Effects of positively charged redox molecules on disulfide-coupled protein folding

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**ABSTRACT**

In vitro folding of disulfide-containing proteins is generally regulated by redox molecules, such as glutathione. However, the role of the cross-disulfide-linked species formed between the redox molecule and the protein as a folding intermediate in the folding mechanism is poorly understood. In the present study, we investigated the effect of the charge on a redox molecule on disulfide-coupled protein folding. Several types of aliphatic thiol compounds including glutathione were examined for the folding of disulfide-containing-proteins, such as lysozyme and prouroguanylin. The results indicate that the positive charge and its dispersion play a critical role in accelerating disulfide-coupled protein folding.

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1. Introduction

The formation of the native conformation of a protein, which is essential for biological activity, is typically a spontaneous process that occurs under thermodynamic control [1]. To understand the mechanism by which proteins undergo folding, a number of folding experiments were carried out on disulfide-containing proteins, since the disulfide-coupled folding of proteins permits the folding intermediates to be trapped and their conformations determined [2,3]. The folding of disulfide-containing proteins into their native conformation is assisted by redox molecules, such as glutathione and cysteine, in vivo and in vitro [4,5]. In general, proteins form a cross disulfide-linked species with the redox molecule at the first step of the refolding reaction and a thiolate group of a Cys residue of a protein molecule intra-molecularly next attacks the cross-disulfide bond, resulting in the formation of an intra-molecular disulfide bond. In the case of multiple disulfide bonds-containing proteins, mis-bridged disulfide species are also produced and the disulfide-exchange reaction into the native conformation is thought to be the rate-determining step of the refolding reaction [6,7]. The mis-bridged disulfide bonds are frequently located at the molecular surface and are exposed to the reducing molecule, although the native disulfide bonds are buried in the protein molecule [8]. This difference in the chemical reactivity between mis-bridged and native disulfide bonds against the redox molecule leads to the formation of the native conformation of a protein. Thus, the final conformation corresponding to its native tertiary structure is basically determined by the thermodynamic stability of the protein molecule [1]. Therefore, the redox potential of the chemical reagents is thought to be an important factor in the process involved in maintaining the native tertiary structure [9,10]. However, the role of the cross disulfide-linked moiety on the disulfide-exchange reaction has not been examined in detail.

Recently, we proposed a mechanism for accelerated disulfide-coupled protein folding using a positively charged glutathione derivative, Arg-Cys-Gly (RCG) [11,12]. The replacement of a Glu residue with an Arg residue in the glutathione molecule resulted in an improvement in the ability of glutathione to function as a
redox reagent for protein folding and accelerated the formation of the native conformation. The results suggest that the positive charge in close proximity to the Cys residue of the redox molecule effectively accelerates the disulfide-exchange reaction. In this study, we further investigate the charge-dependent acceleration mechanism of disulfide-coupled protein folding, a series of aliphatic thiol reagents was examined for their participation in the folding of prouroguanylin and lysozyme which contain 3 and 4 disulfide bonds, respectively [13,14]. The total charge and molecular size of the thiol reagents were considered in the refolding of these two proteins. The results indicate that a positive charge and a larger solvent accessible surface area for the positive charge of the redox molecule are preferred for accelerating the disulfide-exchange reaction in protein folding.

2. Materials and methods

2.1. Materials

Glutathione and lysozyme were purchased from the Peptide Institute, Inc. (Osaka, Japan) and SEIKAGAKU CORPORATION (Tokyo, Japan), respectively. 2-Mercaptoethanol and mercapto-propionic acid were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). 2-Dimethylaminoethanethiol, 2-diethylaminoethanethiol, and 2-diisopropylaminoethanethiol were purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). 2-Aminoethanethiol hydrochloride and Arg-Cys-Gly was obtained from Nacalai tesque, Inc. (Kyoto, Japan) and PH Japan Co., Ltd (Hiroshima, Japan), respectively. All chemicals and solvents used were of reagent grade.

