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Breakthroughs and Views

Multiple roles of cysteine in biocatalysis

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Abstract

In biology, sulfur frequently occurs in the form of cysteine, an amino acid that fulfills a wide range of different functions in proteins including disulfide formation, metal-binding, electron donation, hydrolysis, and redox-catalysis. The 'redox-chameleon' sulfur appears in several oxidation states in vitro, each of them exhibiting specific reactivity, redox-activity, and metal-binding properties. While cysteine-peptidases rely on reduced cysteine to catalyze hydrolytic reactions, many redox-enzymes use distinctively different cysteine redox-couples for exchange, electron, atom, and radical transfer reactions. Although cysteine and cystine can still be considered as the most abundant forms of cysteine in vivo, other modifications such as cysteine acids and sulfur-centered radicals are becoming increasingly important in biochemical research. As such, the biochemistry of sulfur remains a source of continuous investigation and excitement.

Keywords: Cysteine; Sulfur redox-chemistry; Hydrolysis; Metal-binding

Cysteine's ability to occur in up to 10 different sulfur oxidation states in vivo leads to a range of cysteine modifications in peptides and proteins. Thiols, thiolates, thiyl radicals, disulfides, sulfenic, sulfinic, and sulfonic acids (including conjugated bases), disulfide-*S*-oxides and selenodisulfides can all be found in organisms. Each of these cysteine modifications exhibits its own specific chemical and biochemical properties such as stability, redox-behavior, metal-binding, acidity, nucleophilicity, and catalytic activity. The reduced form of cysteine alone can be involved in nucleophilic substitution, metal ion transfer, electron transfer, proton transfer, hydrogen atom transfer, hydride transfer, and oxygen atom transfer. This unique reactivity of cysteine is mirrored by the myriad of functions cysteine fulfills in vivo, including structural stabilization, catalysis, redox-activity, and metal-binding.

This minireview addresses some of the functions cysteine accomplishes as part of the active site of enzymes. Understandably, such a discussion has to be limited to a few selected highlights. We will therefore focus on the underlying mechanistic chemistry of cysteine's diverse roles in hydrolytic and redox-enzymes that reflects the unique reactivity of cysteine and explains the variety of transformations the thiol group can undergo in vivo (Fig. 1).

Peptidases: cysteine as nucleophile and ligand

Cysteine occurs in the active site of a number of cysteine-endoproteases where it acts as a potent nucleophile (i.e., electron pair donor). Cysteine-proteases form a separate class of proteases, distinguished by their particular active site and catalytic mechanism. They have been found in animals, bacteria, plants, and viruses where they fulfill a range of functions. Among the best-known examples are papain, cathepsin B, and human caspases. The hydrolytic mechanism of these enzymes involves two steps [1]: the first step—the acid-catalyzed formation of a thioester acyl-enzyme intermediate with concomitant release of the cleaved amine—proceeds via a tetrahedral transition state formed when the nucleophilic thiolate of the protease's cysteine attacks the carbonyl of the substrate's amide. This is followed by a second step, the base-catalyzed hydrolysis of the

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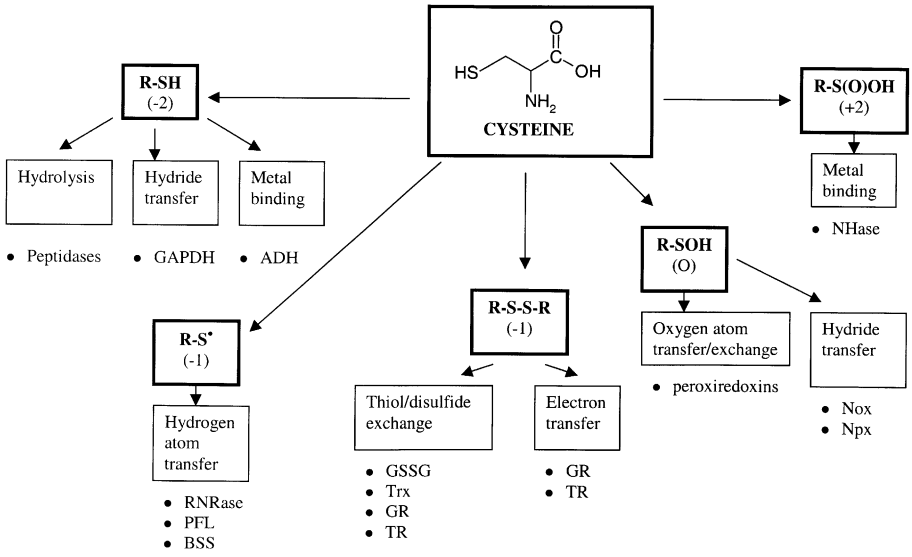

