Nonenzymic Reactivation of Reduced Bovine Pancreatic Ribonuclease by Air Oxidation and by Glutathione Oxidoreduction Buffers*

(Received for publication, June 17, 1974, and in revised form, March 26, 1975)

A. Karim Ahmed,$ Stephen W. Schaffer,§ and Donald B. Wetlauffer~!

From the Department of Biochemistry, University of Minnesota Medical School, Minneapolis, Minnesota 55455

With the glutathione system that leads to rapid regeneration of reduced lysozyme (Saxena, V. P., and Wetlaufer, D. B. (1971) Biochemistry 9, 5015), reduced pancreatic ribonuclease (RNase) regenerated activity in high yield (>90%) but at a considerably lower rate (t½ ~75 min). Systematic examination of the effects upon regeneration of the concentrations and ratios of reduced and oxidized glutathione (GSH and GSSG) showed the same broad optima for RNase as were earlier found for lysozyme: [GSSG] = 5 x 10⁻⁴ M, [GSH] = 5 x 10⁻³ M. Regeneration of reduced RNase by air oxidation was shown to be inhibitable by 10⁻⁴ M EDTA, whereas the glutathione regeneration was unaffected by EDTA. In addition the air-oxidative regeneration showed a strong temperature dependence, in contrast with the glutathione system. The mechanisms of these two kinds of regenerations are therefore different.

Six potentially catalytic metal ions were tested in the air-oxidative regeneration of RNase: Cu⁺⁺, Co⁺⁺, Mn⁺⁺, Fe³⁺, Zn⁺⁺, and Ni⁺⁺. Of these, only Cu⁺⁺ enhanced the rate of regeneration of RNase activity, although both Cu⁺⁺ and Co⁺⁺ catalyzed thiol oxidation of reduced RNase. The rates and yields of RNase regenerations were independent of protein concentration from 3 x 10⁻⁷ M to 1.2 x 10⁻⁵ M in the glutathione system. Preincubation of freshly dissolved reduced RNase under nonoxidizing conditions before adding glutathione did not change the rate or extent of regeneration. Studies of its pH dependence showed that the glutathione regeneration depends on the deprotonation of prototropic groups with 7.5 < pK < 8.0.

The major ion exchange chromatographic peaks from glutathione and air-oxidative regenerations appeared to be identical with native RNase, by the criteria of specific activity, chromatographic mobility, and circular dichroic spectra. The glutathione system permits regeneration at much higher RNase concentration than the air regeneration, with rates and yields comparable to the greatest reported for air regeneration.

The mechanisms governing formation of three-dimensional structure in proteins continue to be a focus for investigation and speculation (1–3). Work from this laboratory has established that thiol disulfide oxidoreduction buffers will facilitate the rapid regeneration of enzymic activity from reduced hen egg lysozyme and reduced human lysozyme (4, 5). It was a matter of considerable interest to investigate glutathione regenerations of reduced pancreatic RNase, since it was with this enzyme that Anfinsen and co-workers carried out their landmark regenerations of native protein from a reduced, disorganized precursor (1). Indeed, RNase was first used to investigate both the air-oxidative regeneration and the enzymic ("shuffle-ase") regeneration (6, 7). Since the physiological role of the enzymic mechanism is still in doubt (3, 8), we have here chosen to compare the air regeneration with glutathione regeneration of reduced RNase. The present paper reports a comparison of the two kinds of regenerations, provides evidence that they are mechanistically different, and shows the results of a first round of optimization of experimental variables in glutathione regenerations.

EXPERIMENTAL PROCEDURES

Materials—Bovine pancreatic RNase A was obtained from Sigma Chemical Co., as type XI-A, Lot 106B-8800. It was used without further purification except in the experiments of Figs. 3 and 5.
β-Mercaptoethanol was an Eastman product and was redistilled before use. Tri(2-hydroxyethyl)aminomethane was obtained as Trizma base from Sigma. All other materials were obtained from Commercial sources.

