A water-soluble selenoxide reagent as a useful probe for the reactivity and folding of polythiol peptides

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A water-soluble selenoxide (DHS\textsuperscript{ox}) having a five-membered ring structure enables rapid and selective conversion of cysteinyl SH groups in a polypeptide chain into SS bonds in a wide pH and temperature range. It was previously demonstrated that the second-order rate constants for the SS formation with DHS\textsuperscript{ox} would be proportional to the number of the free SH groups present in the substrate if there is no steric congestion around the SH groups. In the present study, kinetics of the SS formation with DHS\textsuperscript{ox} was extensively studied at pH 4–10 and 25°C by using reduced ribonuclease A, recombinant hirudin variant (CX-397), insulin A- and B-chains, and relaxin A-chain, which have two to eight cysteine residues, as polythiol substrates. The obtained rate constants showed stochastic SS formation behaviors under most conditions. However, the rate constants for CX-397 at pH 8.0 and 10.0 were not proportional to the number of the free SH groups, suggesting that the SS intermediate ensembles possess densely packed structures under weakly basic conditions. The high two-electron redox potential of DHS\textsuperscript{ox} (375 mV at 25°C) compared to L-cystine supported the high ability of DHS\textsuperscript{ox} for SS formation in a polypeptide chain. Interestingly, the rate constants of the SS formation jumped up at a pH around the pK\textsubscript{a} value of the cysteiny1 SH groups. The SS formation velocity was slightly decreased by addition of a denaturant due probably to the interaction between the denaturant and the peptide. The stochastic behaviors as well as the absolute values of the second-order rate constants in comparison to dithiothreitol (DTT\textsuperscript{red}) are useful to probe the chemical reactivity and conformation, hence the folding, of polypeptide chains.

A small globular protein, which has several disulfide (SS) linkages in the native state (N), spontaneously folds into the functional structure from the denatured and reduced form (R) through SS formation (i.e., oxidation) and thiol (SH)/SS exchange (i.e., SS reshuffling and/or SS rearrangement) reactions [1,2]. By chemically trapping the transient SS intermediates, we can elucidate the folding processes in principle. In such oxidative folding study in vitro, disulfide reagents, such as trans-4,5-dihydroxy-1,2-dithiane (DTT\textsuperscript{ox}) and oxidized glutathione (GSSG), are generally used as a SS-forming reagent, which gradually produces native SS bonds in a polypeptide chain via inter- and intramolecular SH/SS exchange reactions under neutral to weakly basic conditions (ca. pH 7–9) [3–9]. However, under these typical folding conditions, it is not easy to observe the key folding intermediates on the oxidative folding pathways because SS formation (i.e., intermolecular SH/SS exchange) and SS rearrangement (i.e., intramolecular SH/SS exchange) reactions proceed cooperatively.

In contrast, we recently demonstrated that the SS formation...
DHS\textsuperscript{ss} to form a maximum of four, three, two, and one SS bonds, respectively. From the kinetic analysis of the SS formation, it was revealed that the SS formation does not always follow the same kinetic behaviors, indicating that the velocity of SS formation is influenced by several factors, such as the structure formed in a polypeptide chain, the pK\textsubscript{a} of the cysteinyl SH groups, and a denaturant added in the reaction solution. Based on the data obtained, advantageous features of DHS\textsuperscript{ss} as a probe for the structure and reactivity of the folding intermediates of SS-containing proteins are discussed.

2. Materials and methods

2.1. Materials

Bovine pancreatic RNase A and insulin were purchased from Sigma Aldrich, Japan and used without purification. A recombinant hirudin variant (CX-397) [16] was provided from Pharmaceuticals and Biotechnology Laboratory, Japan Energy Co., Ltd. and was used without further purification. Relaxin A-chain (Rlx-A) was obtained by solid-phase peptide synthesis by application of the usual Fmoc protocol. DHS\textsuperscript{ss} [19] and AEMTS [20] were synthesized according to the literature methods. All other reagents were commercially available and used without further purification.

2.2. Preparation of reduced polypeptides

Reduced RNase A and CX-397 were prepared by following the procedure previously described [12,13]. Reduced Ins-A and Ins-B were obtained as follows. To a solution of insulin (3–4 mg) dissolved in 500 μL of a 100 mM Tris–HCl/1 mM EDTA buffer solution at pH 8.5 containing 4 M Gdn-SCN as a denaturant was added an excess amount of DTTred (7–8 mg). The reaction mixture was incubated at 28 °C for 60 min. The resulting solution containing reduced chains of insulin was desalted to 0.1 M aqueous ammonia purged with nitrogen by passing through the column packed with Sephadex G15 resin. The desalted peptide solution (7 mL) was acidified to pH 3 with 100 μL of acetic acid and lyophilized. The resulting white solid materials were dissolved in 2.5 mL of 0.1% aqueous trifluoroacetic acid (TFA) solution containing 20% acetonitrile. Insoluble precipitates were settled out by centrifugation (14,000 rpm) for 10 min. Reduced Ins-A and Ins-B were separated by HPLC equipped with a Tosoh TSKgel ODS-100V 4.6 × 150 mm reverse phase column using a 77:23 (v/v) mixture of 0.1% TFA in water (eluent A) and 0.1% TFA in acetonitrile (eluent B) at a flow rate of 1.0 mL/min. A solvent gradient (a ratio of eluent B linearly increased from 23% to 35% in 0–15 min, from 35% to 37% in 15–20 min, from 37% to 40% in 20–23 min, and from 40% to 100% in 23–24 min) was applied. The separated peptide fractions detected by UV absorption at 280 nm were collected and lyophilized. The resulting white powder materials of Ins-A and Ins-B (purity > 95% estimated by HPLC analysis) were stored at −30 °C.

