Inactivation of Ribonuclease Inhibitor by Thiol-disulfide Exchange*

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Porcine ribonuclease inhibitor (RI) contains 30 1/2-cystinyl residues, all of which occur in the reduced form. Reaction of the native protein with 5,5'-dithiobis(2-nitrobenzoic acid) resulted in the release of 30 mol of the product 5-mercapto-2-nitrobenzoate, and the loss of the RNase inhibitory activity. A linear relationship between the degree of modification and inactivation was observed. The rate of modification was greatly increased in the presence of 6 M guanidinium HCl. Reaction with substoichiometric amounts of 5,5'-dithiobis(2-nitrobenzoic acid) was found to yield a mixture of fully reduced active molecules, and fully oxidized inactive ones, but no partially oxidized forms were detected. This suggests that an "all-or-none" type of modification and inactivation took place. All 1/2-cystinyl residues in the inactive, monomeric inhibitor had formed disulfide bridges, judged by the absence of either free thiol groups or mixed disulfides with 5-mercapto-2-nitrobenzoate. This fully disulfide-crosslinked molecule had an open conformation compared to the native one, as shown by gel filtration and limited proteolysis. Reaction of phenylarsinoxide with vicinal dithiols yields products that are much more stable than those with monothiols. Titration of RI with this reagent yielded complete inactivation at a reagent/thiol ratio of 0.5. Taken together, these observations suggest that the thiol groups in RI have a diminished reactivity due to three-dimensional constraints. After the initial modification of a small number of thiol groups, a conformational change occurs which causes an increase in reactivity of the remaining thiols. The thiol groups are situated close enough together to permit the formation of 15 disulfide bridges in the inactive molecule.

RNase activity in extracts of tissues is generally found in a latent state, due to the presence of a specific inhibitor protein (Blackburn and Moore, 1982). The association of RNase inhibitor (RI) with pancreatic RNase A and homologues like angiogenin is very tight (K, = 0.7-67 nM; Shapiro and Vallee, 1991; Vicentini et al., 1990; Lee et al. (1989)). However, the inhibitor is only active in the presence of reducing agents such as dithiothreitol (DTT) or β-mercaptoethanol. In fact, treatment of enzyme-inhibitor complexes with thiol-modifying reagents like p-hydroxymercuribenzoate (Roth, 1956; Shortman, 1982; Bishay and Nicholls, 1973) or N-ethylmaleimide (Girija and Sreenivasan, 1966; Bates et al., 1985) inactive RI and liberate the enzyme. The primary structure of RI has been determined (Hofsteenge et al., 1988; Lee et al., 1988; Schneider et al., 1988). It consists of 15 homologous leucine-rich repeats and contains 30 (porcine RI) or 32 (human RI) 1/2-cystinyl residues, all of which occur in the reduced form. Most of the cysteinyl residues occur at a constant position within the repeating unit.

The unusually large number of cysteinyl residues and their importance for the function of RI led us to investigate the effect of modification by thiol-disulfide interchange. Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid); DTNB) is a convenient compound for such studies due to the spectral properties of the product, 5-mercapto-2-nitrobenzoate (MNB). We present evidence that DTNB inactivates RI by an all-or-none mechanism: the products consist of only fully modified inactive and unmodified active molecules, and no intermediate forms can be detected. The inactive monomeric protein contains 15 disulfide bridges, but no free thiols or mixed disulfides with MNB. Moreover, it has an open conformation compared to the compact one of the native molecule.

EXPERIMENTAL PROCEDURES

Materials—Recombinant porcine ribonuclease inhibitor was expressed in Saccharomyces cerevisiae and purified as described previously (Vicentini et al., 1990). Bovine pancreatic RNase A and thromosyn from Bacillus thermoproteolyticus were obtained from Boehringer, Mannheim. DTNB, phenylarsinoxide (PAO), DTT, L-cysteine, and iodoacetic acid were from Fluka, Buchs, Switzerland. Guanidinium hydrochloride (GuHCl) was purchased from Pierce.

Reaction with DTNB—To remove dithiothreitol RI was exhaustively dialyzed against 20 mM Tris-Cl buffer, pH 7.5, 150 mM NaCl, 0.1% polyethylene glycol, 1 mM EDTA, which was continuously purged with helium. The effectiveness of this method was evaluated by determining the concentration of DTT in a parallel dialysis experiment without protein. The remaining amount of thiol due to DTT was 0.3% of that due to RI.