2.2. Calculation of the van der Waal surface and the solvent accessible surface area with positive partial charges

A software program, MarvinSketch ver 5.10 (ChemAxon, Inc., Budapest, Hungary), was employed to calculate van der Waals and solvent accessible surface areas with positive partial charges (ASA+) of the alkylamino moiety (–CH2–CH2–NR2: R, alkyl group) of each thiol reagent. The calculation of the surface areas of the alkylamino moiety was performed using 7.5 and 1.4 Å of the pH value and solvent radius, respectively.

2.3. Refolding reactions of lysozyme and prouroguanylin

The reduction of lysozyme was carried out by a previously described method [11]. Folding reactions were carried out in the presence of several types of thiol reagents. The denatured/reduced proteins (lysozyme, 0.1 mg/ml; prouroguanylin, 0.2 mg/ml) were dissolved in 0.1 M Tris/HCl (pH 7.5) and allowed to undergo folding in the presence of 2 mM reductant and 0.2 mM GSSG at room temperature for 48 h. All solutions used in the refolding experiments were flushed with N2 gas, and the reactions were carried out in a sealed vial under an atmosphere of N2. The experiments were performed in duplicate. The folding yield was calculated based on the average of independent experiments.

2.4. Assay of lysozyme activity

Lysozyme activity was measured using Micrococcus lysodeikticus, as previously reported [15,16]. Briefly, the refolding solution of lysozyme (10 μl) was added to a suspension (0.5 mg/ml) of M. lysodeikticus cells in 20 mM sodium phosphate buffer (1 ml, pH 6.5). The absorbance of the reaction mixture was measured at 600 nm using a UV–Vis spectropolarimeter model V-550 (Japan Spectroscopic Co., Tokyo, Japan).

2.5. Reversed-phase high performance liquid chromatography (RP-HPLC)

The HPLC apparatus was comprised of an ELITE system (Hitachi High-Technologies Corporation, Tokyo, Japan), equipped with an L-2400 detector and a D-2500 chromato-integrator. Proteins were separated by RP-HPLC using a Cosmosil 5C18-AR-II column (4.6 × 150 mm, Nacalai, Inc. Kyoto, Japan) and confirmed by MALDI-TOF/MS analyses, as reported previously [11]. Refolding yields were estimated by the HPLC peak area at 220 nm.

3. Results and discussion

The folding mechanisms of proteins possessing multiple disulfide bonds have been extensively studied and the results indicate that a series of folding intermediates, including mis-brided disulfide isomers, are produced during the folding of disulfide-bond-containing proteins [17–19]. However, the mechanism associated with the cross disulfide-linked intermediates with the thiol reagent to produce the native conformation is not fully understood. In a recent paper, we proposed that the positive charge of the redox molecule accelerates disulfide-coupled protein folding [11]. Therefore, to further investigate the mechanism of the cross-disulfide-linked species in protein folding, a series of thiol reagents was examined in terms of their participation in the refolding of lysozyme, a model protein.

First, to confirm the acceleration effect caused by the positive charge on disulfide-coupled protein folding, the folding recovery of lysozyme in the presence of several types of redox reagents was determined. For this purpose, the effectiveness of 2-aminoethanethiol (AET), 2-mercaptoethanol (ME), and 3-mercaptopropionic acid (MPA), as reducing reagents, was examined in the folding of lysozyme. The structural formulas of the thiol reagents are shown in Fig. 1. The net charges of AET, ME, and MPA in the case of a cross-linked species with a protein molecule are +1, 0, and −1 at pH 7.5, respectively. To clearly estimate the ability of a reagent as a redox molecule for protein folding as accurately as possible, the refolding reaction was carried out at pH 7.5, which corresponds to the pH value in the endoplasmic reticulum and also decreases the reaction velocity for disulfide-coupled protein folding [20,21]. In addition, GSSG (the oxidized form of glutathione) was employed for the refolding reaction of lysozyme, since GSSG is commonly used in the folding of disulfide-containing proteins and the oxidized forms (R–S–S–R) of thiol reagents do not significantly affect the refolding.

![Fig. 1. Structures of the thiol reagents. The structures are depicted as a ionic format pH 7.5 except for a thiol group. Total charges are indicated in parentheses.](image-url)
recovery of proteins, compared to GSSG [9]. In fact, R–S–S–R was also spontaneously produced as a reaction product between the thiol reagent (R–SH) and GSSG, as shown in Fig. 2A. The reducing reagent was carried out using the denatured/reduced form of lysozyme in the presence of 2 mM reducing reagent and 0.2 mM GSSG and the refolding recovery and the recovered enzymatic activity of lysozyme were estimated, as shown in Fig. 3.