Reduced Rlx-A was prepared as follows. The Rlx-A obtained by solid-phase peptide synthesis was dissolved in 500 μL of a 100 mM Tris–HCl/1 mM EDTA buffer solution at pH 8.5 containing 8 M urea as a denaturant and was treated with DTTred (7–8 mg) at 28 °C for 60 min. After insoluble precipitates were settled out by centrifugation (14,000 rpm) for 10 min, the resulting solution containing a reduced Rlx-A was desalted to 0.1 M acetic acid through the column packed with Sephadex G15 resin. The desalted peptide solution (7–8 mL) was then lyophilized. The obtained white solid materials were dissolved in 2.5 mL of 0.1% aqueous TFA containing 20% acetonitrile. Insoluble precipitates were settled out by centrifugation (14,000 rpm) for 10 min. Rlx-A was isolated by HPLC equipped with a Tosoh TSKgel ODS-100V 4.6 × 150 mm reverse phase column using a 80:20 (v/v) mixture of 0.1% TFA in water (eluent A) and 0.1% TFA in acetonitrile (eluent B) at a flow rate of 0.7 mL/min. A solvent gradient (a ratio of...
eluent B linearly increased from 20% to 36% in 0–15 min, from 36% to 39% in 15–20 min, from 39% to 40% in 20–23 min, and from 40% to 100% in 23–24 min) was applied. The fractionated reduced Rlx-A detected by UV absorption at 275 nm was collected and lyophilized. The obtained white powder material of Rlx-A (purity > 90% estimated by HPLC analysis) was stored at –30 °C.

To prepare solutions of reduced Ins-A, Ins-B, and Rlx-A at pH 4.0–10.0, 0.5–1.0 mg of each chain in the reduce form (R) was dissolved in 0.5–1.0 mL of a variable pH buffer solution containing 6 M urea. The stock solution was diluted threefold with the same pH buffer solution without urea. The concentration of R was determined by UV absorption at 280 (Ins-A and -B) or 275 (Rlx-A) nm. The recorded signals were integrated and analyzed. After a certain period of time, the oxidation reaction was quenched by the reaction with 109 μL of DTTred (0.5–8.0 mM), and monitored for 1.0–20 s. The obtained data were fitted to a single exponential curve, and the obtained reaction velocities were plotted against the concentration of DTTred to determine the second-order rate constant.

2.5. HPLC analysis

Sample solutions of RNase A and CX-397 from the short-term experiments were analyzed by HPLC using a cation-exchange column according to the literature [12,13]. On the other hand, sample solutions of Ins-A, Ins-B, and Rlx-A were directly injected to HPLC equipped with a 1 mL sample solution loop and a Tosoh TSKgel ODS-100V 4.6 × 150 reverse phase column. A different solvent gradient was applied for each peptide chain. For Ins-A, eluent A was 0.1% TFA in water, eluent B was 0.1% TFA in acetonitrile, and a ratio of eluent B was linearly increased from 22% to 28% in 0–15 min, from 28% to 40% in 15–20 min, and kept constant in 20–23 min at a flow rate of 1.0 mL/min. For Ins-B, the same eluents were employed, and a ratio of eluent B linearly increased from 20% to 36% in 0–15 min, from 36% to 39% in 15–20 min, and from 39% to 40% in 20–23 min at a flow rate of 1.0 mL/min. For Rlx-A, eluent A was 0.1% TFA in water, eluent B was 0.1% TFA in 60% acetonitrile, and a ratio of eluent B was linearly increased from 33.3% to 38.3% in 0–15 min, from 38.3% to 41% in 15–20 min, and kept constant in 20–23 min at a flow rate of 0.7 mL/min. The fractionated folding intermediates were detected by UV absorption at 280 (Ins-A and -B) or 275 (Rlx-A) nm. The recorded signals were integrated and analyzed.

2.6. Characterization of disulfide intermediates

The disulfide intermediates fractionated by HPLC were collected, desalted to 0.1 M acetic acid, and lyophilized. The molecular mass of each separated intermediate was measured on a Jeol JMS-T100LP mass spectrometer operated in the ESI(+) mode. Since modification of the folding intermediates with AEMTS caused an increment of the molecular mass by 76 Da per a free SH group, the number of the SS bonds for each fractionated intermediate could be determined based on the observed mass number.

