A catalytic career: Studies spanning glutamine synthetase, phospholipase C, peroxiredoxin, and the intracellular messenger role of hydrogen peroxide

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I learned biochemistry from P. Boon Chock and Earl Stadtman while working on the regulation of Escherichia coli glutamine synthetase as a postdoctoral fellow at the National Institutes of Health. After becoming a tenured scientist at the same institute, my group discovered, purified, and cloned the first three prototypical members of the phospholipase C family and uncovered the mechanisms by which various cell-surface receptors activate these enzymes to generate diacylglycerol and inositol 1,4,5-trisphosphate. We also discovered the family of peroxiredoxin (Prx) enzymes that catalyze the reduction of H$_2$O$_2$, and we established that mammalian cells express six Prx isoforms that not only protect against oxidative damage but also mediate cell signaling by modulating intracellular H$_2$O$_2$ levels. To validate the signaling role of H$_2$O$_2$, we showed that epidermal growth factor induces a transient increase in intracellular H$_2$O$_2$ levels, and the essential cysteine residue of protein-tyrosine phosphatases is a target for specific and reversible oxidation by the H$_2$O$_2$ produced in such cells. These observations led to a new paradigm receptor signaling, in which protein tyrosine phosphorylation is achieved not via activation of receptor tyrosine kinases alone but also through concurrent inhibition of protein-tyrosine phosphatases by H$_2$O$_2$. Our studies revealed that Prx isoforms are extensively regulated via phosphorylation as well as by hyperoxidation of the active-site cysteine to cysteine sulfinic acid, with the reverse reaction being catalyzed by sulfiredoxin. This reversible hyperoxidation of Prx was further shown to constitute a universal marker for circadian rhythms in all domains of life.

Early years and the move from Korea to the United States

In May 1961, a month after I entered Seoul National University College of Liberal Arts and Sciences as a chemistry major, the fledgling democracy in Korea ended with a military coup. A series of intense antigovernment demonstrations ensued and persisted throughout the 1960s. Our college campus was the center for the student antigovernment movement. Streets surrounding the campus were frequently filled with soldiers and shrouded in clouds of tear gas, and many classes were canceled. However, the classes taught by Professors Kyu Won Choi (analytical chemistry) and Se Hun Chang (physical chemistry) were seldom canceled. These professors, like many others since the Korean independence in 1945, had been teaching without Ph.D. degrees, but they had recently returned to Korea after earning their doctorates in the United States.

Chemistry majors were mocked as the most subservient students on campus. Textbooks used in class were in English, but most students could not afford to buy these books printed in the United States or even cheaper versions printed with the copyright in Japan. Instead, they bought pirated versions printed in Taiwan. The professors encouraged us to go to the United States for graduate studies. Many of us who attended the chemistry department in the 1960s recall how lucky we were to be taught by two such passionate professors with a vision for the future.

I took the Korean equivalent of ROTC courses and was commissioned after graduation in 1965 as a second lieutenant in the Korean Army with a specialty in ammunition supply. I was assigned to an infantry division located near the Korean Demilitarized Zone. Given that I wanted to go to graduate school in the United States after my 2 years of military service, I had to take the Graduate Record Examinations (GRE) and the Test of English as a Foreign Language (TOEFL) while I was in the army. The last chance to take the tests to fulfill the 1967 admission requirements was September of 1966. In that month, there were several instances of North Korean agent infiltration through the area controlled by our infantry division, and all officers were ordered to stay near the military base. I could not take days off to take the tests in Seoul. Next January, I received letters denying me financial assistance from all the graduate schools to which I had applied. One of these graduate schools was Catholic University of America in Washington, D.C., where Gilbert Castellan, the author of a physical chemistry textbook used in our undergraduate course, was a faculty member in the chemistry department. I had a cousin, Jhoon Goo Rhee, who was widely recognized as the “Father of American Taekwondo” for introducing this martial art to the United States. After learning that I was not accepted to Catholic University, my cousin went there to meet Richard Timmons, a young assistant professor of chemistry in charge of graduate admissions. I was then admitted to a teaching assistant slot not...
taken by another applicant, and I arrived in Washington in June 1967. Richard Timmons later told me that he was most impressed with my cousin, who he had seen several times on local television. I chose to do my Ph.D. thesis under the direction of John Eisch, who had recently joined the department as head after moving from the University of Michigan and who was the author of a textbook titled *The Chemistry of Organometallic Compounds*. In the meantime, I married Young Kyu Park, another graduate student whom I met at Catholic University. (The infiltration of North Korean agents thus turned out to be a blessing in disguise for me.) The goal of my Ph.D. project was to elucidate the mechanism of the hydroalumination of alkynes, and the research progressed well. Early in 1971, my thesis advisor told me to write up my results and prepare for graduation in the fall. As my writing skills were not well-honed, this progressed at a painfully slow pace. I would constantly look for excuses to do more experiments. I gave up the prospect of graduating that fall and moved to the State University of New York (SUNY) in Binghamton with John Eisch, who joined the university as chair of the chemistry department. Although I had not formally received my Ph.D. degree, I was appointed to a postdoctoral fellow position. I finally and successfully defended my thesis in 1972, and my work in the Eisch laboratory resulted in eight publications in respected journals.

