# An Unusual Adduct of Dithiothreitol with a Pair of Cysteine Residues of a Protein as a Stable Folding Intermediate

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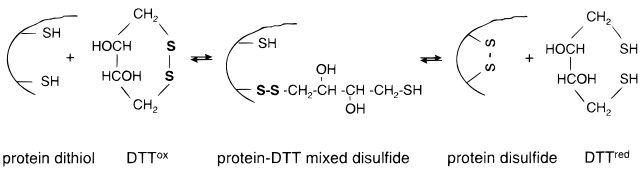
Dithiothreitol ($DTT^{red}$) and its oxidized form, *trans*-4,5-dihydroxy-1,2-dithiane ($DTT^{ox}$), a cyclic disulfide compound, have been used widely in redox-related protein studies,[1] especially those involving protein folding.[2−4] The major advantage of using $DTT^{ox}$ is that, unlike oxidized glutathione (GSSG) and other *linear* disulfide compounds, DTT does not form a stable mixed disulfide with protein thiols because of its rapid rate of recyclization (Scheme 1). This dramatically decreases the number of possible disulfide-bonded intermediates that must be considered, from 7192 to 763 in the case of regeneration of a four-disulfide protein, such as bovine pancreatic ribonuclease A (RNase A), and therefore significantly simplifies protein folding studies.[5,6] We report here a DTT adduct of RNase A in which an intramolecular disulfide loop is formed by a bridging DTT with two intermolecular disulfide bonds. This species serves as the first evidence that DTT can also form stable mixed disulfide intermediates with protein thiols by interacting with specific local residues. This result demonstrates the potential role that specific interactions between the redox reagent and the protein can play in directing folding pathways.

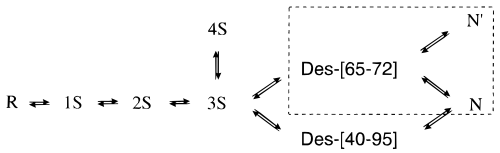
Regeneration of RNase A from its totally reduced form by $DTT^{ox}$ proceeds through parallel pathways requiring the formation of two partially folded native-like three-disulfide intermediates in the rate-determining steps, des-[65-72] missing the native 65-72 disulfide bond and des-[40-95] missing the native 40-95 disulfide bond[7,8] (Scheme 2, where R is the reduced protein and 1S, 2S, 3S, and 4S are ensembles of species containing one, two, three, and four disulfide bonds, respectively). In a series of regeneration experiments, the rate constant for the oxidation of des-[65-72] to native RNase A (N), des-[65-72] → N, was observed to depend on the redox conditions at 25 °C, pH 8.0. This surprising result can be attributed[8] to a species, referred to as N′, that forms during the oxidation of des-[65-72] with $DTT^{ox}$, coelutes with N on a cation exchange column, but can be reduced back to des-[65-72] by $DTT^{red}$ 25 000 times more rapidly than N.

A reduction-pulse experiment, carried out on the coeluting mixture of N and N′ using 1 mM $DTT^{red}$, led to the reduction of N′ without affecting N; this revealed that N′ accounts for 35% of the material obtained from direct oxidation of des-[65-72] that elutes in the position of the native protein on a Hydropore cation exchange column at 25 °C, pH 7. N′ can be separated from N, however, on the same column at an elevated temperature (55 °C) and can be purified further in two forms, N′1 and N′2, which have slightly different retention times. Thermal or guanidine

**Scheme 1**




**Scheme 2**




hydrochloride denaturation of the N and N′ mixture, in the absence of a redox couple, does not affect the distribution of N and N′. Equilibration for 10 days at pH 8 (25 °C) also does not change the distribution. However, the two purified N′ species are readily converted to N when a redox pair is present, indicating that the N′ species are dead ends in the folding pathway (Scheme 2) and that a direct pathway from the N′ species to N without reduction does not exist.

Both N′ species have a molecular mass of 13 835 which is the molecular mass of N (13 683) plus 152, determined by electrospray ionization mass spectrometry coupled with Fourier transform (ESI/FTMS), with a resolving power of $10^5$ (Figure 1). The difference of 152 in molecular mass between N and N′, ± 1.0, is exactly the molecular mass of $DTT^{ox}$, indicating that the N′ species are adducts of RNase A and $DTT^{ox}$ through two intermolecular (mixed) disulfide bonds, namely a bridging DTT.[9] The two N′ isomers, N′1 and N′2, result from the D- and L-forms of DTT. This was confirmed by carrying out an oxidation experiment using L-$DTT^{ox}$; the second N′ peak in the HPLC chromatogram was no longer observed.