2.7. Enzymatic digestion of 3S intermediates of CX-397

To investigate difference in SS-bond components of the intermediate ensembles generated at pH 4.0 and 8.0, the enzymatic digestion of 3S intermediates ensemble of CX-397 was carried out by following the literature [22]. The desalted and lyophilized 3S intermediates (0.3
mg/mL), which were obtained by the reaction of R with five equivalents of DHSox, were digested at 25 °C with thermolysin (30 μg/mL) for 16 h in 50 mM N-ethylmorpholine/acetate buffer at pH 6.4. The concentrations of 3S intermediates and thermolysin were 0.3 mg/mL and 30 μg/mL, respectively in the reaction solution. The sample solution was acidified to pH 2–3 with 15% aqueous TFA, and 10 μL of the resulting solution containing the digested peptide fragments was directly injected to HPLC equipped with a 20 μL sample solution loop and a Tosoh TSKgel ODS-100V 4.6 × 150 reverse phase column using 0.1% formic acid in water (eluent A) at a flow rate of 0.8 mL/min. The concentration gradient (a ratio of eluent B (0.1% formic acid in acetone/titrile) linearly increased from 0% to 10% in 0–30 min, from 10% to 40% in 30–55 min, and from 40% to 50% in 55–58 min) was applied.

2.8. Potentiometric titration

The two-electron reduction potential (E’) for DHSox was determined by the potentiometric titration method described in the literature [23,24] with sodium dithionite (Na2S2O4) as a reducing titrant. Measurement was performed in a two-electrode cell system with a single compartment containing a platinum spiral wire indicator electrode and an Ag/AgCl electrode connected to a millivolt meter (Toa HM-70V). 10 mM of sodium dithionite was titrated into the cell containing 35 mL of 1 mM DHSox in 100 mM sodium acetate buffer solution at pH 5.5, with a 30 min equilibration period between each additional aliquot. The obtained equivalent point was corrected to the redox potential of DHSox with respect to the normal hydrogen electrode (NHE).

2.9. Measurement of CD and fluorescence spectra

To characterize structures of the SS intermediates, the fluorescence spectrum was measured on a Jasco FP-6200 spectrofluorometer by using a quartz cell (light path length = 10 mm) under the conditions of 275 nm for the excitation wavelength, 280–400 nm for the emission wavelength range, 60 nm/min for the scan speed, 1 nm for the band width, 1 s for the response, and 5 nm for the slit widths of both the excitation and emission. The circular dichroism (CD) spectrum was recorded on a Jasco J-820 spectropolarimeter by using a quartz cell (light path length = 1 mm) under the conditions of 245–195 nm for the wavelength range, 50 nm/min for the scan speed, 1 nm for the band width, and 1 s for the response. Sample solutions for the spectral analyses were prepared by using a 10-fold diluted buffer solution with respect to that used for the short-term oxidation experiments.

3. Results

3.1. Short-term oxidation of reduced polythiol peptide chains with DHSox

Short-term oxidation experiments of RNase A at pH 4.0–10.0 and CX-397 at pH 7.0 using DHSox as an oxidant have already been described in the literatures [12,13]. The kinetic analysis revealed that the SS formation reaction took place through a stochastic mechanism, indicating that the SS-formation velocity is proportional to the number of the SH groups present along the chain. In this study, we obtained further evidences for the stochastic SS formation, short-term oxidation experiments using DHSox were carried out at pH 4.0–10.0 for additional polythiol peptide models. We chose peptides with a rather small number of Cys residues in order to perform the kinetic analysis of the SS formation reaction as accurate as possible.

Reduced RNase A (R(N)), and CX-397 (R(CX-397)), with eight and six cysteinyl SH groups, respectively, were obtained by reduction of the native proteins (N) with DTTred in the presence of 4 M guanidine thiocyanate as a denaturant. Similarly, reduced Ins-A (RIns-A) and Ins-B (RIns-B), with four and two cysteinyl SH groups, respectively, were obtained from native bovine pancreatic insulin. On the other hand, reduced Rlx-A (RRLx-A) having four SH groups was synthesized by standard solid-phase peptide synthesis and purified by HPLC after the reductive treatment.

The SS formation (oxidation) reaction of R was initiated by addition of stoichiometric amounts of DHSox (0.5–4 equiv. with respect to R) at 25 °C and pH 4.0–10.0 by using a quench-flow instrument [10]. The reaction was carried out in the presence of urea (2 M) for Ins-A, Ins-B, and Rlx-A in order to prevent aggregation. It should be noted that the experiments could not be performed for Ins-A at pH 4.0 even in the presence of 6 M urea due to aggregation. A reaction time of the oxidation was controlled to be 100 ms–60 s. The generated SS intermediates with one, two, three, or four SS bonds were chemically trapped with AEMTS [20], which can rapidly and irreversibly modify each free SH group into SSCH2CH2NH3+ form. This modification brought an increase in the molecular mass by 76 Da as well as one positive charge per one SH group. Therefore, the number of the blocked SH groups (hence the number of SS bonds) can be easily identified by mass spectrometry as well as HPLC. A cation-exchange column was used in the HPLC analysis for the SS intermediates of RNase A and CX-397, while a reverse-phase column was used for the SS intermediates of Ins-A, Ins-B, and Rlx-A.

