[1]Abbreviations: DTT^{ox}, oxidized dithiothreitol; DTT^{red}, DL-dithiothreitol; GSSG, oxidized glutathione; GSH, reduced glutathione; RNase A, bovine pancreatic ribonuclease A; des[*x*-*y*], a disulfide species having all the native disulfide bonds except that between Cys-*x* and Cys-*y*, e.g., des[65-72] in RNase A; R, the fully reduced disulfide species; *n*S, the ensemble of unstructured disulfide species with *n* disulfide bonds, e.g., the 2S ensemble; 3S*, the ensemble of structured disulfide species of RNase A (with three native disulfide bonds) that are not in quasi-equilibrium with the 3S ensemble, but rather oxidize preferentially to the native protein; N′, a covalent adduct of RNase A in which a DTT molecule crosslinks cysteines 65 and 72; EDTA, ethylenediaminetetraacetic acid; AEMTS, 2-aminoethylmethanethiosulfonate; GdnHCl, guanidine hydrochloride; CD, circular dichroism.

Appendix A: Experimental Methods

Disulfide-bond studies require four basic elements. First, a redox reagent is needed to catalyze the disulfide redox reactions (*1,2*). Second, a method for rapidly quenching redox and reshuffling reactions is required to "freeze" the distribution of disulfide species at various times. Third, methods for fractionating the various disulfide species and ensembles are needed to monitor the concentrations of these species. Lastly, a recombinant DNA expression system is useful for preparing mutant analogs of various disulfide species for kinetic and structural experiments. This Appendix provides a basic introduction to these elements.

Disulfide Redox Reagents

Two redox reagents are commonly used in disulfide-bond studies: dithiothreitol (DTT^{ox}/DTT^{red})[1] and glutathione (GSSG/GSH) (a redox reagent *in vivo*), although novel redox reagents remain an active area of research (*3,4*). The reaction mechanisms of these two redox reagents are described in Figures A1 and A2, while Figure A3 illustrates how disulfide reshuffling can occur through a mixed disulfide intermediate.

Cyclic redox reagents such as DTT are powerful reducing agents; once formed, mixed disulfide species are rapidly reduced by the second free thiolate of the bound redox reagent (Figure A1). Thus, mixed disulfide species have negligible concentrations when cyclic reagents are used, simplifying kinetic modeling and structural interpretations. An exception occurs when a mixed disulfide bond is buried conformationally before it can be reduced by the second free thiolate, as in the N′ species of RNase A (Figure A4).

Linear redox reagents such as glutathione (GSSG) are better oxidizing agents, since their mixed disulfide species are more stable than those of DTT (Figure A2). However, the rate of forming intraprotein disulfide bonds may *decrease* at high concentrations of GSSG, because the protein thiol groups become effectively blocked, i.e., most thiols become involved in

mixed disulfide bonds. Thus, it is helpful to introduce a reducing reagent to allow reshuffling of such blocked species and of fully oxidized, nonnative species (e.g., species of the 4S ensemble of RNase A). Our original investigations of the oxidative folding of RNase A employed glutathione and yielded results qualitatively similar to those obtained with DTT (*7-11*). However, one drawback of linear redox reagents such as glutathione is that disulfide species with mixed disulfide bonds can become populated to a significant extent. Such species can be very numerous (*9,12,13*) and their heterogeneity makes structural interpretations of the regeneration kinetics difficult.

The possibility of contaminating redox reagents should always be considered. To eliminate air oxidation, experiments are carried out anaerobically under argon atmosphere, and chelating agents such as EDTA are added to bind the metal ions that catalyze air oxidation. A related issue is autocatalytic redox reactions, i.e., disproportionation reactions in which one protein molecule serves as a redox reagent for another. For RNase A, this effect becomes significant only in the absence of a redox reagent (e.g., when studying disulfide reshuffling in isolation) and at high protein concentrations near the isoelectric point; this effect can be checked by the concentration dependence of the regeneration. A reversible disulfide-blocking reagent may also act as a redox reagent under some conditions; if the blocking of the protein thiols is too slow, a disulfide bond between a blocking group and a protein thiol can be attacked by a second protein thiolate, forming a new disulfide bond and releasing the blocking group into solution.

Quenching Methods

A thiol-quenching method should have six qualities. First, the thiol-disulfide exchange reactions should be quenched completely and rapidly, so that the concentrations of disulfide species are essentially identical before and after the quenching. Second, the quenching should be long-lived, so that the intermediates do not rearrange while they are being fractionated.

