Thiol-Disulfide Exchange of Ribonuclease Inhibitor Bound to Ribonuclease A

EVIDENCE OF ACTIVE INHIBITOR-BOUND RIBONUCLEASE A

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Ribonuclease Inhibitor (RI) has been purified from porcine testis. It contains 30 half-cystines whose oxidation affects its ability to bind and inhibit ribonuclease (RNase). By N-terminal sequence analyses testis RI showed to be identical to that from porcine liver, for which a characteristic all-or-none type of SH-oxidation by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) has been reported (Fominaya, J. M., and Hofsteenge, J. (1992) J. Biol. Chem. 257, 24655–24660). Under comparable reaction conditions, testis RI bound to RNase A did not exhibit this particular type of oxidation; instead, bound RI got intermediate oxidation degrees (up to 14 thiols oxidized per RI moiety) without dissociating from RNase. Moreover, RNase bound to partially oxidized RI was able to express some (15%) of its potential activity (active complex). Only when DTNB treatments accounted for complex dissociation (>14 thiols oxidized per RI moiety) the released RI molecules exhibited the all-or-none oxidation behavior. By both kinetic and circular dichroism analyses, conformational changes have been evidenced for the transition from the inactive to the active form of RI-RNase complex. Relaxation of RI-RNase binding without major alterations in RI structure is proposed as responsible for complex activation. The results are discussed in terms of a model for the reversible regulation of RNase activity mediated by the redox status of RI.

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† The abbreviations used are: RI, ribonuclease inhibitor; RNase A, pancreatic ribonuclease A; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoic acid; pHMB, para-hydroxymercurobenzoate; PAGE, polyacrylamide gel electrophoresis; CCA, convex constraint analysis.

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Thiol-disulfide exchange, as a general mechanism of enzyme control (28), might be involved in the proposed regulation. Inactivation of porcine RI by exchanging of its thiol groups with the Ellman’s disulfide (DTNB) has been reported (29). This investigation, performed on free RI, evidenced an interesting “all-or-none” mechanism of inactivation by which, in the presence of amounts of DTNB that did not account for a complete oxidation of the thiol groups present in a RI preparation, the resulting RI molecules did not show intermediate oxidation degrees. Instead, a fraction of RI molecules resulted with all their half-cystines oxidized, whereas the rest maintained all of them as free thiols.

According to our working hypothesis, we were interested in...
studying the effect of thiol-disulfide exchange on RI bound to RNase rather than on free RI. Thus, we report herein the effects of the Ellman’s disulfide on the complex between porcine RI and RNase A, showing that RI, while bound, can reach intermediate oxidation degree, at the same time that the bound RNase can express some of its activity. The obtained results provide new ground to the hypothesis about the redox control of RNase bound to RI.

EXPERIMENTAL PROCEDURES

Materials

Ribonuclease Inhibitor was purified from pig testis as described below. Testes were from pigs intended for slaughter (6 months old or older) and were directly collected at the abattoir (GYPISA, Pozuelo de Alarcon, Madrid) immediately after the animal death. Only testes weighing more than 200 g were selected, immediately frozen in liquid nitrogen, and stored at −80 °C until required. Under these storage conditions no decrease of the inhibitor activity was observed, at least after 6 months.

Bovine pancreatic RNase A (type XII-A), DTNB, and dithiothreitol were purchased from Sigma; cytochrome c and SDS were from Serva (Heidelberg, Germany); iodoacetic acid was obtained from Merck (Darmstadt, Germany); and formic acid was from Carlo Erba (Milano, Italy). All other chemicals were at least of reagent grade. The buffers were prepared in glass-distilled water and lyophilized. The digest was redissolved in 20 mM Tris-HCl, pH 7.5, containing 2 mM EDTA, and dialyzed against 20 volumes of the same buffer for 6 and 12 h. The resulting solution is salt-fractionated again under the same conditions. The final recovery of RI was 70% or higher, being its final specific activity of 100,000 units/mg, as found for placenta inhibitor (27).