HPLC chromatograms of the sample solutions obtained for CX-397, Ins-A, Rlx-A, and Ins-B when the Rs were reacted with 2, 1, 1, and 0.5 equiv. of DHSox, respectively, are shown in Fig. 3. R is (with one SS bond), 2S (with two SS bonds), and 3S (with three SS bonds) intermediates were clearly separated for CX-397 depending on the number of the SS bonds by the cation-exchange column (Fig. 3A). A marginal amount (2%) of N, resulting from occasional generation of the three native SS linkages, was observed, suggesting that SS formation reaction almost statistically progressed. A small peak (symbol x) of an impurity, which was originated from R, was also observed. However, since its population was very low, the peak was ignored in the kinetic analysis below. On the other hand, the SS intermediates (R, 1S, and 2S) of Ins-A, Rlx-A, and Ins-B were separated roughly depending on the number of the blocked SH groups by using the reverse-phase column (Fig. 3B–D). Although the peaks of the impurities (~9%) originating from their sources were observed, their populations did not change during the reaction with DHSox. Therefore, these peaks were ignored in the kinetic analysis. It should be noted that the 1S, 2S, and 3S intermediates are not homogeneous but heterogeneous ensembles having one, two, and three SS bonds, respectively. For example, the SS intermediates of Ins-A and Rlx-A having four Cys residues would theoretically contain six and three SS isomers for 1S and 2S, respectively. This can be actually presumed from the HPLC chromatograms. On the other hand, 1S of Ins-B having just two cysteine residues was a sole product. The each SS intermediate fractionated by HPLC was isolated, and the number of SS bond was assigned by ESI(+)-MS spectrometry.

Relative populations of the SS intermediates of CX-397, Ins-A, Rlx-A, and Ins-B as a function of the reaction time are shown in Fig. 4. It is clearly seen that the SS formation reaction with DHSox progresses rapidly, irreversibly, and quantitatively for all model peptides. The comparable results were obtained in the short-term oxidation experiments using different equivalents of DHSox or under different pH conditions (see Supporting information). Similar behaviors could also be observed for RNase A [10,12]. Reaction schemes of the SS formation for RNase A, CX-397, Ins-A, Rlx-A, and Ins-B are given in Eqs. (1)–(5).
The second-order rate constants, $k_1$ for $R \rightarrow 1S$, $k_2$ for $1S \rightarrow 2S$, $k_3$ for $2S \rightarrow 3S$, and $k_4$ for $3S \rightarrow 4S$, were subsequently determined by fitting the experimental data. The obtained rate constants ($k_1$–$k_4$) are listed in Table 1. Good agreement between the simulation curves and the plots of experimental data shown in Fig. 4 clearly indicates accuracy of the postulated reaction schemes. The obtained rate constants $k_1$–$k_4$, which correspond to the second-order rate constants for formation of one SS bond with DHSox$^{ox}$, are plotted against the solution pH. The rate constants monotonously increase with increasing the pH, except for the cases of CX-397 and Ins-B at 10.0. Fig. 5 further shows that $k_{av}$ of RNase A, CX-397, Ins-A, and Ins-B obviously jumps up in a narrow pH range of 7–9.

3.2. Oxidation kinetics of DTT$^{red}$

To compare the second-order SS-formation rate constants of polythiol peptide chains with that of a low molecular-weight dithiol compound, we carried out short-term oxidation of DTT$^{red}$ using DHSox$^{ox}$ as an oxidant (Eq. (6)) [19].

When 0.125–4.0 mM DTT$^{red}$ was reacted with 0.1 mM DHSox at 25 °C and pH 7.0, the UV absorbance at 310 nm due to formation of DTT$^{ox}$ became constant within 10 s, suggesting that the SS formation (oxidation) reaction completed in a short time. The rate constants ($k_1$) for the SS formation of DTT$^{ox}$ (Table 1) were determined by plotting the observed pseudo first-order rate constants ($k_{obs}$) against the concentration of DTT$^{red}$ (Fig. 6). Similar experiments were carried out at pH 4.1, 4.7, 8.0, and 10.0. The obtained second-order rate constants are also plotted in Fig. 5, showing a similar jump of the rate constant at around pH 8 to that observed for the polythiol peptide chains.

