Acceleration of oxidative folding of bovine pancreatic ribonuclease A by anion-induced stabilization and formation of structured native-like intermediates

Lawrence K. Low, Hang-Cheol Shin, Mahesh Narayan, William J. Wedemeyer, Harold A. Scheraga*

Baker Laboratory of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853-1301, USA

Abstract Phosphate anions accelerate the oxidative folding of reduced bovine pancreatic ribonuclease A with dithiothreitol at several temperatures and ionic strengths. The addition of 400 mM phosphate at pH 8.1 increased the regeneration rate of native protein 2.5-fold at 15°C, 3.5-fold at 25°C, and 20-fold at 37°C, compared to the rate in the absence of phosphate. In addition, the effects of other ions on the oxidative folding of RNase A were examined. Fluoride was found to accelerate the formation of native protein under the same oxidizing conditions. In contrast, cations of high charge density or ions with low charge density appear to have an opposite effect on the folding of RNase A. The catalysis of oxidative folding results largely from an anion-dependent stabilization and formation of tertiary structure in productive disulfide intermediates (des-species). Phosphate and fluoride also accelerate the initial equilibration of unstructured disulfide ensembles, presumably due to non-specific electrostatic and hydrogen bonding effects on the protein and solvent.

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Key words: Protein folding; Ribonuclease A; Dithiothreitol; Neutral salt; Stabilized intermediate

1. Introduction

The oxidative folding of the four-disulfide protein bovine pancreatic ribonuclease A (RNase A) is under investigation in our laboratory. Two major [1,2] and two minor [3,4] pathways have been identified at 25°C and pH 8, involving the formation of two three-disulfide species, des-[65–72] and des-[40–95], which lack the 65–72 and 40–95 disulfide bonds, respectively, but which have native-like structure [5,6]. These two species have been found to be obligatory intermediates that oxidize directly to native protein in the presence of oxidized dithiothreitol (DTT^{ox}) [1]. Two additional intermediates with three native disulfide bonds, des-[26–84] and des-[58–110], become observable when oxidative folding is carried out at lower tem-

perature ($\sim 15°C$) [7]; however, it is not known whether these intermediates oxidize to native protein at a significant rate.

It has been proposed that the key step in oxidative folding of RNase A is generally the formation of intermediates with stable tertiary structure that protects the regenerated native disulfide bonds from reduction and reshuffling and, thus, favors oxidation to native protein [1]. Accordingly, our laboratory has been studying the effects on oxidative folding of factors that promote the formation of such stable tertiary structure; in this paper, we examine the effects of stabilizing phosphate salts and other neutral salts.

Salts can affect molecular interactions principally through three effects. First, salts may bind specifically to structured species; we will assume that they cannot bind specifically to unstructured species. Second, salts can weaken electrostatic interactions through charge screening. Generally, charge screening is independent of the nature of the ions and more dependent on the ionic strength of the ions present, e.g. screening distances for electrostatic interactions are believed to decrease with increasing salt concentrations up to 150 mM. Third, salts may influence hydrophobic interactions within a protein presumably by affecting water structure and protein hydration. The non-specific effects act on both structured and non-structured species, and are characterized by the lyotropic, or Hofmeister series. (In this series, anions of high charge density, kosmotropes, are known to bind tightly to water molecules and increase the surface tension of aqueous solutions; they induce hydrophobic interactions by preferentially hydrating a structured or non-structured species [8,9]. On the other hand, chaotropes, or ions of low charge density, have the opposite effect of enhancing the mobility of nearby water molecules and are known to destabilize proteins while increasing their solubility in solution. [8,9].)

Kosmotropes such as phosphate, sulfate, and fluoride are known to stabilize many proteins and polypeptides, presumably through general electrostatic and hydrogen bonding effects on the protein and solvent discussed above [8–14]. In addition, X-ray diffraction experiments show that phosphate and sulfate anions can bind specifically to RNase A sites [15]. Thermal denaturation experiments with phosphate and sulfate anions suggest that the increase in the stability of RNase A, e.g. the increase of its thermal transition temperature T_m by as much as 20°C, results from a combination of both specific binding and non-specific salt effects [10,11,16–18].