Third, the quenching should not cause other covalent modifications of the protein; in particular, blocking groups should be specific for thiols and not participate in side reactions with other residues. Fourth, the quenching method should aid in isolating specific disulfide species. Fifth, the quenching method should (generally) be reversible so that the thiol-disulfide exchange can be restarted from a previously quenched and isolated disulfide species. Finally, the quenching should not unfold or significantly disrupt the structured disulfide species.

Three quenching techniques are in common use: acid quenching, blocking with alkyl halides (e.g., iodoacetamide and iodoacetate), and blocking with alkylalkanethiosulfonates such as aminoethylmethanethiosulfonate (AEMTS). In acid quenching, the pH of the solution is lowered to strongly acidic conditions (e.g., pH 2), where the concentration of the reactive thiolate groups is low. Acid quenching is fast, complete and reversible, but has two drawbacks: thiol-disulfide exchange reactions continue (albeit at very reduced rates) and subsequent analyses must be carried out at low pH. By contrast, blocking with alkyl halides such as iodoacetate is irreversible and slow, being comparable in rate to disulfide redox and reshuffling reactions. Thus, the concentrations of disulfide species after blocking may not correspond to their concentrations immediately prior to blocking. Moreover, at higher concentrations, iodoacetate modifies other nucleophilic groups, e.g., those of His, Met and Tyr (*14*).

A good compromise can be reached by blocking with alkylalkanethiosulfonates, i.e., compounds of the form R-$(CH_2)_m$-S-SO_2-$(CH_2)_n$-CH_3 (*15*). Our laboratory uses AEMTS (*16,17*) which corresponds to the compound with m=2 and n=0, where R is an amino group. When a free thiolate of the protein attacks the disulfide bond of AEMTS, a mixed disulfide bond is formed with cysteamine (Protein-S-S-$(CH_2)_2$-NH_3^+) while methanesulfinic acid (HSO_2CH_3) is released into solution. The AEMTS-blocking reaction is fast [roughly 10^5

times faster than iodoacetate (*16*)], specific for thiols, goes to completion, and may be reversed by adding a reducing agent to liberate the cysteamine blocking group. The terminal amino group of cysteamine introduces an additional positive charge for every blocked thiolate; hence, ion-exchange chromatography or electrophoresis may be used to fractionate the disulfide ensembles.

Fractionation and Characterization of Disulfide Intermediates

The fractionation of disulfide ensembles can be accomplished by several methods. Ion-exchange chromatography is well-suited when the ensemble is blocked with AEMTS, because the aminoalkylalkanethiosulfonates add a positive charge for every blocked thiolate. Ion-exchange columns show very little loss of protein and, thus, the concentrations of the eluted fractions can be regarded as quantitatively accurate. Ion-exchange columns may also be used to separate the protein from the redox reagent and unreacted blocking reagents (*18*).

The individual fractions from the chromatograms can be collected and analyzed to determine the number of thiol groups and disulfide bonds, using a new methodology specifically designed for AEMTS-blocked disulfide intermediates (*19*). The results clearly indicate that the cation-exchange column separates the blocked disulfide species by the number of disulfide bonds, i.e., fractionates the *n*S disulfide ensembles (Figures A5 and A6).

Ion-exchange chromatography has also been used at pH 5.0 to isolate some unblocked disulfide species, particularly des[40-95] and des[65-72] (*18*). Differences in the thermal transition temperatures of the des species are presently being exploited to fractionate these species better. Other fractionation methods have also proven useful, e.g., 2D gel electrophoresis (*20*), capillary electrophoresis (*21*), and gel filtration (*22*).

Methods for fractionating specific disulfide species have also been developed. For example, conformationally unstructured species can be reduced to R by a 2-minute pulse of weakly reducing conditions (5 mM DTT^{red}). Such a pulse will not significantly reduce

conformationally structured species such as des[65-72], since their disulfide bonds are protected from reduction. Thus, the structured species can be isolated as well-separated peaks on the chromatograms and may then be analyzed by peptide mapping methods (*23*).

Site-directed Mutants

Stable analogs of specific disulfide species can be produced by using site-directed mutagenesis of cysteines to alanines or serines (*24,25*). Such analogs are useful for structural studies of these species, or to eliminate specific redox or reshuffling reactions in the disulfide-coupled folding pathways. The choice of alanine or serine depends on the situation of the disulfide bond or thiol group to be replaced, and on whether stability or fidelity is paramount. Alanine is more analogous to nonpolar thiol groups and half-cystines, whereas the polar serine is more analogous to the partially ionized form of the thiol group. Thus, serine may provide a more faithful analog of thiol groups and des species near pH 8, whereas alanine is generally more stabilizing and may be preferable as an analog of thiol groups and des species at neutral pH.