3.3. A redox potential of DHSox$^{ox}$

From the above kinetic analyses of the SS formation, it was obvious that DHSox$^{ox}$ enables rapid, quantitative, and irreversible SS formation for various polythiol substrates. To further confirm the high oxidizing ability of DHSox$^{ox}$, the two-electron redox potential of DHSox$^{ox}$ was measured by potentiometric titration using sodium dithionite (Na$_2$S$_2$O$_4$) as a reducing titrant at 25 °C and pH 5.5 in an aqueous medium. The obtained titration curve is shown in Fig. 7. The value of the redox potential obtained for DHSox$^{ox}$, (375 ± 15 mV vs. NHE) was consistent with previously reported values of several selenoxide compounds (380–490 mV) [23,24]. Compared with the redox potentials of l-cystine (−238 mV) and DTT$^{ox}$ (−327 mV) at pH 7.0 [25], the redox potential of DHSox$^{ox}$ was prominently higher, supporting that DHSox$^{ox}$ has high potential of SS formation for polythiol substrates.

4. Discussion

4.1. Comparison of DHSox$^{ox}$ with previous SS-forming reagents

DHSox$^{ox}$, a water-soluble selenoxide with five-membered ring, was applied as a SS-forming reagent for five polythiol peptides and DTT$^{red}$ in this study. In each case, rapid, quantitative, and irreversible SS
formation was clearly observed. In the previous oxidative folding study of SS-containing proteins, disulfide reagents, such as DTT\textsuperscript{red} and GSG, were usually applied [3–9]. In addition to these conventional reagents, new types of the reagents with improved reactivity, such as polymer-bound SS-containing reagents [26], aromatic SS reagents [27], glutathione derivatives [28], and short peptides with a Cys-Xaa-Xaa-Cys- motif [29], have been developed. Very recently, diselenide (SeSe) reagents, such as oxidized selenoglutathione [25,30], dipyridyl diselenide [31], and selenocystamine [31], have also been applied to oxidative folding of proteins in vitro and in vivo. However, the redox potentials of disulfides (−265 to −236 mV) and diselenides (−407 to −352 mV) are comparable or rather lower than that of t-cystine (−238 mV) [25]. In other examples, sulfoxide reagents with high redox potential, such as dimethyl sulfoxide (160 mV) [32] and polymer-bound methylsulfoxide [33], are sometimes applied for oxidation of thiol substrates.

In contrast, selenoxides possess much higher redox potentials than the aforementioned reagents. May and coworkers [23] and Singh and coworkers [24] reported the redox potentials of various selenoxide reagents (380–490 mV) and suggested that selenoxides can be useful as a SS-forming reagent to thiols, such as benzenethiol and GSH. The redox potential of DSS\textsuperscript{red} (375 ± 40 mV) at 25 °C measured in this study was found to be comparable, supporting the high oxidizing ability of DSS\textsuperscript{red} to generate SS bonds. Indeed, the second-order rate constants (3.5–4.1 mM\textsuperscript{-1} s\textsuperscript{-1} at pH 7.0) reported by May and coworkers [23] for the oxidation of GSH with phenylaminoethyl selenoxide and its derivatives were similar to those determined in this study ($k_{av}$ = 3.8–3.3 s\textsuperscript{-1} at pH 7.0) for the oxidation of polythiol peptides.

Table 1. The second-order rate constants for oxidation (SS formation) of polypeptide chains (RNase A, CX-397, Ins-A, Rlx-A, and Ins-B)* and DTT\textsuperscript{red} with DSS\textsuperscript{red} as an oxidant at 25 °C.