Fig. 1. Oxidation states, properties, reactivity, and occurrences of different cysteine modifications in vivo. Enzymes discussed in the text are shown as examples. Oxidation states of sulfur are given for R in oxidation state +1.

thioester with subsequent release of the carboxylic acid and regeneration of free enzyme.

Although cysteine-proteases are mechanistically similar to serine proteases the switch from oxygen to sulfur endows cysteine-based catalysis with a much stronger nucleophile in the form of a sulfur-centered 'charge relay dyad.' Sulfur's valence electrons, in the third valence shell, are highly flexible and the imidazole ring of a neighboring histidine is able to generate a 'charge relay dyad' in the form of a delocalized electron cloud stretching from sulfur to the aromatic imidazolium ring. This dramatically lowers the pK_a of cysteine (from 8.5 in free cysteine to 3.5 in the active site), allowing enzyme activity to occur over a wide pH range from pH 3 to 8 [1].

The active site cysteine not only facilitates effective catalysis—it also makes these enzymes sensitive to oxidation and metal poisoning [2]. Metal-binding to cysteine is, however, beneficial in another class of peptidases. Metalloproteases such as the zinc-β-lactamases II contain active site zinc/sulfur complexes [3]. Sulfur is not directly involved in catalysis but acts as a ligand for the catalytic metal. This 'dual role' of cysteine in the same family of enzymes, i.e., as catalytic species or as inactive 'keeper' of catalytic metal ions, is also found in oxidoreductases.

Cysteine-based redox-catalysis

While most redox-active proteins contain cofactors such as metals, NAD^+, or FAD, several biological redox-couples are exclusively based on amino acid side chains. Among them, cysteine-based redox-systems are exceptional since sulfur can participate in several mechanistically distinct redox-reactions. These reactions can be classified as thiol/disulfide exchange reactions (e.g., in thioredoxin (Trx)), electron-transfer reactions (e.g., in glutathione reductase (GR)), thiol/thiyl hydrogen radical transfer reactions (e.g., in ribonucleotide reductase (RNRase)), oxygen atom transfer redox-couples (e.g., in NADH oxidase (Nox) and NADH peroxidase (Npx)), and different hydride transfer reactions (e.g., in Npx and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) (Fig. 1).

Thiol/disulfide exchange reactions are common in vivo substitution reactions, with cysteine acting as nucleophile. They are involved in maintaining the cellular redox-balance via the glutathione/glutathione disulfide (GSH/GSSG) redox-couple and are at the heart of thiolation and glutathiolation reactions. Thiol/disulfide exchange reactions are an example of biological redox-reactions that do not involve electron transfer [4]: for instance, the reaction of RSH with GSSG to the mixed disulfide GSSR and GSH involves the formal oxidation of RSH's sulfur and the reduction of one of GSSG's sulfurs. Numerous peptides, proteins, and enzymes with various sulfur redox-potentials undergo this kind of reaction (e.g., glutathione (GSH), thioredoxin (Trx), protein disulfide isomerase (PDI), and glutaredoxin (Grx)) [5]. Among them, GR and thioredoxin reductase (TrxR) are particularly interesting since cysteine undergoes exchange and electron transfer reactions at different stages of the catalytic cycle.