**Becoming a biochemist while working on *Escherichia coli* glutamine synthetase**

While I was preparing for my thesis defense, John Eisch asked me about my future plans. I told him that I intended to return to Korea and to undertake the total synthesis of ginsenosides, triterpene saponins found in the ginseng root, which is considered an herbal panacea in Korea. At that time, I did not comprehend very well the importance of research in an academic career and was not well-prepared to answer Eisch’s question. From time to time, I followed publications on ginseng coming out of a research institute at the University of Tokyo as well as a research institute in Vladivostok, in the former Soviet Union. Russia’s interest in ginsenosides stemmed from the finding of its scientists that these compounds could improve the performance of astronauts and athletes. Knowing how poorly prepared I was for the difficult task of natural product synthesis, Dr. Eisch gently advised me to apply for a postdoctoral fellowship at the National Institutes of Health (NIH) to get more training in enzymology and natural products. The logic behind his advice was that the synthesis of complex molecules like ginsenosides is possible only through a combination of bulk chemistry and the stereospecific action of enzymes. He also mentioned that Richard Timmons from Catholic University was spending a sabbatical at a renowned enzymology lab at NIH, the Laboratory of Biochemistry, within the National Heart, Lung, and Blood Institute (NHLBI), headed by Earl Stadtman. When Richard Timmons heard of my desire to apply for a postdoctoral fellowship at NIH, he introduced me to P. Boon Chock, who had just joined the Laboratory of Biochemistry in a tenure-track position. The latter agreed to be a sponsor for my NIH postdoctoral fellowship application, and, with his generous help, I was accepted for the position.

The regulation of glutamine synthetase (GS)\(^2\) in *E. coli* was a focus of study in Earl Stadtman’s laboratory around the time of my arrival (1). Many later-renowned scientists studied this subject while training in the Stadtman laboratory. I learned fast kinetics from P. Boon Chock, who had done postdoctoral research in the laboratory of Manfred Eigen—a recipient of the 1967 Nobel Prize in Chemistry for his work on measurement of fast chemical reactions—at the Max Planck Institute in Göttingen. I applied the stopped-flow technique to study the kinetics of the GS reaction.

Studies in the 1960s and early 1970s by Stadtman’s group revealed that GS activity is regulated via a bicyclic cascade involving reversible adenylylation (attachment of an AMP moiety) and uridylylation (attachment of a UMP moiety) of protein tyrosine residues (1). In the 1970s, GS and glycogen phosphorylase provided the two best-known examples of post-translational regulation, the former via nucleotidylation of tyrosine in bacteria and the latter via phosphorylation of serine and threonine in hepatocytes.

As I gradually learned enzymology and became acquainted with a number of interesting discoveries made on the NIH campus, my ideas on research and a career began to change. In particular, the story behind how Stadtman’s group discovered the nucleotidylation of GS influenced me strongly. They observed that the catalytic activity of GS varied widely depending on the conditions of *E. coli* growth. From their observations that different GS preparations showed small differences in UV absorbance at 260 nm, they postulated the existence of a nucleotide adduct (2). This was confirmed by the demonstration that treatment of some preparations with snake venom phosphodiesterase induced the release of AMP and a change in the catalytic properties of GS, thus leading to the discovery of a novel regulatory mechanism based on the adenylylation of tyrosine. Subsequently, they found that adenylyltransferase (ATase) could catalyze both the adenylylation and deadenylylation of GS (3) and that the activity of ATase was modulated by the protein PII. They further showed that, like GS, PII exists in two forms: in this case, an unmodified form that in the presence of \(L\)-glutamine stimulates the adenylylation of GS, and a uridylylated form (PII-UMP) that in the absence of \(L\)-glutamine and in the presence of \(\alpha\)-ketoglutarate stimulates the deadenylylation of GS (4).

I joined the research project at this stage of its development. GS is composed of 12 identical subunits, and GS activity is inversely proportional to the state of adenylylation, with the average number of covalently bound adenylyl groups per molecule (\(n\)) ranging from 0 to 12. The adenylylation and deadenylylation reactions are regulated by \(L\)-glutamine and \(\alpha\)-ketoglutarate, which have pronounced reciprocal effects. Theoretical analysis predicted that, in the presence of all adenylylation–deadenylylation cycle components, the value of \(n\) will achieve a steady state that will vary as a function of effector concentra-
tions, with at least 28 kinetic or equilibrium constants being required to describe the reaction cycle. I eventually determined these constants with the help of P. Boon Chock (5), benefitting from his strong theoretical background. This steady work provided the path to my being granted a tenured senior scientist position in 1979 (Fig. 1).

The determination of these constants required the purification of large amounts of GS, ATase, and PII proteins as well as the partial purification of uridylyltransferase (UTase) for preparation of PII-UMP. GS is a relatively abundant protein, and its purification had been well established. For isolation of the other proteins, however, we had to grow kilogram quantities of E. coli and subject the cell homogenates to industrial-scale column chromatography. The preparation of PII was especially difficult, given the extremely low abundance of this protein. We achieved a major breakthrough with the finding that most proteins, but not PII, in E. coli homogenates are precipitated by the addition of β-mercaptoethanol to a final concentration of 26% (v/v). This procedure, whose effectiveness was because PII does not contain any cysteine residues, allowed enrichment of PII by several-hundredfold. Although I was delighted with the discovery of this magical step for PII purification, I became highly unpopular in our building as a result of the unpleasant odor caused by the use of liter quantities of β-mercaptoethanol. Even though I tried to perform the attendant tasks under a chemical fume hood and during the weekend as much as possible, the centrifugation and dialysis of large volumes of material inevitably polluted the entire corridor, with the stench lingering into the workweek. Even my 4-year-old daughter held her nose and tried to stay away from me when I came home. I was warned several times by chemical safety officers on the NIH campus. Looking back, I feel I was somewhat ruthless but that I was lucky to have patient and understanding colleagues.