| pH  | Denaturant | $k_1$ (mM\textsuperscript{-1} s\textsuperscript{-1}) | $k_2$ (mM\textsuperscript{-1} s\textsuperscript{-1}) | $k_3$ (mM\textsuperscript{-1} s\textsuperscript{-1}) | $k_{av}$ (mM\textsuperscript{-1} s\textsuperscript{-1}) |
|-----|------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|
| 4.0 | None       | 7.1 ± 0.4                                         | 4.9 ± 0.3                                         | 3.0 ± 0.2                                         | 1.7 ± 0.1                                         |
| 7.0 | None       | 10.7 ± 0.5                                        | 7.3 ± 0.4                                         | 4.7 ± 0.3                                         | 2.6 ± 0.2                                         |
| 8.0 | None       | 30.6 ± 0.9                                        | 20.1 ± 0.7                                        | 14.9 ± 0.8                                        | 7.4 ± 0.4                                         |
| 10.0| None       | 42.6 ± 2.3                                        | 29.6 ± 2.3                                        | 17.9 ± 1.1                                        | 8.2 ± 0.5                                         |
| 4.0 | None       | 4.3 ± 0.2                                         | 2.6 ± 0.1                                         | 1.2 ± 0.1                                         | 1.4 ± 0.1                                         |
| 7.0 | None       | 9.8 ± 0.5                                         | 6.6 ± 0.3                                         | 2.8 ± 0.3                                         | 3.1 ± 0.2                                         |
| 2 M Gdn-HCl | 2.3 ± 0.2 | 1.4 ± 0.1                                         | 0.63 ± 0.05                                      | 0.7 ± 0.06                                        | 0.7 ± 0.06                                        |
| 7.0 | None       | 3.1 ± 0.2                                         | 1.7 ± 0.1                                         | 1.1 ± 0.1                                         | 1.0 ± 0.1                                         |
| 8.0 | None       | 18.6 ± 1.0                                        | 10.0 ± 0.6                                        | 3.6 ± 0.6                                         | 5.4 ± 0.4                                         |
| 2 M Gdn-HCl | 11.5 ± 0.9 | 6.2 ± 0.5                                         | 2.2 ± 0.1                                         | 3.0 ± 0.2                                         | 3.0 ± 0.2                                         |
| 9.8 | None       | 9.6 ± 0.6                                         | 5.0 ± 0.3                                         | 2.7 ± 0.2                                         | 2.8 ± 0.2                                         |
| 10.0| None       | 12.4 ± 0.7                                        | 7.2 ± 0.5                                         | 3.2 ± 0.5                                         | 3.8 ± 0.3                                         |
| 7.0 | 2 M Gdn-HCl | 3.4 ± 0.3                                         | 1.7 ± 0.2                                         | 1.7 ± 0.2                                         | 1.7 ± 0.2                                         |
| 8.0 | 2 M Gdn-HCl | 8.2 ± 0.3                                         | 4.0 ± 0.3                                         | 4.1 ± 0.2                                         | 4.1 ± 0.2                                         |
| 7.0 | 2 M Gdn-HCl | 17.8 ± 0.3                                        | 8.2 ± 0.3                                         | 8.6 ± 0.2                                         | 8.6 ± 0.2                                         |
| 8.0 | 2 M Gdn-HCl | 11.3 ± 0.8                                        | 5.6 ± 0.4                                         | 5.6 ± 0.4                                         | 5.6 ± 0.4                                         |
| 7.0 | 2 M Gdn-HCl | 1.2 ± 0.1                                         | 1.2 ± 0.1                                         | 1.2 ± 0.1                                         | 1.2 ± 0.1                                         |
| 7.0 | 2 M Gdn-HCl | 6.2 ± 0.2                                         | 6.2 ± 0.2                                         | 6.2 ± 0.2                                         | 6.2 ± 0.2                                         |
| 8.0 | 2 M Gdn-HCl | 7.3 ± 0.5                                         | 7.3 ± 0.5                                         | 7.3 ± 0.5                                         | 7.3 ± 0.5                                         |
| 10.0| 0.2 M urea | 4.7 ± 0.4                                         | 4.7 ± 0.4                                         | 4.1 ± 0.2                                         | 4.1 ± 0.2                                         |
| 7.0 | 4 M urea   | 2.6 ± 0.1                                         | 2.6 ± 0.1                                         | 2.6 ± 0.1                                         | 2.6 ± 0.1                                         |
| 4.1 | None       | 0.55 ± 0.15                                       | 0.55 ± 0.15                                       | 0.45 ± 0.01                                       | 0.45 ± 0.01                                       |
| 4.7 | None       | 0.45 ± 0.01                                       | 0.45 ± 0.01                                       | 0.45 ± 0.01                                       | 0.45 ± 0.01                                       |
| 7.0 | None       | 1.3 ± 0.1                                         | 1.3 ± 0.1                                         | 1.3 ± 0.1                                         | 1.3 ± 0.1                                         |
| 8.0 | None       | 5.1 ± 0.2                                         | 5.1 ± 0.2                                         | 5.1 ± 0.2                                         | 5.1 ± 0.2                                         |
| 10.0| None       | 12.0 ± 1.0                                        | 12.0 ± 1.0                                        | 12.0 ± 1.0                                        | 12.0 ± 1.0                                        |

* The values of $k_{av}$ were determined by fitting the data from the short-term oxidation experiments to the reaction schemes of Eqs. (1)–(5).

† The values of $k_1$ were determined by the stopped-flow experiments.

‡ The averaged second-order rate constants for SS formation of the diithiols with DSS\textsuperscript{red} at 25 °C obtained by using the equations, $(k_1/4 + k_2/3 + k_3/2)/4$ for RNase A, $(k_1/3 + k_2/2 + k_3/3)/3$ for CX-397, $(k_1/2 + k_2)/2$ for Ins-A and Rlx-A, and $k_3$ for Ins-B and DTT\textsuperscript{red}.

* Data quoted from Refs. [10,12].

† Data quoted from Ref. [13].

‡ The oxidation did not follow stochastic reaction.
Fig. 5. The pH dependence of the $k_{av}$ values obtained at 25°C by the short-term oxidation experiments using DHTox as an oxidant. The curves were manually drawn for convenience.

Fig. 6. Linear plots showing the dependence of observed pseudo first-order rate constants ($k_{obs}$) for the oxidation of DTTred (0.125–4.0 mM) with DHTox (0.1 mM). (a) pH 10.0, (b) pH 8.0, (c) pH 7.0, (d) pH 4.7, and (e) pH 4.1.

