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Oxidation of buried cysteines is slow and an insignificant factor in the structural destabilization of staphylococcal nuclease caused by H_2O_2 exposure

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Summary. The oxidation of buried cysteine or methionine residues can destroy the enzyme activity of a protein by disrupting structure. Engineering in such an oxidatively triggered switch for enzyme activity would only be useful if the effects of substitution are relatively minor, while the effects of the oxidized side chain upon structure are significant and the oxidation relatively easy. To assess the feasibility of this strategy for controlling enzyme activity, the effects of such substitutions and their oxidation were studied in a well characterized model protein, staphylococcal nuclease. Stability and enzyme activity of the oxidized proteins was assessed and compared to the stability and enzyme activity of the unoxidized proteins. Cysteines were found to be generally well tolerated in buried positions but these mutants were not more destabilized than wild-type when oxidized. This shows that buried cysteines are difficult enough to oxidize that this is not likely to be a useful protein engineering strategy or a commonly used regulatory modification. Similar effects were observed for methionine.

Keywords: Enzyme activity – Rate – Redox – Regulation – Solvent accessibility

Introduction

Methionine, tryptophan, tyrosine, histidine, and cysteine are among the side chains in proteins susceptible to oxidation. Without question though, the most easily oxidized group in proteins is the thiol of cysteine. This oxidation can be mild and readily reversible, resulting in a disulfide or perhaps a sulfenic acid (R-SOH) (Claiborne et al., 2001; Claiborne et al., 1993; Denu and Tanner, 2002). It can also be more complete, to a sulfinic acid ($R\text{-}SO_2H$) or even a sulfonic acid ($R\text{-}SO_3H$), and difficult to reverse (Barrett et al., 1999; Mallis et al., 2002; Wagner et al., 2002).

Oxidation of proteins is of interest for several reasons. It has been suggested as a causative or contributory factor in many diseases (Davies et al., 1999; Dean et al., 1997). Oxidized proteins have been found to increase in aged organisms, leading to the proposal that protein oxidation contributes to the aging process (Levine and Stadtman, 2001; Linton et al., 2001; Schoneich, 2001; Thomas and Mallis, 2001). However, of particular interest to us is an idea that has recently been gaining currency. It has been proposed that the activity of proteins is regulated *in vivo* by the oxidation state of cysteine residues (Brune and Mohr, 2001; Claiborne et al., 1999; Cunnick et al., 1998; Klotz, 2002; Lipton et al., 2002; Moran et al., 2001; Wilcox et al., 2001). It seems clear that this redox regulation is important in a number of systems, including both those where hydrogen peroxide (Denu and Tanner, 1998; Finkel, 1999; Fu et al., 2001; Griendling et al., 2000; Morad and Suzuki, 2000; Rhee et al., 2000; Ryter and Tyrrell, 1998; Xu et al., 2002) or the signaling molecule nitric oxide (NO) (Brune and Mohr, 2001; Lipton et al., 2002; Stamler et al., 2001) act as oxidants. This regulation of activity is potentially reversible if the oxidation is mild, or likely irreversible if the thiol is taken to a higher oxidation state.

While the evidence is accumulating that cysteine redox regulation is important *in vivo* it is less clear what the molecular mechanism is by which protein activity is altered. In the largest number of cases where the mechanism is known it appears that oxidation of an active cysteine either directly affects activity or substrate binding. However, this is not the only mechanism by which cysteine oxidation could affect the biological activity of a protein. It is well known that chemical modifications of side chains can have profound effects on protein stability

and structure, the prototypical example being phosphorylation. Just as phosphorylation is known to alter the stability of one particular protein conformation relative to another, the oxidation of cysteine could presumably change protein structure and stability by reducing side chain hydrophobicity, increasing the capacity for polar interactions, or simply by altering the size and shape of the cysteine. It is common knowledge that disulfide formation can have a profound influence on protein structure and hence activity. However, in relatively few instances has cysteine oxidation to the sulfenic, sulfinic, or sulfonic acids been reported to alter protein structure (Blazquez et al., 1996; DalleDonne et al., 1999; Meyer et al., 2002; Milzani et al., 2000; Pozdnyakova and Wittung-Stafshede, 2002; Rua et al., 2002; Sluis-Cremer and Dirr, 1995; Volkin et al., 1997). This is somewhat puzzling since such structural effects seem to be more important in activity regulation by phosphorylation than does direct alteration of the active site residues.