*RI Purification*

The rapid procedure described by Blackburn (30) for the purification of RI from human placenta was essentially employed. Testes, once thawed and stripped of skin, were homogenized as described for placenta. After centrifugation of homogenates a high fat content remains in the supernatants, making it necessary to perform the subsequent salt-fractionation twice. For such a purpose the 35%-60% salt saturation precipitate is redissolved in 20 mM Tris-HCl, pH 7.5, containing 2 mM EDTA, and dialyzed against 20 volumes of the same buffer for 6 and 12 h. The resulting solution is salt-fractionated again under the same conditions. The final recovery of RI was 70% or higher, being its final specific activity of 100,000 units/mg, as found for placenta inhibitor (27).

RI eluted from the affinity column was pooled and immediately desalted by gel filtration through Sephadex G-25 M (PD-10 column; Pharmacia LKB, Uppsala, Sweden) equilibrated and eluted with 20 mM Tris-HCl, pH 8.0, containing 2 mM EDTA, 150 mM NaCl, and 15% (v/v) glycerol. The pools so obtained were stored at 4 °C in vials hermetically stopped by Mininet septa (Allitech; Deerfield, IL). The vials were placed in a diafiltration chamber lacking a filtration membrane and kept at 4°C. The solvent outlet was opened in order to allow efficient air displacement. At fixed times the diafiltration chamber was opened, and 1 mM DTNB in 50 mM phosphate buffer, pH 7.0, was injected into the vials through the Mininet septa. In each vial, the injected volume accounted for a DTNB/complex molar ratio of 0.5. In order to monitor the thiol-disulfide exchange, prior to DTNB addition aliquots of the vials were withdrawn and subjected to the same DTNB reaction than the rest remaining in the vials. The aliquots were continuously monitored at 412 nm in a Beckman DU-7 spectrophotometer until reaching constant absorbance values. Then, further additions of DTNB were performed to account for additional DTNB/complex molar ratios of 0.5 and so on. In this way, the accumulated DTNB/complex molar ratios of the reaction mixtures were increased 0.5 at a time. DTNB additions to the vials were repeated until all of the thiol groups in the mixtures were oxidized.

Quantification of Thiol Groups

The number of thiol groups oxidized after each DTNB addition step was evaluated by TNB quantification. For such a purpose an extinction coefficient of 13,600 M−1 cm−1 at 412 nm was used for TNB (35). Additionally, the number of thiol groups remaining after each reaction step was measured in aliquots of the reaction mixtures by the method of Ellman (36), as described by Glazer et al. (37). 15% (w/v) SDS was employed as denaturing agent.

Enzymatic Assay

Ribonucleaseolytic activity, both free and latent (inhibited by RI), was evaluated by using clyclic 2,3-CMP as substrate (30). In all cases the amount of active RNase A in the assay mixture was in the 0.2–0.8 μg range.

Chromatographic Analyses

Gel filtration on a Superdex 75 HR 10/30 column (Pharmacia) was employed to evaluate the dissociation degree of complex preparations, whereas ion-exchange on a Spherisorb-TSK DEAE SPW column (75 × 7.5 mm) (Beckman) was useful when RI-RNase complex had to be separated from released RI. In both cases a high performance liquid chromatography equipment from Beckman Instruments was employed.

Gel Filtration—The column was equilibrated and eluted with 50 mM phosphate buffer, pH 7.0, containing 0.15 M NaCl and 10% (v/v) glycerol. Salt was included in order to avoid nonspecific interactions between the acidic RI and the alkaline RNase A, whereas the presence of glycerol was mandatory for quantitative recovery of RNase A. Samples of 100 μl were applied onto the column. A flow rate of 0.5 ml/min was employed, and the absorbance of the eluate was continuously monitored at 280 nm. For each evaluation two samples were run. They were prepared by incubating for 10 min at room temperature 90 μl of the
CD Measurements and Secondary Structure Analyses