Glass-distilled water (from a still pot containing 0.2 M H₂SO₄, fed with deionized water) was used in all regeneration experiments. Water was obtained from a still using glass-distillation equipment. Glassware was cleaned by a scrubbing procedure, detailed elsewhere (9). The last two steps involve several rinses with 0.1 M HCl, followed by several rinses with glass-distilled water.

RNase Assay—RNase activity was determined by the method of Kalnitsky et al. (10), using yeast RNA as substrate. The assay was carried out at pH 4.0, 30° for 60 min in 0.5 ml of 100 mM both reduced and native RNase. Controls were carried out every day the assay was used, with the calibrating digests containing the same concentrations of reagents as were introduced from the sample aliquot (GSH, GSSG, EDTA, etc.). In a few of the later experiments a continuous recording assay method suggested by Fletcher and Hash (11) was employed. It had the advantages of improved convenience and somewhat better precision. Control assays were carried out to recalculate for each experiment.

Sulfhydryl Assay—The colorimetric method of Ellman (12) was used. Control experiments showed that addition of various reagents employed in the regeneration mixtures did not interfere with the assay. Protein concentration was determined by ultraviolet absorbance (ε, used. Control experiments showed that addition of various reagents advantages of improved convenience and somewhat better precision. Control assays were carried out to recalculate for each experiment.

Metal Ion Catalysis—The regenerations were carried out at 30° with pH 8.2, 0.09 M Tris-acetate buffer, initial concentration of reduced RNase 10⁻⁴ M. The chloride salts of Cu⁺⁺, Cu⁺⁺, Zn⁺⁺, Fe⁺⁺, and Mn⁺⁺ were tested at 10⁻⁵, 10⁻⁶, and 10⁻⁷ M. Ni⁺⁺ was used in the form of its nitrate at the same concentrations.

Circular Dichroic Spectra—Measurements were made on a Durrum-Jasco J-10 circular dichrometer, calibrated according to the method of Cassim and Yang (15). Silica cells of 0.10- and 1.00-cm path length were used. Frequent base-line recordings were made with the same solvent as in the protein solution of interest. RNase from the major peak of each of the three chromatograms of Fig. 5 was examined, each in the solvent in which the peak was eluted from the column: 0.005 M Tris-chloride, pH 8.0/0.10 M NaCl. Spectra were measured on samples at 23-25°.

RESULTS AND DISCUSSION

Regeneration of activity from reduced RNase with the optimal concentrations of GSH and GSSG proceeded to a 4-hour yield of 70% in the early experiments (Fig. 1). In later experiments, yields were commonly in the range 80 to 90% (Figs. 2 and 3). The “half-time” t₅₀, the time for regeneration 50% of the specific activity obtained with unreduced starting material, was generally in the neighborhood of 75 min with the optimal glutathione system (Figs. 1 to 3). Results from the trials for determining optimal concentrations and ratios of GSSG and GSH are shown in Table I. Although the testing here was not as extensive as that employed for optimization of the regeneration of human and hen egg lysozyme (4, 5), the same broad optima appear to apply to RNase as were found for the lysozymes: [GSSG]/[GSH] ranging from 1/10 to 1, and [GSH] + [GSSG] ranging from 1 to 10 mM.

Fig. 1A shows the results of regenerations carried out by Cu⁺⁺-catalyzed air regeneration (t₅₀ = 220 min), by EDTA-inhibited air regeneration where the inhibition is virtually complete, and two sets of glutathione regeneration data which lie on a single curve, showing t₅₀ = 80 min. Since one of the glutathione regenerations was carried out in the presence of 10⁻⁴ M EDTA while the other regeneration contained no EDTA, it is apparent that EDTA is not an inhibitor of the glutathione regeneration.

