Formation and Reactions of Sulfenic Acids in Proteins

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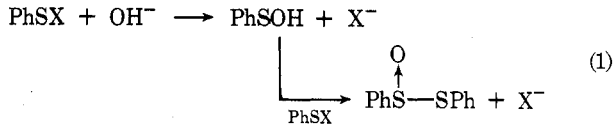
The side chains of cysteinyl residues in proteins either exist in the free sulfhydryl form or are linked covalently to another cysteinyl side chain through a disulfide bond. Disulfide bonds are introduced into proteins as post-translational events. The classic experiments of Anfinsen suggest that the disulfide bonds in native proteins are formed by the oxidation of specific pairs of cysteinyl residues that are brought into close proximity by the spontaneous folding of newly synthesized polypeptide chains.[1]

It is usually assumed that the inactivation by mild oxidants of enzymes which require sulfhydryl groups for activity is due to the formation of disulfide bonds between the essential cysteinyl residue and another sulfhydryl group in the protein.[2] Usually these inactivations can be reversed completely by the addition of thiols, which suggests that the oxidation of the essential sulfhydryl groups is not accompanied by gross conformational changes. To form a disulfide bond in an enzyme during in vitro oxidation under nondenaturing conditions requires the following structural features. An intramolecular disulfide bond will form only when the two participating cysteinyl residues are close to each other in the native protein. If this were the case, one might have expected that a disulfide bond would have been formed between the two cysteinyl residues during the natural folding process. An intermolecular disulfide bond will only form during the oxidation of native proteins when the two participating sulfhydryl groups are located in an environment on the surface of the protein which will allow them to approach each other. During the last several years evidence has accumulated which suggests that sulfhydryl groups in several proteins are converted to sulfenic acids or other sulfenyl derivatives, and not to disulfide bonds, when they react with mild oxidants under nondenaturing conditions.

Sulfenic acids have been suggested to be intermediates in the oxidation of protein sulfhydryl groups but have been considered to be too unstable to accumulate to any extent.[2] Until recently, 1-anthraquinonesulfenic acid, which was originally isolated by Fries in 1912,[3] and two other anthraquinonesulfenic acids synthesized by Bruice were the only sulfenic acids which were characterized.[4] The rare occurrence of sulfenic acids in small organic molecules[5] may be attributed to the high reactivity of this functional group as either a nucleophile or as an electrophile under different reaction conditions. For instance, the alkaline hydrolysis of aromatic sulfenyl halides invariably leads to the formation of the corresponding thiolsulfinates, rather than sulfenic acids, as the first isolable products.[6] Kice has presented evidence which indicates that this behavior is due to the nucleophilic reactivity of the sulfenic acids generated by hydrolysis toward the dicoordinate sulfur in the parent sulfenyl halides as described by eq 1.[7] With the

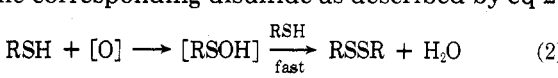


(1)

exception of the successful isolation of 4-methyluracilsulfenic acid, the alkaline scission of disulfides has not proved to be a satisfactory method to synthesize sulfenic acids.[8] When treated with alkali most aromatic disulfides are converted to 3 mol of thiol and 1 mol of sulfinate.[9]

Although alkanesulfenic acids have never been isolated when alkyl disulfides are treated with alkali, they have been identified as products of the thermal decomposition of alkyl sulfoxides and alkyl thiolsulfinates.[10,11] By a combination of NMR and ir spectroscopy and trapping experiments, Shelton and Davis have identified 1,1-dimethylethanesulfenic acid as a product of the thermolysis of di-*tert*-butyl sulfoxide.[10] Block has reported that a number of alkanesulfenic acids can be trapped with alkenes and alkynes during the pyrolysis of alkanethiolsulfinic esters.[11]

Sulfenic acids may be transient intermediates during the oxidation of mercaptans with mild oxidants such as *o*-iodosobenzoate or a stoichiometric quantity of hydrogen peroxide. However, these oxidations invariably proceed with the quantitative formation of disulfides. This behavior may be attributed to the electrophilic character of the sulfenyl sulfur in the intermediate which would react rapidly with the parent thiol to produce the corresponding disulfide as described by eq 2.



(2)

Cysteinyl side chains situated in clefts in globular proteins which are accessible to solvent are uniquely suited for oxidation to sulfenic acids by mild oxidants. When the surface topology of the protein prevents the formation of interchain disulfide bonds and there are no

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(10) (a) J. R. Shelton and K. E. Davis, *J. Am. Chem. Soc.*, **89**, 718 (1967); (b) J. R. Shelton and K. E. Davis, *Int. J. Sulfur Chem.*, **8**, 205 (1973).
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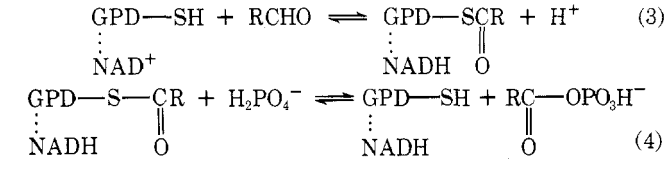
other sulfhydryl groups in the vicinity to form intrachain disulfide bonds, it is obvious that a sulfenic acid should be in an ideal environment to accumulate during the reaction of a cysteinyl side chain with a mild oxidant. It is the purpose of this Account to review the evidence which suggests that stable protein sulfenic acids are formed during the oxidation of cysteinyl side chains in some instances, and to review the unique reactivity that protein sulfenic acids possess.

