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# Native Conformational Tendencies in Unfolded Polypeptides: Development of a Novel Method To Assess Native Conformational Tendencies in the Reduced Forms of Multiple Disulfide-Bonded Proteins

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The term oxidative folding describes the concomitant formation of the native disulfide bonds and the native tertiary structure of a protein from the reduced and unfolded polypeptide.[1] Much interest has centered on how the correct (native) disulfide-pairing is encoded in the linear sequence of the polypeptide chain.[2,3] A method is presented here for the elucidation of the "native tendency" of the reduced polypeptide chain to form the native set of disulfide bonds. We define "native tendency" as the propensity of the polypeptide chain to promote the direct formation of the full native set of disulfide bonds over non-native ones, and it is represented by the ratio of the formed native protein to the fully oxidized non-native (scrambled) species during the course of the oxidation. This definition stands in contrast to the usual oxidative folding experiments and other equilibrium measurements in which the native set of disulfide bonds are favored by another mechanism as well. In these latter experiments, the formed disulfide bonds keep rearranging, and it is the folded structure in the native protein and in some intermediates that favors the native set of disulfide bonds by protecting them from further rearrangement. The essence of our method is to oxidize the cysteines to form the disulfide bonds under conditions in which both reduction and disulfide reshuffling, which are essential to rearrange non-native disulfide bonds, are much slower than oxidation. When applied to bovine pancreatic ribonuclease A (RNase A), the method revealed little or no bias toward the formation of the *full* native set of disulfide bonds in the reduced forms of the protein, in the absence of disulfide rearrangement.

While related methods, such as FRET experiments or disulfide mapping of the 1S ensemble[4−6] (*n*S is an ensemble of disulfide-containing intermediates each having "*n*" disulfide bonds), specifically characterize the conformational ensemble of the reduced forms, "native tendency" reflects the conformational bias toward formation of the subsequent native disulfide bonds not only in the reduced, but also in the native, disulfide-bonded intermediates (1S and 2S in the case of RNase A). Here, we have exploited the ability of *trans*-$[Pt(en)_2Cl_2]^{2+}$ (where "en" is ethylenediamine) to oxidize the thiols faster than the rate at which thiol-disulfide exchange reactions can take place.[7] Because the reduced form of the oxidant, $[Pt(en)_2]^{2+}$, does not reduce disulfide bonds to any detectable extent, it is possible to arrange conditions under which practically no disulfide reduction can take place. *Thus, under our experimental conditions, only oxidation is possible with reduction and reshuffling reactions being inhibited*. We chose conditions, pH 4.7, under which disulfide reshuffling reactions are sufficiently slow (about 1000-fold less than at pH 8) but, under which, conformational folding of RNase A and its intermediates, which have been characterized,[8] is still favored. Because the rate of both the platinum oxidation and the reshuffling reactions depends on the thiolate concentration, and hence on the pH, the oxidation rate is thus expected to be faster than the reshuffling rate at higher pH as well. Therefore, the experiment can be carried out at pH's where the difference in the p$K_a$'s of the cysteines of the protein of interest has a smaller impact on the results. In fact, the experiments were also carried out at pH 7 and pH 8, and similar results were obtained as at pH 4.7.

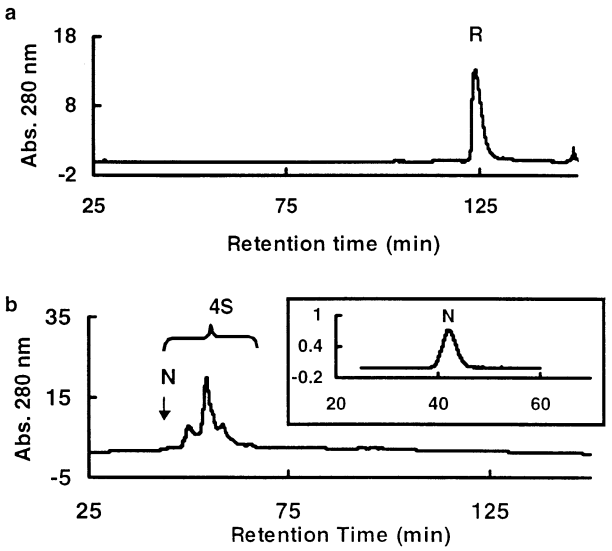


***Figure 1.*** (a) An aliquot of the starting reaction mixture containing reduced RNase A that was blocked with AEMTS. (b) An AEMTS-blocked aliquot of the above solution upon addition of the oxidant, *trans*-$[Pt(en)_2Cl_2]^{2+}$. The fully oxidized scrambled species 4S (indicated by a bracket) is the only ensemble that can be observed, with a small native peak indicated as N. The inset to Figure 1b shows an AEMTS-blocked aliquot of the reaction mixture (as in the main part of the figure) after adding 5 mM $DTT^{red}$. For clarity, only the region in which N elutes in the chromatogram has been enlarged and shown because N constitutes 0.6% of R and hence only 0.6% of the total four-disulfide containing species. The chromatograms in Figure 1a and b were obtained by injecting the desalted protein onto a Rainin Hydropore SCX cation-exchange column (pH 7) using HPLC as described previously.[8,9] The salt concentration was increased linearly from 50 to 150 mM NaCl over a period of 150 min in all cases.

