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Prolyl isomerases catalyze antibody folding in vitro

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Abstract

Some slow-folding phases in the in vitro refolding of proteins originate from the isomerization of prolyl-peptide bonds, which can be accelerated by a class of enzymes called prolyl isomerases (PPIs). We used the in vitro folding of an antibody Fab fragment as a model system to study the effect of PPI on a folding reaction that is only partially reversible. We show here that members of both subclasses of PPIs, cyclophilin and FK 506 binding protein (FKBP), accelerate the refolding process and increase the yield of correctly folded molecules. An acceleration of folding was not observed in the presence of the specific inhibitor cyclosporin A, but still the yield of correctly folded molecules was increased. Bovine serum albumin (BSA) increased the yield comparable to cyclophilin but, in contrast, did not influence the rate of reactivation. These effects were observed only when cyclophilin or BSA were present during the first few seconds of refolding. However, the rate-limiting reactivation reaction is still accelerated when PPI is added several minutes after starting refolding. In contrast, the prokaryotic chaperone GroEL influences the refolding yield when added several minutes after initiating refolding. The results show that PPIs influence the folding of Fab in two different ways. (1) They act as true catalysts of protein folding by accelerating the rate-limiting isomerization of Xaa–Pro peptide bonds. Proline isomerization is obviously a late folding step and has no influence on the formation of aggregates within the first seconds of the refolding reaction. (2) PPI and BSA are able to increase the yield of refolding of Fab by reducing the formation of aggregates or the adsorption to the surface of the reaction vessel in an unspecific manner. This behavior is clearly distinct from the mechanism of action observed with the chaperone GroEL.

Keywords: antibodies; BSA; chaperone; cyclophilin; Fab fragment; folding catalyst; GroE; protein folding; peptidyl-prolyl isomerase

Peptidyl-prolyl-cis-trans-isomerases are conserved and abundant proteins located in the cytosol and organelles of eukaryotic cells and in the cytosol and periplasm of bacteria. They were first described as PPIs by Fischer et al. (1984) and Handschumacher et al. (1984). There are two unrelated subclasses of PPIs: cyclophilins, which are specifically inhibited by the immunosuppressive cyclic peptide cyclosporin A, and FK 506 binding proteins, which are inhibited by the immunosuppressive drug FK 506 (cf. Walsh et al., 1992; Schmid et al., 1993; Stein, 1993). The PPIs of both subclasses have been demonstrated to catalyze the isomerization of Xaa–Pro peptide bonds both in small peptides and during the in vitro refolding and unfolding of proteins (cf. Mücke & Schmid, 1992; Walsh et al., 1992; Schmid et al., 1993). Thereby, both PPI subclasses accelerate slow folding phases of proteins that are determined by the isomerization of prolyl-peptide bonds (Lang et al., 1987; Lin et al., 1988; Kiefhaber et al., 1990; Jackson & Fersht, 1991; Mücke & Schmid, 1992). Catalysis of refolding by PPI was first observed with the light chain of the murine monoclonal antibody MAK 33 (Lang et al., 1987). The folding of antibody domains comprises fast- and slow-folding phases. The slow phases were attributed to the isomerization of proline residues (Goto & Hamaguchi, 1982a,b) and could indeed be accelerated by PPI, whereas the fast-folding phase remained unaffected (Lang et al., 1987).

Refolding reactions of proteins used so far in model studies on PPI function were completely reversible pro-

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Abbreviations: BSA, bovine serum albumin; CK-MM, dimeric human muscle-specific creatine kinase; CsA, cyclosporin A; ELISA, enzyme-linked immunosorbent assay; Fab, proteolytically derived antibody fragment consisting of the entire light chain and the two N-terminal domains of the heavy chain linked by an interchain disulfide bridge; FKBP, FK 506 binding protein; GdmCl, guanidinium chloride; MAK 33, murine antibody of subclass κ/IgG1 directed against muscle-specific human creatine kinase; pNA, *p*-nitroanilide; PPI, peptidyl-prolyl cis/trans isomerase.

cesses (Lang et al., 1987; Kiefhaber et al., 1990; Jackson & Fersht, 1991; Mücke & Schmid, 1992) with the exception of carbonic anhydrase (Freskgard et al., 1992). In this case, cyclophilin has been proposed to act as a chaperone suppressing the aggregation of the refolding protein.