Electron transfer to disulfides allows these enzymes to link the mechanistically different redox-systems of electron transfer and nucleophilic exchange. In GR, reduction of glutathione disulfide (GSSG) by two of the enzyme's reduced cysteines proceeds via a thiol/disulfide exchange reaction at the active site of the enzyme, resulting in GSH and an intramolecular disulfide at the

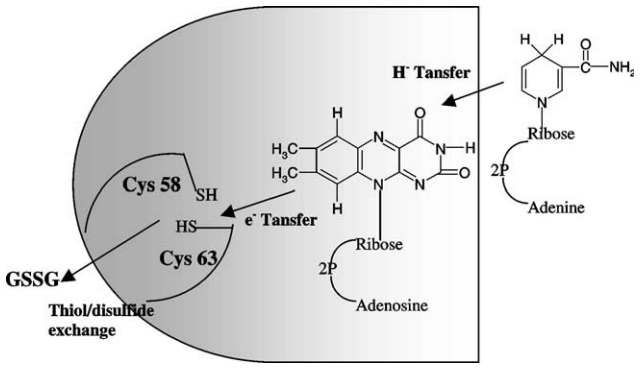

Fig. 2. The reduction of GSSG by NADPH catalyzed by glutathione reductase. The chain reaction consists of exchange, electron transfer, and hydride transfer. Active site Cys-58 and Cys-63 are oxidized to the disulfide via electron-free thiol/disulfide exchange and subsequently reduced via electron transfer from $FADH_2$.

active site of GR [6]. This intermolecular disulfide is re-reduced by direct *electron transfer* from $FADH_2$ to the disulfide bond and $FADH_2$ is regenerated by NADPH to complete the overall reduction of GSSG by NADPH (Fig. 2). TrxR follows a similar mechanism, reducing the disulfide in Trx by consuming NADPH. Interestingly, human TrxR contains a Gly–Cys–SeCys–Gly sequence and, unlike GR, operates with a thiol, selenol/selenyl-sulfide rather than a thiol, thiol/disulfide redox-couple [7].

Radical formation and transfer provide enzymes with an additional cysteine-based redox-pathway. The thiol/thiyl radical redox-couple occurs in important human enzymes such as the RNRase superfamily, pyruvate formate lyase (PFL), and benzylsuccinate synthase (BSS). The thiyl radical is either formed by a long-range one-electron transfer from the thiol (with subsequent loss of H^+) or by short-range hydrogen atom (radical) abstraction from the thiol. These reactions are distinctly different from exchange and electron-transfer reactions. For example, the aerobic Fe-dependent (class I) RNRase from *Escherichia coli* generates a thiyl radical by long-range electron transfer from cysteine to an active site tyrosyl radical [8]. The thiyl radical is reduced back to thiol by hydrogen atom abstraction from the 3′ position of the ribonucleotide substrate. In contrast, the formation of the thiyl radical in anaerobic formate-dependent (class III) RNRase from bacteriophage T4 and in anaerobic pyruvate formate lyase (PFL) from *E. coli* proceeds via a short-range hydrogen atom transfer from cysteine to a glycyl radical (in PFL the initial radical at Gly734 is transferred to Cys-418 via Cys-419 in two proton abstraction steps). In the case of the anaerobic RNRase this thiyl radical is then reduced back to cysteine by hydrogen abstraction from the ribonucleotide. In PFL, the thiyl radical is ultimately reduced to a thiol by hydrogen abstraction reaction from Gly734 that regenerates the active site [8].

Oxygen atom transfer from oxidizing species to cysteine results in sulfur acids. Enzymes containing active site sulfenic acids (RSOH) include human peroxyredoxin (Pox), Npx from *Enterococcus faecalis*, and Nox from *Enterococcus faecalis 10C1*. In a reaction resembling thioester formation, the enzyme's thiolate attacks the peroxide with subsequent formation of sulfenic acid and release of hydroxide. The following reduction of the sulfenic acid to regenerate the active site follows two different mechanisms: The disulfide reduction pathway involves the reaction of RSOH with two equivalents of substrate thiol to release disulfide and water. It proceeds via a mixed disulfide intermediate and is characteristic of the peroxyredoxins (Pox) [9]. In contrast, the sulfenic acids in Nox and Npx are reduced by hydride transfer from $FADH_2$ to RSOH with release of hydroxide [9].