Additional evidence for a covalent DTT adduct of RNase A came from a reduction-pulse experiment using dithioerythritol (DTE). One equivalent of $DTT^{ox}$ was released from the N′ species by incubation for 5 min with 3.3 mM DTE and quantitatively detected on a YMC $C_{18}$ minibore column. Pure $DTT^{ox}$, $DTT^{red}$, $DTE^{ox}$, and $DTE^{red}$, which have different retention times on the column, were used as references in the experiment.

No DTT adducts were detectable when similar oxidation experiments were carried out by starting with des-[40-95]. Des-[40-95] has a thermal transition temperature of 42 °C at pH 6.4, 2 °C lower than that of des-[65-72],[10] and its AEMTS-blocked form retains 5% RNase A activity in comparison to 75% for des-[65-72] [7]. Obviously, the formation of a stable mixed disulfide intermediate of DTT is highly sequence- or structure-dependent.

Scheme 3 shows the obligatory pathway by which N′ is formed from des-[65-72]. The first step is the formation of an intermediate, I′, containing a mixed disulfide bond between DTT and one of two cysteine thiols in des-[65-72] (I′ is also an obligatory intermediate in the formation of N). This mixed disulfide species undergoes another bimolecular reaction with a second $DTT^{ox}$ to form another intermediate, I″, which contains two DTT moieties and two mixed disulfide bonds. An intramolecular step ($k_{intra}$) results in formation of N′.

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(9) While a blocking agent, 2-aminoethyl methanethiosulfonate (AEMTS), was used in the preparation of the N′ species, and an AEMTS-blocked des-[65-72] with two cysteamine blocking groups would also give a molecular mass of 13 835, the retention time of such a blocked species on a cation exchange column is characteristically different from that of N and would not coelute with N (see ref 7).

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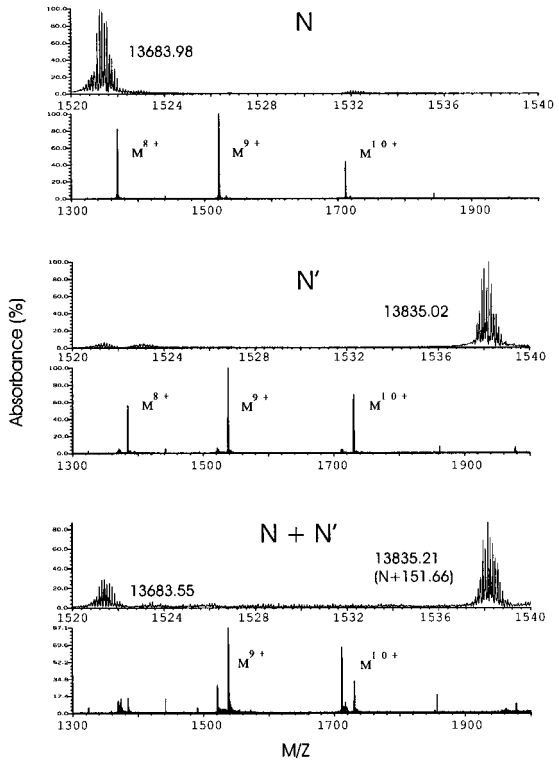
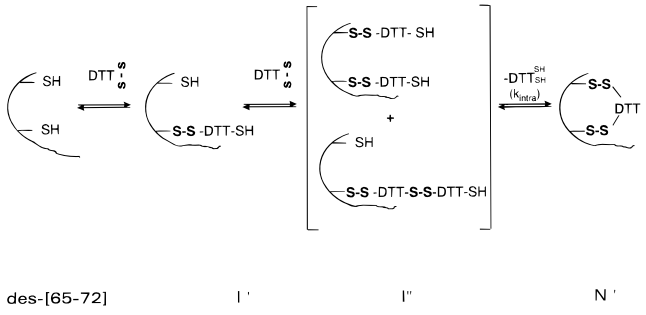



**Figure 1.** Mass determination of N′ using ESI/FTMS, sum of 25 scans. Lower panel: mixture of N and N′ material that coeluted on HPLC at 25 °C. Middle panel: N′ separated from the mixture at 55 °C. Upper panel: N separated from the mixture at 55 °C. The sample was prepared by direct oxidation of 32 $\mu$M des-[65-72] by 200 mM DTT$^{ox}$ at 25 °C, pH 8.0, followed by HPLC separation on a Rainin Hydropore SCX column at 25 °C, pH 7. The material eluting in the position of native RNase A was further fractionated on the same column, however, at 55 °C. The purity of the N′ species was better than 95%, determined by a 5-min reduction pulse with 1−5 mM DTT$^{red}$.