Late in 1979, Emilio Garcia joined my laboratory as a postdoctoral fellow from the University of California at Davis. Taking advantage of the fact that PII was now readily available, Emilio and I purified UTase (6). The purification was facilitated by the use of an E. coli strain that harbors multiple copies of the UTase gene and overproduces the enzyme by a factor of 25. This strain was selected by screening the Clarke and Carbon collection of 2000 E. coli strains that carry ColE1 plasmids containing small random segments of the bacterial chromosome. Using the purified enzyme, which comprises a single polypeptide, we showed that it catalyzes the uridylylation as well as the deuridylylation of PII, similar to the bifunctionality exhibited by ATase (Fig. 2).

Transition to the study of receptor signaling via phospholipase C

One day in 1982, Earl Stadtman came to my office and casually mentioned that it might be time for me to move on to a new area of research, adding that he would continue to get undeserved credit for the work I published as long as I continued to work on GS. He also mentioned that he was gradually shifting his focus to the role of protein oxidation and degradation in aging. I sensed he was telling me that I had become too complacent with my work—around that time we had cloned all four genes for the protein components of the GS regulatory cascade (GS, ATase, PII, and UTase), and we continued to publish follow-up papers on the regulation of GS in E. coli. Appreciative of his generosity and wisdom, which he had shared with many of his trainees (Stadtman mentored two Nobel laureates, multiple members of the National Academy of Sciences, and many current leaders in the biomedical community), I took his advice seriously, and I decided to look for a new research topic.

One easy choice was to study GS in yeast, which had been shown by others to be subject to complex regulation as in E. coli, but not through either nucleotidylation or phosphorylation. Kanghwa Kim joined my lab as a postdoctoral fellow from Korea in 1983 and devoted his full efforts to this topic. Although our studies in this area were largely unfruitful, an astute observation made by Kanghwa during the purification of yeast GS led us to discover a family of unconventional antioxidant enzymes, which we later named peroxiredoxins. This discovery led me to concentrate my last 20 years of research on the intracellular messenger function of hydrogen peroxide (H₂O₂) and its regulation by peroxiredoxins (more on this later).

Ultimately, although intrigued by the similarities and dissimilarities between the GS enzymes of E. coli and Saccharomyces cerevisiae, I was not satisfied with our progress on regulation of
EROL activates protein kinase C (7, 8).

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of Ca²⁺ receptors, that inositol 1,4,5-trisphosphate induces the release (PLC) in response to the activation of various cell-surface phosphatidylinositol 4,5-bisphosphate by phospholipase C erol. Through further reading, I came to comprehend that these second messengers inositol 1,4,5-trisphosphate and diacylglycerol are of research and was introduced to the two newly crowned what was drawing such interest, I stepped out of my immediate crowded and often overflowed to the entrance. Curious about conferences in 1984 and 1985, I noticed certain sessions were investigation. While attending a number of NIH and national meetings asked me why I pursued the seemingly unrewarding

Figure 2. The bicyclic cascade that regulates GS activity. Emilio Garcia (left) and Sue Goo Rhee (right) purified UTase, the last protein component of the bicyclic cascade to be isolated. Reprinted from Ref. 1. This research was originally published in the Journal of Biological Chemistry. Stadtman, E. R. The story of glutamine synthetase regulation. J. Biol. Chem. 2001; 276:44357–44364. © the American Society for Biochemistry and Molecular Biology.

...the yeast enzyme, and I continued to look for a new area of investigation. While attending a number of NIH and national conferences in 1984 and 1985, I noticed certain sessions were crowded and often overflowed to the entrance. Curious about what was drawing such interest, I stepped out of my immediate area of research and was introduced to the two newly crowned second messengers inositol 1,4,5-trisphosphate and diacylglycerol. Through further reading, I came to comprehend that these two messengers are generated as a result of the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C (PLC) in response to the activation of various cell-surface receptors, that inositol 1,4,5-trisphosphate induces the release of Ca²⁺ from the endoplasmic reticulum, and that diacylglycerol activates protein kinase C (7, 8).

I also learned that one critical question that remained regarding this bifurcating signaling pathway was how various receptors are coupled to PLC. I felt that purification of PLC and the cloning of its cDNA would be necessary to address this question. Several earlier studies suggested that the coupling of receptor function to PLC is mediated by an unspecified G protein, in a manner analogous to the modulation of adenylate cyclase via the G proteins G_s and G_i, which couple receptor function to synthesis of the second messenger cAMP (9). Alfred Gilman’s finding that adenylate cyclase is an intrinsic membrane protein strongly suggested that the association of PLC with the plasma membrane would also be a prerequisite for its regulation by cell-surface receptors. Indeed, PLC activity had been detected in various membrane preparations by several investigators.