Appendix B: Sub-Millisecond Conformational Ordering in Disulfide and Folding Intermediates

Several independent methods indicate that the fully reduced disulfide species R of RNase A has significant conformational order (*26-31*; and A. Navon, V. Ittah, H. A. Scheraga and E. Haas, to be published). This conformational order may clarify a recent controversy in studies of the conformational folding of RNase A (*32*).

Kinetic studies of the conformational folding of GdnHCl-denatured RNase A with native disulfide bonds and native cis/trans proline isomers (denoted U_{vf}) under a broad range of folding conditions indicated the presence of an intermediate species (denoted I_{ϕ}) populated on the submillisecond time scale (*33*). The presence of this intermediate seemed to be

confirmed by a stopped-flow circular dichroism (CD) study, which exhibited a "burst phase", i.e., a large change in the CD signal on the submillisecond time scale (*34*). The amount of secondary and tertiary structure in I_ϕ were estimated (roughly 40%) from the ratio of the burst-phase amplitude to the total change in CD that occurs in folding (*34*). (This estimate corresponds to a simple "two-state" approximation: residues are either fully structured in the native form or fully unstructured, and the unstructured residues are assumed to give no CD signal.) The specific structure of this intermediate was then investigated by pulse-labeled H/D exchange (*35,36*), which indicated significant protection in regions of high local hydrophobicity: near the 26-84 disulfide bond (where the second α-helix packs against the major β-hairpin in the native structure) and in the hydrophobic cluster near the C-terminus (residues 106-109 and 115-118).

This scenario was called into question by a recent study, in which similar stopped-flow CD experiments were carried out on the fully reduced species R and the equilibrium unfolded (but disulfide-intact) species U (*32*). Both proteins also exhibited a large burst phase by CD and, more importantly, the amplitudes of these burst phases agreed with each other and with that of the U_{vf} burst phase. The authors then interpreted thermal transition and H/D exchange data (*37*) to argue that the burst phase of R corresponded to the disordered collapse of a random coil, i.e., produced no conformational order besides a low radius of gyration. From this interpretation and the near-equivalence of the burst-phase amplitudes, the authors argued that the burst phases of U and U_{vf} similarly produced no conformational order (besides a general collapse), contrary to the conclusions of the original kinetic, CD and pulse-labeled H/D exchange experiments (*33-36*). Based on this conclusion and earlier studies of cytochrome c (*38*), the authors hypothesized that submillisecond burst

phases in proteins almost always correspond to the formation of a collapsed random coil, and not to the formation of an ordered folding intermediate.

However, the detection of significant conformational order in R cited above should prompt a re-evaluation of these conclusions. If the CD experiments are interpreted as showing that similar ordering of the peptide backbone occurs in the burst phases of R, U and U_{vf} (*32*), the presence of native-like tertiary topology in R implies a similar ordering in I_{ϕ}. Moreover, the CD data are not precise enough to exclude the presence of small structured regions as seen in the pulse-labeled H/D exchange experiments. It should also be noted that submillisecond burst-phase ordering and even structure formation have been observed in several other proteins and in peptide fragments (*39,40*). It is by no means obvious that the observed CD burst phase corresponds to the formation of a collapsed random coil.

However, the new experiments (*32*) do highlight an important pitfall in the interpretation of burst-phase amplitudes. The "two-state" approximation cited above may overestimate the mean structural content, since it neglects the CD signal resulting from conformational ordering without the formation of structure.

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Figure Captions

Figure A1. Mechanism of disulfide-bond formation by cyclic redox reagents such as dithiothreitol (DTT), through two thiol/disulfide exchange reactions. In the first step, a protein thiolate attacks the disulfide bond of DTT, opening the ring and forming a mixed disulfide bond with a dangling thiolate group. The recyclization reaction is very rapid, establishing an equilibrium in which the mixed disulfide form has a very low population. In the second step of oxidation, the mixed disulfide bond is attacked by another thiolate of the protein, forming the disulfide bond and releasing reduced DTT. In the reverse reaction, a disulfide bond can be attacked by a reduced DTT molecule to form the mixed disulfide bond, followed by rapid recyclization of the DTT to fully reduce the protein disulfide bond. Thus, the rapid recyclization reaction makes DTT and other similar cyclic redox reagents strong reducing agents and relatively weak oxidizing agents (*1,2*).