**Scheme 3**




The significant population of an adduct of DTT with a pair of cysteine residues of a protein is quite unexpected. The rate constant for recyclization of DTT[11] in a mixed disulfide at pH 8 (25 °C) is $1.5 \times 10^5$ min$^{-1}$. This corresponds to an *effective concentration*[11−14] of the second thiol of DTT in a mixed disulfide intermediate[11] of 1670 M. To form a species containing a bridging DTT (e.g., N′), it is necessary to form two mixed disulfides with DTT (as shown in Scheme 3). This should result in the rate of formation of N′ being ∼160-fold[11] slower than that of N, rather than the ∼2-fold which is observed experimentally. There are several mechanisms which can explain this discrepancy. From the kinetic analysis of the regeneration of RNase A, it has been determined that the formation of both N and N′ have a linear dependence on [DTT$^{ox}$]. This information along with the knowledge of the experimentally determined rate constants for formation of N and N′ can be used to distinguish among possible mechanisms. The rate constant for formation of the 65-72 disulfide bond in des-[65-72] is ∼1.5 times larger than the average rate constant for the formation of a disulfide bond in reduced RNase A. This indicates that the population of N′ does not result from a slow rate of formation of N. In addition, such an explanation would require that the rate of formation of N′ would have a second-order dependence on [DTT$^{ox}$]. The rapid formation of N′ could result from hyper-reactivity of Cys-65 and/or Cys-72, but this would fail to explain the observed [DTT$^{ox}$] dependence and would also be expected to accelerate the rate of formation of N to an equal extent. A third possibility, that the loop closing reaction ($k_{intra}$) is unusually rapid in N′, can also be excluded by the observed linear [DTT$^{ox}$] dependence. The simplest mechanism that fits the data is that the mixed disulfide bond in I′ (Scheme 3), once formed, is stabilized by a local conformation switch induced by specific interactions between the covalently bound DTT and the protein. This switch in the local conformation makes the mixed disulfide bond less accessible for the attack of the second thiol of DTT and retards its recyclizing to form DTT$^{ox}$ (and N). This first step, formation and stabilization of the mixed disulfide, is rate determining. Given that the formation of N′ from I′ is analogous to the formation of N from des-[65-72], it is likely that the I″ → N′ process is only slightly more rapid than the formation and stabilization of I′.

Prior to this paper, no stable mixed disulfides between proteins and DTT had ever been reported. The formation of the unusual bridging DTT adduct of RNase A appears to be the result of a stabilized DTT−protein mixed disulfide intermediate. The specific interactions that stabilize the intermediate are as yet unknown. However, the strength of the interaction presumably arises because it involves a unimolecular process, i.e., the effective concentrations of the interacting groups are quite high. Since the formation of mixed disulfides is required for the folding of disulfide-containing proteins, it is likely that strong specific interactions between the redox couple and the refolding protein are a common occurrence. Consequently, it is not the existence of significant interactions between the covalently bound redox reagent and the protein that is unusual but rather the ability to detect them. In the situation presented here, this arises as a result of a unique set of circumstances, viz., the binding of DTT covalently to the protein protects the mixed disulfide intermediate from exchange; the bound DTT contains a second thiol permitting formation of a bridging species which results in a fully oxidized protein thereby preventing intramolecular disulfide exchange with other protein thiols.

It is important to emphasize that this process is not restricted to DTT; other redox reagents such as glutathione (which is significantly larger than DTT) are likely to exhibit even more extensive interactions with the protein. Moreover, given the likelihood that such interactions will be local in nature, there is no reason to assume that they are restricted to highly structured species. Such interactions could occur at any stage in the folding process and, by preferentially protecting one cysteine from oxidation, direct the folding toward a particular pathway.

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**Author-Supplied Registry Numbers.** DTT$^{ox}$, 14193-38-5; DTT$^{red}$, 3483-12-3; L-DTT$^{red}$, 16096-97-2; DTE $^{red}$, 6892-68-8; RNase A, 9001-99-4.

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