Evidence for Sulfenic Acids in Proteins

Since sulfenic acids are unstable to acid hydrolysis and do not possess distinguishing spectroscopic features that can be used for their identification in intact proteins, the existence of protein sulfenic acids rests on indirect evidence. The elusiveness of simple sulfenic acids has prevented the detailed study of the reactivity of this fundamental functional group in aqueous solution. Therefore, the reactions that have been used to identify sulfenic acids in proteins cannot be directly correlated with similar reactions in model systems.

The most convincing evidence that specific protein sulfhydryl groups can be oxidized to stable sulfenic acids has been provided by studies on the catalytically active sulfhydryl groups of papain and glyceraldehyde-3-phosphate dehydrogenase (GPD for the remainder of this discussion). Both papain and GPD have sulfhydryl groups that participate directly in catalysis.[12,13] Papain catalyzes the hydrolysis of peptide bonds and the hydrolysis of synthetic amides and esters, all of which proceed with an enzyme thiolester intermediate. In solution, papain exists as a single polypeptide chain made up of 212 amino acids which contains three disulfide bonds.[12] The elucidation of the structure of the enzyme by x-ray crystallography has revealed that the side chain of Cys-25, the single sulfhydryl group in the enzyme, is located in a groove on the surface of the enzyme.[14] GPD catalyzes the oxidative phosphorylation of 3-phosphoglyceraldehyde using NAD^+ as cofactor, as illustrated by eq 3 and 4. During catalysis a thiolester



intermediate is formed when hydride is transferred to the noncovalently bound coenzyme from a thiohemiacetal derivative of the catalytically essential sulfhydryl group. The thiolester intermediate is then phosphorylyzed to form the acyl phosphate product.[15] The active form of the enzyme is an oligomer composed of four identical polypeptide chains. The monomeric unit of the enzyme isolated from pig muscle is composed of 332 amino acids.[16] In addition to Cys-149, the catalytically active sulfhydryl group, the monomeric unit of pig muscle GPD contains three additional sulfhydryl groups. There are no disulfide bonds in the enzyme.

That specific sulfhydryl groups in some proteins can be oxidized to stable sulfenic acids by mild oxidants rests on the following lines of evidence. (1) There are precedents for the formation of other derivatives of protein sulfhydryl groups at the sulfenic acid oxidation state. Stable protein sulfenyl iodides and sulfenyl thiosulfates have been characterized. (2) The stoichiometry of oxidation of sulfhydryl groups in some proteins is consistent with the formation of sulfenic acids and not disulfides. (3) The oxidation of the catalytically active sulfhydryl groups of papain and GPD by mild oxidants introduces an electrophilic center in each of the enzymes which has properties similar to sulfenyl halides.

In 1955 Fraenkel-Conrat presented the first conclusive evidence that the sulfhydryl groups in proteins can be converted to stable derivatives at the sulfenic acid oxidation state.[17] He demonstrated that the lone sulfhydryl group of the tobacco mosaic virus coat protein is converted to a sulfenyl iodide when the protein is treated with triiodide. Treatment of simple thiols with iodine or triiodide normally converts them to disulfides. The evidence which suggests that the tobacco mosaic virus coat protein is converted to a sulfenyl iodide during its reaction with triiodide is based on stoichiometry and the binding of ^{131}I. Each sulfhydryl group which disappeared consumed 2 equiv of iodine and not one, as occurs during the oxidation of simple thiols to disulfides. Moreover, the disappearance of the sulfhydryl group was accompanied by the binding of ^{131}I which could be released by the addition of cysteine or by denaturation of the protein. Stable sulfenyl iodide derivatives of β-lactoglobulin and ovalbumin have also been characterized by Cunningham.[18]