Figure A2. Mechanism of disulfide bond formation by linear disulfide reagents such as glutathione (GSSG). In the first step, a protein thiolate attacks the GSSG disulfide bond, forming a mixed disulfide bond and releasing a reduced glutathione molecule into solution. In the second step, a free protein thiolate can attack the mixed disulfide, forming the disulfide bond and releasing another reduced glutathione molecule. Since glutathione does not undergo a rapid recyclization reaction, the mixed disulfide bond is more stable with glutathione than with DTT (Figure A1), making glutathione a better oxidizing agent and a worse reducing agent (*1*). However the absence of the auto recycling mechanism allows every protein thiol group to become blocked under strongly oxidizing conditions.

Figure A3. Mechanisms of intramolecular thiol/disulfide exchange (disulfide reshuffling). (a) In the simplest version, a protein thiolate group attacks a protein disulfide bond, transferring the disulfide bond to the attacking thiolate. (b) The same effect can also occur through a mixed disulfide bond intermediate.

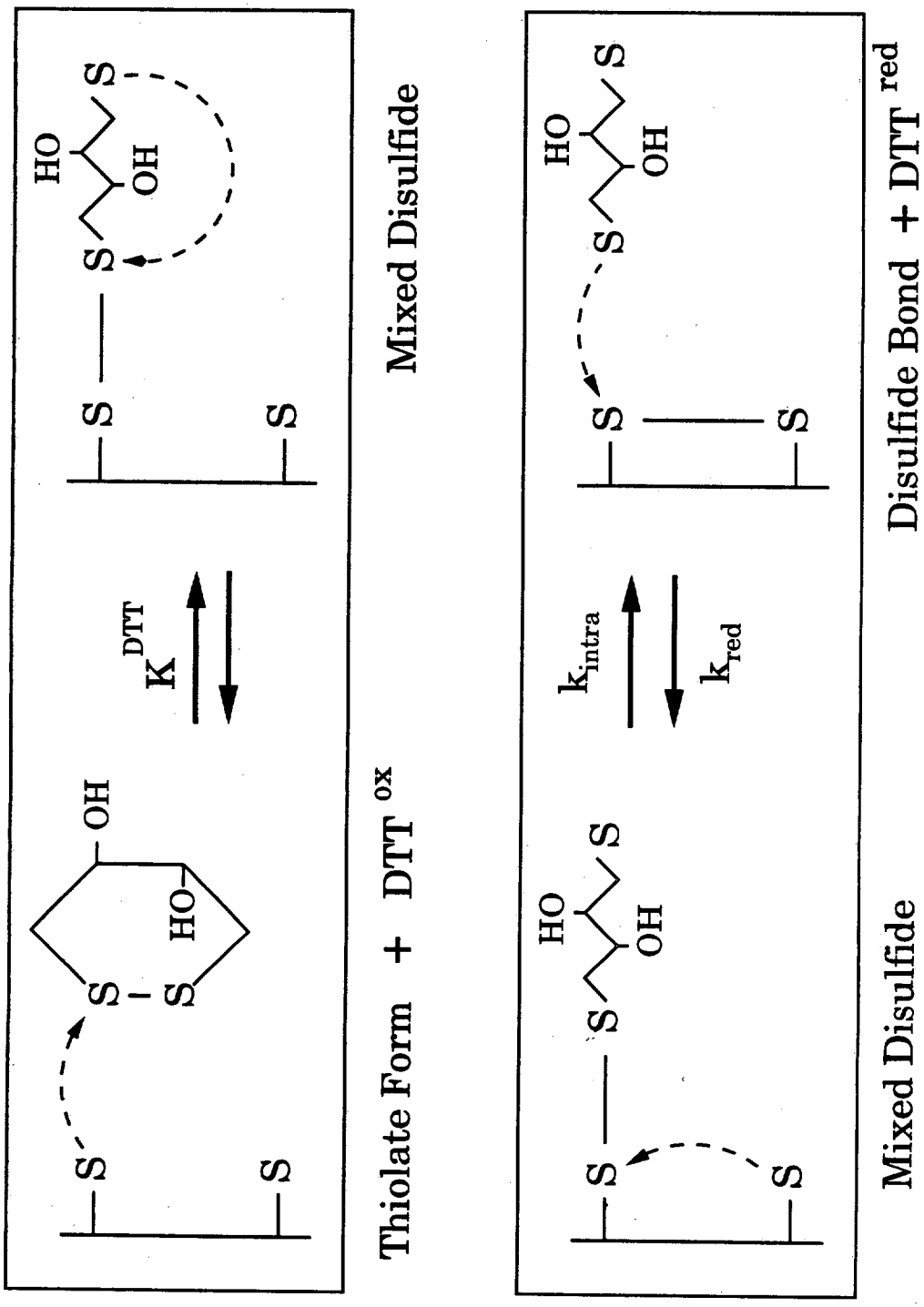
Figure A4. A three-step mechanism for the formation of N′ that is consistent with the experimental data (*5*). (a) In the first step, the protein forms a mixed disulfide bond with a DTT molecule, which is then buried in the protein (indicated by the oval in the diagram). Some evidence points to Cys72 as the cysteine involved in this buried mixed disulfide bond (*6*). (b) In the second step, the remaining thiolate of the bound DTT molecule forms a disulfide bond to another DTT molecule. (c) In the final step, the remaining free thiolate of the protein attacks the DTT-DTT disulfide bond, forming the crosslinked covalent adduct N′.

