolding of HCK were summarized and shown in Fig. 6.

The effects of antibodies on folding of their antigens have been subject of a number of investigations. Blond-Elguindi and Goldberg (1990) studied the kinetics of regaining immunoreactivity toward five mAbs during the folding of the guanidine-unfolded β2 subunit of tryptophan synthase. Similar methods were also used to study the folding of horse cytochrome c (Allen et al., 1994), lysozyme (Jarrett et al., 2002), β-galactosidase (Hamlin and Zabin, 1972), hemagglutinin (Braakman et al., 1991) and tailspike protein of phage P22 (Friguet et al., 1994). In these studies, antibodies were mainly used as tools to study conformation transitions during the folding of proteins. The influence of antibodies on the folding process of target proteins was also studied. Morris et al. (1987) studied the effect of two mAbs on chick breast muscle creatine kinase refolding and found that one of the antibodies (CK-2A7) inhibited the reactivation of CK. Grigorieva et al. (1999) observed that the addition of two mAbs blocked the spontaneous renaturation of d-glyceraldehyde-3-phosphate dehydrogenase. In contrast, Xu et al. (2004) found that three mAbs significantly increased the yield of reactivation and eliminated the formation of aggregates of luciferase. Similar results were obtained when studying the effect of antibodies on refolding of reduced BSA (Chavez and Benjamin, 1978), the ribonuclease A fragment (Carlson and Yarmush, 1992) and carboxypeptidase (Solomon and Schwartz, 1995; Katzav-Gozansky et al., 1996). The chaperone-like effect of antibodies was discussed by Ernolenko et al. (2004) in their review published in 2004. Despite various data on the ability of antibodies to influence folding and inhibit polypeptide aggregation, the phenomenon of the chaperone-like activity of antibodies has not been thoroughly investigated. Moreover, based on the limited data in the literature, there seems to be no universal mechanism by which antibodies have effects on folding, but rather the mechanism is dependent on the properties of the epitope and folding pathway of each particular protein. The effect of different antibodies on the refolding of their target proteins is a topic in need of further exploration. In our study, phage display technology was used to discover one scFv antibody to HCK, which exhibited similar chaperone-like activity as the mAbs reported in the literature. It is the first time that a scFv antibody with dual-chaperone-like function was found. The novel method that we use to screen antibodies with chaperone-like function will provide insight on finding new chaperone-like proteins with high specificity for target proteins.

**Conclusions**

In this study, we performed selection of scFv antibodies from a high-capacity phage antibody library using HCK as a target ligand. The scFv A4 was determined to inhibit aggregation and favor recovery of native conformation of HCK during its refolding. This antibody also increased the stability of HCK during its heat-induced unfolding process.

In the literature, antibodies have been used to study protein folding and some antibodies were found to show chaperone-like effects. However, full-length mAbs were mainly used in these studies, and the mechanism of the chaperone-like activity resulted from specific antibody–antigen interaction has not been thoroughly investigated. In this study, it is the first time that a scFv antibody with dual-chaperone-like function was found. A molecular mechanism by which this antibody exhibits chaperone-like effects was also proposed. Our study demonstrates that phage antibody libraries can lead to the discovery of chaperone-like antibodies, and the specificity of the resulting antibody toward its antigen could provide new molecular details regarding how chaperones interact with the protein’s unfolding and unfolding pathways. Most importantly, these results provide guidance for our future research to discover potent antibody-based specific proteins with chaperone-like activities. In the future, epitope mapping of scFv will be carried out, to understand relationship between the binding specificity of the antibodies and their chaperone-like function. The presence of scFv A4 led to very slow but eventually greater recovery of HCK’s activity. Therefore, for designing future selection experiments, we will focus not only on specific binding, but also on a faster $k_{off}$ rate, which, interestingly, is contrary to the concept in designing therapeutic antibodies as neutralization agents or the antibody-dependent cell-mediated cytotoxicity.

**Supplementary data**

Supplementary data are available at PEDS online.

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