DTNB was dissolved in 0.2 M phosphate buffer, pH 7.2, 4 mM EDTA, and its concentration was determined by reaction with an excess of dithiothreitol. Reactions were performed at 25°C in the same buffer in polystyrene cuvettes. The release of MNB was monitored at 412 nm using a Hewlett-Packard 8452A spectrophotometer and quantitated using an extinction coefficient of 14,150 M-1 cm-1 (Jocelyn, 1987). Controls without protein were performed to measure the rate of background reactions. If required, 6 M GuHCl was included to obtain denaturing conditions. Different concentrations of RI and DTNB were used as specified in the particular experiments.

To measure the effect of the modification on the activity of RI, aliquots from the reaction mixture were diluted 450-fold in 0.1% bovine serum albumin in H2O, and used directly in the RNase assay. The dilution of the reagent, as well as the excess thiol from bovine serum albumin were sufficient to stop the reaction.

Affinity Chromatography on RNase-Sepharose—RNase-Sepharose was prepared following the procedure of Burton and Fucci (1982). A column of 0.5 ml was used containing 0.18 mg of RNase A/ml of hydrated Sepharose 4B. The column was equilibrated in 0.2 M phosphate buffer, pH 7.2, containing 4 mM EDTA. Samples from the DTNB modification reaction were loaded onto the column which was
that obtained by quantitative amino acid analysis (ratio using the method of Bradford (1976) as adapted by Bio-Rad. The with P-mercaptoethanol and carboxymethylation with iodoacetic acid 0.083; n = 8).

**RESULTS**

**Reaction of RI with DTNB** — Reaction of native RI with micromolar concentrations of DTNB resulted in the release of 29.5 mol of MNB per mol of RI (Fig. 1). In the presence of 6 mM GuHCl an equivalent amount of MNB was liberated (31.2 mol/mol), but the rate of reaction was considerably increased. In fact, it was similar to that observed with the thiol groups of low molecular weight substances like DTT and L-cysteine (Fig. 1, inset). These results indicated that the thiol groups in native RI are either poorly accessible to DTNB or have a low reactivity. Since both effects could depend on the three-dimensional structure of the protein, it was of interest to examine the effect of DTNB on the activity and structure of RI.

**Effect of DTNB on the RI Activity** — To study the effect of modification of thiol groups by DTNB on the activity of RI, the inhibitor (0.4 μM) was incubated with the reagent (12 μM) (i.e. a 1:1 protein thiol to reagent ratio) and RI activity was measured in parallel with the release of MNB as a function of time. The results presented in Fig. 2 indicated a close correlation between the number of thiol groups modified (increase of the absorbance at 412 nm) and the degree of inactivation. Extrapolation of the data showed that complete inactivation occurred when 29.4 mol of SH per mol of RI had reacted with DTNB (Fig. 2). This observation could be explained by two different mechanisms: 1) RI can only be completely inactivated by modifying all thirty thiol groups, or 2) DTNB initially reacts with one or a few thiol groups, leading to a change in conformation and a large increase in the rate of reaction of the remaining thiol groups (all-or-none reaction (Friedman, 1975)). In the latter case, inactivation could occur at either stage of the reaction. To distinguish between the above-mentioned mechanisms, RI was reacted with substoichiometric amounts of DTNB, and the reaction products were examined by affinity chromatography on RNase-Sepharose.

**Effect of DTNB on the RI Activity** — To study the effect of modification of thiol groups by DTNB on the activity of RI, the inhibitor (0.4 μM) was incubated with the reagent (12 μM) as described under "Experimental Procedures." The number of modified thiol groups was calculated from the increase in the absorbance at 412 nm and the known protein concentration. At timed intervals aliquots were removed, diluted, and assayed for activity. Controls incubated under the same conditions without DTNB did not show a loss of activity. The solid line represents the result of linear regression analysis of the data.

**Fig. 1. Time course of the reaction of RI with DTNB.** The reaction was carried out as described under "Experimental Procedures" using 0.4 μM RI and 12 μM DTNB. N indicates the results obtained starting with native RI, whereas † indicates those obtained in the presence of 6 mM GuHCl. A, MNB liberation after adding an extra 10-fold excess of DTNB to the reaction mixture. The arrowhead on the right indicates the absorbance value expected when 30 thiols react per molecule of RI. Experimental errors were 5% or less. The inset shows the results obtained with two low molecular weight thiol compounds (DTT, 6 μM; and L-cysteine, 12 μM) under the same conditions as employed with native RI.