To address the above questions, a series of thiol reagents (AET, DMAET, DEAET, and DPAET) was examined for the refolding of lysozyme. AET, DMAET, DEAET, and DPAET were ranked according to their molecular size, as shown in Fig. 3 and summarized in Table 1. As expected, among the thiol reagents tested, AET showed the best refolding recovery for lysozyme, indicating that the positive charge of the cross disulfide-linked moiety in a protein molecule accelerates disulfide-coupled protein folding via the thiol-disulfide-exchange reaction (Fig. 2B).

The refolding recoveries for Arg-Cys-Gly (RCG) were similar to GSH under the conditions used in this study (Fig. 3 and Table 1) although our previous study indicated that refolding in the presence of RCG was more rapid compared to the standard glutathione redox system. The differences between the present and previous conditions for the refolding of lysozyme are mainly the pH value (7.5 versus 8.0, respectively) and the concentration of the oxidized form (0.2 and 1 mM, respectively). As described above, disulfide-coupled protein folding under the conditions used in this study occurs much more slowly than that under the previous conditions. Therefore, it was possible to estimate the ability of the thiol reagents more accurately, although it was not possible to determine the difference between RCG and GSH under the conditions used in this study. The findings suggest that AET, as a component of a redox molecule, confers more activity compared to RCG. The net charge of both AET and RCG is +1 but RCG also carries a local negative charge at its C-terminal carboxylate group. In addition, the positive charge of the amino group of AET is spatially located much closer to the cross disulfide bond compared to the amino and guanidino groups of RCG. Therefore, compared to RCG, AET was able to accelerate the refolding reaction of lysozyme much more effectively. This result also raises the question of how steric hindrance or spatial environments around the positive charge affect disulfide-coupled protein folding.

To address the above questions, a series of thiol reagents (AET, DMAET, DEAET, and DPAET) was examined for the refolding of lysozyme. AET, DMAET, DEAET, and DPAET were ranked according to their molecular size, as shown in Fig. 3 and Table 1. As expected, among the thiol reagents tested, AET showed the best refolding recovery for lysozyme, indicating that the positive charge of the cross disulfide-linked moiety in a protein molecule accelerates disulfide-coupled protein folding via the thiol-disulfide-exchange reaction (Fig. 2B).

Table 1

<table>
<thead>
<tr>
<th>Charge (pH 7.5)</th>
<th>pK_a Value of amino group</th>
<th>pK_a Value of thiol group</th>
<th>Size</th>
<th>Surface area^a (Å²)</th>
<th>Refolding yield^b (%)</th>
<th>Reference(^c) number</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>–1</td>
<td>4.43</td>
<td>8.56</td>
<td>van der Waals</td>
<td>45.3 ± 2.1</td>
<td>22</td>
</tr>
<tr>
<td>ME</td>
<td>0</td>
<td>9.5</td>
<td>+</td>
<td>ASA^a</td>
<td>35.5 ± 3.5</td>
<td>23</td>
</tr>
<tr>
<td>MPA</td>
<td>–1</td>
<td>10.24</td>
<td>+</td>
<td></td>
<td>30.0 ± 2.8</td>
<td>24</td>
</tr>
<tr>
<td>AET</td>
<td>+1</td>
<td>3.36</td>
<td>8.27</td>
<td></td>
<td>112</td>
<td>154 ± 5.5</td>
</tr>
<tr>
<td>DMAET</td>
<td>+1</td>
<td>3.3</td>
<td>7.95</td>
<td></td>
<td>183</td>
<td>200 ± 1.4</td>
</tr>
<tr>
<td>DEAET</td>
<td>+1</td>
<td>3.25</td>
<td>7.8</td>
<td>+++</td>
<td>244</td>
<td>241 ± 3.5</td>
</tr>
<tr>
<td>DPAET</td>
<td>+1</td>
<td>3.2</td>
<td>8.0</td>
<td>+++</td>
<td>304</td>
<td>251 ± 2.8</td>
</tr>
<tr>
<td>RCG</td>
<td>+1</td>
<td>5.01^d, 1.52^e</td>
<td></td>
<td></td>
<td>44.5 ± 0.7</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) The pK_a value was calculated using the equation: pK_a = pK_b – 14.