By accelerating rate-determining Xaa-Pro isomerization reactions during protein folding, PPIs may in principle be able to decrease the life-times of nonnative folding intermediates, which are susceptible to aggregation. Therefore, catalysis of Xaa-Pro peptide bond isomerization may limit wrong intra- and intermolecular interactions and, consequently, increase the number of molecules that reach the native state.

In order to elucidate the effects of PPI on a system in which unproductive side reactions compete with correct folding, we used the refolding of the MAK 33 Fab fragment as a model reaction. As mentioned before, the light chain of this antibody, which contains two cis-prolines (at amino acid positions 8 and 141), had been used earlier to establish the catalytic function of PPI in protein folding (Lang et al., 1987). The Fab fragment of the murine monoclonal antibody MAK 33 consists of the entire light chain and the two N-terminal domains of the heavy chain (Fd). The Fd-chain contains two cis-prolines at amino acid positions 157 and 159, respectively, as deduced from comparison with X-ray structures of a homologous Fab fragment (He et al., 1992). The two polypeptide chains are linked by a disulfide bridge located at the respective C-terminal ends.[1]

In contrast to the unfolding of the isolated light chain, unfolding of the entire Fab molecule is not completely reversible. Under our conditions, about 30% of the unfolded molecules reach the native state (Schmidt & Buchner, 1992). Here we address the questions whether and how PPI affects the refolding of MAK 33 Fab. We show that PPI accelerated the folding of the denatured oxidized Fab fragment in a concentration-dependent way. Interestingly, PPI also increased the yield of functionally refolded molecules. However, this potential chaperone effect of PPI could also be observed in the presence of the inhibitor CsA. Furthermore, BSA showed a comparable influence on the yield of Fab refolding.

Results

PPIs accelerate Fab folding

Incubation of MAK 33 Fab in 6 M GdmCl leads to the complete loss of the ordered three-dimensional structure as monitored by fluorescence and CD spectroscopy (data not shown). However, the disulfide bridges remain intact, and therefore the two polypeptide chains of the Fab fragment are still linked covalently.[1] Renaturation of these molecules comprises domain-folding and domain-pairing reactions, which compete with nonproductive aggregation. The slow-folding phases of oxidized antibody domains are limited in rate by Xaa-Pro isomerization reactions (Goto & Hamaguchi, 1982a,b) and are effectively catalyzed by cyclophilin (Lang et al., 1987). To investigate the catalytic effect of PPIs on the folding of the more complex MAK 33 Fab fragment, which contains four cis-prolines (He et al., 1992), we performed refolding experiments in the presence of increasing amounts of cyclophilin and FKBP. Both the uncatalyzed and the catalyzed refolding reactions exhibited apparent first-order kinetics as monitored by the regain of activity. As shown in Table 1, both PPIs increased the overall rate constant severalfold when present in excess over the antibody fragment (cf. also Fig. 1 and see below). In all experiments, FKBP was slightly less efficient than cyclophilin in catalyzing the recovery of antigen-binding capacity.

To analyze whether we achieved maximum acceleration of proline isomerization in the presence of excess PPI, we performed "double-jump experiments." This means that refolding was initiated after a 20-s pulse of denaturation at 10 °C. This pulse is sufficient for complete denaturation as indicated by fluorescence and CD spectroscopy (data not shown), but the proline residues largely maintain their native configuration (Lang et al., 1987). As a consequence, the observed refolding kinetics should not be influenced by proline isomerization. The kinetic analysis of the refolding of this denatured species gave rate constants identical with those observed for refolding in

Table 1. *Rate acceleration of Fab folding by PPI*[a]

	k_{app} (min^{-1})		
	Without cyclophilin	With cyclophilin	With FKBP
Long-term denaturation	0.032 ± 0.003		
Short-term denaturation	0.120 ± 0.010		
Ratio PPI/Fab			
1		0.050 ± 0.003	0.040 ± 0.003
5		0.100 ± 0.005	0.053 ± 0.002
10		0.110 ± 0.005	0.063 ± 0.005
20		0.110 ± 0.010	0.069 ± 0.007
30		0.110 ± 0.008	0.064 ± 0.003
30 + 20 μM CsA		0.032 ± 0.001	–

[a] Refolding of denatured Fab was carried out as described in the legend to Figure 1. The apparent rate constants were determined by fitting the kinetic data obtained by ELISA to a first-order reaction. Long-term denaturation was carried out by incubating the Fab sample for >2 h in GdmCl buffer as described in the Materials and methods. Short-term denaturation was achieved by incubation of the Fab sample for 20 s at 10 °C in GdmCl. In the Materials and methods section short-term denaturation and subsequent refolding is referred to as double jump.

