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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.

Keywords: conformational switch/cysteine/cystine/oxidation/ribonuclease

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

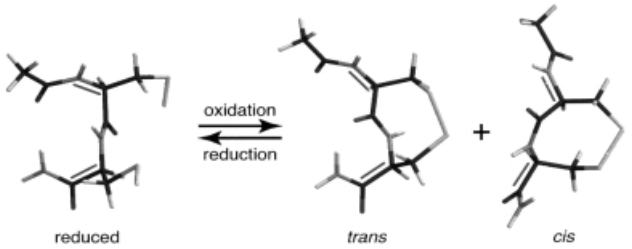

Fig. 1. Depiction of adjacent cysteine residues in a model peptide, $AcCysCysNH_2$, as a redox switch. The structure of reduced $AcCysCysNH_2$ is based on the ϕ and ψ main-chain dihedral angles of Ala5 and Ala6 in crystalline RNase A (Protein Data Bank entry 1RCN). The structures of oxidized $AcCysCysNH_2$ have a *trans* ($\omega = 180°$) or *cis* ($\omega = 0°$) peptide bond, and are depicted in a conformation of minimal energy according to the MMFF94 force field in MacSpartan Pro v.1.3.5 (Wavefunction, Irvine, CA). The *trans* and *cis* forms have $C^{\beta}SSC^{\beta}$ dihedral angles of –84° and +80°, respectively. The bars denote the direction of the peptide chain as it extends from C^{α} of each cysteine or half-cysteine residue.

		1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0
Wild-type	RNase A	K E **T A A A K F E R Q H M** D S S T S A A...
A5C/A6C	RNase A	K E **T A C C K F E R Q H M** D S S T S A A...
S15C/S16C	RNase A	K E **T A A A K F E R Q H M** D C C T S A A...

Fig. 2. N-terminal amino acid sequence of wild-type RNase A and the A5C/A6C and S15C/S16C variants. The engineered adjacent cysteine residues and His12, a critical active-site residue, are underlined. Residues 3–13, which form an α-helix, are in bold-faced type.

and reduction on the catalytic activity and conformational stability of the variants.

Materials and methods

Production of A5C/A6C and S15C/S16C variants

Plasmids that direct the production of the two variants, A5C/A6C RNase A and S15C/S16C RNase A, were created by site-directed mutagenesis (Kunkel *et al.*, 1987) using oligonucleotides CTACCGCTCAAACTTGCAGCATGCAGTTTCCTTGCC (for A5C/A6C) and GGAGCTGCTGGCAGCACTAGTACAACAGTCCATGTGCTG (for S15C/S16C). The proteins were produced, folded and purified as described elsewhere (delCardayré and Raines, 1994; Park and Raines, 2000), with the following modification. After the protein was folded overnight in a solution containing reduced glutathione (GSH; 1.5 mM) and oxidized glutathione (GSSG; 0.3 mM), DTT was added to 1 mM to reduce any mixed disulfides between the newly introduced sulfhydryl groups and glutathione or disulfide bonds formed by adjacent cysteines. The buffers for chromatography also contained 1 mM DTT to keep the introduced sulfhydryl groups reduced during purification. The catalytic activity of RNase A (and, presumably, the integrity of its native disulfide bonds) is not affected by 1 mM DTT.

Assays of enzymatic activity

RNase A catalyzes the cleavage of poly(cytidylic acid) [poly(C)]. Poly(C) was purified by ethanol precipitation. Assays of poly(C) cleavage were performed at 25°C in 0.10 M MES–NaOH buffer (pH 6.0) containing NaCl (0.10 M). Rates of poly(C) cleavage were determined by monitoring absorption at 250 nm and using $\Delta\varepsilon = 2380$/M/cm (delCardayré *et al.*, 1994). Enzyme concentrations were determined by using $\varepsilon = 0.72$ ml/mg/cm at 277.5 nm (Sela *et al.*, 1957). Kinetic parameters were determined by nonlinear regression analysis using the Michaelis–Menten kinetic mechanism.