In 1962 Pihl and Lange demonstrated that a stable sulfenyl thiosulfate derivative of GPD is formed when the enzyme is inactivated with tetrathionate.[19] Simple thiols are oxidized to their corresponding disulfides by tetrathionate, presumably with the formation of a sulfenyl thiosulfate intermediate. When GPD is inactivated with tetrathionate labeled with ^{35}S, radioactive thiosulfate becomes covalently bound to the enzyme which can be released by the addition of simple thiols or by denaturation of the enzyme by mild heat.[19,20] Tetrathionate reacts with Cys-149, the catalytically active sulfhydryl group at the active site of the enzyme, to form a sulfenyl thiosulfate derivative, as described in Scheme I. The addition of simple thiols to the inactivated enzyme releases thiosulfate; this is accompanied by the restoration of activity. The sulfenyl thiosulfate derivative of the enzyme is stable at 0 °C. However, when it is incubated at 37 °C or dissociated with urea, thiosulfate is released from the enzyme and a disulfide bond is formed between Cys-149 and a cysteine residue four amino acid residues removed from it in the primary sequence, as shown in Scheme I. The formation of the intramolecular disulfide bond, which is brought about by heat treatment, irreversibly inactivates the enzyme, i.e., the enzyme cannot be reactivated by the addition of thiols.[20,21] This is apparently caused by a conforma-

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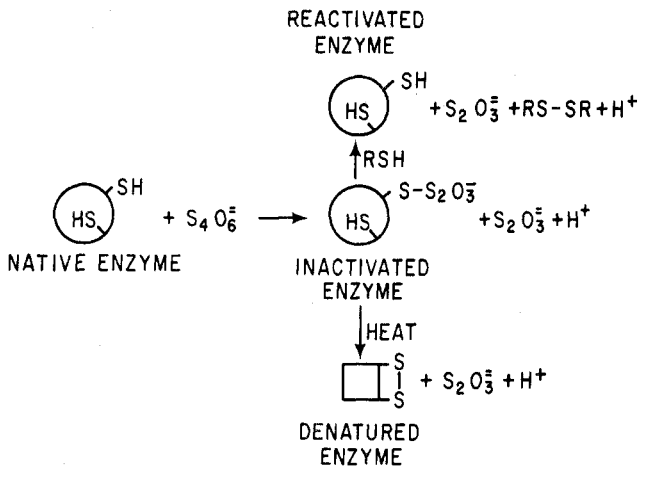
Scheme I



tional change which pulls Cys-153 from its native environment and allows it to displace thiosulfate from the sulfenyl thiosulfate derivative of Cys-149. X-ray crystallographic studies have shown that the side chain of Tyr-311 lies between the side chains of Cys-149 and Cys-153 in the native enzyme.[22] This would prevent the formation of a disulfide bond between these two residues under nondenaturing conditions.

The catalytically active sulfhydryl group of GPD reacts stoichiometrically with *o*-iodosobenzoate. The addition of *o*-iodosobenzoate to the enzyme, equivalent to the amount of active sulfhydryl group present, completely inactivates the enzyme. This inactivation can be reversed by the addition of thiols. With the use of *o*-iodosobenzoate labeled with ^{125}I it has been shown that a complex between *o*-iodobenzoate and the enzyme is not responsible for this inactivation.[20] Ehring and Colowick have shown that 1 mol of thiol, rather than 2, disappears per mol of *o*-iodosobenzoate added during the inactation of the enzyme.[23] This stoichiometry suggests that *o*-iodosobenzoate oxidizes the sulfhydryl group of Cys-149 to a sulfenic acid and not to a disulfide.

Similar considerations suggest that the active-center cysteine residue of papain is oxidized to a sulfenic acid by hydrogen peroxide. Low concentrations of hydrogen peroxide inactivate papain, and this inactivation can be reversed by the addition of thiols.[24] Ultracentrifugation of the inactivated enzyme failed to reveal dimers.[24a] Therefore, an intermolecular disulfide bond is not formed when papain is inactivated by hydrogen peroxide. The stoichimetric addition of hydrogen peroxide to papain leads to 70% inactivation of the enzyme. This indicates that the catalytically active sulfhydryl group is converted to a sulfenic acid and not to a sulfinic acid.[24c]

When the essential sulfhydryl group of GPD is oxidized with mild oxidants, it is converted to a derivative which undergoes reactions that are not characteristic of disulfides. However, these reactions are easily explained if the inactive derivative of the enzyme were a sulfenic acid. The oxidized sulfhydryl group in the enzyme is reduced by arsenite at neutral pH, while disulfides are not.[20] The oxidized form of GPD also reacts with a number of nucleophiles which do not react with disulfides under neutral and slightly acidic conditions. Following oxidation of its catalytically active sulfhydryl group with *o*-iodosobenzoate, GPD also reacts covalently with olefins. The reaction of oxidized GPD with nucleophiles and olefins, which will be discussed in detail, are reactions which are characteristic of sulfenyl halides.[25a]

The inactive derivative of the essential sulfhydryl group of papain produced by oxidation with hydrogen peroxide is also reduced to a sulfhydryl group by arsenite.[24c] The oxidized derivative of papain also reacts with nucleophiles which normally do not react with disulfides.[24c]

When the preceding lines of experimental evidence are considered together, a strong argument can be made to support the existence of stable sulfenic acid residues in papain and GPD following the inactivation of these enzymes by mild oxidants. Trundle and Cunningham have presented similar evidence which suggests that the reactive sulfhydryl group of creatine kinase is oxidized to a sulfenic acid by iodine.[26] Presumably, the sulfenyl iodide which is formed as the first product is in an aqueous environment and is hydrolyzed to form the sulfenic acid, as described by eq 5.