Nevertheless, we decided to purify PLC from the cytosolic fraction of bovine brain because the cytosolic fraction showed a much higher phosphoinositide-hydrolyzing activity than did the membrane fraction. Several people I later met at various meetings asked me why I pursued the seemingly unrewarding purification of cytosolic PLC. The answer was ignorance—I simply did not know enough about G proteins and adenylate cyclase at that time to try to account for the coupling of PLC function to cell-surface receptors. Just as I was launching this project, two postdoctoral fellows, Sung Ho Ryu (in late 1985) and Pan-Ghill Suh (in early 1986), joined my laboratory. They were not at all familiar with G proteins, adenylate cyclase, or, for that matter, PLC. I am sure that if they had been fully informed about what they were getting into, they would have said no to my suggestion. Fortunately, I at least had the prior experience of purifying GS, and I felt both physically and mentally prepared to embark on the large-scale purification of PLC. Early each Monday morning, one of us would drive 50 miles round trip to purchase 16 fresh bovine brains from the J. W. Treuth & Sons slaughterhouse in Catonsville, MD, near Baltimore. It was usually past midnight by the time we had prepared a cytosolic fraction and started to apply it to an ion-exchange chromatography column, which yielded three partially resolved peaks of PLC activity. After processing 400 bovine brains through five chromatography steps, we succeeded in 1986 to 1987 in purifying to homogeneity three distinct forms of PLC with molecular masses of 150, 145, and 85 kDa. We initially named these bovine brain enzymes PLC-I, PLC-II, and PLC-III, respectively (10).

Two small incidents (although perhaps not small from the perspective of the NIH safety officers) occurred at this time. We use to homogenize one brain at a time in a Waring blender with 1 liter of buffer containing two protease inhibitors, phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate. After a number of weeks of this operation, we all began to complain that it was rather dark in the cold room and asked building maintenance to change the fluorescent light bulbs. One of us then noticed that our pupils were all markedly constricted. Although we had thought that we were dealing with the neurotoxic diisopropyl fluorophosphate (a cholinesterase inhibitor) with extreme care, we were performing the fume-generating homogenization process 16 times in 1 day in a poorly ventilated cold room. Fortunately, after we stopped using diisopropyl fluorophosphate, our vision returned to normal. A second incident, which took longer to resolve, occurred while we were preparing ³²P-labeled phosphoinositide for use in our PLC assay. While harvesting yeast grown in a medium containing [³²P]orthophosphate, a bottle broke inside the centrifuge. My license for isotopes was suspended, and the centrifuge was sealed for several months.
Comparison of the predicted amino acid sequences of PLC-\(\beta\), PLC-\(\gamma\), and PLC-\(\delta\) revealed that, although these three types of enzyme showed a low overall sequence similarity, they shared marked similarity in two regions designated \(X\) (~150 amino acids) and \(Y\) (~120 amino acids). We therefore predicted that the \(X\) and \(Y\) regions constitute the catalytic site, with this prediction ultimately being corroborated when the crystal structure of PLC-\(\delta\) was determined. Whereas PLC-\(\beta\) and PLC-\(\delta\) contain short sequences of 50–70 amino acids separating the \(X\) and \(Y\) regions, PLC-\(\gamma\) has a long sequence of 400 amino acids separating the two regions that contain both Src homology (SH) 2 and SH3 domains, the domains first identified as noncatalytic regions common to a variety of Src-family tyrosine kinases (14). PLC-\(\gamma\) was the first nontyrosine kinase protein found to harbor SH2 and SH3 domains. The subsequent identification of SH2 and SH3 domains in several other proteins that do not possess tyrosine kinase activity contributed substantially to our understanding of the role of protein–protein interactions in signal transduction pathways.

Surprisingly, the sequence of PLC-\(\alpha\) showed no similarity to those of the other PLC enzymes. The isolated cDNA was subsequently found to encode a protein–disulfide isomerase devoid of PLC activity. The putative PLC-\(\alpha\) cDNA was obtained with the use of an antibody directed against the 62-kDa uterus enzyme. Apparently, however, the enzyme preparation was contaminated with a protein–disulfide isomerase that is highly antigenic. The PLC-\(\alpha\) protein was subsequently shown to be a fragment of PLC-\(\delta 1\) (15). Thereafter, the designation PLC-\(\alpha\) ceased to exist.

As I have already mentioned, an important issue in the area of receptor signaling concerned the mechanism by which PLC activity is coupled to various receptors. Although we had the PLC clones in hand and not being familiar with cell-surface receptors, we were unsure how best to tackle this question. Luckily, as the result of fruitful collaboration with Graham Carpenter, Joseph Schlessinger, Tony Hunter, and Gordon Guroff, we were able to show that treatment of a number of cell types with epidermal growth factor (EGF), platelet-derived growth factor (PDGF), or nerve growth factor induced the tyrosine phosphorylation of PLC-\(\gamma 1\), but not that of PLC-\(\beta 1\) or PLC-\(\delta 1\), and that the tyrosine phosphorylation of PLC-\(\gamma 1\) correlated well with the increased turnover of phosphatidylinositol 4,5-bisphosphate (13). We then demonstrated that treatment of cells with EGF or PDGF elicited a redistribution of PLC-\(\gamma 1\) from a predominantly cytosolic localization to the membrane. This was achieved by the binding of PLC-\(\gamma 1\) through its SH2 domain to the autophosphorylated form of the corresponding growth factor receptor. In 1991, we showed that phosphorylation of PLC-\(\gamma 1\) on tyrosine 783 is essential for its activation (16). In the same year, we added the T cell antigen receptor, membrane IgM, and the high-affinity IgE receptor to the ranks of cell-surface receptors that induce PLC-\(\gamma 1\) phosphorylation on tyrosine 783 (Fig. 3) (13).