Figure A5. Expanded kinetic model of the oxidative folding of RNase A, showing individual disulfide species within each ensemble (*18*). The distribution of disulfide species within the 1S, 2S and 3S ensembles are in quasi-equilibrium, because the disulfide reshuffling reactions within these ensembles are much faster than the redox reactions between them. (The relative equilibrium populations of some species are indicated in the Figure.) Thus, the 1S, 2S and 3S ensembles can each be treated as a single kinetic species. By contrast, there is no equilibration among the disulfide species of the structured 3S* ensemble; each species lies on a separate kinetic pathway.

Figure A6. Cation-exchange chromatogram of the oxidative folding intermediates of RNase A in the absence and presence of 400 mM phosphate, respectively, after 90 minutes of regeneration at pH 8.0 and 25°C. The disulfide ensembles are cleanly separated. Two des species, des[40-95] and des[65-72] (indicated by diamonds in the upper chromatogram), are observed in the absence of phosphate, whereas the two other des species, des[26-84] and des[58-110], are not populated significantly. By contrast, all four des species (indicated by diamonds in the lower chromatogram) are populated in the presence of 400 mM phosphate; the indicated species are des[58-110], des[65-72], des[26-84] and des[40-95], in order of increasing elution times. Interestingly, the anomalous elution of des[40-95] in the 1S region

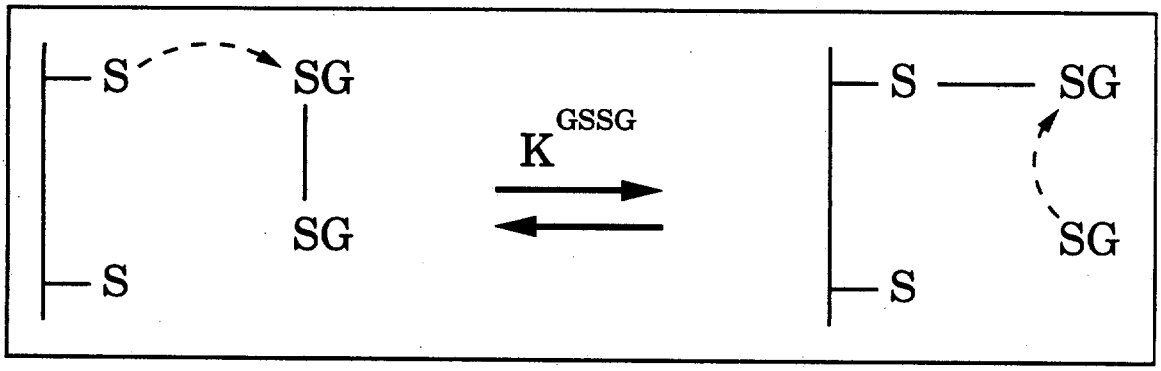
varies with the age of the column, eluting earlier (even in the 2S region) on newer columns (*18*).