Hydride transfer to sulfenic acid is yet another cysteine-based redox-mechanism recently discovered in vivo. In enterococcal Npx a hydride is transferred from NADH to CysSOH of Cys-42 via FAD [9]. This enzyme strictly bypasses the disulfide redox-state as confirmed in a landmark study by Miller et al. [10]. The creation of an active site disulfide by the introduction of an additional cysteine (Cys-40) forced the formation of a disulfide from the thiol group of Cys-40 and the sulfenic acid of Cys-42. In this case the k_{cat} of the mutant enzyme dropped to 0.1% of wild-type Npx, also indicating that hydride transfer to the disulfide does not occur in this enzyme.

Hidden activities of cysteine in redox-catalysis

In several oxidoreductases, cysteine does not directly partake in but still facilitates catalysis, e.g., as a ligand for catalytic metal ions or as a hydride transfer 'facilitator.' Hydride transfer from substrate to NAD^+ is found in dehydrogenases such as alcohol dehydrogenase (ADH) and GAPDH. GAPDH catalyzes the oxidation and phosphorylation of aldehydes to acyl-phosphate while reducing NAD^+ to NADH [11]. The active site of GAPDH contains a cysteine that attacks the aldehyde to form a tetravalent intermediate. A hydride is then transferred from the intermediate to NAD^+ to form a thioester at the active site in a step vaguely resembling cysteine-catalyzed proteolysis where the tetravalent intermediate releases an RNH^- anion rather than H^-. The latter is then cleaved by nucleophilic attack of phosphate resulting in the formation of a phosphoester with regeneration of the active site. In contrast, ADHs from yeast and human liver contain a zinc/sulfur complex that catalyzes the oxidation of ethanol to acetaldehyde (with an additional, structural zinc/sulfur site in close vicinity to the active site). Zinc is bound by two cysteines and a histidine and binding of alcohol substrate via its OH group completes the zinc coordination and effectively locks the alcohol into position for hydride transfer to

NAD^+, hence oxidizing the alcohol without changes in the redox-state of zinc or sulfur [12].

Other metal ions commonly bound to cysteine ligands are iron, nickel, copper, manganese, and molybdenum. More recently, the identification of a sulfinic acid (RS(O)OH) ligand in bacterial nitrile hydratase (NHase) from *Rhodococcus* sp. N-771 has caused considerable excitement. This enzyme catalyzes the hydration of nitriles to the corresponding amides and NHase can coordinate non-heme Fe(III) or a non-corrinoid Co(III) via the oxygen of its cysteine sulfinic acid [13,14]. Compared to reduced cysteine, the oxidized amino acid is therefore capable of a very different metal-binding. Rather than binding metals such as zinc via sulfur, the sulfinic acid binds metals such as iron and cobalt via the 'hard' oxygen. Oxidized forms of cysteine are also being investigated in transcription factors such as BPV-1 E2 protein and activator protein-1.

Conclusion

This minireview has touched on the complexity of cysteine's thiol group in vivo. The amino acid's various redox-transformations, oxidation states, metal-binding properties, and its ability to act as nucleophile make it one of the most exciting and equally complex systems to study. Among the reactions cysteine undergoes in vivo, thiol/disulfide exchange and radical reactions, electron, oxygen and hydride transfer reactions, and metal-binding have featured most prominently here. This does not imply that other occurrences of cysteine, e.g., as part of structural disulfides or zinc/sulfur complexes, are less important. The recent discovery of in vitro sulfinamide formation from cysteine sulfenic acid and lysine in S100A8 under oxidative stress conditions [15] has added just one more structure to the long list of cysteine's in vitro and in vivo modifications. One can therefore confidently predict that the biochemistry of cysteine will provide plenty of unheralded opportunities for future research [16].

Acknowledgments

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