$$\mathrm{ESH + I_2 \rightleftharpoons ESI + HI} \xrightarrow{\mathrm{H_2O}} \mathrm{ESOH + HI} \qquad (5)$$

Acyl Phosphatase Activity Catalyzed by the Sulfenic Acid Form of GPD

It has been shown that the specific conversion of Cys-149 in GPD to a sulfenic acid converts the enzyme from a dehydrogenase to an acyl phosphatase.[23,27] Since other sulfenyl derivatives of the enzyme, such as the sulfenyl thiosulfate derivative, will not hydrolyze acyl phosphates, the sulfenic acid derivative of Cys-149 is specifically required for this activity.[27a] The reaction mechanism described by eq 6 and 7 has been proposed for the acyl phosphatase reaction catalyzed by the sulfenic acid form of the enzyme.

$$\mathrm{GPDSOH + R{-}\overset{\overset{\mathrm{O}}{\|}}{\mathrm{C}}{-}OPO_3H^-} \rightleftharpoons \mathrm{GPDSO{-}\overset{\overset{\mathrm{O}}{\|}}{\mathrm{C}}{-}R + H_2PO_4^-} \qquad (6)$$

$$\mathrm{GPDSO{-}\overset{\overset{\mathrm{O}}{\|}}{\mathrm{C}}{-}R + H_2O \longrightarrow GPDSOH + R\overset{\overset{\mathrm{O}}{\|}}{\mathrm{C}}{-}O^- + H^+} \qquad (7)$$

Equation 4 suggests that the conjugate base of the sulfenic acid at the active site of the enzyme displaces inorganic phosphate from the carbonyl carbon of the acyl phosphate substrate to form an enzyme-bound acyl sulfenylate intermediate. In the second step of the reaction scheme, the acyl sulfenylate reacts with water to

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Table I
Nucleophiles Which Inactivate the Acylphosphatase Activity Catalyzed by the Sulfenic Acid Form of Glyceraldehyde-3-phosphate Dehydrogenase

Nucleophile	Excess Required for 100% Inactivation	Sulfenic Acid Reduction Observed	Ref
Dithiothreitol	1–2	Yes	27a
β-Mercaptoethanol	1–2	Yes	27a
$S_2O_3^{2-}$*	1	Yes	27a
Thiouracil	20	No	27a
Thiourea*	100	Yes	27a
HSO_3^-	1	No	27a
CN^-	1	No	29
Dimedone*	2	No	33
Semicarbazide	10	No	30b
Azide	10	Yes	29
Benzylamine*	1000	Yes	35
Phenylhydrazine	10	Yes	36
Isopropylhydrazine	200	Yes	36
Ascorbate	10	Yes	29

release the carboxylate anion and the regenerated sulfenic acid form of the enzyme. The mechanism described by eq 6 and 7 is consistent with a variety of experimental observations.[27,28]

Since 1,3-diphosphoglycerate, the product of the dehydrogenase reaction catalyzed by GPD, is hydrolyzed at a much faster rate than acetyl phosphate by the oxidized enzyme, the conversion of Cys-149 to a sulfenic acid apparently does not destroy the specific binding site for 1,3-diphosphoglycerate.[23] This is not surprising since the conversion of Cys-149 to a sulfenic acid modifies the active site of GPD by the incorporation of a single oxygen atom.

The acyl phosphatase activity only appears when Cys-149 of GPD is oxidized to a sulfenic acid and disappears when this sulfenic acid residue is modified chemically. Thus, monitoring of this activity has proved useful for the identification of reagents which oxidize protein sulfhydryl groups to sulfenic acids and to identify reagents that react with protein sulfenic acids. Other oxidizing agents which convert GPD to an acyl phosphatase and thus oxidize Cys-149 to a sulfenic acid are iodine monochloride,[27a] triiodide,[23] hydrogen peroxide, and trinitroglycerin.[29] Molecular oxygen also oxidizes the essential cysteine residue of GPD to a sulfenic acid by the reaction described by eq 8.[30]

$$\text{GPD-SH} + O_2 + H_2O \longrightarrow \text{GPD-SOH} + H_2O_2 \qquad (8)$$

Reaction of Nucleophiles with Protein Sulfenic Acids

Foss was the first to recognize that dicoordinate sulfur compounds such as sulfenyl halides can be attacked by nucleophiles.[31] These nucleophilic substitution reactions are represented by eq 9 and have been studied in detail by Kice and his colleagues.[7,32]

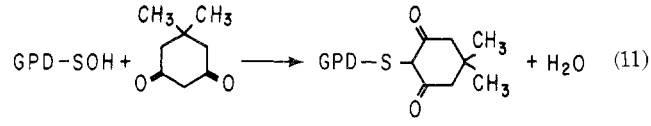
$$RSY + Nu^- \longrightarrow R\text{-}S\text{-}Nu + Y^- \qquad (9)$$