In this article, we report how variations in temperature and ionic strength of different salts can affect the oxidative folding of RNase A. To our knowledge, this is the first study provid-

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*Corresponding author. Fax: (1)-607-254 4700.
E-mail: has5@cornell.edu

ing evidence that anions of high charge density accelerate the folding of a protein by stabilizing and increasing the formation of productive intermediates.

2. Materials and methods

2.1. Materials

Native RNase A (type 1-A, Sigma) was reduced with Reduced dithiothreitol (DTT[red]; ultrapure, Sigma) as described previously [19]. DTT[ox] was obtained from Sigma and purified by the method of Creighton [20]. 2-Aminoethyl methanethiosulfonate (AEMTS) was prepared according to the method described by Bruice and Kenyon [21]. All other reagents obtained were of the highest grade commercially available.

2.2. Oxidative folding experiments with RNase A

Folding experiments involving RNase A were initiated by placing fully reduced RNase A (35 μM) under the following folding conditions: pH 8.1, 100 mM DTT[ox], 100 mM tris (hydroxymethyl) aminomethane hydrochloride (Tris–HCl), 2 mM ethylenediaminetetraacetic acid (EDTA), and 0–400 mM of a neutral salt (K_2HPO_4, $(NH_4)_2HPO_4$, Na_2HPO_4, NaCl, or KCl) or 400 mM of another neutral salt (NH_4Cl, NaF, $NaClO_4$, or $MgCl_2$), at temperatures ranging from 15 to 37°C. During the folding process, aliquots of the folding mixture were removed at various times, and the unreacted protein thiols were blocked with AEMTS [19], which allows the folding mixture to be fractionated by cation exchange HPLC on the basis of the number of cysteines blocked by AEMTS [19]. In addition to the conventional blocking procedure, some aliquots of the folding mixture were subjected to a 30-s reduction pulse of DTT[red] (final concentration of 15 mM) prior to AEMTS blocking as described previously [1]. The reduction pulse is weak enough to leave structured disulfide species intact, but unstructured species are reduced rapidly to the fully reduced state, R.

3. Results

3.1. Phosphate catalyzes the overall regeneration of native protein

Results from the oxidative folding experiments in the presence of phosphate salts showed that the rate of formation of native protein, N, is well approximated by a first-order rate equation, $\ln[1-N] = -kt$, where $[1-N]$ is the fractional concentration of all non-native species, k represents the rate constant for the formation of native protein, and t is the regeneration time (Table 1).

Once the concentration of phosphate exceeds 100 mM, the regeneration rate begins to increase (Fig. 1). At 400 mM phosphate, the folding rate increases by 2.5-fold at 15°C, 3.5-fold at 25°C, and 20-fold at 37°C, relative to the rate in the absence of phosphate (Table 2). This behavior was observed for all folding conditions between 15 and 37°C regardless of which cation was present in the phosphate salt (NH_4^+, K^+, or Na^+) in the folding mixture (Table 3). Our data sug-
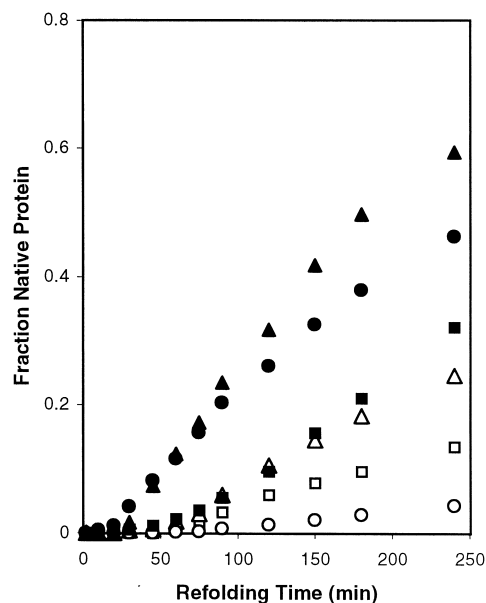

Fig. 1. Appearance of native protein as a function of folding time. The conditions were: 100 mM DTT[ox], 35 μM reduced RNase A, 100 mM Tris–HCl, 2 mM EDTA (pH 8.1). Folding is shown in the absence of phosphate at 15°C (□), 25°C (△), and 37°C (○), and in the presence of 400 mM phosphate at 15°C (■), 25°C (▲), and 37°C (●).

gest that the increase in the rate of formation of native protein results largely from the presence of phosphate anions in solution.

