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Short communication

In vitro-refolding of a single-chain Fv fragment in the presence of heteroaromatic thiols

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Abstract

The aim of the present work was to explore the use of heteroaromatic thiol compounds, namely derivatives of pyridine and pyrimidine, as redox reagents for the *in vitro*-refolding of a recombinantly expressed single-chain Fv fragment (scFvOx). The mixed disulfide of scFvOx with glutathione was used as a starting material, while reduced glutathione, 4-mercaptopyridine, 2-mercaptopyrimidine, 2-mercaptopyridine N-oxide, and the mercaptobenzene derivative thiosalicylic acid, respectively, served as catalysts for the formation of native disulfide bonds during renaturation. In contrast to thiosalicylic acid, and despite their significantly lower thiol pK_a values, none of the heteroaromatic thiol compounds accelerated the apparent kinetics of *in vitro*-refolding compared to the naturally occurring peptide glutathione. However, significantly improved renaturation yields were observed in the presence of 4-mercaptopyridine and 2-mercaptopyrimidine, demonstrating the usefulness of aromatic thiol compounds as reagents for the *in vitro*-refolding of antibody fragments.

Keywords: *In vitro*-refolding; Redox buffer; Antibody fragments; Mixed disulfide; Aromatic thiol compounds

For the formation of disulfide bonds during *in vitro* protein refolding, redox buffer systems have to be included in the reaction mixture. The pair of reduced monomeric and oxidized dimeric glutathione (GSH/GSSG) are the most commonly used thiol exchange reagents for disulfide bond formation and isomerization. Since the thiolate anion is the active species in thiol-disulfide exchange, the pH of the refolding buffer has to be kept close to the pK_a of the cysteine thiol in glutathione. For some proteins, this limitation may result in less than optimal refolding. Several low-molecular-weight thiol compounds have been designed with the aim of improving thiol-disulfide exchange during the oxidative refolding of proteins. The synthetic dithiol Vectrase-P, which has pK_a values of 8.3 and 9.9, respectively, and a standard redox potential of −0.24 V, was designed to mimic the active site of protein disulfide isomerases (Woycechowsky et al., 1999). This compound was more efficient in activating disulfide-scrambled RNAse A than monothiols and was found to improve the refolding yield of human proinsulin (Winter et al., 2002). Series of aromatic monothiols, characterized by low thiol pK_a values, have been tested for their effect on rate and yield of the renaturation of scrambled RNAse A (Gough et al., 2002, 2003, 2006; Gough and Lees, 2005). At slightly acidic to slightly basic pH values (pH 6.0–7.7), these aromatic thiols significantly increased the refolding rate compared to glutathione.

Heteroaromatic thiol compounds have so far not been tested as catalysts for disulfide bond formation. The electron-withdrawing effect of more electronegative atoms in *ortho* or *para* should provide additional stabilization of thiolate anions and consequently lower the thiol pK_a value in these compounds. If the pK_a indeed is a decisive factor in determining the effectiveness of thiol compounds as disulfide bond-forming catalysts, heteroaromatic thiols should have highly favorable properties as reagents for protein renaturation.

In the present work, we set out to investigate the effect of heteroaromatic thiols, namely derivatives of pyridine and pyrimidine, on the *in vitro*-refolding of a single-chain antibody fragment, scFvOx (Fiedler and Conrad, 1995). Antibody fragments are among the most technologically relevant targets for *in vitro*-refolding (Holliger and Hudson, 2005). Heterologous expression in bacteria provides yields in excess of 1 g l^{-1} (Chen et al., 2004; Sletta et al., 2004). However, these proteins are frequently obtained in the form of insoluble inclusion bodies,

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which need to be solubilized and refolded *in vitro* (Buchner and Rudolph, 1991; Glockshuber et al., 1992; Martineau and Betton, 1999). Although strategies exist that are potentially able to circumvent this problem (Skerra and Plückthun, 1988; Carter et al., 1992; Frisch et al., 1994; Proba et al., 1998; Martineau et al., 1998; Shaki-Loewenstein et al., 2005), expression into cytoplasmic inclusion bodies followed by *in vitro*-refolding still represents the most generally applicable methodology for the production of antibody fragments, as well as for many other recombinant proteins.

The protocol for the *in vitro*-refolding of scFvOx that had been previously used in our group (Lange et al., 2005) employed GSH/GSSG as redox buffer at pH 8.5. In order to optimize the renaturation conditions, we examined the effect of proton activity on renaturation yield in the range from pH 4.0 to 9.5 (Fig. 1, black bars). A maximum yield of 11% was obtained at pH 7 for the reduced denatured protein, while the yield at pH 8.5 was below 8%. A parallel series of experiments was performed using the mixed disulfide of scFvOx with glutathione as starting material for the renaturation reaction (Fig. 1, gray bars). In these experiments, GSH was used at a total concentration of 0.2 mM to catalyze the formation of disulfide bonds by elimination of glutathione from the protein. This concentration had been found to result in the highest refolding yields in preliminary experiments (not shown). For the mixed disulfide of scFvOx, the useful pH range was apparently shifted to the slightly acidic region, with a maximum renaturation yield of 12% at pH 6. Apart from determining the pH optima of the respective renaturation reactions, these results established the renaturation *via* the mixed disulfide as a satisfactory test system for studying the influence of alternative thiol compounds on the *in vitro*-refolding of scFvOx.