**Fig. 2. Effect of modification with DTNB on the activity of RI.** RI (0.4 μM) was reacted with DTNB (12 μM) as described under "Experimental Procedures." The number of modified thiol groups was calculated from the increase in the absorbance at 412 nm and the known protein concentration. At timed intervals aliquots were removed, diluted, and assayed for activity. Controls incubated under the same conditions without DTNB did not show a loss of activity. The solid line represents the result of linear regression analysis of the data.
The reaction of DTNB with proteins such as RI, which contain more than one thiol group can follow two pathways. Each of the thiol group reacts with one molecule of MNB and thiol-MNB-mixed disulfide (Reaction 1, Scheme I). Alternatively, initial formation of a mixed disulfide with MNB can be followed by a thiol-disulfide exchange reaction with a second protein thiol group leading to the formation of a disulfide bridge and the liberation of MNB (Reaction 2, Scheme I). Obviously, the disulfide bridge may be intra- or intermolecular.

The results of the reaction of RI with DTNB at different ratios of DTNB to protein thiol presented in Table I clearly demonstrated that, with RI, the reaction does not stop after the first step but proceeds completely to the second step. At each ratio, the amount of MNB liberated was twice as high as expected from Reaction 1 (Scheme I) alone. In addition, the degree of inactivation was precisely twice as high as expected from a reaction between DTNB and protein thiol alone. The reaction products were investigated by affinity chromatography. For this purpose, RI (1.67 nmol) was reacted with 12.5 nmol of DTNB (0.25 mol/mol of protein thiol), which resulted in the inactivation of 51.5% of the RI and the liberation of 24.9 nmol of MNB (49.7% of total thiol modified). The mixture was applied to a column of RNase-Sepharose (Fig. 3). The material eluting in the nonbound fraction contained inactive RI (RI') and 17.6 nmol of MNB (recovery: 74%). Characterization of RI' by SDS-polyacrylamide electrophoresis showed it to be largely monomeric. Furthermore, addition of DTT did not release MNB (<0.2 mol/mol) after adding an excess of DTNB. Taken together these findings suggest that RI' contains only disulfide bridges.

The RI bound to the RNase-Sepharose could be eluted from the column with an excess of DTNB (Fig. 3). The eluate contained RI' and 18.5 nmol of MNB. The recovery of an equimolar amount of MNB in this eluate (18.5 nmol; recovery: 74%) compared to that in the unbound fraction (17.6 nmol) showed that 48.5% active molecules that were initially retained on the column, contained all thiol groups in the reduced state. This was confirmed by the finding that RI that had been eluted from the RNase-Sepharose with 10% acetic acid contained 30.3 mol of thiol/mol of RI. In a separate experiment it was found that treatment of the bound RI with a solution containing 1 mM DTT released <0.4 mol of MNB/mol of RI, indicating the absence of a significant amount of mixed disulfides. Thus, reaction of RI with DTNB (0.25 mol/mol protein thiol) resulted in the formation of 51.5% inactive molecules that contained only disulfide bridges and 48.5% active molecules that contained only thiol groups. These observations show that the inactivation of RI by DTNB under these conditions occurred via an all-or-none type of reaction. Only the two extreme situations were observed: fully modified inactive molecules, and fully unmodified active ones. No intermediate stages were apparent.

Reaction with Phenylarsinoxide—The results described above strongly suggested that the thiol groups in RI are situated closely enough to allow formation of disulfide bridges. PAO reacts to form a stable complex with vicinal dithiols (Torchinskii, 1974a) by bridging them. The product of the reaction with monothiols, however, is readily reversible (Torchinskii, 1974a). RI was incubated with increasing amounts of PAO, and at different time points aliquots were diluted and assayed for RI activity (see "Experimental Procedures"). To obtain complete inactivation of the inhibitor 0.5 mol of PAO/mol of protein thiol was required, whereas 67% inactivation was observed after the addition of 0.33 mol of PAO/mol of protein thiol (Fig. 4). These results are in agreement with the notion that the thiol groups are situated closely enough together to be bridged pairwise by PAO, confirming the interpretation of the results obtained with DTNB (Table I and Fig. 3).