Circular dichroism spectra were recorded in the 240–200-nm wavelength range by using a J. A. Yvon Mark III dichrograph fitted with a 250-watt xenon lamp. The scanning speed was of 0.5 nm/s and 0.05-cm optical path cells were employed. Ellipticity values were expressed in units of degree-cm²-dm⁻¹ residue, considering that the mean residue mass for RI-RNase complex is 107.5 Da, as calculated from its amino acid composition. Prior to the spectroscopic measurements, DTNB-treated samples were chromatographed on Sephadex G-25 M (PD-10 columns, Pharmacia) in order to remove the released TNB. Under the employed DTNB reaction conditions no conformational changes should be expected for RNase A. Therefore, its calculated contribution was subtracted from each CD spectrum in order to simplify the subsequent conformational analyses. Thus, a set of CD curves (one curve for each accumulated DTNB/complex ratio) was generated, mostly reflecting the CD behavior of the RI subunit as increasingly oxidized. This set was appended to a set of 240–200 nm CD spectra from 18 reference proteins (Table VIII in Ref. 38) in order to deconvolute the whole data set according to the convex constraint analysis (CCA) method (39). For such a purpose three conformational components were allowed, in accordance with the known three-dimensional structure of RI (1, 2), which can be taken as a repetition of three structural motifs. The shapes of the three pure CD curves so resulting were in good agreement with the known CD spectra of α-helix, β-sheet, and unordered secondary structure (Fig. 1). Goodness of the deconvolution analysis was additionally checked with six proteins of known three-dimensional structure, whose CD spectra were among the reference data set: myoglobin, lactate dehydrogenase, lysozyme, ribonuclease A, α-chymotrypsin, and elastase. Correlations between their secondary structural percentages, as well as from x-ray diffraction results (Table III in Ref. 40), and the respective estimations from the applied CCA method were quantified for each secondary structure through the Pearson product-moment correlation coefficient (41). Very good correlation was found for α-helix estimations (r = 0.995); on the other hand, the correlation coefficients for β-sheet and unordered form, 0.802 and 0.587, respectively, though poorer, were still significant. For these calculations β-turn percentages were not taken into account. The CD contribution of this structural element should be obscured in the pure CD curves of the other conformations and so its conformational weights added in some extent to the CCA-estimated percentages. Most likely this contamination affects the estimated contributions of β-sheet and unordered form, so explaining their lower correlation coefficients.

Kinetics of the Reactions in Scheme I

The differential equations that govern the production of both TNB molecules and the evolution of the intermediate mixed disulfide are as follows.

\[
\frac{d[\text{TNB}_1]}{dt} = k_c[\text{complex}][\text{DTNB}] - k_c[\text{complex-SS-TNB}] \] (Eq. 1)

\[
\frac{d[\text{TNB}_2]}{dt} = k_c[\text{complex-SS-TNB}] \] (Eq. 2)

\[
\frac{d[\text{complex-SS-TNB}]}{dt} = -k_c[\text{complex}][\text{DTNB}] + k_c[\text{complex-SS-TNB}] \] (Eq. 3)

Where Equation 1 can easily be integrated, integration of Equation 2 requires to previously integrate Equation 3. In both cases, an integral similar to that numbered as 501 in the integral table of “CRC Handbook of Chemistry and Physics” (43) must be solved. The exact integrated equation contains a sum of squared and higher order terms that can be discarded because of their nonsignificant effect on the final results. Thus, the following integrated equations are obtained.

\[
[\text{TNB}_1](t) = \frac{AB}{[A]^2 - B^2} \ln \left(\frac{[A]^2}{[A] - B}\right) e^{kt} \] (Eq. 4)

\[
[\text{TNB}_2](t) = B[1 - e^{kt}] \] (Eq. 5)

where A = [complex], number of SH, B = [DTNB], m = A – B; zero subindex refers to concentrations at zero time.