To test whether air-oxidative regeneration may also be taking place in a glutathione regeneration we carried out the regenerations of Fig. 1B. Here three glutathione regenerations were compared, one an optimal glutathione system, the second the same as the first plus 10⁻⁷ M Cu⁺⁺, and the third the same as the first plus 10⁻⁴ M Cu⁺⁺ and 10⁻⁴ M EDTA. The results of all three regenerations are seen to give the same regeneration curve. Neither deliberate “contamination” of a glutathione regeneration with cupric ion nor the addition of EDTA to such
Fig. 1. Regeneration of reduced RNase by air oxidation and by glutathione systems. All experiments were carried out at 30° in 0.09 M Tris-acetate, pH 8.2, and the protein concentration was 0.020 mg/ml. In Panel A, X represents an "air oxidation" regeneration in which (CuSO₄) = 10⁻⁴ M; • represents an "air oxidation" regeneration to which 10⁻³ M Na₂EDTA had been added, ○ represents a glutathione-facilitated regeneration system in which [GSSG] = 3 x 10⁻⁴ M, [GSH] = 3 x 10⁻³ M, (Na₂EDTA) = 10⁻³ M. In Panel B, ○ represents a glutathione-facilitated regeneration in which (GSSG) = 3 x 10⁻⁴ M, (GSH) = 3 x 10⁻³ M, (CuSO₄) = 10⁻³ M; in □ (GSSG) = 3 x 10⁻⁴ M, (GSH) = 3 x 10⁻³ M, (CuSO₄) = 10⁻³ M, and (Na₂EDTA) = 10⁻³ M.

FIG. 2. Reactivation of reduced RNase at different temperatures by air oxidation (Panel A) and by a glutathione system (Panel B). Both sets of reactivation solutions contained 0.09 M Tris-acetate, pH 8.2 at 25°, protein concentration 0.020 mg/ml. The experiments of Panel A had no other components added, while those of Panel B contained [GSSG] = 3 x 10⁻⁴ M, [GSH] = 3 x 10⁻³ M.

Table I: Dependence of reactivation of reduced RNase on GSSG and GSH concentrations

<table>
<thead>
<tr>
<th>[GSH]</th>
<th>[GSSG]</th>
<th>[GSH]/[GSSG]</th>
<th>[GSH]/[Protein]</th>
<th>[GSSG]/[Protein]</th>
<th>Percent activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>M</td>
<td>30 min</td>
<td>90 min</td>
<td>30 min</td>
<td>90 min</td>
</tr>
<tr>
<td>3 x 10⁻⁴</td>
<td>0</td>
<td>400</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>3 x 10⁻³</td>
<td>3 x 10⁻³</td>
<td>5</td>
<td>400</td>
<td>80</td>
<td>15</td>
</tr>
<tr>
<td>3 x 10⁻²</td>
<td>3 x 10⁻⁴</td>
<td>0.5</td>
<td>400</td>
<td>80</td>
<td>22</td>
</tr>
<tr>
<td>3 x 10⁻¹</td>
<td>3 x 10⁻⁴</td>
<td>5</td>
<td>40</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>6 x 10⁻¹</td>
<td>3 x 10⁻⁴</td>
<td>0.1</td>
<td>80</td>
<td>80</td>
<td>16</td>
</tr>
<tr>
<td>0</td>
<td>3 x 10⁻⁴</td>
<td>0</td>
<td>0</td>
<td>80</td>
<td>0</td>
</tr>
</tbody>
</table>

*These ratios are expressed on the basis of equivalents of thiol and disulfide.

a "contaminated" glutathione regeneration has an effect on the regeneration. These results show that metal ion-catalyzed regeneration does not occur to any detectable extent during an optimal glutathione regeneration. It is likely that Cu²⁺ and other potentially catalytic metal ions are chelated either by GSH or by GSSG, or both, in a catalytically impotent form.