The acyl phosphatase activity catalyzed by the sulfenic acid form of GPD is very sensitive to inactivation by nucleophiles. These nucleophiles are listed in Table I. The nucleophilic reagents were incubated with 0.03–0.10 mM oxidized GPD expressed as the active-site concentration. The column, Excess Required for 100% Inactivation, indicates the concentration of each of the nucleophiles which is required to completely inactivate the acyl phosphatase reaction within 30 min when they were incubated with the oxidized enzyme at 23 °C. The classical carbonyl reagents phenylhydrazine, cyanide, bisulfite, and dimedone are nearly stoichiometric inhibitors of the acyl phosphatase activity as shown in Table I. Two lines of evidence have shown that the nucleophiles which inactivate the acyl phosphatase activity catalyzed by the GPD do so by reacting with the sulfenyl sulfur of Cys-149 as described by eq 10. One line

$$\text{GPD-SOH} + Nu^- \rightleftharpoons \text{GPD-SNu} + OH^- \qquad (10)$$

of evidence is based on the covalent binding of radioactivity to the enzyme when the acyl phosphatase activity is inactivated by the radioactive nucleophiles marked by an asterisk in Table I. These radioactive reagents do not react covalently with reduced GPD, nor do they inactivate the dehydrogenase activity catalyzed by the reduced form of the enzyme. Moreover, the incorporation of radioactive cyanide, dimedone, and benzylamine into the oxidized enzyme is stoichiometric with the degree of inactivation of the acyl phosphatase activity that these nucleophiles produce.

It has been shown directly by amino acid sequence analysis that [^{14}C]dimedone forms a covalent derivative of Cys-149 when it inactivates the acylphosphatase reaction catalyzed by the sulfenic acid form of the enzyme.[33] Since the covalent label is not removed from the enzyme by dithiothreitol, it appears that the C-2 carbanion of dimedone reacts with the sulfenic acid derivative of Cys-149 to form a thioether as described by eq 11. If an enolate oxygen of dimedone had reacted with



(11)

the protein sulfenic acid in a nucleophilic displacement reaction to form a sulfenyl ester, the label would have been removed by dithiothreitol.

The observation that a number of nucleophiles listed in Table I lead to the reactivation of the dehydrogenase activity catalyzed by reduced GPD is the second line of evidence that suggests that they react with the sulfenyl sulfur of Cys-149. Since the reduction of the sulfenic acid derivative of GPD by these various nucleophiles may have bearing on the possible participation of enzyme-bound sulfenic acids in biological oxidations, the reduction of the sulfenic acid at the active site of oxidized GPD by these reagents will be commented upon in detail.

The reduction of the sulfenic acid at the active site of GPD by thiols, thiosulfate, and thiourea presumably occurs by a double displacement mechanism.[27a] For example, the addition of a stoichiometric amount of thiosulfate to the oxidized enzyme completely inactivates the acylphosphatase reaction catalyzed by this

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form of the enzyme. The dehydrogenase activity is not reactivated under these conditions. When the oxidized enzyme is treated with a 100-fold molar excess of thiosulfate, partial reactivation of the dehydrogenase activity is observed. The double displacement mechanism described by eq 12 and 13 accounts for these observations. The sulfenyl derivative of the enzyme produced in the first displacement reacts with excess thiosulfate to produce the sulfhydryl form of the enzyme and tetrathionate in the second displacement.

$$\text{GPD-SOH} + S_2O_3^{2-} \rightleftharpoons \text{GPD-S-}S_2O_3^- + OH^- \quad (12)$$

$$\text{GPD-S-}S_2O_3^- + S_2O_3^{2-} \rightleftharpoons \text{GPD-S}^- + S_4O_6^{2-} \quad (13)$$

The addition of a 10-fold molar excess of azide to oxidized GPD at pH 6.0 nearly quantitatively reduces the sulfenic acid at the active site of the enzyme.[29] With the use of azide labeled with ^{15}N and mass spectrometry, it has been shown that nitrogen is evolved when the oxidized enzyme is reduced by excess azide.

^{15}N–^{15}N is not evolved from solutions of reduced GPD when ^{15}N-labeled azide are added to them. Therefore, the evolution of nitrogen which occurs upon the addition of azide to GPD is a characteristic of the sulfenic acid form of the enzyme. Jirousek has established that the sulfenyl iodode derivative of β-lactoglobulin is reduced by excess azide with the stoichiometry shown in eq 14.[34] However, Jirousek did not detect the production of molecular nitrogen when the sulfenyl iodide derivative of β-lactoglobulin was treated with equivalent quantities of azide.[34]

$$\text{Pr-SI} + 2N_3^- + 2H^+ \longrightarrow \text{Pr-SH} + HI + 3N_2 \quad (14)$$