A related area of interest is determination of the factors which control the ease of cysteine oxidation. To the best of our knowledge only one group (Maleknia et al., 1999; Maleknia et al., 2002) has examined the influence of cysteine side chain environment upon rates of oxidation. Just as in the better studied case of methionine oxidation (Estell et al., 1985; Kornfelt et al., 1999; Levine et al., 1996; Nguyen et al., 1993), it was suggested that solvent accessibility is probably a major factor in determining the rate of oxidation. While the rate is slower than for solvent exposed residues, hydrogen peroxide still can oxidized buried methionines (Keck, 1996). The rate of oxidation of buried cysteines is an important question since oxidation of buried cysteines would be expected to have a larger impact on protein structure than solvent exposed residues. In effect oxidation introduces a very polar group into a generally nonpolar environment, an unfavorable situation likely to lead to structural rearrangement or severe destabilization of the native state. If oxidation of cysteine can indeed regulate protein activity through conformational changes this raises the prospect of engineering proteins to introduce such redox regulatory control into them. If, however, they are very slow to oxidize, buried cysteines might be less suitable to be used for redox regulation.

Therefore we felt it worthwhile to examine the effects of cysteine oxidation in a well characterized model protein, staphylococcal nuclease, both wild-type and various mutants. The results of these studies are reported here.

Materials and methods

Highly polymerized salmon testes DNA was purchased from Sigma. Tris(2-carboxyethyl)phosphine-HCl (TCEP) was purchased from Molecular Probes, Inc. 2-Mercaptoethanol (BME) was purchased from Millipore. Hydrogen peroxide (H_2O_2, nominal 30%) was purchased from Fisher Scientific. All mutants of staphylococcal nuclease were prepared by procedures previously described (Byrne et al., 1995).

Procedure for oxidation

Hydrogen peroxide, a well known oxidizing agent fairly specific for methionine and cysteine (Shechter et al., 1975) was used. We have previously established that these conditions do not oxidize tryptophan (Kim et al., 2001). To 0.12 μmole (192.3 μl) of wild-type or mutant (single mutants V23C, K48C, T62C, T62M and K64C; double mutant T62C/V23C) staphylococcal nuclease at a concentration of 2.6 mg/ml (pH 7.0, 25 mM sodium phosphate, 100 mM NaCl) was added 11.2 μl of 31.6% w/w hydrogen peroxide (approximately 630 equivalents assuming each of the 4 methionines in wild-type is oxidized to sulfoxide and a single cysteine is oxidized to the sulfinic acid state). This solution was stirred for 30 minutes at room temperature. The reaction mixture was then quenched with a large excess (25.2 μl, approximately 2000 equivalents) of β-mercaptoethanol. The oxidized proteins were stored at -20°C until stabilities and activities were determined.

Protein stability determination

Guanidine hydrochloride denaturation and data analysis for unoxidized proteins were carried out as previously described (Kim et al., 2001; Schwehm and Stites, 1998; Stites et al., 1995). Briefly, in this procedure the sole tryptophan of staphylococcal nuclease is excited at 297 nm and emission at 325 nm is followed as function of guanidine hydrochloride concentration in an Aviv ATF-101 automated titrating fluorometer. (Fluorescence emission spectra of proteins did not change upon oxidation other than what was consistent with the presence of some fraction of denatured protein, data not shown.) The apparent equilibrium constant, K_{app}, is calculated from the fluorescence intensity, I, at each guanidine hydrochloride concentration according to the equation: $K_{app} = (I_N - I)/(I - I_D)$, where I_N and I_D are the fluorescence intensities of the native and denatured states, respectively. An extrapolation of $-RT\ln K_{app}$ from the easily measured transition region back to zero denaturant concentration gives the stability difference between the native and denaturated states in the absence of guanidine hydrochloride, ΔG_{H_2O}. The other parameters, m_{GuHCl} and C_m, are the slope of this extrapolated line and the midpoint concentration of the denaturation. In one minor alteration of previously used procedures, all unoxidized cysteine mutants were pre-treated with a reducing agent, *tris*-carboxyethylphosphine (5 mM concentration), to prevent disulfide formation.