The effects of the monomeric, reduced forms of 4-mercaptopyridine (4MPY), 2-mercaptopyrimidine (2MPYM),

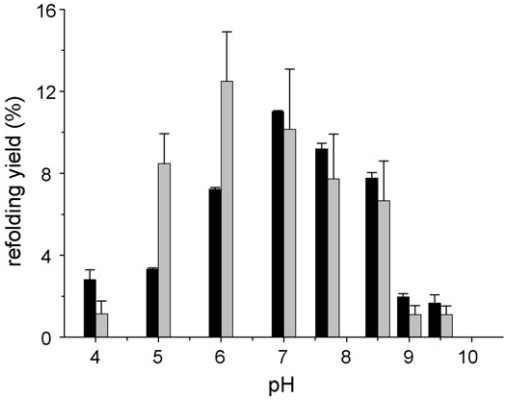
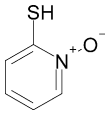
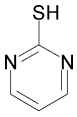
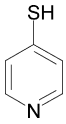
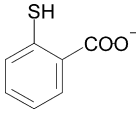


Fig. 1. pH dependence of the *in vitro*-refolding of scFvOx. Renaturation of reduced-denatured scFvOx (black bars) in the presence of 2.5 mM GSH/2.5 mM GSSG, and of the mixed disulfide of scFvOx with glutathione (gray bars) in the presence of 0.2 mM GSH, at the indicated pH values. The renaturation reactions were performed at 15 °C with a protein concentration of 140 μg ml^{-1} in either 0.1 M sodium acetate buffer (pH 4.0–5.0), 0.1 M bis-Tris/HCl (pH 6.0), 0.1 M Tris/HCl (pH 7.0–8.5), or 0.1 M sodium borate buffer (pH 9.0–9.5). All buffers contained 1 M L-arginine hydrochloride and 1 mM EDTA. Renaturation yields were analyzed after 96 h by ELISA (Lange et al., 2005). The mixed disulfide of scFvOx with glutathione was prepared as previously described for lysozyme (Reddy et al., 2005).

Table 1
Aromatic thiol compounds used in this work

Compound[a]	pK_a[b]
2-Mercaptopyridine N-oxide 	−1.95[c] (4.67)[c]
2-Mercaptopyrimidine 	1.31[d]
4-Mercaptopyridine 	1.50[d]
Thiosalicylic acid (2-mercaptobenzoic acid) 	(3.5[e]) 8.3[f]

[a] The aromatic thiol compounds were procured from TCI (Tokio, Japan) and used without further purification. Stock solutions of 10 mM of the respective thiol compounds in 5% methanol were prepared freshly before use.

[b] pK_a values are given for the thiol proton; values in brackets correspond to the pK_a values of other protonable groups.

[c] Jones and Katritzky (1959).

[d] Akrivos et al. (1996).

[e] Calculated using Advanced Chemistry Development Software V8.14 for Solaris (ACD/Labs, Toronto, Canada) in SciFinder Scholar v.2006.

[f] Gough et al. (2006).

and 2-mercaptopyridine N-oxide (2MPYox) (Table 1) on the *in vitro*-refolding of the mixed disulfide of scFvOx with glutathione were tested over the range of pH values from pH 4 to 8.5 (Fig. 2A). Thiosalicylic acid (ThioSA; compound 1 from Gough et al., 2006) was included for comparison. In these experiments, the thiol compounds were employed at a total concentration of 0.2 mM. Therefore, the relative concentrations of the protonated and the active thiolate form, respectively, were not fixed but varied as a function of thiol pK_a and solution pH. The renaturation yields showed a similar pH dependence in all cases, with observed pH optima ranging from pH 6 (ThioSA) to pH 7.7 (4MPY). For three of the four tested compounds, significantly enhanced yields of *in vitro*-refolding in comparison to glutathione were observed at least at one pH value (marked by asterisks in Fig. 2A), while using 2-mercaptopyridine N-oxide resulted in significantly reduced refolding yields above pH 6. The highest renaturation yield (22%) was observed in the presence of 4-mercaptopyridine at pH 7.7, which represents an increase of 80% over the best renaturation yield obtained with glutathione. Thiosalicylic acid significantly and consistently enhanced the yield of *in vitro*-refolding over a broad pH range from pH 5 to 7.7, with a maximum of 20% at pH 6.

We tried to follow the elimination of glutathione from the mixed disulfide of scFvOx over the course of the renaturation reactions. Regrettably, these attempts were unsuccessful, due to