The reaction scheme described by eq 15–17 has been postulated to account for the reduction of the sulfenic acid at the active site of GPD by excess azide.[29] The reduction is suggested to be initiated by the displacement of hydroxide from the sulfenyl sulfur by azide to form a sulfenyl azide. Nitrogen is then eliminated from the sulfenyl azide and a sulfenyl nitrene derivative of the enzyme is formed as described by eq 16. When excess azide is present the sulfenyl nitrene can react with another azide ion to form an unstable intermediate which eliminates two additional molecules of nitrogen with the formation of the reduced enzyme. This reaction scheme is consistent with the experimental observation that N_2 is evolved when the oxidized enzyme is treated with a stoichiometric amount of azide.

$$\text{GPD-SOH} + N_3^- \rightleftharpoons \text{GPD-S}N_3 + OH^- \quad (15)$$

$$\text{GPD-S}N_3 \longrightarrow \text{GPD-S}\ddot{N}: + N_2 \quad (16)$$

$$\text{GPD-S}\ddot{N}: + N_3^- \longrightarrow [\text{GPD-S}N_4^-] \longrightarrow \text{GPD-S}^- + 2N_2 \quad (17)$$

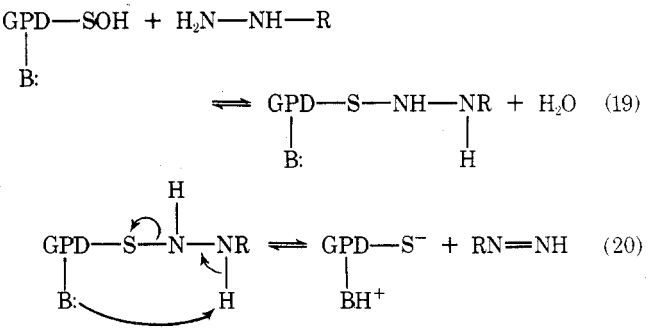
Benzylamine,[35] phenylhydrazine, and isopropylhydrazine[36] inactivate the acylphosphatase activity catalyzed by oxidized GPD, presumably by reactivating with the sulfenic acid at the active site of the enzyme to form the corresponding sulfenamide and sulfenyl hydrazide derivatives of the enzyme. There is evidence for the formation of a sulfenamide derivative of GPD when the acylphosphatase activity is inactivated with [^{14}C]benzylamine.[35] When the sulfenic acid form of GPD is treated with [^{14}C]benzylamine, radioactivity becomes covalently bound to the enzyme, proportional to the degree of inactivation of the acylphosphatase activity. Since the reduced enzyme does not bind [^{14}C]benzylamine, and [^{14}C]benzylamine is discharged from the oxidized enzyme by dithiothreitol, it has been concluded that benzylamine reacts with the protein sulfenic acid to form a sulfenamide derivative, as described by eq 18.

$$\text{GPD-SOH} + H_2NCH_2Ph \rightleftharpoons \text{GPD-S-NH}CH_2Ph + H_2O \quad (18)$$

It has also been reported that benzylamine reacts with the sulfenic acid form of papain to yield a derivative that cannot be reactivated by thiols. This evidence has been interpreted to indicate that benzylamine reacts with the sulfenic acid form of papain to form a sulfenamide derivative of Cys-25.[24c]

When the sulfenic acid form of GPD is incubated with phenylhydrazine or isopropylhydrazine under anaerobic conditions, the acylphosphatase activity disappears and the dehydrogenase activity catalyzed by the sulfhydryl form of the enzyme reappears.[36] These reactions were carried out anaerobically to prevent the autoxidation of the substituted hydrazines to their corresponding diimides. This is especially true for phenylhydrazine, which reacts rapidly with molecular oxygen in aqueous solution to form phenyldiimide and hydrogen peroxide.[37]

To explain the reduction of the sulfenic acid at the active site of GPD by the substituted hydrazines, the reaction sequence described by eq 19 and 20 has been proposed. In the first step of the reaction sequence the substituted hydrazine displaces hydroxide from the sulfenic acid at the active site of the enzyme to form a sulfenyl hydrazide. Then a base, B:, initiates the shuttle of electrons to the sulfenyl sulfur by removing a proton from the sulfenyl hydrazide, as shown in eq 20. Stable sulfenyl hydrazides have been prepared by the reaction of trichloromethylsulfenyl chloride with substituted hydrazines.[38] In addition there is evidence for the formation of a stable sulfenyl phenylhydrazide derivative of papain.[39] For these reasons the reduction of the sulfenic acid at the active site of GPD by substituted hydrazines may be a consequence of the arrangement of amino acid side chains at this active site which participate in the physiological reaction catalyzed by the enzyme. A schematic representation of the hydride transfer reaction which occurs at the active site of GPD during physiological catalysis is shown by eq 21. A thiolester intermediate is formed when hydride is



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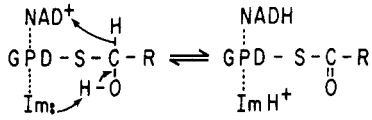
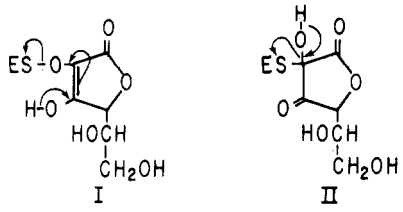