As previously reported (Kim et al., 2001) the denaturation data for oxidized proteins fits the two-state model relatively poorly, probably because of the presence of multiple species with different levels of methionine oxidation. Fortunately, of the three parameters, m_{GuHCl}, C_m, and ΔG_{H_2O}, it is the latter, the stability difference between the denatured and native states in the absence of denaturant, that is both most important for our purposes and the most accurate. This accuracy is due to the fact that after the oxidation the resulting mixture of proteins is often sufficiently destabilized that a reasonable estimation of the average equilibrium constant between the denatured and native states can be made directly without any extrapolation. For virtually all mutants or oxidized proteins, the relative fluorescence intensities of the native and denatured states are constant, very near a ratio of 5.7 to 6.0. The quantum yield of the tryptophan decreases in the denatured state and the emission maxima shifts from approximately 335 nm to a broader peak with a maxima around 350, all due to greater exposure to solvent in the denatured state. The

denatured state intensity at high guanidine hydrochloride concentrations, I_D, can therefore be used to estimate the native state intensity, I_N, and then in combination with the intensity at zero guanidine hydrochloride concentration, an equilibrium constant and, thus, ΔG_{H_2O} were determined using the equation $K_{app} = (5.85I_D - I)/(I - I_D)$. Note that ΔG_{H_2O} is in these cases a concentration weighted average value of the stability of all the different protein species present.

Enzyme activity

Degradation of DNA by both unoxidized and oxidized proteins (0.26 μg of each enzyme added to 2.0 ml of DNA) was used was measured using an HP 8452 Diode Array Spectrophotometer at room temperature of approximately 24°C by previously published methods (Cuatrecasas et al., 1967).

Results and discussion

We wished to explore how the environment of the cysteine influenced its reaction with peroxide and how large of an impact any oxidation had on structural stability. We chose as our model system the protein staphylococcal nuclease. This protein is especially suited for such work for several reasons. There are no cysteines found in the wild-type protein so it is simple to substitute a cysteine at any given position and attribute the results to that single thiol. Equally important, we have previously examined the impact of methionine oxidation in this protein (Kim et al., 2001) and therefore have a clear understanding of the effects of this other oxidation in the system. There are four methionines in wild-type staphylococcal nuclease, all partly solvent exposed. These methionines and, possibly, other side chains such as tryptophan and histidine can oxidize and destabilize the protein. The question we address here is whether cysteines increase the susceptibility of the protein to oxidative destabilization.

Accordingly we made the single mutants V23C, K48C, T62C, and K64C as well as the double mutant T62C/V23C. In addition, T62M was included to compare the effects of another potentially oxidizable side chain. Positions 23 and 62 are completely buried in the major hydrophobic core. Threonine 62 is unusual in that while buried in a largely hydrophobic environment, it is a polar residue hydrogen bonded to main chain carbonyls. Positions 48 and 64 are both completely solvent exposed. Positions 23 and 62 are located so that any disulfide bond, if formed, in the native state of double mutant T62C/V23C would be very strained.

Wild type and mutant proteins were oxidized with hydrogen peroxide followed by addition of excess β-mercaptoethanol (BME) as a quenching agent. This quenching agent removes any excess peroxide, preventing further oxidation reactions, and also reduces disulfides and sulfenic acids back to the thiol state. Only the higher oxidation states are stable in the presence of excess BME. Intermolecular or, in the case of the double mutant, intramolecular disulfide crosslinking would certainly alter structure and stability, but this would be a rather trivial result. Sulfenic acid derivatives are expected to be rather unstable in the absence of specialized structural features stabilizing them and are prone to further reaction (Claiborne et al., 1999). We therefore chose to focus on the more stable oxidation forms. We first established the amounts of peroxide and reaction times just sufficient to result in significant loss of protein stability, but not so overly harsh as to completely oxidize everything and obscure difference in oxidative susceptibility (Figs. 1 and 2). Other side chains in the