Characterization of the Disulfide-cross-linked RI—The amount of multimeric forms in the preparations of fully disulfide cross-linked RI varied with the concentrations of both RI and DTNB. Under optimal conditions ([RI] = 8.3 μM, and [DTNB] = 150 μM) the monomeric form constituted 98.7% of the RI. This protein was purified by gel filtration

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**TABLE I**

| [DTNB] | Thiol modification | RI activity | Expected* |
|--------|-------------------|-------------|-----------|
| mol of MNB/mol of RI | Total SH reacted | % inactive | % |
| 1:6 | 9.95 | 33.2 | 32.5 | 16.7 |
| 1:4 | 14.87 | 49.7 | 51.5 | 25.0 |
| 1:2.7 | 21.21 | 70.7 | 73.6 | 37.5 |
| 1:2 | 30.56 | 101.9 | 98.1 | 50.0 |

*The percentage of inactivation was calculated assuming the formation of a mixed disulfide between 1 mol of thiol and 1 mol of MNB.
and found to contain neither free thiol, nor mixed disulfides with MNB. This form of RI which is inactive and contains only disulfide bridges will be designated cl-RI.

Modification of proteins often leads to alterations of the three-dimensional structure. To establish whether the formation of disulfide bridges had caused a conformational change, the elution behavior of different forms of RI were examined by gel filtration on a Superose 12 column. The elution volume of cl-RI ($K_v = 0.57$) was slightly lower than that of native RI ($K_v = 0.61$), while RI that had been completely denatured and carboxymethylated (cm-RI) was found to elute with a $K_v$ value of 0.35 (Fig. 5). Thus, cl-RI seems to have a slightly increased hydrodynamic volume compared to native RI.

Additional evidence for a change in conformation was obtained from experiments using limited proteolysis. RI contains 135 potential cleavage sites for thermolysin. Nevertheless, native RI was stable for at least 30 min in the presence of this enzyme (1% w/w; Fig. 6). As a control, cm-RI was observed to be completely digested under these conditions (also cm-RI in a 1:1 mixture with native RI was completely digested, ruling out an inhibitory effect of native RI on thermolysin; data not shown). In contrast to native RI, cl-RI was much more sensitive to proteolysis. Under identical conditions cl-RI was degraded to products that migrated with the ion front on a 12.5% SDS-polyacrylamide gel (Fig. 6). These results suggest that the increased hydrodynamic volume of cl-RI was due to a change from the compact native structure to a more open conformation.

**DISCUSSION**

The sulfhydryl moiety is one of the most reactive groups encountered in proteins. Several factors can influence the reactivity of these groups in proteins (Torchinskii, 1974b). They can be buried in the protein interior, or their chemical environment can affect their reactivity. The reactivity of the 30 cysteinyl residues of native RI toward the thiol-modifying reagent DTNB was diminished compared to that of RI in the presence of 6 M GuHCl, or small molecular weight compounds (Fig. 1). RI is a strongly negatively charged molecule containing a total of 58 aspartic and glutamic acid residues. These groups could attenuate the modification reaction due to electrostatic repulsion of the negatively charged DTNB molecule. In addition, they could also decrease the amount of thiolate ion formed on the cysteinyl residues (increase of $pK_a$), but this effect is thought to be small (Gilbert, 1990). A third possible reason could be the interaction of the side chains of the cysteinyl residues with other residues, e.g. through the formation of hydrogen bonds (Gregoret et al., 1991).

The complete inactivation of RI coincided with the modification of all 30 thiol groups (Fig. 2). This could be explained by an all-or-none type of reaction in which one or a small number of residues react, inducing a conformational change which greatly enhances the reactivity of the remaining residues (summarized in Fig. 7). Whether the inactivation is
caused directly by this conformational change, or by the subsequent modification of the remaining thiols groups is yet unclear. A salient feature of the modified inactive molecule was that all of its free thiols groups had been converted into disulfides and that no mixed disulfides were present. This raises the question as to whether the initial modification with DTNB (step 1, Scheme I), or the formation of the first disulfide (step 2, Scheme I) is the cause of the conformational change.

Another question that remains to be answered concerns the number of cysteinyl residues that have to react before the conformational change takes place, and whether this constitutes a specific set of residues. Alkylation of RI with iodoacetate followed by the reaction of the resulting residual S-S groups with DTNB results in inactivation. It is tempting to speculate that the formation of disulfide-linked aggregates (Srivastava and Beutler, 1973; Roy and Spector, 1978). Under the same pathological condition the RI activity completely disappears (Cavalli et al., 1979). It is of interest to note that the formation of disulfides in RI increased its sensitivity toward proteolysis (Fig. 6). Wolff et al. (1979) have hypothesized that the rate of proteolysis of oxidized proteins is a factor in the pathogenesis of cataract formation. It is tempting to speculate that the formation of cl-RI, followed by proteolysis, is responsible for the disappearance of RI in this situation.

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