Ample evidence indicated a mandatory role for a G protein in transduction of the signal from certain receptors to PLC, and such evidence further suggested that both pertussis toxin-sensitive and pertussis toxin-insensitive G proteins might actually play such a role. The identification of the PLC-activating G protein had to wait until 1991, when previously uncharacterized \(G\alpha\) subunits, designated \(G\alpha\) subunits, were discovered. Through collaboration with John Exton and Melvin Simon, we showed that all four members of the \(G\alpha\) subfamily activate PLC-\(\beta\) isozymes but not PLC-\(\gamma\) or PLC-\(\delta\) (13). None of the \(G\alpha\) subunits is a substrate for ADP-ribosylation catalyzed by pertussis toxin. In contrast, the \(\alpha\) subunits of \(G\alpha\) and \(G\delta\) do undergo ADP-ribosylation by pertussis toxin, which prevents dissociation of the \(G\beta\gamma\) subunits from the \(G\alpha\) subunit. In 1993, we reported that \(G\beta\gamma\) dimers specifically activate PLC-\(\beta\) isoforms and that the region of PLC-\(\beta\) that interacts with \(G\alpha\) differs from that responsible for interaction with \(G\beta\gamma\), providing a mechanistic basis for pertussis toxin-sensitive and -insensitive PLC activation (13).

**Figure 3. Sung Ho Ryu (left) and Pan-Ghill Suh (right) purified and cloned the first three prototypical members of the PLC family.** The regulation of PLC-\(\beta\) and PLC-\(\gamma\) by a G protein–coupled receptor and a receptor tyrosine kinase, respectively, is also shown.
In 1995, I was appointed chief of the Laboratory of Cell Signaling within NHLBI. By 1996, the total number of PLC isoforms identified had increased to 10 (four β, two γ, and four δ isoforms), six of which were discovered in our laboratory (13). At this time, we began to lose interest in searching for new PLC isozymes, as we found that PCR-based cloning tended to identify already known isoforms. Our desertion proved premature, however, given that three new types of PLC (ε, ζ, and η), all with the conserved X and Y regions, were later identified between 1998 and 2002.

**Discovery of peroxiredoxin**

Another reason we had started to move away from PLC involved both our discovery of a family of H₂O₂-reducing enzymes named peroxiredoxins (Prxs) in the mid-1980s and our observation in the mid-1990s that H₂O₂ might serve as an intracellular messenger in signaling by peptide growth factors such as EGF. These findings ultimately served as a springboard for a new avenue of investigation as described below.

While studying the regulation of GS in *S. cerevisiae* in 1983, Kanghwa Kim noticed that the yeast enzyme, which had been purified to apparent homogeneity, lost activity and underwent partial degradation when stored in a buffer containing DTT (17). Neither the addition of various protease inhibitors nor further purification on several HPLC columns had an effect on the rate of GS inactivation and degradation. It therefore seemed unlikely that a contaminating protease was responsible for this damage. Around this time, Earl Stadtman and Rodney Levine, a section head in the Laboratory of Biochemistry, were studying the oxidative modification of bacterial GS, which precedes its proteolysis (18). Influenced by their studies on reactive oxygen species, I started to wonder whether the inactivation of yeast GS was related to free radicals. One day I asked Kanghwa to flush a tube of the purified enzyme with nitrogen to see whether the enzyme was protected. We found that the enzyme was protected.

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Investigation prompted by these observations revealed that the damage to GS was actually caused by hydroxyl radicals (HO·) generated by the following four sequential reactions in the presence of a thiol (RSH), O₂, and catalytic amounts of containing Fe³⁺: 

\[
\text{Fe}^{3+} + \text{RSH} \rightarrow \text{Fe}^{2+} + \text{R}^{-} + \text{H}^{+} \\
\text{Reaction 1}
\]

\[
\text{Fe}^{2+} + \text{O}_2 \rightarrow \text{Fe}^{3+} + \text{O}_2^{-} \\
\text{Reaction 2}
\]

\[
2\text{O}_2^{-} + 2\text{H}^{+} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \\
\text{Reaction 3}
\]

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{HO}^{-} + \text{HO}_2^{-} \\
\text{Reaction 4}
\]

The fact that GS in crude extracts of yeast remained active in the presence of thiol and Fe²⁺, whereas under similar conditions the purified enzyme was rapidly inactivated, suggested the existence of a yeast component that protected GS from inactivation by the thiol-dependent oxidation system. This protective component was purified and found to be a 25-kDa protein (17). This protein did not exhibit detectable superoxide dismutase or catalase activity. Given that generation of the superoxide anion (O₂⁻) and H₂O₂ in Reactions 2 and 3 above is obligatory for the production of HO⁻, the elimination of either O₂⁻ or H₂O₂ by such activity would have provided protection.

Soon thereafter, Kanghwa Kim returned to Korea, and Ho Zoon Chae, another postdoctoral fellow from Korea, inherited this project and set out to examine the nature of the oxidant eliminated by the 25-kDa protein and the mechanism of its action. Ho Zoon found that the 25-kDa yeast protein reduces H₂O₂ and contains a cysteine residue at its active site (19). The electron for the reduction of H₂O₂ was found to come from NADPH via thioredoxin reductase and thioredoxin (Trx) (20). We referred to this 25-kDa protein initially as thiol-specific antioxidant (TSA) and subsequently as Trx-dependent peroxidase (TPx) before settling on the name of peroxiredoxin (Prx), given that not all members of this family rely on Trx as an electron donor (21).