The high redox potential of DHTox is advantageous for temporary separation of the SS formation phase from the SS rearrangement phase in oxidative folding of SS-containing proteins. Indeed, by applying DHTox for the folding of RNase A, we have already succeeded in separation of the two folding phases clearly and characterization of the key folding intermediates much easier than when the conventional reagents with a low redox potential were applied [10–12]. On the other hand, formation of N is usually very slow by using DHTox because it does not catalyze the SS rearrangement. To overcome this limitation of DHTox as an oxidative folding reagent, a thiol reagent, such as cysteine and glutathione (GSH), should be added to the reaction solution after completion of the SS formation.

4.2. Stochastic SS formation

The SS formation reaction of polythiol peptides with DHTox would take place in a stochastic manner if there is no steric congestion [13]. This was confirmed for various polythiol peptides in this study (see Table 1). The rate constants, $k_1$ and $k_2$, for Ins-A were roughly in a ratio of 2:1 in a range of pH 7.0–10.0. Similarly, the $k_1$ and $k_2$ for Rlx-A at pH 7.0 and 8.0 were proportional to the number of the free SH groups existing in the reactive species. The same behaviors can be seen for the cases of RNase A at pH 4–10 and CX-397 at pH 4.0 and 7.0. However, the rate constants $k_1$–$k_3$ of CX-397 obtained at pH 8.0 and 10.0 were not in a ratio of 3:2:1. The reasons for the non-stochastic behaviors are discussed in the following sections.

Provided that the SS formation of peptides with DHTox proceeds stochastically, the averaged second-order rate constants ($k_{av}$) obtained for any polypeptides should be similar to that obtained for small dithiols under the same conditions. This is indeed the case as the values of $k_1$ obtained for DTTred are roughly comparable to the $k_{av}$ values for the polypeptide models (Fig. 5), supporting the stochastic SS formation mechanism. However, it would also be notable that the value of $k_1$ for DTTred at pH 10.0 is significantly larger than the $k_{av}$ values determined for polythiol peptides. This may reflect the redox potential of DTTred ($−327$ mV), which is lower than that of l-cystine ($−238$ mV) [24].

4.3. Effects of pH on the SS formation velocity

Acidity (deprotonation liability) of a SH group is an important factor for the SS formation velocity because the thiolate (S$^-$) form is much sensitive to an oxidant than the neutral thiol (SH) form. The $pK_a$ value of a cysteinyl SH group is 8.22 [34], and the ratio of the reactive thiolate species rapidly increases in a narrow pH range around the $pK_a$ value. Therefore, the rate constant of the SS formation would increase significantly at the pH. In fact, when the averaged rate constants ($k_{av}$) were plotted against the pH, jumps of the $k_{av}$ values were clearly observed at around the $pK_a$ value for the SH group (Fig. 5). However, the magnitude of the $k_{av}$ jump was too small if the $k_{av}$ value was proportional to the thiolate concentration. This suggests that the SS formation is accelerated by protonation of DHTox [13].

Fig. 5 also shows that the behavior of the $k_{av}$ jump is slightly different between the polythiol peptide substrates. In the case of RNase A and Ins-A, the $k_{av}$ jumps were observed at around pH 8, which is close to the $pK_a$ values for a cysteinyl SH group ($pK_a = 8.22$) [34]. The pH range was also close to that of the $k_{av}$ jump observed for DTTred
the SH groups irrespective of the length of the polypeptide chain. A denaturant (2 M urea). The obtained rate constants of this assumption, short-term oxidation experiments of CX-397 and subsequent unimolecular process (step 2), is disturbed by local or global formation of SS intermediates shown in Fig. 1 (step 1), or the subsequent thermodynamic subphases (Fig. 8). Thus, under acidic conditions, the kinetic intermediates (1S, 2S, and 3S) with no rigid structure would form from R because only stochastic SS formation proceeds. The produced kinetic intermediates are then converted to the thermodynamic intermediates (1S, 2S, and 3S) through SS reshuffling by changing the solvent pH or standing the solution for a long time. Given the reaction scheme of Fig. 8 for CX-397, the SS components of the 3S intermediates should be different under acidic and basic conditions. From the profile of the HPLC chromatograms, however, the difference in the relative populations of the 3S isomers between the kinetic and thermodynamic intermediates (i.e., 3S and 3S) was not obvious. We, therefore, applied enzymatic digestion for 3S reshuffling at pH 4.0 and 8.0, respectively. As a result, it was found that the populations of the digested peptide fragments are significantly different (see Supporting information), suggesting the existence of some structures under weakly basic conditions. To confirm the structural difference between the kinetic and thermodynamic intermediates of CX-397, CD and fluorescence spectra were measured at pH 4.0 in different reaction times.

The kinetic intermediates (2S as a major component) were prepared by the oxidation of RCX-397 with two equivalents of DSS® at pH 4.0 for 5 min, and the thermodynamic intermediates (2S as a major component) were obtained by the subsequent incubation at 25 °C for 24 h. During the incubation, the kinetic intermediates would slowly transform to the 2S via SS reshuffling even at pH 4.0 as previously observed in the case of RNase A [12]. The CD spectra of these intermediates did not exhibit any significant differences from that of R, indicating that no secondary structure is present in both kinetic and thermodynamic intermediates (Fig. 9A). In the fluorescence spectra, on the other hand, the relative intensity at around 310 nm increased obviously during the incubation, suggesting that the structures of the intermediates become compact during the kinetic to thermodynamic transition by SS reshuffling (Fig. 9B). The result supported that the SS formation processes of CX-397 are composed of kinetic and thermodynamic subphases like the case of RNase A [12].