[1] Because proteolytic digestion of the antibody by papain is performed in the presence of cysteine (Johnstone & Thorpe, 1987), a number of molecules will be present as noncovalently linked dimers. The preparations used for the experiments described here contained about 15% of this species.

the presence of excess cyclophilin after long-term denaturation (Table 1). Furthermore, the kinetics of refolding of short-term denatured Fab were not influenced by the presence of PPI (data not shown). Therefore, in the presence of excess PPI proline isomerizations do not contribute to the rate-limiting events of refolding.

To characterize the influence of both PPIs on the folding of the Fab fragment further, renaturation in the presence and in the absence of PPI was performed in the temperature range 10–25 °C. At all temperatures, cyclophilin accelerated the folding reaction (Table 2). Similar effects were obtained with FKBP (data not shown). An Arrhenius plot of the measured rate constants yielded an apparent activation energy of about 67 kJ/mol for spontaneous refolding and 45.6 kJ/mol for refolding in the presence of cyclophilin. Because appearance of the antigen-binding properties of the antibody fragment was monitored in these experiments, only apparent rate constants could be determined. These reaction rates are most likely composed of different individual rate-determining events and therefore, the values obtained do not allow discrimination between activation energies for different folding steps.

PPI influences the yield of Fab folding

If the competition between correct and nonproductive folding is influenced by the rate of proline isomerization, it should be possible to increase the fraction of correctly folded molecules by adding PPI during renaturation. As shown in Figure 1, both cyclophilin and FKBP accelerated the folding reaction of oxidized MAK 33 Fab and increased the yield of functional molecules from 30 to 40%. To test whether the increase in yield was caused by the catalysis of Xaa-Pro peptide bond isomerization by PPI, the Fab fragment was refolded in the presence of cyclophilin and its competitive inhibitor CsA. CsA alone had no effect on Fab reactivation under the experimental conditions used (Fig. 1B). In the presence of both CsA and cyclophilin, cyclophilin did still affect the yield of functionally renatured molecules, but folding was no longer accelerated. This demonstrates that the active site of PPI is not required for the increase in functional reconstitution. Recently, it has been proposed that cyclophilin may act as a molecular chaperone in the refolding of carbonic anhydrase (Freskgard et al., 1992). Molecular chaperones seem to influence folding by binding nonnative protein, thus preventing unproductive side reactions (Gething & Sambrook, 1992; Jaenicke & Buchner, 1993). However, for several proteins, the presence of "protective" proteins, such as serum albumin during refolding, also resulted in an increased reactivation yield (Jaenicke & Rudolph, 1989; Buchner et al., 1991; Zhi et al., 1992). In order to discriminate between effects of "binding proteins" such as BSA, and specific chaperone properties, we examined the influence of cyclophilin on the yield of reactivation in further detail. We performed refolding experiments, in which cyclophilin was added in the absence or in the presence of the specific inhibitor CsA either immediately before or at different times after initiation of refolding. As controls for unspecific or chaperone effects, we added the same molar amounts of BSA or lysozyme on