At the time, all antioxidant proteins were thought to be dependent on a cofactor such as a heme, flavin, manganese, or selenium group at their active center. The 25-kDa yeast protein had no such group, but rather had a cysteine at its active site. Heme- or selenium-containing peroxidases were well known, but the concept of a thiol-dependent peroxidase was new. We had trouble convincing our colleagues that the cysteine residue could be involved in the peroxidase reaction. Given that most proteins contain at least one cysteine residue, it was difficult for them to accept that the cysteine in Prx could be so specific for H₂O₂.

This conundrum appeared to be resolved when the first crystal structure of a Prx family member was obtained. The Prx structure revealed that the sulfur atom of the active-site cysteine (later designated the peroxidatic cysteine (Cₚ)) is surrounded by two basic amino acid residues that lower the pKₐ value for CP–SH (where Kₐ is the acid constant) of the CP–SH thiol by stabilizing the thiolate anion and thereby facilitate nucleophilic attack on hydroperoxide and the consequent formation of Cₚ–SOH (22). The pKₐ value for Cₚ–SH was subsequently determined to be between 5.2 and 6.3 for several Prxs, whereas that of a typical proteinaceous thiol group is ~8.3. Kinetic experiments revealed, however, that the second-order rate constant for the reaction of Cys–SH with peroxide increased only ~20-fold when the thiol was ionized to the thiolate anion, whereas the Cₚ–SH of Prx reacted with peroxide 5–6 orders of magnitude faster than did other proteinaceous cysteine residues, suggesting that the low pKₐ alone could not account for the exceptional reactivity of Cₚ–SH (23, 24).

Prxs were also found to possess a high-affinity binding site for H₂O₂ that is lacking in catalase and GSH peroxidases. Structural studies carried out later by several laboratories showed that the transition state for the reaction of Prx with H₂O₂ is characterized by an extensive hydrogen bond network that includes the Cₚ thiolate anion, H₂O₂, and several conserved residues of Prx (25, 26). This network of hydrogen bonds thus provides a binding site for H₂O₂ that properly aligns the substrate for attack by the Cₚ thiolate sulfur and lowers the activation energy of the reaction.
tion energy for breaking of the O–O bond of H$_2$O$_2$ by stabilizing the transition state intermediate (24).

It is now known that Prx enzymes constitute a large family of peroxidases that are present in multiple isoforms in most organisms. All Prx enzymes are obligatory dimers and contain a conserved C$_p$ in the N-terminal region of the molecule. Most, but not all, Prxs contain an additional conserved cysteine residue (designated the resolving Cys (CR)) in the C-terminal region of the protein. On the basis of the presence or location of the C$_n$ residue, Prxs are classified into 2-Cys, atypical 2-Cys, and 1-Cys subfamilies (27).

During the catalytic cycle, C$_p$–SH reacts with H$_2$O$_2$ to form sulfenic acid (C$_p$–SOH). In 2-Cys Prxs, the resulting unstable sulfenic intermediate forms an intersubunit disulfide with CR–SH of the other subunit in the Prx dimer, and the disulfide is subsequently reduced by Trx (Fig. 4). Mammalian cells express six Prx isoforms: four 2-Cys Prxs (PrxI to PrxIV), one atypical 2-Cys Prx (PrxV), and one 1-Cys Prx (PrxVI). Identification of mammalian Prxs and their reaction mechanisms was largely the work of Ho Zoon Chae and another postdoctoral fellow, Sang Won Kang, in my laboratory.

While studying the kinetics of yeast Prx and human Prxl, we observed that their peroxidase activity deceased gradually with time. We subsequently found that this inactivation was due to selective oxidation of C$_p$–SH to sulfenic acid (C$_p$–SOH) (28). The Cys–SOH generated as an intermediate during catalysis thus appeared occasionally to undergo further oxidation to Cys–SO$_2$H, a reaction that cannot be reversed by Trx and thus leads to Prx inactivation. The presence of H$_2$O$_2$ alone was not sufficient to induce the oxidation of C$_p$–SH to C$_p$–SO$_2$H; instead, all the catalytic components (H$_2$O$_2$, Trx, thioredoxin reductase, and NADPH) were required, indicating that such hyperoxidation occurs only when Prxl is engaged in the catalytic cycle. Kinetic analysis indicated that Prxl was hyperoxidized at a rate of 0.07% per turnover.

In 2000, Hyun Ae Woo, a graduate student from Ewha Womans University in Korea, joined my lab to carry out her doctoral research with the support of a collaborative research program between NHLBII and the university. Hypothesizing that the accumulation of H$_2$O$_2$ as a result of Prx inactivation might lead to cell death, I suggested to Hyun Ae that she monitor Prx hyperoxidation and examine its relation to apoptosis. Several weeks later, she told me that the sulfenic Prxl produced during exposure of cells to H$_2$O$_2$ was gradually reduced back to the thiol form after removal of H$_2$O$_2$. This demonstration of the ability of mammalian cells to reduce protein sulfenic acid thus contradicted the general belief that oxidation to the sulfenic state is an irreversible process in cells (29). Subsequently, Michel Toledano’s laboratory identified sulfiredoxin (SrX) as the enzyme responsible for the reduction of sulfenic Prx and showed that this reaction relied on ATP as an energy source (Fig. 4) (30).