(21)

transferred from a thiohemiacetal, formed between the thiol of Cys-149 and 3-phosphoglyceraldehyde, to NAD^+, during the dehydrogenase reaction catalyzed by GPD.[15] Crystallographic studies have shown that the imidazole side chain of His-176 is in the proper orientation to assist this hydride transfer by removing a proton from the thiohemiacetal as shown in eq 21.[22] It is possible that the imidazole side chain of His-176 might participate in the reduction of the sulfenic acid at the active site of GPD by substituted hydrazines. It might be in the right orientation to remove a proton from sulfenyl hydrazide derivatives at the active site and thus initiate the shuttle of electrons to the sulfenyl sulfur which would result in the formation of the reduced enzyme and the corresponding diimide as described in eq 20. It should also be noted that the sulfenic acid form of GPD is reduced very slowly in the presence of benzylamine.[35] This reduction might occur by a mechanism similar to the one described by eq 19 and 20 in which His-176 abstracts a proton from the sulfenamide derivative of Cys-149.

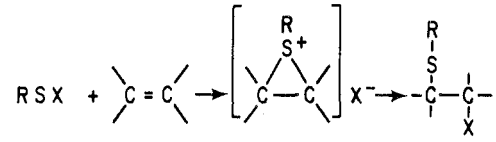
The acylphosphatase activity catalyzed by oxidized GPD is also inactivated by ascorbate, which is accompanied by the partial reactivation of the dehydrogenase activity catalyzed by the sulfhydryl form of the enzyme.[29] Ascorbic acid is an enediol with a pK_a of 4.2.[40] To initiate the reduction of the protein sulfenic acid, either the C-3 enolate or the C-2 carbanion of ascorbate could attack the sulfenyl sulfur with the displacement of hydroxide to form, respectively, either the intermediate illustrated by structure I or the intermediate illustrated by structure II. I is based on the observation



that, of the two enolic groups of ascorbic acid, the one on C-3 is the most acidic.[40] Electrons could then shuttle to the sulfenyl sulfur in either intermediate to produce dehydroascorbate and the sulfhydryl form of the enzyme. The observation that the complete inactivation of the acyl phosphatase reaction catalyzed by the sulfenic acid form of GPD is not accompanied by the full reactivation of the dehydrogenase activity suggests that a covalent derivative between the oxidized enzyme exists under certain conditions.

Reaction of the Sulfenic Acid Form of GPD with Olefins

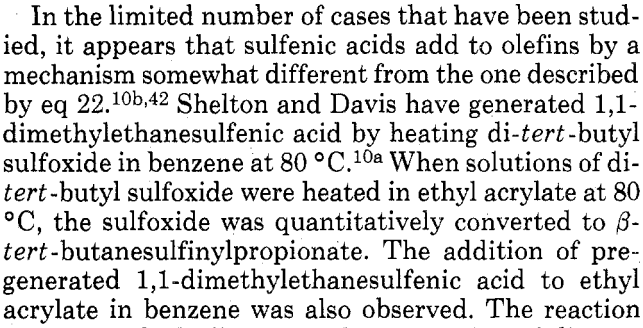
Sulfenyl derivatives including sulfenic acids are characteristically trapped by olefins. The addition of sulfenyl halides to olefins has been studied in detail.[41] The reaction of sulfenyl halides with *cis*- and *trans*-2-butenes invariably proceeds with the formation of the corresponding trans adducts.[41b] This stereospecificity is consistent with the formation of an episulfonium ion intermediate upon the attack of the sulfenyl sulfur on the olefinic bond, as shown in eq 22. The intermediate



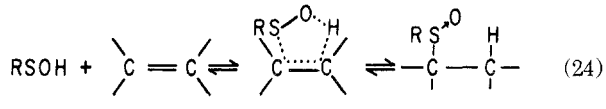
(22)

then reacts with the leaving group X^- or a component of the solvent to produce a trans-β-substituted thioether.

In the limited number of cases that have been studied, it appears that sulfenic acids add to olefins by a mechanism somewhat different from the one described by eq 22.[10b,42] Shelton and Davis have generated 1,1-dimethylethanesulfenic acid by heating di-*tert*-butyl sulfoxide in benzene at 80 °C.[10a] When solutions of di-*tert*-butyl sulfoxide were heated in ethyl acrylate at 80 °C, the sulfoxide was quantitatively converted to β-*tert*-butanesulfinylpropionate. The addition of pregenerated 1,1-dimethylethanesulfenic acid to ethyl acrylate in benzene was also observed. The reaction sequence which illustrates the conversion of di-*tert*-butyl sulfoxide to ethyl β-*tert*-butanesulfinylpropionate when it is heated in the presence of ethyl acrylate is presented in eq 23. Shelton and Davis have also demonstrated the addition of 1,1-dimethylethanesulfenic acid and other sulfenic acids generated by the thermal decomposition of sulfoxides to methyl propiolate and ethynylbenzene. Since the addition of the sulfenic acids to methyl propiolate proceeds with stereospecific cis addition to give the methyl *trans*-sulfinylacrylates, the mechanism described by eq 24 has been proposed for



