

Adjacent cysteine residues as a redox switch

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Oxidation of adjacent cysteine residues into a cystine forms a strained eight-membered ring. This motif was tested as the basis for an enzyme with an artificial redox switch. Adjacent cysteine residues were introduced into two different structural contexts in ribonuclease A (RNase A) by site-directed mutagenesis to produce the A5C/A6C and S15C/S16C variants. Ala5 and Ala6 are located in an α -helix, whereas Ser15 and Ser16 are located in a surface loop. Only A5C/A6C RNase A had the desired property. The catalytic activity of this variant decreases by 70% upon oxidation. The new disulfide bond also decreases the conformational stability of the A5C/A6C variant. Reduction with dithiothreitol restores full enzymatic activity. Thus, the insertion of adjacent cysteine residues in a proper context can be used to modulate enzymatic activity.

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Introduction

The modulation of enzymatic activity is of great importance in biology (Cleland and Craik, 1996; Alberghina, 1999). The non-equilibrium nature of biological systems necessitates temporal or spatial suppression or enhancement of specific biochemical pathways according to cellular metabolism or exogenous signals. For example, conformational regulation by phosphorylation is a recurring theme in cellular signal transduction pathways (Munico and Miras-Portugal, 1996; Sefton and Hunter, 1998). Indeed, it is common to find that an enzymatic activity is enhanced significantly by phosphorylation of a single residue. Such *in vivo* regulation through conformational change inspired us to devise a new 'on-off' switch.

Adjacent cysteine residues in a protein form a strained eight-membered ring upon oxidation to a cystine (Figure 1). The peptide bond in the eight-membered ring was predicted by early theoretical calculations to have a *cis* conformation (Ramachandran and Sasisekharan, 1968; Chandrasekaran and Balasubramanian, 1969). However, studies with model peptides showed that this peptide bond is in a conformational equilibrium, with either the *cis* conformation (Capasso *et al.*, 1977; Sukumaran *et al.*, 1991) or the *trans* conformation (Garcia-Echeverría and Rich, 1997) being preferred according to context. In either conformation, the ring alters the structure of the polypeptide chain (Figure 1).

The oxidation of naturally occurring adjacent cysteine residues can impair protein function. For example, oxidation of Cys558 and Cys559 in the active site of mercuric reductase abolishes its catalytic activity (Miller *et al.*, 1989). When the disulfide bond is reduced with dithiothreitol (DTT), the enzyme recovers its activity.

The oxidation of adjacent cysteine residues can also destabilize protein conformation. The oxidation of cysteine residues in human ribonuclease inhibitor (hRI) inactivates the protein. Of the 32 cysteine residues in hRI, four belong to two pairs of adjacent cysteine residues: Cys94, Cys95, Cys328 and Cys329. When either pair is replaced with alanines, the resistance of the protein to oxidation is enhanced (Kim *et al.*, 1999).

Disulfide bonds between adjacent cysteine residues are known to exist even in the native conformation of proteins. Janus-faced atracotoxins (Wang *et al.*, 2000) and a variant of α -conotoxin GI (Gehrmann *et al.*, 1998) have adjacent half-cystines linked by a distorted *trans* peptide bond. Likewise, methanol dehydrogenase from both *Methylophilus* W3A1 (Ghosh *et al.*, 1995) and *Methylobacterium extorquens* (Xia *et al.*, 1999) has a distorted *trans* peptide bond between adjacent half-cystines in its active site. The resulting eight-membered ring is necessary for function, as the enzyme loses its catalytic activity when the disulfide bond is reduced by DTT (Blake *et al.*, 1994). A regulatory role for the disulfide bond between adjacent cysteine residues in the ligand-binding site of acetylcholine receptor has also been proposed (Kao and Karlin, 1986; Mosckovitz and Gershoni, 1988). Interestingly, a disulfide bond between adjacent cysteine residues is more stable to reduction than is one in a CXC motif—two cysteines with one intervening residue (Zhang and Snyder, 1989).

Together, these studies suggested to us that adjacent cysteines could serve as an artificial 'redox switch' by which enzymatic activity is turned on and off according to the reduction potential of the medium. If the native conformation of a protein necessitates the *trans* conformation of a peptide bond, imposing a distorted or *cis* conformation by the oxidation of adjacent cysteine residues would lead to inactivation. Reduction of the disulfide bond between adjacent cysteines would switch the protein back to its native conformation.