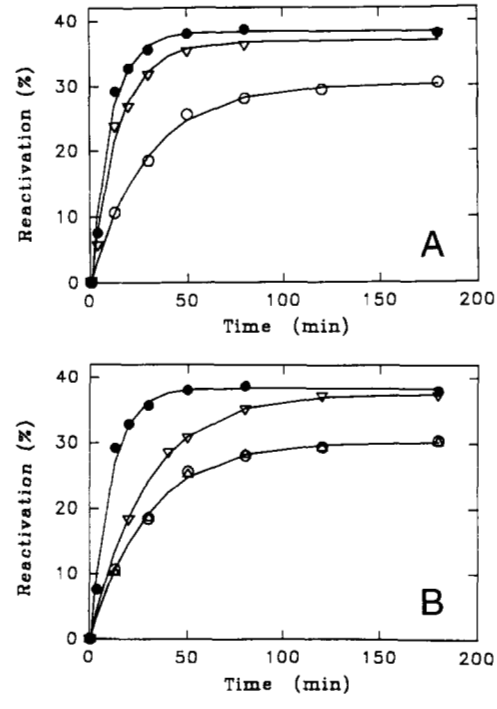

Fig. 1. Influence of PPIs on Fab refolding. Denatured Fab was refolded in 0.1 M Tris-HCl, 2 mM EDTA, pH 8, at 10 °C and a final concentration of 0.2 μM in the presence of PPIs. At times indicated, samples were taken and regain of activity was monitored by ELISA. **A:** Renaturation in the absence of PPIs (○), in the presence of 6 μM cyclophilin (●), and in the presence of 6 μM FKBP (▽). **B:** Renaturation of Fab in the absence of PPI (○), in the presence of 6 μM cyclophilin (●), in the presence of 20 μM CsA (△), and in the presence of 6 μM cyclophilin and 20 μM CsA preincubated for 30 min at 10 °C (▽).

Table 2. *Temperature dependence of Fab folding*[a]

	k_{app} (min^{-1})	
T (°C)	Spontaneous	Catalyzed
9.3	0.035	0.110
11.5	0.043	0.130
12.5	0.070	0.200
20.0	0.099	0.219
24.9	0.149	0.296

[a] Denatured Fab was refolded in 0.1 M Tris-HCl, 2 mM EDTA, pH 8, at temperatures varying between 10 and 25 °C in the absence of PPI or in the presence of 6 μM cyclophilin. The final concentration of Fab was 0.2 μM. The kinetics of reactivation were determined by ELISA and fitted according to a first-order reaction.

the one hand, or stoichiometric amounts of GroEL, a well-characterized prokaryotic chaperone (cf. Jaenicke & Buchner, 1993), on the other hand to the refolding Fab fragment. The influence of these proteins on the rate and yield of reactivation is shown in Figure 2. Whereas lysozyme had no effect on yield and rate, refolding in the presence of BSA resulted in an increase in yield similar to that obtained with PPI (Fig. 2A). With both proteins the maximum effect on the yield of refolding was observed at a 50-fold molar excess over the refolding Fab fragment (data not shown). The influence of BSA on the yield decreased rapidly when BSA was added several seconds after the initiation of refolding. As expected, BSA had no effect on the overall rate constant of reactivation (Fig. 2B). Similar to BSA, the ability to influence the yield dropped significantly when cyclophilin was added several seconds after initiating refolding (Fig. 2A). The accelerating effect of cyclophilin on the kinetics remained the same, regardless of the time of addition (Fig. 2B). The effect on the yield of Fab refolding was also observed in the presence of the inhibitor CsA. Furthermore, renaturation in the presence of cyclophilin after short time denaturation also resulted in an increase in yield but not in an acceleration of the folding reaction (data not shown). Thus, catalysis of proline isomerization seemed not to be involved in the increase in yield.