(23)



(24)

the addition of sulfenic acids to olefinic and acetylenic multiple bonds. The cyclic transition state depicted with carbon–hydrogen bond formation lagging behind carbon–sulfur bond formation is based on an argument which takes microscopic reversibility into account.[42] The cis elimination of sulfenic acids from sulfoxides proceeds through an identical transition state in which carbon–sulfur bond breakage lags behind the cleavage of the carbon–hydrogen bond.

Barton and his colleagues have trapped sulfenic acid intermediates generated by heating penicillin *S*-oxides with norbornadiene, dimethyl acetylenedicarboxylate, and dihydropyran.[43] Norbornadiene and dimethyl

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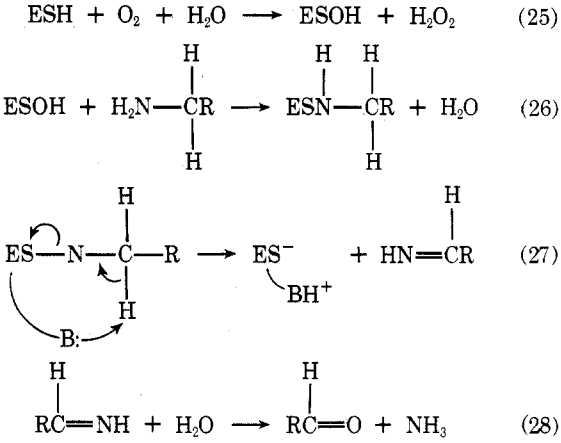
acetylenedicarboxylate react with the sulfenic acids by addition to produce sulfoxides while dihydropyran reacts by substitution which is accompanied by loss of water to produce vinyl sulfides.

The sulfenic acid derivative of GPD has been shown to react with olefins.[33] The acylphosphatase activity catalyzed by the sulfenic acid derivative of the enzyme is inactivated by 3-cyclohexene-1-carboxylate, tetrahydrophthalimide, and dihydropyran. Once the acylphosphatase activity catalyzed by the oxidized enzyme is inactivated by the olefins, dithiothreitol no longer reactivates the dehydrogenase activity catalyzed by the reduced form of the enzyme. This suggests that the olefins inactivate the acylphosphatase by reacting with the sulfenic acid at the active site of the enzyme to form a covalent derivative of Cys-149. Amino acid sequence analysis of the tryptic peptide containing Cys-149 following the inactivation of the acyl phosphatase with tetrahydrophthalimide has provided direct evidence for such a covalent modification.

Conclusions

The fact that sulfhydryl groups in proteins are easily oxidized to sulfenic acids under in vitro conditions makes it reasonable to suggest that protein sulfenic acids might play a role in enzyme catalyzed oxidations. Both hydrogen peroxide and molecular oxygen which are biological oxidants have been shown to oxidize protein sulfhydryl groups to sulfenic acids. The reactivity of the sulfenic acid derivative of GPD with a variety of nucleophiles has shown that protein sulfenic acids are excellent electrophilic centers that are well suited for participation in oxidative catalysis. An example of a biological oxidation in which a protein sulfenic acid could participate is the oxidative deamination of primary amines catalyzed by the nonflavin amine oxidases. These enzymes which require Cu^{II} for activity are inactivated by carbonyl reagents such as substituted hydrazines.[44] For this reason it has been postulated that pyridoxal phosphate or a closely related compound is a prosthetic group for these enzymes. However, this has not been rigorously established.[45,46] The electrophilic center in these enzymes which reacts with carbonyl reagents could be a sulfenic acid which could participate in catalysis by the reaction scheme illustrated by eq 25–28. The reaction scheme suggests that a sulfhydryl



group at the active site of the enzyme reacts with molecular oxygen to form a sulfenic acid and hydrogen peroxide. This oxidation might be aided by the copper ion which is required for catalysis. The amine substrate then reacts with the sulfenic acid to form a sulfenamide intermediate which then disproportionates with the aid of a base to form the reduced enzyme and the imide product. The imide is then hydrolyzed to form the corresponding aldehyde and ammonia. The reaction scheme is consistent with the stoichiometry exhibited by the nonflavin amine oxidases. The very slow reduction of the sulfenic acid derivative of GPD in the presence of benzylamine may proceed by the reaction sequence described by eq 26 and 27. Although there is no direct evidence for the participation of a sulfenic acid during catalysis by the nonflavin amine oxidases, the reaction scheme described by eq 25–28 is presented here to illustrate the type of enzyme-catalyzed oxidation in which a protein sulfenic acid could participate.

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