Fig. 2 shows the contrasting responsiveness to temperature of the air oxidation (Fig. 2A, left panel) and the glutathione system (Fig. 2B, right panel). The air-oxidative regeneration is inhibited strongly above room temperature: at 25° t₅₀ = 75 min, while at 30° t₅₀ = 190 min. These findings confirm the general trend reported (6) for air regenerations of RNase. Regeneration via the glutathione system is much less temperature-sensitive: at 14°, t₅₀ = 140 min, while at 37° t₅₀ = 70 min. Of the temperatures examined, 37° is optimal for rapid regeneration, but the optimum is clearly broad, with only minor variations in rate between 25 and 40°. The finding of a maximum regeneration rate of reduced RNase at or near 37° is in contrast with the inhibition of the air oxidation in this temperature range, and increases the physiological plausibility of the glutathione system. The temperature dependence of RNase regeneration by the protein thiol oxidoreductase does not appear to have been determined. The strong difference observed in temperature dependence between air regenerations and glutathione regenerations also implies that the two regenerations operate by different mechanisms. This conclusion was reached earlier for regenerations of hen egg lysozyme (4).

Of the six potentially catalytic metal ions examined, only Cu²⁺ and Co²⁺ showed a clear-cut acceleration of thiol oxidation (at concentrations between 10⁻⁷ and 10⁻⁴ M). In a comparison of these ions' effects on regeneration of RNase activity (Table II) we found that 10⁻⁴ M Cu²⁺ accelerated both —SH oxidation and regeneration of RNase activity, while Co²⁺ catalyzed —SH oxidation without affecting regain of activity. Of the metal ions tested, it is clear that only Cu²⁺ is
effective in catalyzing the air regeneration of RNase. Other experiments showed that the regeneration rate is invariant and reproducible over the [Cu²⁺] range from 10⁻¹⁷ to 10⁻⁶ M, but is strongly inhibited at 10⁻¹⁷ M.

Since catalysis of regeneration is marked at 10⁻¹⁷ M, it would not be surprising to find detectable catalysis at substantially lower Cu²⁺ concentrations. It also seems likely that copper is the trace metal commonly responsible for adventitious catalysis of air regenerations of reduced proteins (6, 16, 17). Our experiments showed that the regeneration rate is invariant and reproducible over the [Cu²⁺] range from 10⁻¹⁷ to 10⁻⁶ M.

Comparison of effects of Cu²⁺ and Co²⁺ on air oxidation and regeneration of reduced RNase

<table>
<thead>
<tr>
<th>Regeneration solution*</th>
<th>—SH/mol protein at 70 min</th>
<th>Percent activity regained at 120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.4</td>
<td>16</td>
</tr>
<tr>
<td>+ 10⁻⁹ M Co²⁺</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>+ 10⁻⁸ M Co²⁺</td>
<td>1.7</td>
<td>16</td>
</tr>
<tr>
<td>+ 10⁻⁷ M Cu²⁺</td>
<td>1.3</td>
<td>44</td>
</tr>
<tr>
<td>+ 10⁻⁶ M Cu²⁺</td>
<td>1.0</td>
<td>52</td>
</tr>
<tr>
<td>+ 10⁻⁵ M Cu²⁺</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>+ 10⁻⁴ M Cu²⁺</td>
<td>1.4</td>
<td>5</td>
</tr>
</tbody>
</table>

*Regeneration solutions all contained 1.4 x 10⁻⁶ M reduced RNase in 0.10 M Tris-acetate buffer, pH 8.2; reactions were carried out at 30°. The results tabulated above are representative values from a much larger body of data (9).

The effect of protein concentration on the glutathione regeneration was examined. Regenerations were carried out at reduced RNase concentrations ranging from 3 x 10⁻⁷ to 1.2 x 10⁻⁵ M. The results of these experiments, shown in Fig. 3, display some scatter but show no systematic trend in the kinetics and yield of regenerated RNase. This contrasts with the reported rate inhibition in the air regeneration at concentrations >2 x 10⁻⁴ M reduced RNase (6). We have not determined the upper limits of protein concentration for acceptable rate and yield in the glutathione regeneration, but the present work shows that a glutathione regeneration can be carried out at protein concentrations at least 10-fold higher than the air regeneration, without diminished yield or reduced rate. This offers a considerable preparative advantage.