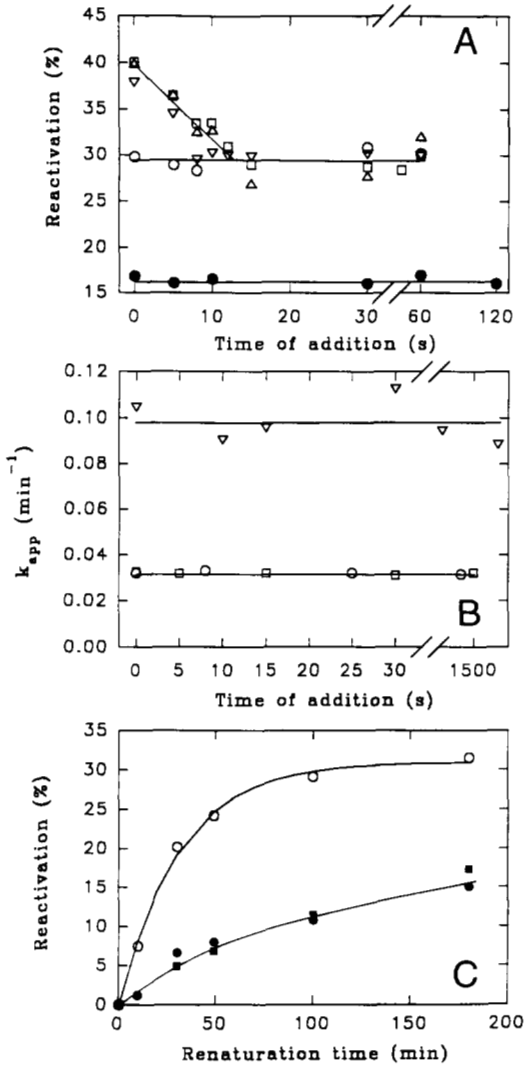

Fig. 2. Influence of various proteins on Fab folding. MAK 33 Fab was denatured and refolded as described in Figure 1. At the times indicated, lysozyme (○), BSA (□), cyclophilin (▽), or cyclophilin preincubated for 20 min with 20 μM CsA (△) were added to the refolding antibody fragment at a final concentration of 6 μM. GroEL (●) was added at a final concentration of 0.2 μM based on the 14-mer complex. Reactivation was assayed by ELISA. **A:** Influence of effector proteins on the yield of reactivation. The yield of functionally reactivated molecules was determined after 190 min of refolding. **B:** Influence of effector proteins on the overall rate constant of reactivation. **C:** Influence of GroEL on Fab refolding. Renaturation was performed in the absence of GroEL (○), in the presence of GroEL (●), and with GroEL added 2 min after initiating refolding (■).

Previously, we have shown that the GroE chaperone system interacts with the MAK 33 Fab fragment during refolding (Schmidt & Buchner, 1992). Binding of nonnative Fab to GroEL in the absence of GroES and ATP results in an initial inhibition of refolding. However, the complex formed dissociates even in the absence of GroES and ATP, resulting in a decelerated reactivation kinetic and thus in an apparent decrease in the yield during the initial phase of refolding (Schmidt & Buchner, 1992). Here, we used GroEL to examine the ability of this chaperone to interact with the refolding protein if added after initiating reactivation. As shown in Figure 2A and C, GroEL did influence the refolding kinetics resulting in decreased yields of refolding (measured after 190 min) even when added several minutes after initiating refolding. Thus, there is a clear difference between the effect of this chaperone and cyclophilin in their ability to bind the nonnative Fab fragment.

Discussion

Antibody Fab fragments are complex molecules that consist of two different polypeptide chains linked via a disulfide bond. Each polypeptide consists of two domains. Only part of these molecules regain their native conformation upon in vitro refolding (Schmidt & Buchner, 1992). Here we show that the overall folding process of an antibody Fab fragment can be accelerated up to threefold by cyclophilin and more than twofold by FKBP. Because there is a significant difference in the ability of cyclophilin and FKBP to catalyze the isomerization of Xaa-Pro peptide bonds in oligopeptides (Harrison & Stein, 1990), there may also be a specificity in the isomerization of Xaa-Pro peptide bonds in proteins. Previously it had been demonstrated that folding of the isolated light chain of MAK 33 can be accelerated about sevenfold by cyclophilin (Lang et al., 1987). Both the spontaneous and the catalyzed folding kinetics of the isolated light chain are

about 10 times faster than the folding kinetics of the Fab fragment described here. The rate-limiting step in the reactivation of the Fab fragment can be assumed to be complex with contributions of prolyl isomerization. The refolding of the Fab fragment in the presence of PPI is still a slow reaction compared to the refolding of the respective light chain. Because the rate constants obtained from double-jump experiments are the same as for the PPI-catalyzed reaction, additional slow steps in the refolding of Fab-like domain pairing of the two constituent polypeptide chains may contribute to the rate-limiting reaction.