3.2. Phosphate catalyzes the pre-equilibration of the unstructured ensembles

The initial stage of oxidative folding involves an equilibration among the unstructured disulfide ensembles of RNase A [19], in which the protein is gradually oxidized without forming stable tertiary structure; however, some conformational order is evident in the unstructured ensembles [22]. In the absence of phosphate at 25°C and pH 8.1, the time to reach the pre-equilibrium steady state is approximately 90–100 min. However, when the phosphate concentration exceeds 100 mM, this pre-equilibration time begins to decrease; at 400 mM phosphate, the pre-equilibration time is roughly 60 min, indicating that the initial equilibration is also catalyzed by phosphate. Similarly, a decrease in pre-equilibration time (by 30–40 min) was observed for oxidative folding experiments with 400 mM phosphate at 15 and 37°C.

Table 1
Comparison of rate constants[a] for the regeneration of native protein at different concentrations of K_2HPO_4 at 25°C

Phosphate concentration (mM)	Rate constant $k \times 10^4$ (min^{-1})
0	12.2 ± 0.8
25	13.3 ± 0.9
50	13.2 ± 1.1
100	15.0 ± 1.2
250	28.2 ± 0.9
400	42.1 ± 1.2

[a]Initial folding conditions: 35 μM reduced RNase A, 100 mM DTT[ox], 100 mM Tris–HCl, 2 mM EDTA, pH 8.1.

Table 2
Observed rate constants[a] for the regeneration of native protein at several temperatures and two different concentrations of K_2HPO_4

Phosphate concentration (mM)	Temperature (°C)	Rate constant $k \times 10^4$ (min^{-1})
0	15	6.9 ± 0.6
	25	12.2 ± 0.8
	37	1.2 ± 0.2
400	15	17.8 ± 1.4
	25	42.1 ± 1.2
	37	24.5 ± 0.9

[a]The regeneration conditions were the same as in Table 1 except that only two different phosphate concentrations were used.

Table 3
Observed rate constants[a] for the regeneration of native protein in the presence of various neutral salts at 25°C

Neutral salt (400 mM)	Rate constant $k \times 10^4$ (min^{-1})
No neutral salt added	12.2 ± 0.8
NaCl	12.3 ± 0.8
KCl	12.5 ± 0.9
K$_2$HPO$_4$	42.1 ± 1.2
Na$_2$HPO$_4$	42.5 ± 0.9
(NH$_4$)$_2$HPO$_4$	40.4 ± 1.4
NaF	24.3 ± 1.1
NH$_4$Cl	6.3 ± 0.9
MgCl$_2$	6.2 ± 0.8
NaClO$_4$	2.0 ± 1.0

[a]Regeneration conditions were the same as in Table 1 except that the type of neutral salt was varied.

3.3. Phosphate stabilizes the structured des-species

Phosphate does not appear to change the distribution of disulfide bonds in the unstructured ensembles significantly, judging from their HPLC elution profiles (data not shown); however, the concentrations of the structured des-species (Fig. 2) relative to the unstructured three-disulfide (3S) ensembles are increased at all temperatures when the phosphate concentration exceeds 100 mM. This is consistent with the strong stabilizing effect of phosphate on the folded (native) wild-type protein. Furthermore, at concentrations of phosphate above 100 mM, the distribution of the three-disulfide des-species with native-like structure is changed significantly at all three temperatures studied (Fig. 2).

At 15°C, all four structured des-species are populated, both in the presence and in the absence of phosphate [7]. The effect of adding phosphate (>100 mM) is to increase the population of des-[40–95] relative to the other des-species.