Fig A1



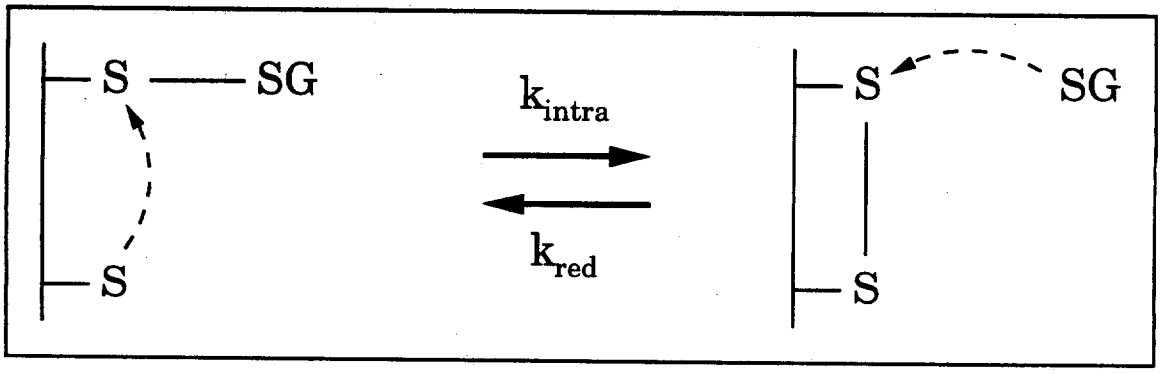
16

Fig A2



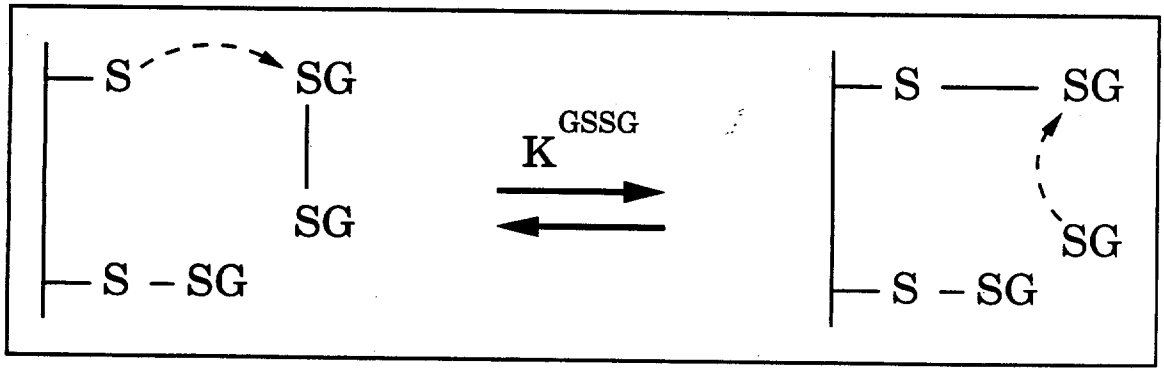
Thiolate Form + Oxidized Glutathione

Mixed Disulfide + Reduced Glutathione



Mixed Disulfide

Disulfide Bond + Reduced Glutathione



Mixed Disulfide + Oxidized Glutathione