Catalysis of proline cis/trans isomerization is achieved by lowering the activation energy of the reaction. Activation energies for protein-folding reactions in which proline isomerizations are rate limiting were previously found to be 67–79 kJ/mol (Brandts et al., 1975; Schmid & Baldwin, 1978; Jackson & Fersht, 1991). In this study, the apparent activation energy for the reactivation of the MAK 33 Fab fragment was determined to be 67 kJ/mol for the uncatalyzed and 45.6 kJ/mol for the cyclophilin-catalyzed reaction. However, because several processes probably contribute to the rate-limiting step, it is not possible to attribute this change solely to prolyl isomerization.

Renaturation experiments after short time denaturation, which were not limited by the isomerization of incorrect Xaa–Pro peptide bonds in the presence and absence of cyclophilin, gave the same rate constants as observed for the cyclophilin-catalyzed folding reaction after long-term denaturation. However, still about half the molecules were not functionally refolded. These results, together with the fact that catalysis by PPI reached a plateau, led to the conclusion that proline isomerization does not seem to be the only critical step in the overall structure formation process. Because each antibody domain has a large interactive surface that allows association with its respective partner domain, correct pairing reactions could be another limiting step in the reactivation process.

The presence of PPI during refolding not only resulted in a several-fold acceleration of the overall folding process, but also in an increased yield of native molecules formed. Previously we have demonstrated that the GroE chaperone system of *Escherichia coli* consisting of GroEL, GroES, and ATP (Schmidt & Buchner, 1992) and eukaryotic Hsp90 (Wiech et al., 1992) influence the refolding of MAK 33 Fab. Both chaperones do not accelerate the folding process. Instead, they bind the nonnative protein, thus lowering the concentration of folding intermediates that are susceptible to aggregation. As a consequence, a larger number of molecules reaches the native structure and assembly.

A chaperone-like effect had also been reported in the case of PPI-catalyzed carbonic anhydrase refolding (Freskgard et al., 1992). In the refolding of Fab, in contrast to carbonic anhydrase, the complex of cyclophilin and CsA was still able to increase the number of native molecules. We performed a series of experiments in which we addressed the question of the specificity of this chaperone effect because it is known that a number of additives, including BSA, can also increase the yield of refolding. In the case of the refolding of citrate synthase it has been shown previously that both the GroE chaperone system and BSA improved reactivation yields significantly (Buchner et al., 1991; Zhi et al., 1992). However, the mechanism by which BSA affects the yield differs from that of chaperone proteins. Whereas chaperones recognize specifically nonnative properties of protein molecules, BSA seems to cover surfaces of vessels to which unfolded proteins may stick. Furthermore, as a transport protein, BSA has hydrophobic pockets that may convey binding properties for hydrophobic substances like tryptophan (He & Carter, 1992). Our experiments confirmed the ability of BSA to affect refolding yields positively; on the other hand, they clearly showed that BSA had an effect only when present in the initial phase of refolding. Similarly, cyclophilin influences the yield when added during the first 10 s of refolding only. Because the effects on the yield and the time range in which cyclophilin is effective in increasing the yield were the same in the presence or absence of CsA, and because the same effect of PPI was observed in double-jump experiments in which the predominant number of prolines was still in the native configuration, we are led to assume that proline isomerization is not responsible for increasing the yield of native molecules. In structural studies two notably hydrophobic clefts on the surface of cyclophilin were identified (Gallion & Ringe, 1992). The CsA binds predominantly to the major cleft so that at least part of the minor cleft maybe available for hydrophobic interaction with other molecules including nonnative proteins.

Under our conditions, even in the presence of excess PPI, not all denatured Fab molecules could be shifted to the productive folding and association pathway. Clearly, the effects on the yield observed in the presence of cyclophilin are different from those in the presence of GroEL, the protein-binding component of the GroE system. The yield of the refolding reaction was markedly influenced by GroEL at stoichiometric concentrations as previously described (Schmidt & Buchner, 1992): the apparent kinetics of reactivation was decelerated and as a consequence the yield of reactivation (measured after 190 min) was decreased even if the chaperone was added several minutes after initiating refolding (Fig. 2A,C). Thus, the chaperone GroEL is able to recognize nonnative Fab for several minutes after initiating refolding. Taken together, the results suggest that the effect of cyclophilin on the refolding of Fab is very similar to that of BSA and clearly different from that of GroEL.