Oxidation of cysteine to sulfenic acid is not restricted to Prx enzymes. Indeed, ~1.4% of the cysteine residues of soluble proteins in rat liver were detected in the sulfenic form, but we found that reduction of Cys–SO$_2$H by SrX is specific to 2-Cys Prx isoforms (31). Furthermore, prokaryotic 2-Cys Prx enzymes were found to be insensitive to hyperoxidation, and prokaryotes were found not to contain SrX. The inactivation of 2-Cys Prx via cysteine hyperoxidation has therefore been suggested to be the result of structural features acquired during evolution to accommodate a specific cellular function of H$_2$O$_2$ (32).

Whereas the detection of hyperoxidized Prxl had initially relied on complex proteomics analysis involving two-dimensional PAGE followed by MS, we subsequently devised a simple immunoblot assay for the detection of hyperoxidized 2-Cys Prxs with antibodies that bind to the conserved sequence surrounding C$_p$–SO$_2$H of these enzymes. With the use of these antibodies, we could pursue the physiological relevance of the seemingly wasteful inactivation–reactivation cycle of 2-Cys Prxs (see below).

**Intracellular messenger function of H$_2$O$_2$ and its regulation by Prx**

Through 1996, our research efforts were largely focused on the receptor-mediated activation of PLC, with only moderate attention being devoted to the identification and biochemical
characterization of mammalian Prx members. This pattern was slowly reversed as we became interested in the potential signaling role of H₂O₂. While Yun Soo Bae, a postdoc in my lab also from Korea, was studying the activation of PLC-γ1, he noticed that EGF induced a transient accumulation of H₂O₂ in A431 cells and that prevention of this accumulation resulted in inhibition of EGF-dependent tyrosine phosphorylation of several proteins, including PLC-γ1 (33). Prior to this observation, the production of H₂O₂ had been detected in a variety of cell types stimulated with transforming growth factor-β1, interleukin-1, tumor necrosis factor-α, PDGF, or angiotensin II (34, 35).

These findings led several groups, including mine, to advance the proposal that H₂O₂ might serve as an intracellular messenger (36). However, little was known about either the mechanism by which H₂O₂ is generated in response to receptor stimulation, the molecules on which H₂O₂ acts to propagate the signal, or the controlled pathway by which H₂O₂ is removed in a timely manner. By the time of our observation of EGF-induced H₂O₂ accumulation, there were a number of indications that an NADPH oxidase-like enzyme is responsible for agonist-induced H₂O₂ production in non-phagocytic cells. Convincing evidence for this was provided in 1989 after David Lambeth’s group identified a new type of NADPH oxidase isozyme that can be coupled to the activation of growth factor receptors (37).

The targets of H₂O₂ action in cells had previously been studied mainly in relation to oxidative injury, which led to the identification of cellular components irreversibly damaged by hydroxyl radicals. No serious efforts to identify targets of H₂O₂ signaling could be found in the literature. We reasoned that H₂O₂ signal propagation is likely not mediated by the reversible binding of H₂O₂ to target proteins. Rather, our studies on Prx enzymes taught us that protein sulfhydryl groups with a low pKₐ value can be selectively oxidized by H₂O₂. Among several proteins known to have a low-pKₐ cysteine at their active site, we focused on protein-tyrosine phosphatases (PTPs), given that the reversible inactivation of PTP family members might explain why the production of H₂O₂ is required for EGF- or PDGF-induced tyrosine phosphorylation, as had been shown by us (33) and Toren Finkel’s group (35). All PTPs share a Cys–X₅–Arg signature motif (where X is any amino acid), in which the invariant, low-pKₐ cysteine residue functions as a nucleophile in catalysis (38).

During a symposium in Maratea, Italy, in 1996, I had a chance to seek advice regarding the possible role of H₂O₂ in the regulation of PTPs from Edmond Fischer, the 1992 Nobel laureate in physiology or medicine, in whose laboratory Nick Tonks first purified and cloned a PTP family member (PTP1B). He encouraged me to pursue my idea by directing me to a review article on PTPs in which he described the necessity for negative regulation of these enzymes for growth factor signaling (38). By taking advantage of the fact that reduced but not oxidized thiol can be alkylated with ¹⁴C-labeled iodoacetic acid, Seung Rock Lee in my lab showed that PTP1B is reversibly oxidized by H₂O₂ produced in response to EGF stimulation in A431 cells (Fig. 5) (39).

On the basis of this finding, we proposed that the activation of receptor tyrosine kinases by their cognate growth factors may not be sufficient to increase the steady-state level of protein tyrosine phosphorylation in a cell; rather, concurrent inhibition of PTPs by H₂O₂ may also be required for this effect. Although we were very excited about such a novel mechanism for PTP regulation, it did not gain much recognition until 4 years later when Tonks’ group showed that the PTP known as SH2–PTP undergoes reversible oxidative inactivation in PDGF-stimulated cells (40). For our PTP1B studies, we had to prepare a mAb to selectively precipitate PTP1B, whereas Tonks’ group adopted the less cumbersome in-gel assay method, which we were not familiar with at the time of our work. We also showed that the tumor suppressor protein PTEN (phosphatase and tensin homolog), another member of the PTP family, is an effector of H₂O₂ in PDGF-stimulated cells (41). Subsequently, not only PTPs but also many other signaling proteins, including protein kinases, ion channels, and transcription factors, have been shown to be regulated through the reversible oxidation of their cysteine residues by H₂O₂ (42).