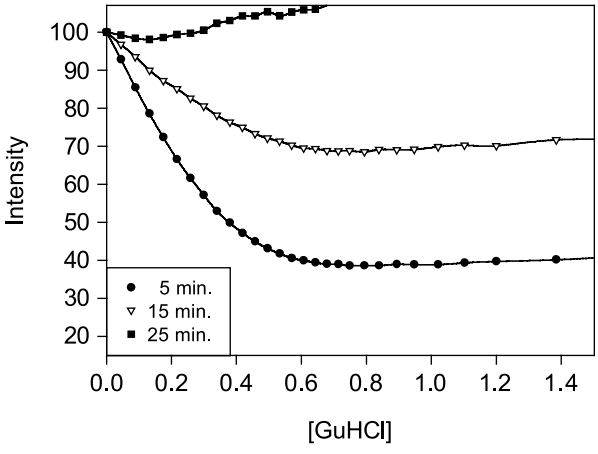

Fig. 1. Time course studies using T62C. The T62C mutant was treated with 630 equivalents of H_2O_2 as described in Materials and Methods save that the time at when quenching agent was added was varied as indicated

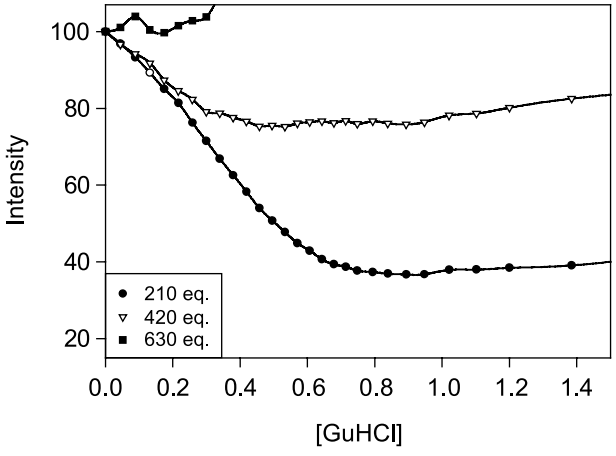

Fig. 2. Peroxide concentration studies using T62C. The T62C mutant was treated for 30 minutes as described in Materials and Methods save that the number of equivalents of H_2O_2 added was varied as indicated

Table 1. Guanidine hydrochloride denaturation parameters for mutants

Mutant	Unoxidized proteins			Oxidized proteins			$\Delta\Delta G^e$
	$\Delta G_{H_2O}{}^a$	$C_m{}^b$	$m_{GuHCl}{}^c$	$\Delta G_{H_2O}{}^d$	C_m	m_{GuHCl}	
Wild-type	5.4	0.82	6.53	1.5	0.4	3.8	3.9
V23C	4.2	0.56	7.30	1.2	–	–	3.0
K48C	5.2	0.81	6.32	0.8	0.24	3.3	4.4
T62C	4.0	0.64	6.23	0.6	–	–	3.4
T62M	4.4	0.75	5.92	0.9	–	–	3.5
K64C	4.9	0.76	6.40	1.5	0.32	4.7	3.4
T62C/V23C	3.5	0.46	7.47	–[f]	–	–	>5.0

[a] Free energy difference between native and denatured states in the absence of denaturant. Units of kcal/mol. Error estimated to be ± 0.1 kcal/mol based on experience of experimental reproducibility

[b] Midpoint concentration (concentration of guanidine hydrochloride at which half of the protein is denatured) in units of molar. Error estimated to be ± 0.01 M

[c] Slope value (change in free energy with respect to change in guanidine hydrochloride concentration) in units of kcal/(mol · M). Error is estimated to be ± 0.13

[d] Parameters returned from fit of guanidine hydrochloride denaturation data to two state model when C_m and m_{GuHCl} are given, but direct estimation where not. Error is introduced by this approximation of a more complex denaturation process of a heterogeneous mixture of oxidized species. Values are approximate only

[e] Approximate difference in stability of oxidized and unoxidized proteins where $\Delta\Delta G = \Delta G_{unoxidized} - \Delta G_{oxidized}$. Units of kcal/mol

[f] Native state completely non-detectable and, while no direct estimation of stability is possible, this indicates ΔG_{H_2O} is -1.5 kcal/mol or less

protein, in particular, the four methionines, will oxidize under these conditions. The question of interest is how a new cysteine side chain elsewhere in the protein alters the overall rate of oxidative destabilization. Once suitable conditions were determined all proteins were subjected to oxidation.