The formation of aggregates during the fast-folding reaction within the first seconds of refolding is not influenced by the configuration of Xaa–Pro peptide bonds. This result is in good correlation with investigations of the

refolding mechanism of complex reversible folding proteins like ribonucleases (Lang & Schmid, 1990; Kiefhaber et al., 1992). In very early and fast reactions during refolding, these proteins form hydrophobic cores and at least part of the native secondary structures even in the presence of incorrect proline isomers. The formation of aggregates or the adsorption to vessel surfaces during refolding of Fab obviously occurs in competition with these early folding events, which are independent of Xaa-Pro peptide bond isomerization. The effect of these processes can be reduced by unspecific interaction with BSA or cyclophilin. In addition to these fast-folding reactions and the slow isomerization of Xaa-Pro peptide bonds, one has to assume that a third folding reaction occurs that cannot be influenced by BSA or PPIs. Because each antibody domain has a large interactive surface that allows pairing with its respective partner domain, correct association reactions could be the third limiting step in the reactivation process.

Materials and methods

Reagents

Human cyclophilin and FK 506 binding protein were produced recombinantly in *E. coli* and purified as described (Liu et al., 1990; Standaert et al., 1990). The isomerase activity of the recombinant proteins was assayed as described using the peptides Suc-Ala-Ala-Pro-Phe-pNA and Suc-Ala-Leu-Pro-Phe-pNA for cyclophilin and FKBP, respectively (Fischer et al., 1984; Lang & Schmid, 1988). The k_{cat}/K_m value was $7.2 \times 10^6\ M^{-1}\ s^{-1}$ for cyclophilin and $2.1 \times 10^6\ M^{-1}\ s^{-1}$ for FKBP. The values obtained are in good agreement with data previously published for the recombinant human proteins (Schönbrunner at al., 1991).

The MAK 33, a murine antibody of subclass κ/IgG1 directed against CK-MM (Buckel et al., 1987), and the respective Fab fragment that was produced by proteolytic digestion (Johnstone & Thorpe, 1987) were obtained from Boehringer Mannheim GmbH. The concentrations of the Fab fragment, cyclophilin, and FKBP were determined spectrophotometrically using the following extinction coefficients at 280 nm: $\epsilon_{Fab} = 80{,}000\ M^{-1}\ cm^{-1}$, $\epsilon_{Cyclophilin} = 8{,}250\ M^{-1}\ cm^{-1}$, and $\epsilon_{FKBP} = 9{,}860\ M^{-1}\ cm^{-1}$ (Standaert et al., 1990).

ELISA reagents including biotinylated CK-MM, fatty acid-free BSA, and the test peptides were obtained from Boehringer Mannheim GmbH.

Unfolding and refolding of the Fab fragment

The 50-kDa Fab fragment (20 μM) was denatured in 6 M GdmCl, 0.1 M Tris-HCl, pH 8, for 2 h at 25 °C. For double-jump experiments (see Results), denaturation was performed for 20 s at 10 °C. Renaturation was initiated by diluting the denatured protein 100-fold into 0.1 M Tris-HCl, pH 8, 2 mM EDTA, under manual stirring at 10 °C in plastic vessels. The concentration of Fab fragment during renaturation was 0.2 μM; the residual GdmCl concentration was 60 mM.

To assure that a stable complex between cyclophilin and CsA is formed, renaturations in the presence of cyclophilin and its inhibitor CsA were performed after preincubating cyclophilin and CsA for 30 min at 10 °C (cf. Kofron et al., 1991).

ELISA

The ELISA assay for MAK 33 was carried out as described previously (Buchner & Rudolph, 1991; Schmidt & Buchner, 1992). In short, CK-MM was attached to tubes using the biotin/streptavidin system. At times indicated, aliquots of refolded Fab fragment were withdrawn and further reactivation was prevented by incubation on ice in the presence of high concentrations of trypsin (400 μg/mL). Native Fab was not degraded by trypsin under the conditions used. Then, the samples were diluted in ELISA buffer containing 30 mM sodium phosphate, supplemented with 0.3 M NaCl. Binding of functionally refolded Fab fragments to CK-MM was detected by using peroxidase-coupled sheep anti-mouse-IgG antiserum. The amount of reactivated protein was quantitated by comparison with standard curves obtained with the authentic Fab fragment.

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