Preincubation Experiments—These experiments were to test for the existence of conformation changes that could be part of the rate-limiting process in regeneration of activity in reduced RNase following its dissolution from a lyophilized form. Preincubation of reduced RNase, 0.20 mg/ml, was carried out for several hours at 23-25° in two different solvents: 0.10 M acetic acid containing 10⁻⁴ M EDTA, and 0.10 M Tris-acetate buffer, pH 7.0, containing 10⁻⁴ M EDTA. Zero time was the time at which lyophilized reduced RNase was dissolved in the preincubation solvent. After varying times of preincubation, aliquots were transferred to a regeneration solution at 30°, whose composition (after aliquot addition) was 0.10 M Tris-acetate buffer, pH 8.2. [GSSG] = 3 x 10⁻⁴ M, [GSH] = 3 x 10⁻⁸ M, 10⁻⁴ M EDTA. Enzyme activity measured after 60 min of regeneration was independent of preincubation times from 10 min to 8 hours, for both preincubation solvents. Control determinations of —SH titer on these samples showed no detectable oxidation over the 8-hour time interval. The results rule out rate-limiting conformation changes following dissolution of the lyophilized reduced protein.

pH Dependence—Fig. 4 shows the pH dependence of the regeneration rate of reduced RNase for two sets of regeneration experiments differing only in total glutathione concentration (same [GSSG]/[GSH]). The results obtained at the lower glutathione concentration appear to plateau at a higher rate at pH >8, but the difference is of marginal significance. Similarly, although the data obtained with the two buffer systems (Tris and bis-tris) has been represented with separate curves, there is no substantial discontinuity from one buffer to the other.

The pH dependence found here for glutathione regenerations is similar to that reported for air regeneration of RNase (6). This is in contrast with the oxidoreductase system for RNase regeneration, which showed (in the partly purified system) virtual pH independence from pH 7.4 to 8.2 (19). This difference suggests that the enzymatic and the nonenzymatic regenerations operate by different mechanisms.

Characterization of Regenerated Products—Fig. 5 shows the column chromatographic behavior of glutathione-regenerated and air-regenerated RNase. The middle chromatogram, obtained with glutathione-regenerated material, showed a major peak (no. 4) chromatographically identical with the purified fraction taken from chromatography of RNase obtained from the supplier (top curve, Peak 6). The identity is confirmed by our finding of the same specific activity in these two peaks, and by the identity of their dichroic spectra (see below). Protein from Peak 3 in the middle chromatogram showed 70% the specific activity of Peak 4 material. The unfractionated glutathione-regenerated material showed 93% the specific activity of the initial purified fraction. The above facts are interpreted as showing that glutathione facilitation gives a high yield of native RNase.

For comparison, the bottom chromatogram of Fig. 5 was obtained from an air regeneration of reduced RNase. The specific activity of the regenerated material before fractionation was 65%. Combined Peaks 3 and 4 showed 19% specific activity. Peak 5 showed 52% specific activity, and Peak 6 showed 100% specific activity, all referred to the initial purified RNase (Peak 6, top curve). The protein of Peak 6 of the lower curve of Fig. 5 appears to be identical with that of Peak 6, upper curve, by chromatographic criteria, by specific activity, and by CD spectra (see below). We carried out the air oxidation at protein concentrations considerably above those claimed in early work to provide optimal rates and yields (6). However, Pfumm and Beychock (17) were unable to repeat those findings and indeed showed considerable chromatographic heterogeneity in their preparations of air-regenerated RNase. The concentration of reduced RNase here chosen for the preparative air regeneration was a compromise. On the one hand, high dilution favors a high regeneration rate and yield. On the other hand, high dilution leads to excessive loss of S/N (signal amplitude/noise amplitude) in the operation of the ultraviolet column monitor. Even if higher dilution should produce a higher frac-

The abbreviations used are: bis-tris, 2,2'-bis(hydroxymethyl)-2',2'-&-nitriloethanol; dansyl, 5-dimethylaminonaphthalene-1-sulfonil.
to RNase and stabilize it against "thermal effects." We believe that changes, but that glutathione binding to a rate-limiting intermediate glutathione binding to RNase is not likely to be responsible for rate changes. Evidence on this point is not presently available.