To explore the use of this motif as a redox switch, we introduced adjacent cysteine residues into ribonuclease A (RNase A; EC 3.1.27.5), a pyrimidine-specific ribonuclease from bovine pancreas (D'Alessio and Riordan, 1997; Raines, 1998). The plethora of information available on the structure and catalytic mechanism of this enzyme makes RNase A a suitable model system for designing and testing artificial switches (Hamachi *et al.*, 2000; Messmore *et al.*, 2000). We created two RNase A variants in which adjacent solvent-exposed residues were replaced with alanine: A5C/A6C RNase A and S15C/S16C RNase A (Figure 2). Each has a pair of adjacent cysteine residues within a distinct context of the three-dimensional structure. We report the effect of oxidation

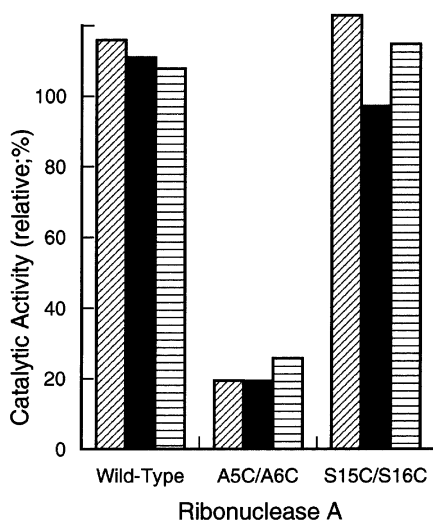


Fig. 3. Effect of oxidation on the ribonucleolytic activity of wild-type RNase A and the A5C/A6C and S15C/S16C variants. Each enzyme (10 nM) was incubated at 50°C for 1 h with dissolved oxygen (hatched), oxidized glutathione (20 nM; solid) or dithionitrobenzoic acid (20 nM; horizontal stripe) as an oxidant. Remaining activities were determined with 0.12 mM poly(C) as substrate in 0.10 M MES–NaOH buffer (pH 6.0) containing NaCl (0.10 M). The relative activities are the ratios of the remaining activities to the original activities determined before oxidation.

replaced with cysteine residues to create A5C/A6C RNase A. The α -helix formed by residues 3–13 contains His12, which is important for the binding and turnover of substrate (Thompson and Raines, 1995; Park *et al.*, 2001). Thus, a *cis* or distorted *trans* conformation of the peptide bond between Cys5 and Cys6 was expected to be detrimental to catalysis. Ser15 and Ser16 were also replaced with cysteine residues to create S15C/S16C RNase A. Ser15 and Ser16 are located in a long surface loop. Because of the likely flexibility of this loop, disulfide bond formation between the adjacent cysteine residues in S15C/S16C RNase A was not expected to disturb catalytic activity significantly.

Catalytic activity of A5C/A6C and S15C/S16C variants

The designed variants, A5C/A6C RNase A and S15C/S16C RNase A, were produced by site-directed mutagenesis. The extra sulfhydryl groups did not interfere significantly with the proper folding of the variants. After purification, the activities of the reduced variants were determined with poly(C) as substrate. The k_{cat} values of A5C/A6C RNase A and S15C/S16C RNase A were ~80% that of wild-type RNase A. The catalytic activities of the variants indicate that any interference caused by the introduced cysteines is negligible when these residues are reduced.

Modulation of catalytic activity by oxidation

Three distinct methods were used to oxidize the engineered variants. Wild-type RNase A and the two variants were oxidized at 50°C by dissolved $\text{O}_2(\text{g})$, GSSG and DTNB. The catalytic activity of A5C/A6C RNase A decreased to 20% of its initial value, whereas the activities of wild-type RNase A and the S15C/S16C variant were unaffected (Figure 3). The efficiencies of the three different oxidation methods were indistinguishable.

To reactivate A5C/A6C RNase A oxidized by GSSG, DTT was added to a final concentration of 1 mM. The variant recovered 52% of its original activity 1 min after adding DTT. In 20 min, the recovered activity reached 61% (Table I). These

Table I. Enzymatic activity of oxidized and reactivated A5C/A6C RNase A prepared with different methods^a

Oxidation method	Enzymatic activity (relative) (%)	
	After oxidation	After reactivation
1 h at 50°C with GSSG	19	61
Overnight dialysis at 23°C	28	100
Carboxymethylation after overnight dialysis	32	105

^aEnzymatic activity was determined with poly(C) (0.12 mM) as substrate in 0.10 M MES–NaOH buffer (pH 6.0) containing NaCl (0.10 M). Relative activity is the ratio of activity remaining to that before oxidation.

data suggest that the loss of catalytic activity by oxidized A5C/A6C RNase A results from the formation of a disulfide bond between Cys5 and Cys6 and that the reduction of this disulfide bond leads to the recovery of activity. Still, reactivation was not complete.