Stability of both the unoxidized and oxidized proteins is shown in Table 1. The experiment was repeated numerous times and, as expected by our selection of reaction conditions that do not devastatingly over oxidize the protein, there were somewhat variable results. The oxidation of the double mutant (V23C/T62C) always progressed sufficiently to apparently completely disrupt the native state. However for the T62C single mutant in just twelve out of sixteen oxidations did complete disruption of structure result. For V23C only two out twelve trials resulted in complete native state disruption, and for T62M as well as for wild-type and the solvent exposed cysteines mutants, complete disruption of structure never occurred and there was relatively less variability in the titration curves. Therefore in Fig. 3 we show representative titration curves before oxidation (which are highly reproducible) and the titration curve after the oxidation trial which resulted in the least destabilized protein mixture for T62C, V23C, and T62M. The stabilities given in Table 1 for these three mutants are calculated from the titration curves shown in Fig. 3.

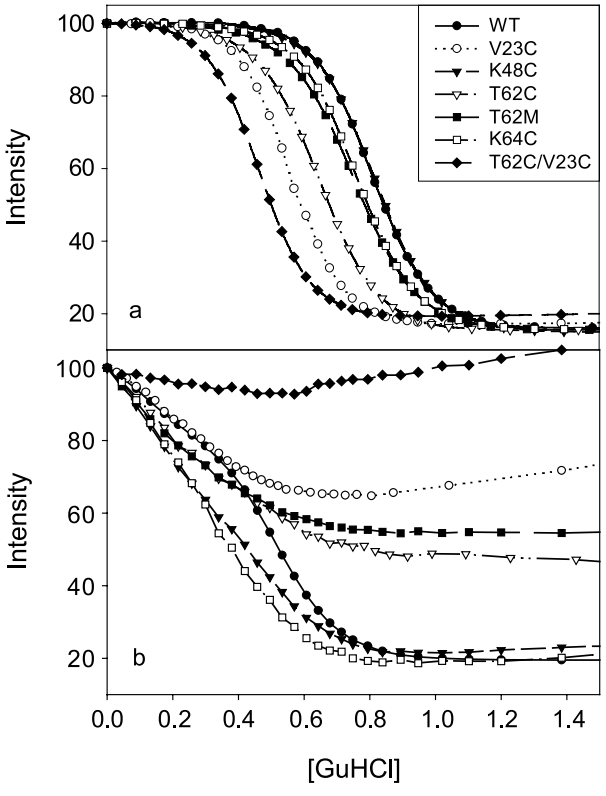

Fig. 3. Plot of relative fluorescence intensity versus guanidine hydrochloride concentration. **a** Unoxidized proteins. **b** Proteins after oxidation under conditions described in Materials and methods

Table 2. Enzyme activities of oxidized and unoxidized proteins

Mutant	Unoxidized protein		Oxidized protein	
	K_{obs}[a]	% rel. to WT	K_{obs}[a]	% rel. to WT
Wild-type	4.5	100	4.8	107
V23C	1.2	22	0.6	13
K48C	2.2	49	2.9	64
T62C	5.5	122	2.6	58
T62M	6.5	144	6.4	142
K64C	1.0	22	1.4	31
T62C/V23C	6.3	140	0.2	4

[a] Enzyme activity measured under pseudo-first order conditions by monitoring change in absorbance of DNA at 260 nm. All K_{obs} values are in units of 10^{-3} $\Delta A_{260\,nm}$/sec. The K_{obs} for wild-type (WT, average of 5 determinations) was 4.5×10^{-3} $\Delta A_{260\,nm}$/sec with a standard deviation of 1.7×10^{-4}. The standard deviations for 3 determinations for V23C, K48C, and K64C were respectively, 7.1×10^{-5}, 3.3×10^{-4}, and 2.4×10^{-4}. However, the variation of K_{obs} for WT is about 2 fold from day to day and differences in activity of less than this are not considered significant

Of course, each mutation affects the stability of the unoxidized protein differently. We have also previously shown (Kim et al., 2001) that our experimental procedure largely, but not completely, oxidizes the four methionines in wild-type staphylococcal nuclease to sulfoxides. Therefore, the difference in the stability of each unoxidized and oxidized mutant protein must be compared to change in stability due to the oxidation of the methionines in the unmutated wild-type in order to reveal the relative sensitivity to oxidation.