A rate constant k of CX-397 at pH 10.0 was smaller than that at pH 8.0. This indicates that R at a high pH already has some structures. The phenomenon that the reduced state (R) has stable conformation under specific conditions has sometimes been reported for SS-containing proteins, such as ribonuclease T1 [39].

Fig. 5 further shows that the rate constant (ksp) for Ins-B at pH 10.0 is obviously smaller than that at pH 8.0, suggesting that R has also densely packed structure even in the presence of 2 M urea. Indeed, when the fluorescence spectra of RIns-B in the presence of 0.2, 2, and 4 M urea were measured, intensity of the fluorescence at 310 nm was enhanced (Fig. 10), suggesting more structuring in a lower concentration of urea. The presence of such a structure was previously reported in the 30% trifluoroethanol solution [40].

4.5. Effects of a denaturant

Urea and Gdn-HCl are frequently used as a denaturant as well as an additive preventing aggregation of polypeptides. In this study, 2 M urea was added in the reaction solutions of Ins-A, Rlx-A, and Ins-B, since they are highly liable to aggregate. Therefore, the effects of the
the oxidation of RCX-397 with DHSox were 5 min and 24 h for the kinetic and thermodynamic intermediates, respectively. For comparison, the respective spectra of R (green lines) and N (black lines) are shown. (A) CD spectra at \([R]_0 = 30.0 \mu M\) in all conditions. The concentration of RIns-B was 20.5 \(\mu M\) in all conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Fig. 9. CD and fluorescence spectra of kinetic (blue lines) and thermodynamic (red lines) SS intermediates of CX-397 obtained at pH 4.0 and 25 °C. The reaction times for the oxidation of R_X−397 with DHSox were 5 min and 24 h for the kinetic and thermodynamic intermediates, respectively. For comparison, the respective spectra of R (green lines) and N (black lines) are shown. (A) CD spectra at \([R]_0 = 30.0 \mu M\). (B) Fluorescence spectra at \([R]_0 = 30.0 \mu M\). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Fig. 10. Fluorescence spectra of RIns-B at 10.0 in the presence of (a) 0.2, (b) 2, and (c) 4 M urea. The concentration of RIns-B was 20.5 \(\mu M\) in all conditions.

denaturant on the concentration of RIns-B was 20.5 \(\mu M\) in all conditions.

Similarly, when the concentrations of urea were changed from 0.2 to 4 M, a decrease of the rate constants was observed for Ins-B at pH 10.0, although the robust structure of RIns-B would be significantly destroyed (Fig. 10). To understand the observed SS formation velocity the both acceleration and deceleration effects of a denaturant should be considered. The former effect would be caused by denaturation of the robust structure of the substrate, whereas the latter effect would be brought by the interaction of the denaturant with the substrate. According to the observed decrease in the SS formation velocity for Ins-B in the presence of 4 M urea at pH 10.0, the deceleration effect of a denaturant must be slightly predominant for polythiol peptide chains.

5. Conclusions

In this study, we have applied DSHox to the SS formation reactions of five polythiol peptides (i.e., reduced species of several SS-containing proteins) and a small dithiol DTTred. Detailed kinetic analysis of the SS formation reactions revealed several features of DSHox as a selective SS-forming reagent.

(1) DSHox allows rapid, quantitative, and irreversible SS formation for a wide range of polythiol substrates.

(2) The reaction velocity jumps up at around pH 8, which is near the \(pK_a\) value of cysteinyl SH groups. However, the pH range of the velocity jump would shift lower when the basic amino acid residues, such as Lys and His, which would modify the \(pK_a\) of the SH groups, are located in the proximity.

(3) The SS-formation rate constants are simply proportional to the number of the free SH groups present in the reduced or partially oxidized polythiols, confirming that the SS formation normally proceeds stochastically (13). However, the formation of global or local folded structures in the substrates would decelerate the SS formation reaction and modify the stochastic nature.

(4) The velocity of the SS formation slightly decreases in the presence of a denaturant, such as urea and Gdn-HCl, due probably to the interaction between the denaturant and the peptide molecules.

The strong ability of DSHox to generate SS bonds was indeed supported by measurement of the redox potential (375 mV vs. NHE at 25 °C), which is much higher than disulfides, diselenides, and sulfoxides that are utilized in the folding study of SS-reduced proteins. To summarize, DSHox with high oxidation ability is a useful reagent for formation of SS bonds in polythiol peptides. The stochastic behaviors of SS formation with DSHox and the absolute values of the second-order rate constants can be utilized to probe the chemical reactivity and conformation of peptides. Thus, selenoxide reagents would provide valuable information on the folding of versatile SS-containing proteins.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fob.2012.12.004.

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