At 25°C, only des-[65–72] and des-[40–95] are observable in the absence of phosphate. The effects of adding phosphate (>100 mM) are to populate the other two structured des-species (des-[26–84] and des-[58–110]) and again to enhance the populations of structured des-[40–95] and des-[65–72] relative to the 3S ensemble.

At 37°C, the two less stable des-species (des-[26–84] and des-[58–110]) are not observable at any phosphate concentration studied. In the absence of phosphate, des-[40–95] and des-[65–72] likewise have very low populations; this is presumably because 37°C is close to their thermal transition temperatures [23]. However, these species become significantly populated in the presence of phosphate (>100 mM).

The concentrations of the structured des-species relative to each other undergo slow changes even after the equilibration of the unstructured ensembles (Fig. 3). At 15°C, des-[26–84] and des-[58–110] slowly decrease in concentration, while des-[40–95] slowly increases. At 25°C, des-[26–84] and des-[58–110] also decrease in concentration while des-[40–95] and des-[65–72] increase. These slow changes in concentration continue throughout the oxidative folding.

3.4. Threshold phosphate concentration

The various effects of phosphate on the oxidative folding of RNase A are evident only when the phosphate concentration
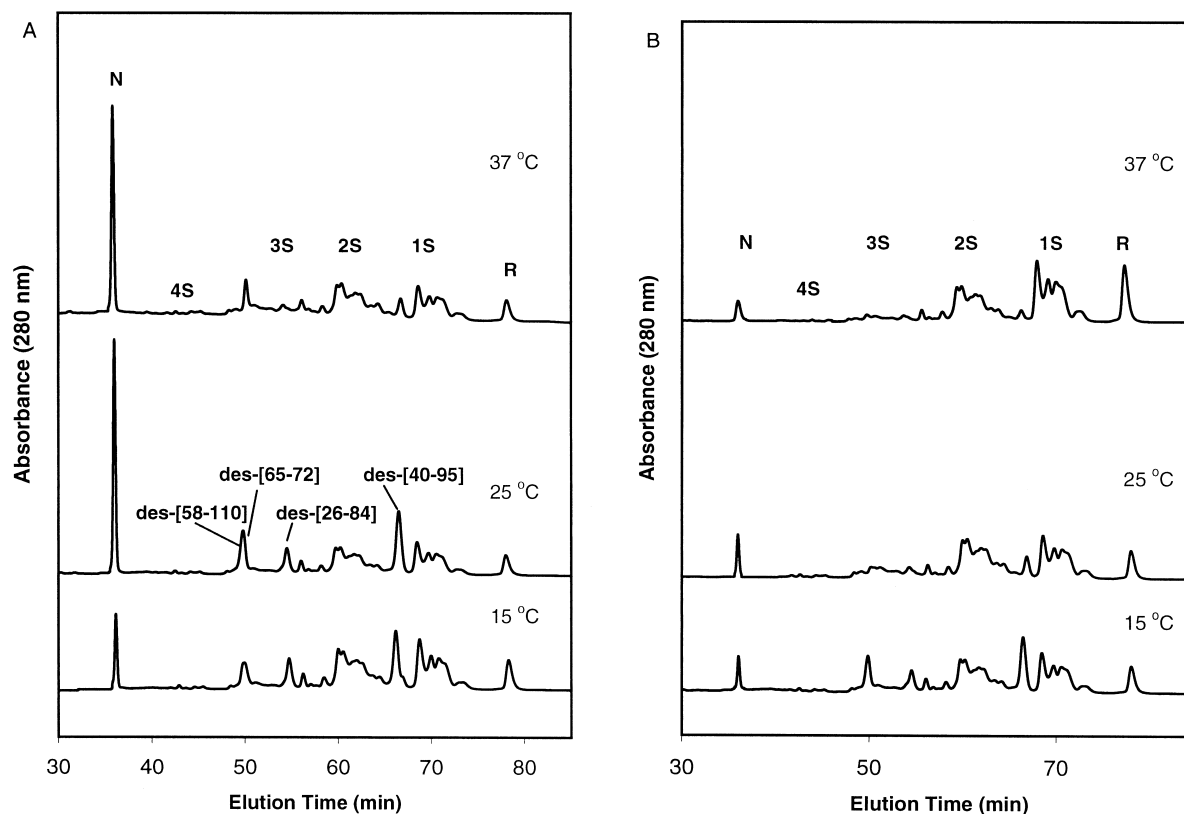