For each DTNB addition, the k_c and k_c values could be obtained by nonlinear regression fitting of the experimental data (A_{412} versus time) to the following equation.

\[
A_{412} = 13,600[\text{TNB}_1](t) + [\text{TNB}_2](t) \] (Eq. 6)

where [TNB_1](t) and [TNB_2](t) are given by Equations 4 and 5, respectively, and 13,600 refers to the molar extinction coefficient of TNB (35).

Statistical Models for DTNB Oxidation of RI-RNase Complex

As a first approach, the same reactivity can be assumed for all the SH groups of the RI-RNase complex. Actually this is not the case, as deduced from the fluctuations detected in the evaluated rate constants (Table I under “Results”); however, it can be demonstrated (data not shown) that, while dissociation is not significant, the slight fluctuations measured for the rate constants are compatible with such an assumption. Accordingly, the probability of a particular SH group to be modified by DTNB will depend on the DTNB/SH molar ratio. Thus, for each global DTNB/complex ratio, r, reached in the reaction mixture, the probability P(m) of finding a complex molecule with m of its 30 thiol groups modified, whichever they can be into the amino acid sequence, will follow a binomial distribution.

\[
P(m) = \binom{30}{m} g^m (1-g)^{30-m} \] (Eq. 7)

where g is the average oxidation degree of the reaction mixture. Obviously, g = 2r/30, since each DTNB molecule reacts with two thiol groups.
ri could be expected, we decided to assess this issue by comparing the N-terminal sequence of testis RI with that of liver RI. As already found for the latter (20), RI from pig testis was not susceptible to automatic Edman degradation, so suggesting that its N-terminal residue was blocked. The amino acid analysis of testis RI revealed the occurrence of two methionyl residues (data not shown). Therefore, we could use the same strategy employed for the determination of the amino acid sequence of liver RI (20). Thus, CNBr cleavage of testis RI yielded two major peptides of 36 and 14 kDa, which could be separated by SDS-PAGE (Fig. 2B). Two similar peptides were also seen after CNBr cleavage of liver RI (20). In addition, this pattern can be taken as an evidence of the occurrence of a blocked methionine as the N-terminal residue; upon CNBr hydrolysis this methionine would migrate, as blocked homoserine, with the front in SDS-PAGE. The two peptides, once electroeluted from the gel were subjected to automatic Edman degradation, rendering the following N-terminal sequences: CB1, Asn-Leu-Asp-Ile-Cys-Glu-Gln-Leu and CB2, Leu-Thr-Glu-Asn-Lys-His-Leu-Asp-Glu-Leu-Gln-Leu. These two sequences are identical to the respective N-terminal sequences of the fragments CB2 and CB3 of liver RI (20). These results constitute strong evidences about the identity of both porcine inhibitors, although they proceed from different organs. Consequently, the knowledge gained in this work about the redox properties of porcine RI bound to RNase A can be taken as an extension of the thiol-disulfide studies carried out with free porcine RI (29).

Thiol-Disulfide Exchange of RI Bound to RNase A

Extended from 3 to 30 of DTNB (0.5 mol of DTNB/mol of complex), as described under “Experimental Procedures.” In this way a collection of “A412 versus time” curves were obtained; as many as addition steps were required to get the complete modification of the 30 cysteinyl residues of bound RI (the eight cysteine residues of RNase A are forming four disulfide bridges; see Ref. 41). For each reaction step the absorbance at 412 nm reached a final value which accounted for the formation of 2 mol of TNB/mol of added DTNB. This behavior was also seen by Fominaya and Hofsteenge (29) for the reaction of free RI with DTNB These authors already indicated that such a behavior can only be interpreted as the consequence of a two-step reaction between DTNB and the protein thiol groups (Scheme I). As a consequence each DTNB molecule transforms two protein thiol groups into one disulfide bridge. This point was confirmed by determination under denaturing conditions of the thiol groups remaining in the complex after completion of the successive reactions with substoichiometric amounts of DTNB. The results of these evaluations are plotted in Fig. 3. The number of unmodified thiol groups plus the number of the modified ones (as calculated from the increase in the absorbance at 412 nm of