Blocked Thiols + Reduced Glutathione

Fig A3

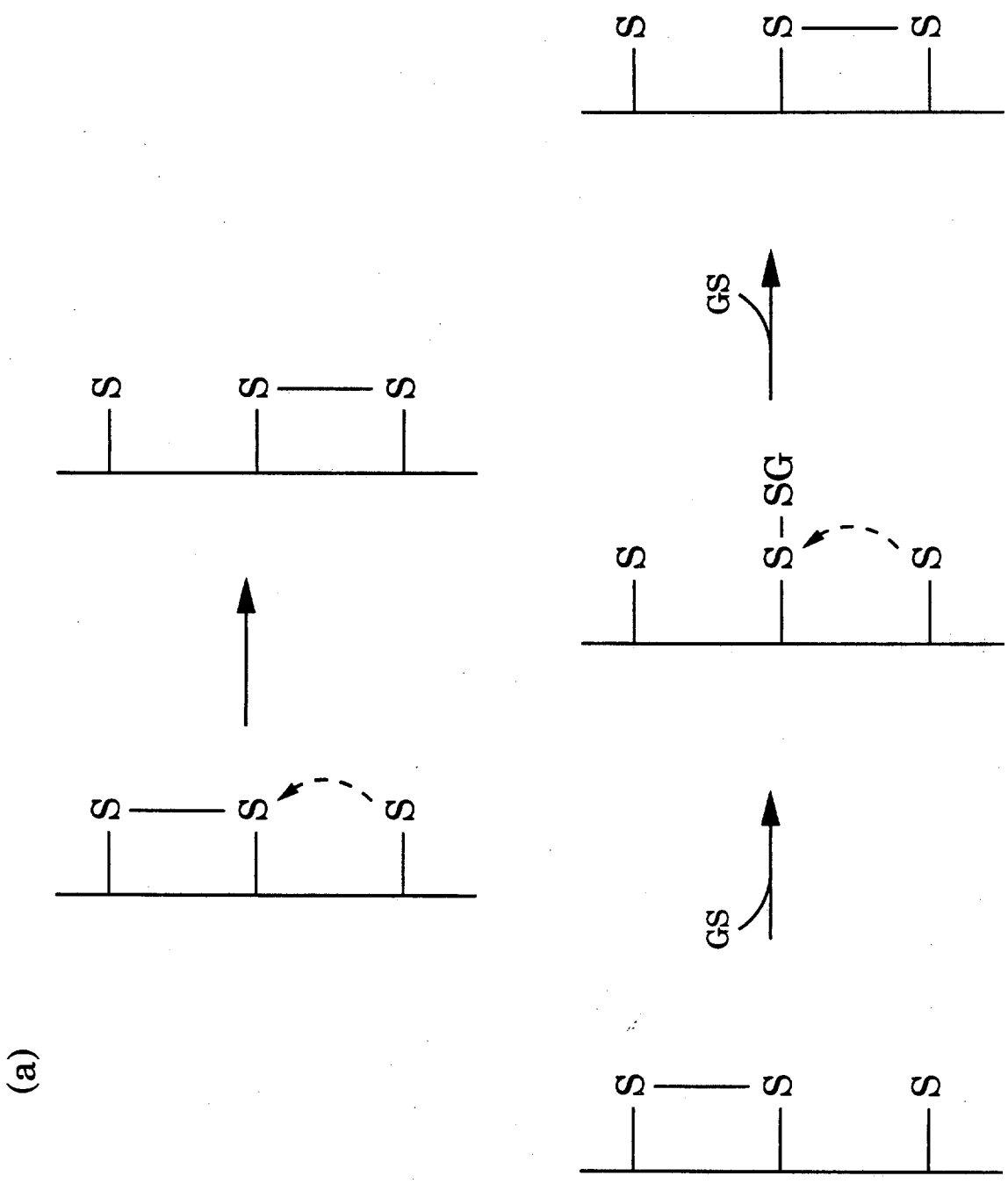
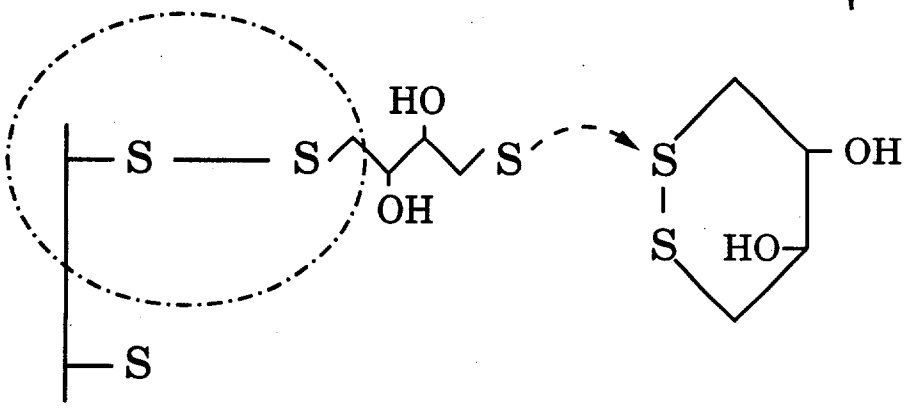


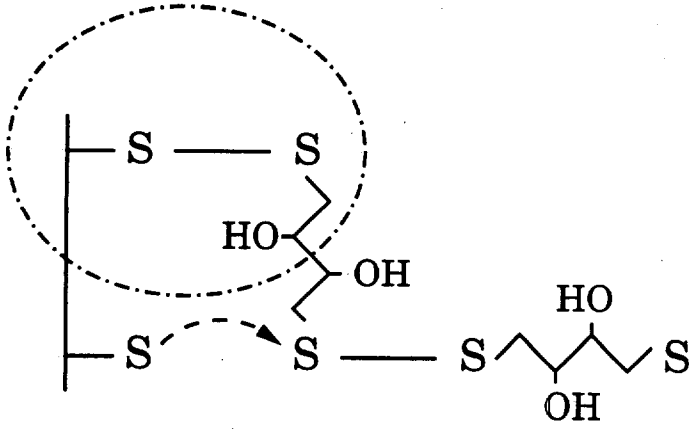
Fig A4

(a)



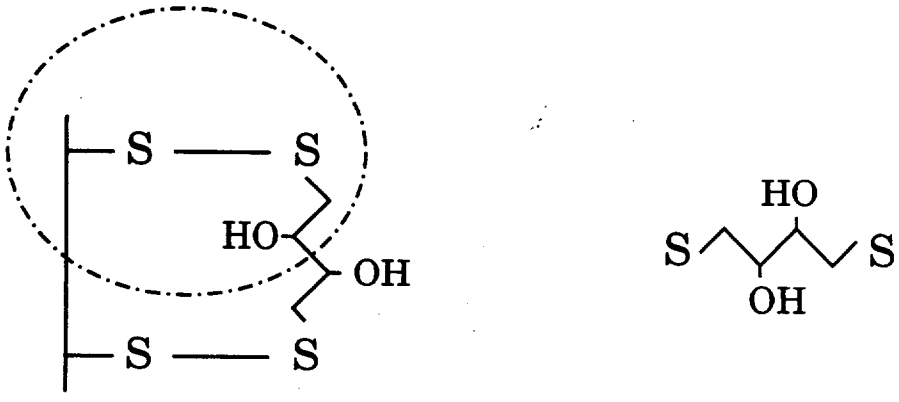
Buried Mixed Disulfide + DTT ^{ox}

(b)