Fig. 2. Distribution of folding intermediates at different temperatures for folding experiments in the presence of 400 mM (A) and 0 mM phosphate (B), at 120 min. Reduced protein and native protein are represented by R and N, respectively. An Arabic numeral preceding S denotes the number of disulfide bonds in a grouping of intermediates. Conditions are the same as those specified in Table 1 with a phosphate concentration of 400 mM.
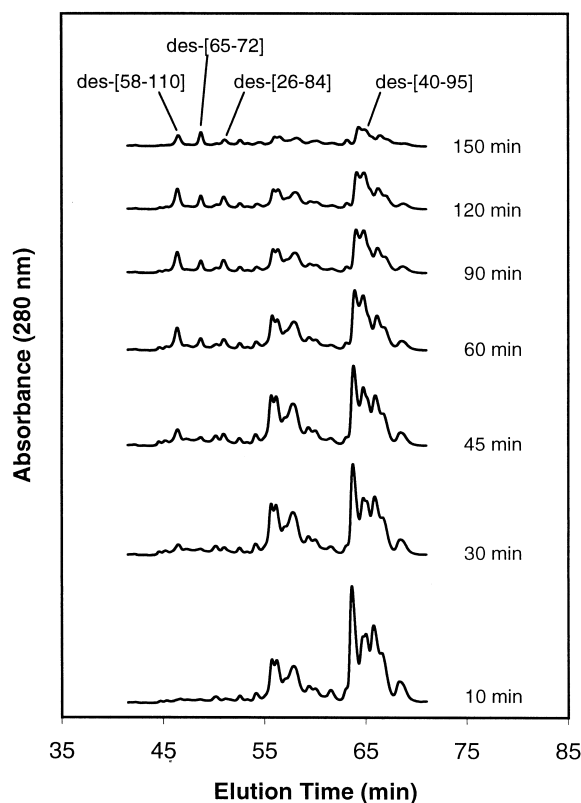
Fig. 3. Change in the distribution of three-disulfide intermediates as a function of folding time in the presence of 400 mM phosphate. Conditions are otherwise the same as in Table 1 (at 25°C). HPLC gradient conditions were modified to separate des-[58–110] and des-[65–72] which overlap in Fig. 2A. It should be noted that des-[40–95] co-elutes with the 1S grouping of intermediates.

exceeds a certain threshold (∼100 mM) (Fig. 4). Below this concentration, phosphate does not appear to affect the overall rate of regeneration, the rate of equilibration among the unstructured ensembles, or the distribution of 3S intermediates.

3.5. Effects of other neutral salts

Other experiments suggest that fluoride anions have a similar effect on the oxidative folding of RNase A. In the presence of 400 mM fluoride at pH 8.1 and 25°C, the pre-equilibration time for intermediates is roughly 60 min. Furthermore, the accelerated regeneration of native protein with 400 mM fluoride is accompanied by a stabilization of the productive native-like intermediates (Table 3 and Fig. 5). The stabilization of productive intermediates with fluoride is consistent with the results obtained from the phosphate experiments and previous studies showing that anions of high charge density can stabilize native conformations of proteins [11–14,16].

In contrast, neutral salts containing cations of high charge density (Mg^{2+}) or ions with low charge density (NH_4^+, ClO_4^-) appear to have an opposite effect on the folding of RNase A (Table 3 and Fig. 5). There was a large decrease in the rate of regeneration of native protein (2–6-fold decrease) in the presence of these ions at 400 mM ion concentration. Interestingly, the decrease in the rate of regeneration of native protein is accompanied by an additional lag time for intermediates to pre-equilibrate (45–60 min longer than refolding experiments without the ions present) and a significant decrease in the

formation of productive native-like intermediates. Populations of des-[40–95] and des-[65–72] are reduced in the presence of such ions.