![Fig. 2. A, silver-stained (44) SDS-PAGE. Lane a, kit of standard proteins, preincubated in the presence of 1% (v/v) 2-mercaptoethanol; lane b, purified testis RI preincubated in the absence of 2-mercaptoethanol; lane c, purified testis RI preincubated in the presence of 2-mercaptoethanol. The “smile” effect of the band in lane b should be attributed to the action of 2-mercaptoethanol diffusing from the neighboring lanes during electrophoresis. B, reverse-stained (33) SDS-PAGE. Lane a, kit of standard proteins of the specified molecular masses; lane b, testis RI preparation subjected to CNBr cleavage as described under “Experimental Procedures”; lane c, purified testis RI used as control. In all cases samples were preincubated in the presence of 1% (v/v) 2-mercaptoethanol.](Image 83x593 to 272x732)

![Scheme I](Image 314x588 to 556x732)
The reaction mixtures accounted for the 30 SH of the complex, so corroborating the two steps reaction of Scheme I. In this scheme the formed disulfide bridges are considered to be intramolecular, although the formation of intermolecular bridges cannot be discarded. Nevertheless, under our experimental conditions, no significant amounts of intermolecular bridges are formed, as deduced by SDS-PAGE analyses under nonreducing conditions of the reaction mixtures (data not shown).

Kinetic Evaluation of SH Reactivity—A careful analysis of the $A_{412}$ versus time curves obtained for each DTNB modification step revealed subtle but significant differences among them. This fact suggested that the reactivity of the thiol groups of the complex varies as its oxidation degree increases. These variations can be evaluated by fitting the experimental curves to the integrated rate equation which account for the production of total TNB (Equation 6 under “Experimental Procedures”). The results of these measurements for both rate constants are shown in Table I. As can be seen these values fluctuate as the complex is increasingly modified by the successive DTNB additions. While [DTNB]/[complex] ratios were lower than 6.0, these fluctuations were small, 1.5 and 4.0 being the ratios for which both rate constants reached minimum values. At higher [DTNB]/[complex] ratios, major changes could be detected. Since such high ratios accounted for considerable complex dissociation, these major changes should be interpreted as due to the unfolding of released RI after massive oxidation (see next paragraphs).

Leaving aside the effects of complex dissociation, one would predict that at [DTNB]/[complex] ratios for which dissociation could be neglected, thiol groups of the complex should result sorted out in their reaction with DTNB; that is, the higher reactivity of a cysteinyl residue the quicker its modification and vice versa. Therefore, a continuous decrease of the fitted $k_2$ values could be expected. Alternatively, if the SH reactivity was not affected by the complex conformation, nonsignificant variations in the rate constants should be expected all through the modification. However, the observed behavior in which SH reactivity fluctuations are detected should be interpreted as a consequence of conformational changes of the complex induced by thiol-disulfide exchange.

Circular Dichroism Studies of RI-RNase Complex as Increasingly Oxidized by DTNB—In order to evaluate the conformational changes of the complex predicted by the observed transitions in the fitted rate constants, aliquots of the DTNB complex reaction mixtures were withdrawn after reaction completion for each step of DTNB addition. Once dialyzed, their CD spectra were registered in the 240–200 nm range and analyzed as described under “Experimental Procedures.” Thus, conformational weights of $\alpha$-helix, $\beta$-sheet, and unordered structure were obtained for each discrete step throughout the gradual DTNB modification. These results are plotted in Fig. 4. In order to facilitate their comparison with the evolution of the fitted rate constants, the values in Table I are also plotted in Fig. 4. The first transition observed for the rate constants (accumulated DTNB/complex ratio = 1.5–2.5) seems to correlate with a conformational change in which the initial decrease in $\alpha$-helix occurs at the expense of an increase in unordered structure, whereas the subsequent increase in $\alpha$-helix is parallel to a decrease in both $\beta$-sheet and unordered structure. On the other hand, the second transition kinetically detected (accumulated DTNB/complex = 4.0–5.0) is correlated with a loss of $\alpha$-helix at the same time as unordered structure increases.