Tris buffer) are appreciable (21), it is hard to see how the heats of ionization of thiol groups and amino groups (as in Epstein et al. (6) and confirmed in Fig. 2A. Similarly, although one regeneration. * Atmospheric gases have a negative tempera-
tion of major peak material in an air regeneration, such high dilutions pose serious problems for protein recovery. The present work shows that there is no such requirement for high dilutions with a glutathione regeneration (cf. also Fig. 3).

CD spectra were obtained from the major peaks of each of the chromatograms of Fig. 5 over the wavelength region 240 to 200 nm. The spectra of these three fractions were the same within experimental uncertainty (±3% at λ > 205 nm), and have a strong qualitative similarity to the CD spectrum of native RNase reported by Pflumm and Beychok (17). In all three cases minima in [θ], mean residue ellipticity, were found at 210 nm. The units of [θ] are degrees cm² per dmol. The values found were as follows: RNase from Peak 6, top curve, [θ]₂₅₀ = –10,400; RNase from Peak 4, middle curve, [θ]₁₉₀ = –10,300; RNase from Peak 6, bottom curve, [θ]₁₃₀ = –10,400. These values are in very good agreement with the amplitudes of the lyophilized material. The preincubation experiments showed that there is no time-dependent change in freshly dissolved reduced RNase that affects the regeneration kinetics. In glutathione-regenerated RNase from Peak 4 (middle curve), we also observed a weak positive peak with a broad maximum from 240 to 242 nm, [θ]₂₅₀ = +80. This peak has been observed in native RNase, but in preparations of air-oxidized RNase it was displaced negatively so that the maximum had a negative value (17).

GENERAL DISCUSSION

The strong inhibitory effect of temperature increase on the air regeneration is puzzling. It cannot be simply an effect on the protein, since no parallel inhibition is seen in the glutathione and air regenerations. Eₐ is the same for the GSH/GSSG couple at 25° and at 37° (22, 23). Thus no single factor appears to be capable of accounting for the large thermal inhibition of the air regeneration of RNase, and no multiple factor hypothesis can be offered that is not totally speculative.

Several common metal ions can catalyze the air oxidation of cysteine, thioglycolic acid, and other low molecular weight thiols (24, 25). However, the present studies show that of the common potential catalysts, only Cu²⁺ and Co²⁺ catalyze the oxidation of the cysteinyl residues of reduced RNase. This finding is in parallel with the report that reduced glutathione is also catalytically oxidized by salts of the same two metals, and not by other transition metal ions (26). Although both Co³⁺ and Cu²⁺ catalyze air oxidation of reduced RNase, only Cu²⁺ catalysis increases regeneration of RNase activity. This merits further investigation. It appears possible that inhibition of regeneration by near-stoichiometric [Cu²⁺] (–10⁻⁵ m) was the result of mercaptide formation, but this was not investigated specifically. Metal ion catalysis of the oxidation of reduced RNase is treated in greater detail elsewhere (9).

There is good evidence for the existence of slowly interconverting conformational isomers of RNase in solution (27). There is also good evidence for the production of stable active dimers and higher oligomers following lyophilization and dissolution of RNase (28). There is also evidence, from fluorescence depolarization studies of reduced, dansylated RNase, for substantial regions of structure in the reduced protein (29). With the foregoing evidence in mind, it appeared possible that the regeneration of reduced RNase might involve rate-limiting conformational changes. The possibility seemed greater because we conventionally stored reduced RNase as a lyophilized solid and initiated regeneration immediately after dissolving the lyophilized material. The preincubation experiments showed that there is no time-dependent change in freshly dissolved reduced RNase that affects the regeneration kinetics.