Experiments carried out with chloride and a counterion, potassium or sodium, at ion concentrations below 1 M showed no changes in the rate of formation of native protein (Table 3) and no changes in the distribution of intermediates (data not shown) compared to the control refolding experiments without the addition of the neutral salts.

4. Discussion

Our data suggest that des-[26–84] and des-[58–110] do not contribute significantly to the regeneration of native protein. The increases in overall regeneration rate upon addition of phosphate are similar at 15°C and 25°C (2.5-fold and 3.5-fold, respectively). However, the concentrations of des-[26–84] and des-[58–110] change significantly at 25°C while they do not at 15°C. Thus, the stabilization of these species does not seem to enhance the regeneration of native protein significantly. Moreover, the 20-fold increase in the regeneration rate at 37°C with phosphate is not associated with an observable stabilization of these two des-species. A slow oxidation rate for these species is structurally plausible because the [26–84] and [58–110] disulfide bonds are buried in the core of the native protein [15]; presumably, the corresponding thiol groups are likewise buried in the des-protein and, thus, difficult to oxidize.

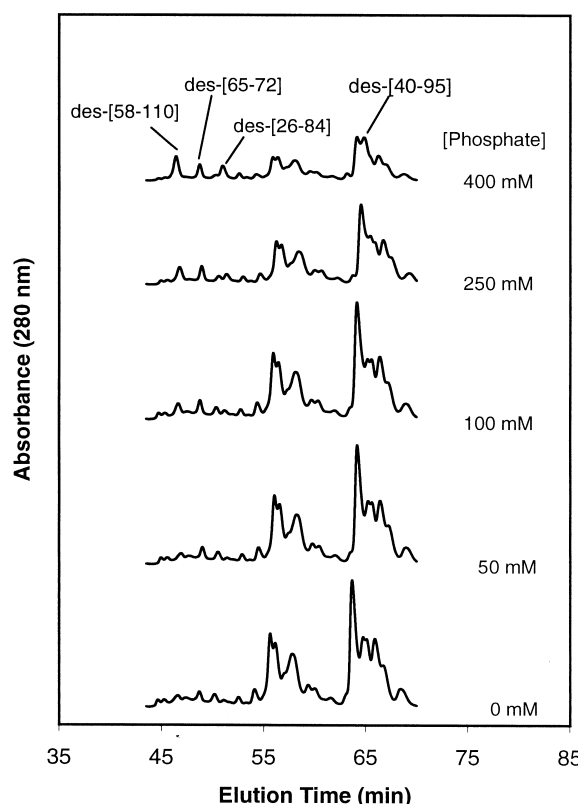Accordingly, our data suggest that the conformational sta-

Fig. 4. Change in the distribution of three-disulfide intermediates at 120 min folding time as a function of phosphate concentration at 25°C. Conditions are the same as specified in Table 1. HPLC gradient conditions are the same as in Fig. 3.
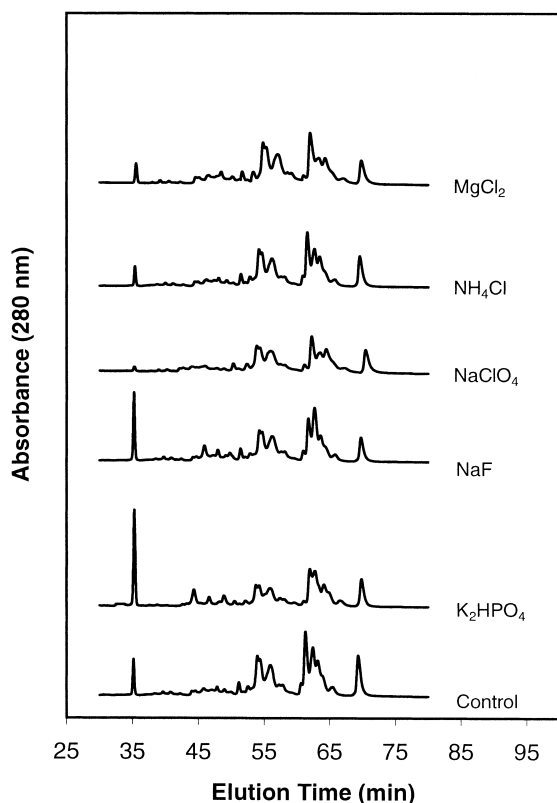
Fig. 5. Distribution of intermediates for different neutral salts (400 mM) at 90 min folding time and 25°C. Conditions are the same as those in Table 1 except that the type of neutral salt content was varied. The gradient conditions are the same as in Figs. 3 and 4.