Inactivation of A5C/A6C and S15C/S16C variants by oxidation

The effect of oxidation on the enzymatic activity of wild-type RNase A, A5C/A6C RNase A, and S15C/S16C RNase A was determined as follows. Enzymes were incubated at 50°C for 1 h in 0.10 M Tris–HCl buffer (pH 8.0) with oxidants. The elevated temperature was used to facilitate any conformational changes necessary to effect cystine formation. The low enzyme concentration, 10 nM, was used to prevent intermolecular disulfide bond formation. Three distinct oxidation conditions were used: (i) O_2(g) dissolved naturally in the buffer, (ii) oxidized glutathione (GSSG; 20 nM) and (iii) 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; 20 nM). Only a twofold excess of GSSG and DTNB was used to minimize the formation of two mixed disulfides between the enzyme and the oxidant. After oxidation, the remaining ribonucleolytic activity of each sample was determined by monitoring the cleavage of poly(C).

Oxidation of A5C/A6C variant by dialysis

Oxidized A5C/A6C RNase A was prepared in a large quantity as follows. The A5C/A6C variant (40 μM) was dialyzed overnight against 0.10 M Tris–HCl buffer (pH 8.0) with bubbling air at ambient temperature. To remove any dimer formed by intermolecular disulfide bonds, the dialyzed solution was applied to a gel filtration column that had been equilibrated with 0.050 M sodium acetate buffer (pH 5.0) containing NaCl (0.10 M). Monomeric protein was applied to a cation-exchange column that had been equilibrated with 0.050 M sodium acetate buffer (pH 5.0) and protein was eluted with a gradient of NaCl. One peak in the chromatogram from the cation-exchange column accounted for >90% of the total protein. The protein in this peak was used for further study.

Carboxymethylation of oxidized A5C/A6C variant

The completeness of the oxidation of A5C/A6C RNase A upon dialysis overnight was determined by carboxymethylation. A solution of iodoacetic acid was neutralized with one equivalent of aqueous sodium hydroxide prior to use. The resulting solution of sodium iodoacetate was added to a final concentration of 0.010 M to A5C/A6C RNase A that had been oxidized by overnight dialysis. After incubation for 15 min at ambient temperature, the protein was purified as described above.

Determination of conformational stability of A5C/A6C variant

As RNase A is denatured, its six tyrosine residues become exposed to solvent and its molar absorptivity near 280 nm decreases significantly. Protein solutions were dialyzed against 0.10 M Tris–HCl buffer (pH 8.0) with bubbling Ar(g), which prevents oxidation. The dialyzed protein solutions were sealed in quartz cuvettes with stopcocks. Conformational stability was assessed by monitoring the change in absorbance at 286 nm as the temperature was increased from 20 to 75°C at 0.2°C/min.

Results and discussion

Design of an RNase A variant with a redox switch

To minimize the adverse effect imposed by the installed cysteines in their reduced state, we selected solvent-exposed alanine and serine residues as mutagenesis targets (Figure 2). Ala5 and Ala6 in the N-terminal α-helix of RNase A were

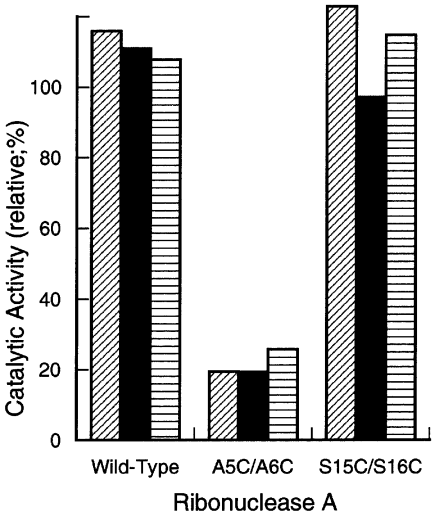

Fig. 3. Effect of oxidation on the ribonucleolytic activity of wild-type RNase A and the A5C/A6C and S15C/S16C variants. Each enzyme (10 nM) was incubated at 50°C for 1 h with dissolved oxygen (hatched), oxidized glutathione (20 nM; solid) or dithionitrobenzoic acid (20 nM; horizontal stripe) as an oxidant. 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 O_2(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 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.

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

[a]Enzymatic 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.

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 ± 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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