The three-dimensional structure of RI molecule, both free and bound to RNase, is basically formed by the repetition of three structural elements (1, 2): 16 $\alpha$-helices (12 residues long in average), 17 $\beta$-strands (3 residues long in average) forming a curved parallel $\beta$-sheet, and 32 loops (containing between 4 and 9 amino acids) connecting the individual $\alpha$ and $\beta$ segments; in these loops several types of $\beta$-turns are present. From these figures it can be calculated that $\alpha$-helices contain 42% of all RI residues, whereas only 11% are in the parallel $\beta$-sheet. Although the percentages of $\alpha$-helix estimated from the CD measurements are in good agreement with the x-ray results, the same cannot be said for $\beta$-sheet, which is overestimated probably due to some contribution from bends (see “Experimental Procedures”).

Effects of Oxidation with DTNB on the Ribonucleolytic Activity and Association Degree of RI-RNase Complex—The dissociation degree of RI-RNase complex as increasingly oxidized by
DTNB was evaluated by gel filtration on a Superdex 75 HR column. The results of these evaluations together with the percentages of released RNase activity are shown in Fig. 5. As can be seen, both activation and dissociation percentages do not evolve in a parallel fashion. In other words, RNase dissociation does not give an entire account of RNase activation. The obtained results point to the occurrence of partially oxidized RI that remains bound to RNase without completely abolishing its activity. We have called this complex showing ribonucleolytic activity, active complex. The minimum number of thiol groups that have to be modified for the complex to become active should then be less than the minimum number required for it to dissociate. In order to investigate this, we have modeled the oxidation of RI bound to RNase.

Our first model assumed a random oxidation mechanism, in which all the thiol groups in the complex have the same reactivity. According to this model predicted activation and dissociation curves can be calculated by Equations 8 and 9. Dotted and dashed lines in Fig. 5 show, respectively, these predicted curves when \( f = 0.15, u = 5, \) and \( v = 15 \). Although they fit well, the experimental data up to a global DTNB/complex ratio of 5.0, a clear discrepancy is observed beyond this value. Thus, the experimental data grow slower than the predicted ones as the global ratio increases. It should be noticed that such a discrepancy becomes patent when the dissociation percentage reaches a significant value (>5%). This fact suggests that random modification may not be a valid model for DTNB oxidation of released RI.

The all-or-none type of reaction reported for the DTNB oxidation of free porcine liver RI (29) suggests that in the reaction mixtures the first RI molecules partially oxidized become more susceptible to subsequent DTNB oxidation. Thus, partially oxidized RI acts as a DTNB monopolizer until all of its thiol groups result modified. This particular behavior of free RI may explain the discrepancies of our model. Free RI molecules with some partial oxidation degree will appear in our reaction mixtures as a result of accumulated DTNB oxidation. They would behave as DTNB monopolizers in a subsequent DTNB addition, so lowering the amount of DTNB available to increase the modification degree of the remaining complex molecules. As a consequence, activation and dissociation of complex preparations will be slowed down once they reach accumulated DTNB/complex ratios, which account for a significant dissociation percentage (e.g., \( r = 5.0 \)).

It is feasible to calculate theoretical activation and dissociation curves in which this monopolizer effect of the released RI is considered. Thus, for each DTNB/complex ratio the fraction of molecules with \( v \) or more of their thiol groups modified (free RI fraction) is calculated by using Equation 8. To determine the activating and dissociating effect of a subsequent DTNB addition, top priority as DTNB consumer is given to this population. For such a purpose, the amount of DTNB that this population consumes to fully modify its remaining thiol groups is subtracted from the DTNB added, so yielding the DTNB which is really available to increase the oxidation degree of the complex molecules. The activation and dissociation percentages can finally be calculated through Equations 8 and 9 by using the “effective” DTNB so determined.