\(^{b}\) The van der Waals and the solvent accessible surface areas with positive partial charges (ASA^+\) were calculated only for the alkylamino moiety of each thiol reagent [28].

\(^{c}\) Refolding yield of lysozyme using thiol reagents was estimated by the HPLC peak area.

\(^{d}\) The pK_a value of the amino group of arginic acid is indicated.

\(^{e}\) The pK_a value of the guanidino group of arginic acid is indicated.

\(^{f}\) References for pK_a values of amino and thiol group.
the pK_a value of the alkylamino group and the pK_b value of the thiol group, were considered. However, as summarized in Table 1, no significant difference was found between the pK_b values of the amino group of the thiol reagents, DMAET, DEAET, and DPAET, or the pK_a values of the thiol groups of the reagents.

In addition, the local hydrophobicity in the cross disulfide-linked species was also considered in the folding reaction. Local hydrophobic environments may suppress the conversion of a thiol to a thiolate group by decreasing the permittivity, thus decelerating the disulfide-exchange reaction in a cross disulfide-linked protein molecule. Therefore, the hydrophobicity of the redox molecule may actually suppress disulfide-coupled protein folding. However, DPAET showed the highest ability among the reagents, even though it contains the largest hydrophobic moiety (a diisopropyl group), indicating that the (local) hydrophobicity of the redox molecule is not a significant factor in disulfide-coupled protein folding. Therefore, factors in addition to positive charge and hydrophobicity need to be considered to explain the accelerating effect of DPAET on protein folding. These are discussed in the next section.

We previously reported that Arg-Cys-Gly intra-molecularly promotes the thiol-disulfide exchange reaction in cross disulfide species by its positive charge, resulting in the acceleration of disulfide-coupled protein folding [11]. The local electrostatic environment around a cysteine residue in a protein can have a strong effect on the disulfide-coupled folding of proteins [23]. Snyder et al. reported that a cysteine residue adjacent to a positively charged moiety forms a disulfide bond more rapidly in a disulfide-exchange reaction, indicating that the adjacent positive charge accelerates disulfide formation and the exchange reaction [25]. In addition, our results reported herein suggest that the local intra-molecular interaction between the positively charged alkylamino group and the negatively charged thiol group (thiolate ion) in the cross disulfide-linked species produced during the refolding reaction is preferred for accelerating disulfide formation (Fig. 2) although steric hindrance is also a factor. Considering these collective findings, we propose a mechanism for the acceleration in the disulfide exchange reaction in the cross disulfide-linked intermediates during folding as follows; in general, the rate-determining step for the disulfide-coupled folding reactions of proteins is thought to be the step involving the disulfide rearrangement of mis-bridged disulfide species to the native conformation. The disulfide exchange/reaction formation is predominantly promoted by the thiolate anion of a Cys residue in a protein molecule. Therefore, the pK_a value of the Cys residues in a protein molecule during the folding reaction should be an important factor in the formation of a disulfide bond. It is known that an amino group stabilizes the thiolate anion by its positive charge and decreases the pK_a value of the thiol group within a molecule [22]. The positively charged moiety of the cross-linked folding intermediates is located in close proximity to the Cys residue when the thiol group of a Cys residue of a protein molecule intra-molecularly attacks the cross-disulfide bond between the reagents and the protein molecule, as shown in Fig. 2B. Therefore, the amino group (alkylamino group) of the cross-linked moiety may decrease the pK_a value of the thiol group of the Cys residue of the cross-linked protein molecule and induce the dissociation of the thiol group to the thiolate form of the Cys residue within a protein molecule (Fig. S1), resulting in an overall acceleration in disulfide-coupled protein folding. The disopropylaminoethyl group of DPAET is physically much closer to the disulfide bond of the cross disulfide species than the guanidino group of Arg-Cys-Gly. Therefore, DPAET possesses a superior folding ability compared to RCG, although the guanidino group possesses a lower pK_b value (stronger basicity) than that of the disopropylaminoethyl group, as summarized in Table 1.