Fully reduced RNase A (Figure 1a) dissolved in 100 mM acetic acid was incubated at room temperature in a Na-acetate buffer (pH 4.7, 0.4 M) containing *trans*-$[Pt(en)_2Cl_2]^{2+}$ at a final protein concentration of 170 $\mu$M. The concentration of the reagent was in 2-fold (or greater) excess above the stoichiometric concentration of RNase A thiols which allowed for the full oxidation of the protein in less than 5 min. To show that the oxidation was complete, any possible unreacted thiols were then rapidly blocked with 20 mM aminoethylmethylthiosulfonate (AEMTS)[8] at pH 8 and analyzed as described previously.[8,9] (*Trans*-$[Pt(en)_2Cl_2]^{2+}$ was prepared by chlorination of *trans*-$[Pt(en)_2Cl_2]$ and used directly in solution without further purification. The final concentration of *trans*-$[Pt(en)_2Cl_2]^{2+}$ was estimated by reacting it with $DTT^{red}$ at pH 5 and by absorbance at 332 nm. AEMTS was prepared as described previously.)[8] The blocking facilitates the detection of any partially oxidized intermediates (e.g., the 1S, 2S, or 3S species). As a control,


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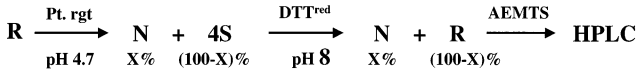
***Scheme 1***




the native protein, rather than the reduced form, was subjected to the same conditions with the reagent. No modification or reduction of the protein was detected, consistent with previous studies.[7,9]

Only the 4S and N (native ribonuclease A) species are observable on the chromatogram in Figure 1b, indicating the presence of only the terminally oxidized species of the protein. Because certain peaks of the scrambled 4S species partially overlap with the native peak on the chromatogram, the 4S species were converted to R (reduced RNase A) by a reduction pulse as described previously,[9] with the latter species well separated from the native protein after blocking with AEMTS (see Scheme 1).

This facilitates a more accurate quantitative estimation of the native protein (inset to Figure 1b). (The inset is enlarged to show the presence of N after a reduction pulse.) The ratio of native protein (N) to the fully oxidized scrambled ensemble (4S) can be estimated by comparing the areas represented by N and R (the latter being the amount of scrambled species originally present). This ratio was found to be 0.006.

Considering that there could theoretically be 104 possible fully oxidized (non-native) species, we found that the experimentally determined percentage of N does not seem to differ significantly from what it would be in a randomly formed disulfide distribution (ignoring the actual locations of the cysteine residues, this constitutes about 1%). Thus, little or no bias toward the formation of the native set of four disulfide bonds can be discerned in the reduced forms of the protein.

These results are of interest in the context of data gathered on the reduced forms of RNase A by other approaches. The reduced protein is known to have a nonrandom distribution of conformations which is biased toward a native-like topology under folding conditions,[10] and mapping of the 1S ensemble [when reduced and oxidized dithiothreitol, ($DTT^{red}$ and $DTT^{ox}$, respectively), were used as the redox couple] showed that some enthalpic bias toward the native disulfide bonds, especially toward the 65−72 disulfide bond, is present in the unfolded reduced protein.[4,5] Almost 50% of all disulfides present in the 1S ensemble have native pairings under folding conditions.[4] By extrapolation, the one-disulfide distribution of the 2S ensemble suggests that almost 90% of the fully oxidized 4S species have at least one native disulfide bond.[5] However, the native tendencies are apparently not sufficiently strong to favor the full set of native disulfide bonds over non-native ones without rearrangement of the disulfide bonds. Our data indicate that formation of the first native disulfide bond(s) does not increase the conformational bias toward the second and, successively, the third native disulfide bonds and that the lack of this mutual support between formation of disulfide bonds and conformational bias leads to formation of an overwhelming amount of fully oxidized, non-native (scrambled) species.

This is consistent with a picture of oxidative folding of RNase A[11] in which most of the intermediates lack stable structure, and conformational folding takes place only with the formation of a specific set of three native disulfide bonds.[1,9,11] By contrast, our results do not support an oxidative folding mechanism in which the native three-dimensional structure forms gradually, in parallel with the formation of the native disulfide bonds.[12]

Finally, we discuss the generality and limitation of the method. A limitation is that the oxidation reaction depends on factors other than the proximity of the cysteines, such as the $pK_a$'s of the individual cysteines. At lower pH, for example, 4.7, the histidines are protonated, which may affect the conformations of the reduced forms of the protein. This problem is solved by also carrying out the experiment at higher pH.

Not many multiple-disulfide-containing proteins have been characterized in a sufficiently detailed manner to help assess the reliability of this method. We chose RNase A because it is one of the best characterized proteins, which allowed our results to be cross-checked with data from other methods such as mapping of the 1S and 2S species. Thus, although the method was tested on only one protein, on the basis of this result, it appears to be applicable to any multiple disulfide-bond-containing protein, the only requirement being that the native protein be distinguishable from the scrambled fully oxidized species, a condition that does exist in most cases.

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