The column chromatographic characterization appears sufficient to establish that the major component regenerated is native RNase, both in the glutathione and air regenerations. However, one could argue that the experiments of Fig. 5 may not provide a fair comparison, because the glutathione-regenerated material was concentrated by ultrafiltration while the air-regenerated material was concentrated by adsorption onto the carboxymethylcellulose ion exchanger. This dissimilarity in concentration procedures, and the occurrence of appreciable losses in both cases, could lead to fractionation. However, the differences in concentration procedures did not involve operations as severe as lyophilization, which leads to polymerization of RNase under some conditions. Ultrafiltration would be expected to enrich oligomers at the expense of monomer, decreasing apparent homogeneity, but the regenerated sample subjected to ultrafiltration proved to be the most nearly homogeneous. Therefore, we believe that there was no significant fractionation in the ultrafiltration step and that the chromatographic differences between air-regenerated and glutathione-regenerated RNase fairly represent the heterogeneity of the products in both cases.

We were surprised to find that the same glutathione system that regenerates reduced hen egg and human lysozyme with 4ₐ = 5 min regenerates reduced RNase 15-fold more slowly. This provoked a search for agents that further accelerate the...
The "shuffle-ase" enzyme system, complete with unidentified evidence from another laboratory for substantial differences. Returning, however, to the rate differences for the problem for nucleases: inactive pro-nucleases have not that of nucleolytic and proteolytic digestion of the ribosome, mRNA, and tRNA, a kind of molecular suicide. A solution of this problem for proteases is their synthesis as inactive enzyme cofactor, whose presence is inhibitory. In trying to relate these dialyzable cofactor, rapidly regenerates reduced RNase (30), but the enzyme regenerates lysozyme only in the absence of the cofactor, whose presence is inhibitory. In trying to relate these in vitro regenerations to in vivo biosynthetic mechanisms we see that the biosynthesis of nucleases and proteases poses a particular problem to the biosynthetic system. The hazard is that of nucleolytic and proteolytic digestion of the ribosome, mRNA, and tRNA, a kind of molecular suicide. A solution of this problem for proteases is their synthesis as inactive enzyme precursors. There is no evidence for the analogous solution of the problem for nucleases: inactive pro-nucleases have not been found. One may argue that premature action of nascent nucleases is avoided by an early compartmentalization of the nuclease, physically separating the enzyme from physiologically inappropriate substrates. This may be so, but compelling evidence does not appear to be at hand. Our argument is, in short, that there are good reasons for expecting that the biosynthesis or folding of nucleases, or both, may involve unique components or operations, or both, not shared in the formation of other proteins. From this perspective it is less surprising that the kinetics of regeneration of reduced RNase and reduced lysozyme show substantial differences.

The results of the present investigation bear on the question of what mechanism is involved in the biosynthetic folding of RNase, but give no clear answer. While the essential components of the glutathione system are found in most tissues, including pancreas (31, 32), the rates found here with the glutathione system are slower than seems plausible for biosynthesis of RNase. The alternative mechanism, i.e. folding facilitated by the "shuffle-ase" enzyme, is plausibly rapid for RNase, but this enzyme could not be found in the bovine pancreas (22). Thus both candidates for physiological facilitation of reduced RNase have that potential, but at present both show a serious weakness.

With the present work, RNase becomes the fourth protein whose regeneration from the disulfide-reduced form is shown to be facilitated by a glutathione system. Rapid regenerations have already been shown for hen egg lysozyme (4), human lysozyme (5), and the Bowman-Birk protease inhibitor (33). Thus the nonenzymatic glutathione system is gaining in functional generality, and in plausibility as a biosynthetic mechanism.

Acknowledgments—We thank Mr. G. Bratt for skilled technical assistance and Mr. Peter Holmberg for assistance in determining the pH dependence of the glutathione regeneration.

REFERENCES

27. Craig, L. C., King, T. P., and Crestfield, A. M. (1963) Biopolymers 1, 231-238
Nonenzymic reactivation of reduced bovine pancreatic ribonuclease by air oxidation and by glutathione oxidoreduction buffers.
A K Ahmed, S W Schaffer and D B Wetlauffer


Access the most updated version of this article at http://www.jbc.org/content/250/21/8477

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/21/8477.full.html#ref-list-1