In Fig. 5 the predicted activation and dissociation curves so calculated are also plotted. The best results were obtained when \( f, u, \) and \( v \) took the previous values of 0.15, 5, and 15, respectively. The accuracy of the fit supports the proposed model of random DTNB oxidation for RI-RNase complex, in conjunction with the all-or-none type of reaction for released
RI. Additional support would require experimental evidence about the presence in the reaction mixtures of complex species showing variable oxidation degrees, whereas released RI, if present, should have all its thiol groups oxidized.

Chromatographic Analysis of Complex Partially Oxidized by DTNB—The RI-RNase complex can be resolved from released RI by anionic exchange. A complex preparation subjected to an accumulated DTNB/complex ratio of 7.5. Elution conditions are described under "Experimental Procedures." Fractionation of the eluate followed by measurement of both thiol and protein contents of each fraction allowed to determine the average number of free thiols per molecule along the elution profile. The results of these determinations are also shown in c as bar plots.

DISCUSSION

The role of RI in the regulation of intracellular RNases remains to be proved. In fact, it has been questioned whether RI-RNase complexes have any implication in the catabolism of RNA, or, on the contrary, if their in vitro detection is only an artifact due to organelle disruption during tissue homogenization.
bridges is considered as occurring between cysteinyl residues which are close to each other in the RI molecule. This consideration has been derived from the results of the kinetic analyses carried out for the reaction between RI-RNase complex and DTNB (Table I). Two values of rate constants were evaluated for each addition of substoichiometric amounts of DTNB: the second order constant, k₁, for the formation of a mixed disulfide between RI and DTNB and the first order constant, k₂, for the formation of an intramolecular disulfide. Both rate constants fluctuate only slightly, provided that the complex does not dissociate. Thus, the second order constant fluctuates around 10 M⁻¹min⁻¹ and the first order constant around 0.4 min⁻¹. One would expect that DTNB oxidation would yield mixed disulfides only if their subsequent transformation to intramolecular disulfides was very slow. The intramolecular reaction will be the rate-determining step if k₂ < k₁ [DTNB]. Therefore, mixed disulfides would accumulate if [DTNB] > 10 M (0.4 min⁻¹). This is not the case in the present study, since in order to assess substoichiometric levels of DTNB, the concentrations of this reagent were always in the micromolar range. In such conditions, the formation of intramolecular disulfides is around 3 orders of magnitude faster than that of mixed disulfides, so explaining that only intramolecular disulfides are measurably formed. In the study carried out on free RI (29), the employed DTNB concentrations were also in the micromolar range, which would explain the disulfide formation also found.

This formation of intramolecular disulfide bridges allows us to conclude that in the mixed disulfide intermediates the adjacent thiol groups behave as if their "effective concentrations" were higher than 10 M. This should be the consequence of the high number of thiol groups in the RI molecule, which occurs mostly at constant positions in the internal repeats of RI (20–22). The susceptibility of RI to form internal disulfide bridges could be considered as an interesting property for its regulation by the cellular redox status. As stated previously (46), under the reducing intracellular conditions, the oxidation of an intracellular protein should be faster than its reduction if the oxidized form plays some role in vivo. Thus, the formation of intramolecular disulfide bridges in RI can act as a driving mechanism that increases the rate of oxidation in comparison with that of reduction, so allowing both active and inactive forms of the RI-RNase complex to coexist at equilibrium.

The experimental evidence obtained about the existence of an "active RI-RNase complex" allows us to maintain that RI, binding RNases, can have a role in reversibly switching them between active and inactive forms. Certainly, the active form may be considered as a poor enzyme, since it only expresses 15% of its potential activity; but, does the cell need the high ribonucleolytic activity that its RNases are able to exert? On the other hand, if the RI-RNase complex really exists in vivo, would the cell ever reach the strong oxidizing conditions required for dissociation? These questions will probably be answered when redox conditions similar to those found in vivo are employed in studying thiol-disulfide exchange of the RI-RNase complex. With this in mind, we are currently investigating the oxidation of the RI-RNase complex by biological disulfides (e.g. GSGS), as well as their thiol counterparts (e.g. GSH) for the reverse reaction.

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