bilization of des-[65–72] and des-[40–95] contributes to the catalysis of the regeneration of native protein. This is particularly evident at 37°C, where the regeneration rate in the absence of phosphate is low, due to the conformational instability of these des-species [1]. In the absence of stable tertiary structure, the disulfide bonds of these species can easily be reduced and/or reshuffled. However, when phosphate or fluoride is added, the tertiary structure is stabilized, protecting the native disulfide bonds and thus enhancing the populations of these des-species. The enhanced population then oxidizes rapidly to native protein, since these species are known to be productive intermediates, especially des-[40–95] [1].

In addition, our data indicate that phosphate and fluoride anions enhance the population of these des-species not only by slowing their reduction and reshuffling but also by promoting their formation. This is evident at low temperatures, where the backward rate (des-species → 3S) is already negligible compared to the forward rate [24]. Based on previously fitted rate constants [2], setting the backward rate to zero at 15°C yields a roughly 20% increase in the regeneration rate which is much smaller than the observed 2.5-fold increase with phosphate.

Given the pre-equilibration data, neutral salts also appear to have a general non-specific effect of catalyzing early stages in the oxidative refolding of RNase A. A 50% increase in pre-equilibration rate is observed when oxidative folding is initiated at several temperatures in the presence of phosphate or fluoride anions. Apparently, since the kosmotropes cannot stabilize the unstructured intermediates involved in the pre-equilibration process, they may influence the folding behavior of the unstructured species by condensing the protein through increased hydrophobic interactions, thus increasing the effective intramolecular concentrations of thiol and disulfide groups for oxidation and reshuffling. Chaotropes (NH_4^+, ClO_4^-) would have the opposite effect of slowing the pre-equilibration rate (a 50% decrease was observed) by disfavoring hydrophobic interactions, thus resulting in a more expanded conformation of the protein.

Specific binding of kosmotrope anions to intermediates with native-like structure may occur, and this would help stabilize the des-species from reshuffling back to the unstructured 3S intermediates at higher temperatures ($> 37°C$). However, as noted above, this specific binding effect accounts for only a 20% increase in regeneration rate at lower temperatures ($< 25°C$); along with a 50% rate increase contributed from the faster pre-equilibration of unstructured intermediates, the combined rate increase accounts for a 70% overall rate increase which is lower than the 2–3.5-fold increase in regeneration rate observed with phosphate and fluoride. This suggests that kosmotrope anions have a general effect of catalyzing the forward reaction rate from 3S to des-species, judging from the increase in formation of des-species observed in the presence of kosmotrope anions (chaotropes would have the opposite effect of slowing the forward reaction). Such anions may aid in stabilizing precursor intermediate states involved in the reshuffling step or in folding intermediate species as suggested by previous conformational studies and thermodynamic considerations [25,26]; however, additional experiments are being carried out to confirm the actual mechanism of catalysis by various anions.

In conclusion, kosmotrope anions have several important effects on the oxidative folding of RNase A. They accelerate the initial equilibration among the unstructured ensembles. They also catalyze the regeneration of native protein by stabilizing productive intermediates such as des-[40–95] and des-[65–72], both by promoting their formation and by inhibiting their unfolding and subsequent reduction/reshuffling. Our studies suggest that the stabilization and accelerated formation of productive native-like intermediates by kosmotropes is caused by a combination of both specific and non-specific effects on the solvent and the protein.

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