Incubation at 50°C could cause irreversible inactivation of an oxidized variant. Hence, reduced A5C/A6C RNase A was also oxidized at 23°C by overnight dialysis against an alkaline buffer with bubbling air. After purification, the activity of the oxidized A5C/A6C RNase A was 28% of its original activity. The oxidized A5C/A6C RNase A recovered 80% of its original activity within 1 min when DTT was added to a final concentration of 1 mM. After incubation with 1 mM DTT for 45 min, the variant recovered full activity (Table I). Thus, the loss in activity upon oxidation is fully reversible, as expected for the formation (by oxidation) and reduction of a disulfide bond between the side chains of Cys5 and Cys6 (Figure 1). Moreover, because the four native disulfide bonds of RNase A are important for catalytic activity (Klink *et al.*, 2000), these data suggest that the new disulfide bond between Cys5 and Cys6 can be reduced selectively.

Verification of completeness of oxidation

Carboxymethylation of a sulfhydryl group adds an extra negative charge to a protein near neutral pH. When oxidized A5C/A6C RNase A so treated was subjected to cation-exchange chromatography after gel filtration, only one protein eluted and this protein appeared at the salt concentration at which the oxidized variant elutes normally. The protein had 32% of the activity of reduced A5C/A6C RNase A. This activity increased to 105% when DTT was added to 1 mM (Table I). These activities are virtually identical with those of the oxidized and reactivated variant prepared without carboxymethylation, indicating that the amount of reduced enzyme is undetectable after overnight dialysis against alkaline buffer.

Effect of oxidation on conformational stability

The difference in the activities recovered from a 1-h incubation at 50°C and from dialysis overnight implied that the oxidized variant may denature irreversibly at 50°C (Table I). Thermal denaturation of reduced and oxidized A5C/A6C RNase A in 0.10 M Tris–HCl buffer (pH 8.0) was monitored with UV spectroscopy. Reduced A5C/A6C RNase A was observed to have a well-defined T_m (the temperature at the midpoint of the thermal denaturation) of 55°C. However, oxidized A5C/A6C RNase A showed an unusual non-co-operative thermal denaturation pattern (data not shown) and its T_m could not be determined with a two-state unfolding model. Moreover, oxidized A5C/A6C RNase A denatures irreversibly at high

temperature. This irreversibility, as well as the inability of the heated, oxidized enzyme to recover full activity (Table I), indicates that the conformational stability of A5C/A6C RNase A has been compromised by oxidation.

cis–*trans* Isomerization of the peptide bond between adjacent cysteine residues

The catalytic activity of oxidized A5C/A6C RNase A is ~30% that of the reduced enzyme. This residual activity could result from the interconversion of the *trans* and *cis* isomers of the peptide bond within the eight-membered ring of the oxidized enzyme. An NMR analysis of a heptapeptide with oxidized adjacent cysteines showed that such an equilibrium exists, and that $30 \pm 5\%$ of the oxidized heptapeptide has a *trans* conformation (Sukumaran *et al.*, 1991). Interestingly, this value is similar to the residual activity of the oxidized A5C/A6C RNase A. Nonetheless, this similarity is likely to be a coincidence, as the *cis*–*trans* equilibrium in a peptide is likely to differ from that in a folded protein. Moreover, it is unlikely that the *trans* isomer of oxidized A5C/A6C RNase A has the same catalytic activity as the reduced variant, as the two have different conformations (Figure 1).

Conclusions

Engineering novel disulfide bonds in proteins has been used to increase conformational stability or to regulate enzymatic activity (Perry and Wetzel, 1984; Matsumura and Matthews, 1989; Klink and Raines, 2000). Recently, thiol–disulfide interchange has also been identified as an *in vivo* molecular on–off switch in signal transduction pathways that control gene expression (Kim and Mayfield, 1997; Zheng *et al.*, 1998). Here, we demonstrated that adjacent cysteine residues are also capable of acting as an on–off redox switch to modulate enzymatic activity. The effectiveness of the regulation by adjacent cysteine residues is sensitive to the structural context of the motif. The adjacent cysteines in A5C/A6C RNase A do indeed modulate enzymatic activity upon oxidation and reduction. In contrast, those in S15C/S16C RNase A do not have any influence, despite their proximity to a critical active-site residue. We anticipate that adjacent cysteines introduced into other contexts could produce an even greater decrease–increase in enzymatic activity.

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