Mixed Disulfide with DTT dimer

(c)



Covalent Adduct N$'$ + DTT ^{red}

Fig A5

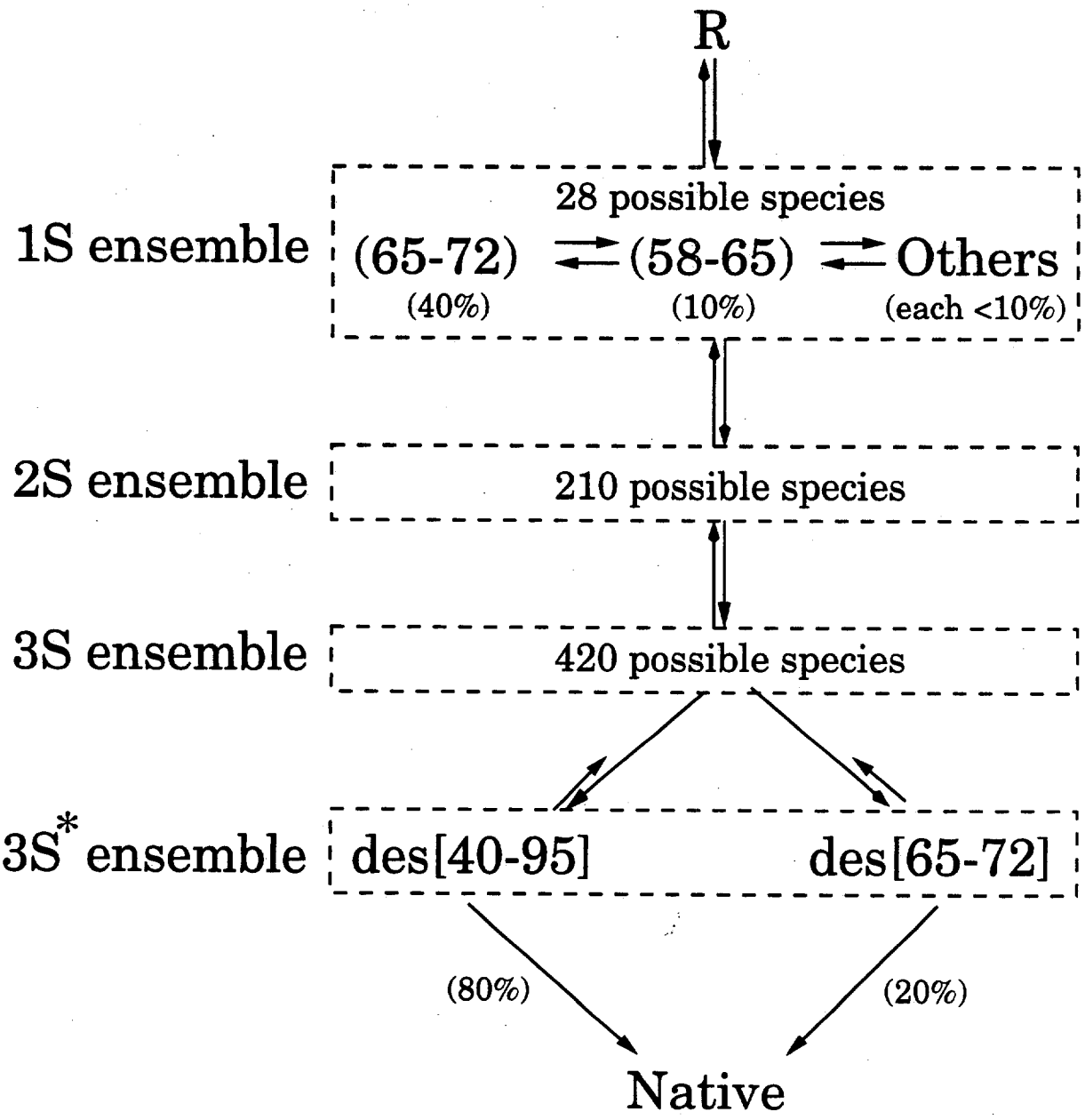


Fig 4b