There is variation apparent in Table 2 from mutant to mutant for the value of $\Delta\Delta G$, the difference in stability between the oxidized and unoxidized protein, but it is not enormous considering the error inherent in estimating the stability of the oxidized species. Wild-type was destabilized by oxidation, as expected, by the oxidation of methionine (Kim et al., 2001). The two mutants, K48C and K64C, both with solvent exposed cysteines, were not destabilized much more than wild-type by oxidation. This is expected since the polar oxidation product should be relatively well tolerated on the surface of the protein. In contrast, it was found that oxidation completely disrupted the native structure of mutant T62C/V23C, indicating a $\Delta\Delta G$ of greater than 5 kcal/mol. However, V23C, T62C, and T62M do not have $\Delta\Delta G$ values significantly different from that of wild-type.

To some degree the $\Delta\Delta G$ values listed in Table 1 for these latter three single mutants are deceiving. Recall that these values are derived from the oxidation trial which had the least effect on their stability. Looking at the number of trails which resulted in complete disruption of native structure it is clear that T62C is the most sensitive to oxidation, followed by V23C, and with T62M being the least sensitive and not terribly different from wild-type. Nevertheless, none of these mutants has had a dramatic increase in sensitivity to oxidation. In theory, this could be either because the rate of cysteine oxidation in buried position is slow or because the products of the oxidation are well tolerated in these positions. The latter can be ruled out since oxidation of cysteine will result in a charged side chain at the pH of our assay and we have demonstrated that buried charged side chains in the major hydrophobic core of staphylococcal nuclease are severely destabilizing (Dwyer et al., 2000; Garcia-Moreno et al., 1997; Stites et al., 1991). The idea that cysteine oxidation in buried positions is slow is also supported by the similar effect of oxidation upon T62C and T62M, since the oxidation of buried methionines by hydrogen peroxide is known to be slow (Estell et al., 1985; Kornfelt et al., 1999; Levine et al., 1996; Nguyen et al., 1993).

Once oxidation of the partly solvent exposed methionines present in all these staphylococcal nuclease variants has sufficiently destabilized the protein, unfolded it, and exposed the formerly buried cysteine to solvent and oxidant, the oxidation of that cysteine should proceed rapidly. To complement stability measurements, enzyme activity of the oxidized proteins was assessed and compared to the activity of the unoxidized proteins. It was found that oxidation virtually completely inactivated the enzyme activity of double mutant, T62C/V23C (two buried cysteines). On the other hand, the activity of all other proteins were relatively unaffected by the same conditions of oxidation. The presence or absence of reducing agents (TCEP or BME) made no difference to the enzyme activities.

This result supports the conclusion that oxidation of the buried cysteines is slow. A variety of staphylococcal nuclease mutants, including very unstable truncation mutants, have been shown to have activity very similar to that of wild-type (Flanagan et al., 1992). Substrate binding pulls the folding equilibrium toward native structure. Alone among the proteins studied here, the double mutant T62C/V23C, which if fully oxidized would have to accommodate two negative charges in close proximity in the low dielectric hydrophobic core of the native state, can not be pulled into the native state even by the large substrate excesses of the activity assay. Clearly then, oxidation of these cysteines, if it occurs, is very destabilizing. However, under the conditions employed here, it does not appear to occur very rapidly.

In summary, it appears that adding buried cysteines to this protein does increase oxidative sensitivity, but the

effect is relatively small. The simplest interpretation of the data presented here is that this lack of additional oxidative sensitivity is because oxidation rates for buried cysteines are quite slow. In contrast, the oxidation of solvent exposed cysteines, while expected to be rapid, has relatively insignificant stability effects since the polar oxidized side chain is likely well tolerated in such positions. This may explain in part why redox regulation of enzyme activity appears to be largely confined to oxidation of solvent exposed cysteines in active sites.

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