In addition, to understanding why DPAET is superior to AET as a folding reagent, solvent accessible surface areas with positive partial charges (ASA^+ of the alkylamino moiety of the thiol reagents, AET, DMAET, DEAET, and DPAET, were calculated using the MarvinSketch software program [28] and the results are summarized in Table 1. DPAET provided the largest ASA^+ (251 Å^2) among the thiol reagents and the ASA^+ values for the alkylamino moiety of the thiol reagents (DMAET, DEAET, and DPAET) showed a good relationship to the folding recoveries of lysozyme, indicating that DPAET effectively provides a positively charged environment for the stabilization of the thiolate anion of a Cys residue of the cross-linked protein molecule, regardless of the fact that it has a larger volume. Indeed, CPK modeling experiments of the cross-disulfide bond carrying an alkylamino group suggested that sufficient spaces is still available for the moiety with DPAET to react with the thiolate anion of the cross-disulfide-linked protein molecule (Fig. S1). In this aspect, AET possesses the smallest ASA^+ value but provides the lowest steric hindrance. Therefore, it can be concluded that, not only the positive charge, but also a larger ASA^+ is preferred for accelerating disulfide-coupled protein folding and that the combination of ASA^+ and the steric hindrance of the thiol reagent need to be considered in terms of regulating the chemical reactivity of the cross-disulfide-linked moiety (intra-molecular disulfide-exchange reaction).

Among the thiol reagents tested, DPAET showed a superior activity for the disulfide-coupled folding of lysozyme. Therefore, we further investigated the mechanism of DPAET-mediated protein folding. For this purpose, prouroguanylin (three intra-molecular disulfide bonds) was employed, since the folding intermediates can be easily separated by HPLC [11]. The fully reduced form (R) of prouroguanylin could still be observed at 60 min in the case of GSH (Fig. 4A) but disappeared within 60 min in the presence of RCG (Fig. 4B) in the refolding reaction, revealing that RCG accelerates the formation of disulfide bonds, as reported previously [11]. The effect of DPAET on the refolding of prouroguanylin was dramatic. The fully reduced form of prouroguanylin disappeared immediately and the native form was observed within 5 min, as shown in Fig. 4C. The refolding reaction using DPAET was essentially complete in 20 min. The rate-determining step for disulfide-coupled protein folding is thought to be the disulfide exchange of mis-bridged disulfide species [6,7]. Therefore, our results indicate that the dispersed positive charge of the disopropylaminoethyl moiety in the transiently produced cross-disulfide-linked species promotes the formation of a thiolate ion via dissociation of the thiol group of the Cys residue of the protein molecule, resulting in the very rapid formation of the native conformation of prouroguanylin. This result was also supported by the results of refolding experiments of prouroguanylin using AET, DMAET, and DEAET (Fig. S2). The use of DPAET resulted in the quick production of intermediates (3SS) and the native conformation was found much faster than when AET was used, indicating the superior ability of DPAET as a redox reagent for disulfide-coupled protein folding. Detailed structural analyses of the folding intermediates in the presence of those thiol reagents are currently in progress.

The findings herein show that the acceleration effect of thiol reagents for disulfide-coupled protein folding can be largely attributed to their positive charge. Recently, a number of proteins have been prepared by recombinant DNA technology for medical and biophysical use. However, proteins are often expressed in the form of an inactive inclusion body, especially when the Escherichia coli expression system is used. Therefore, the recombinant proteins need to be refolded into the native conformation in the presence of the appropriate chemical additives in vitro [12]. In such cases, DPAET represents an ideal compound for converting inactive recombinant proteins to the active forms and would permit a
superior recovery of the native conformation in the refolding reaction.

In conclusion, a positive charge in close proximity to the Cys residue of the redox molecule effectively accelerates the disulfide-exchange reaction. A reducing agent with a larger ASA+ (solvent accessible surface area with positive partial charges) is preferred for accelerating disulfide-coupled protein folding by inducing/stabilizing the thiolate form of the Cys residue of the protein molecule in the cross-disulfide-linked molecule.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.09.031.

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