Mammalian cells express three types of H₂O₂-eliminating enzymes: catalase, GSH peroxidase (GPx), and Prx. Catalase is localized exclusively in peroxisomes, whereas GPx and Prx isoforms are found in various organelles. Prxs are much more abundant than GPxs, typically constituting 0.1 to 0.8% of total soluble protein in cells. Prx isoforms were therefore prime candidates for regulators of H₂O₂ signaling initiated by cell-surface receptors. Indeed, overexpression or partial depletion of Prxs in various cell types was shown to affect the intracellular level of H₂O₂ produced in response to growth factor or cytokine stimulation (43).

Even though the target thiol groups of H₂O₂ effector proteins often have a low pKₐ, they react with H₂O₂ several orders of magnitude more slowly than does C₉–SH of Prx. Furthermore, Prxs have a high-affinity binding site for H₂O₂.
We thus felt that we had to come up with some explanation for how the effector proteins can overcome such a competitive disadvantage and become oxidized by H₂O₂ that is produced only transiently.

In 2005, I returned to Korea to lead a newly established research institute at Ewha Womans University. This female-only university was one of the top-ranked institutions for undergraduates in Korea, but its graduate science program was not as strong as its undergraduate program. I felt that I could make some contribution. Through the NHLBIEwha collaborative program, I had already mentored several excellent graduate students from the university. In addition, I took the opportunity to bring four of my bright postdoctoral fellows as faculty to the institute. A year after I arrived at Ewha University, I was elected a First National Honor Scientist of Korea, a title that brought with it sufficient research funding to share with new young investigators. Back in Korea, one of my priorities was to explain how the kinetic disadvantage of H₂O₂ effector proteins relative to Prx can be overcome. We hypothesized that Prx molecules near these effector proteins must be transiently inactivated to allow the effectors to react with H₂O₂. After struggling for several years, we uncovered two examples of such a scenario.

One example is the reversible inactivation of mammalian Prxl through phosphorylation on tyrosine 194 by Src-family protein-tyrosine kinases in cells stimulated with PDGF or EGF or via immune receptors such as the T cell and B cell receptors (44). Only ∼0.3% of total Prxl was found to be phosphorylated in PDGF-stimulated NIH 3T3 cells, as a result of the fact that phosphorylation is confined to Prxl molecules associated with lipid rafts in the plasma membrane. The spatially confined inactivation of Prxl thus provides a means for generating a favorable H₂O₂ gradient around lipid rafts, where signaling proteins are concentrated, while preventing the global accumulation of H₂O₂ to toxic levels.

The second example is the reversible inactivation of Prxl through phosphorylation on threonine 90 by cyclin-dependent kinase 1 (Cdk1) during cell cycle progression (45). The threonine-phosphorylated Prxl was found at the centrosome during early stages of mitosis but not during interphase or late mitotic stages. The accumulation of H₂O₂ around the centrosome that results from the inactivation of centrosome-associated Prxl facilitates oxidative inactivation of centrosome-bound Cdc14B, a PTP family member, and thereby prevents premature degradation of mitotic activators such as cyclin B, Polo-like kinase 1, and Aurora A (46).

Hyperoxidation of 2-Cys Prx and the circadian clock

While the exciting discoveries on Prx regulation via phosphorylation were being made, I had not forgotten my goal to understand why eukaryotic 2-Cys Prx enzymes have evolved to undergo hyperoxidation during catalysis and subsequent reduction through an energy-consuming process. When we examined various tissues of mice maintained under normal conditions, an electrophoretic band corresponding to the hyperoxidized form of PrxlIII, which is found only in mitochondria, was detected at high intensity in the adrenal cortex and at lower intensity in brown adipose tissue, heart, lung, and ovary, whereas the hyperoxidized forms of other 2-Cys Prxs were not detected in any of the tissues examined.

The adrenal cortex is the site of sequential oxidation of cholesterol to corticosterone catalyzed by four cytochrome P450 enzymes (CYPs) in response to stimulation with adrenocorticotropic hormone (ACTH). We found that H₂O₂ molecules produced by CYP11B1 in mitochondria during corticosterone synthesis are reduced by PrxlIII, resulting in the hyperoxidation of PrxlIII, the consequent buildup of H₂O₂ in mitochondria, and its eventual overflow into the cytosol (47). The released H₂O₂ triggers the phosphorylation of p38 mitogen-activated protein kinase (MAPK), resulting in down-regulation of corticosterone production. It was known that the circulating concentrations of ACTH and corticosterone oscillate during the 24-h daily cycle, with these oscillations being controlled by the master circadian clock in the hypothalamus. We found that the abundance of PrxlIII–SO₂H as well as that of phosphorylated p38 in the adrenal gland of mice also underwent circadian oscillation, whereas the total amounts of PrxlIII and p38 remained unchanged (47). Ablation of Srx prevented the circadian changes in the levels of both PrxlIII–SO₂H and corticosterone (47). The amount of PrxlIII–SO₂H that accumulates during the reduction of H₂O₂ is proportional to the amount of H₂O₂ removed, which in turn is proportional to the amount of corticosterone synthesized. The release of mitochondrial H₂O₂ into the cytosol as a result of PrxlIII–SO₂H accumulation thus signals that a sufficient amount of corticosterone has been synthesized by triggering the activation of p38 and thereby inhibiting further corticosterone synthesis. Our study therefore showed that the seemingly wasteful processes of H₂O₂ production by CYP11B1 and reversible hyperoxidation of PrxlIII by its own substrate together represent an evolutionary adaptation that provides a mechanism for feedback inhibition of steroidogenesis (47).

Daily timekeeping by living organisms is thought to be driven by transcriptional–translational feedback loops that give rise to rhythmic expression of clock genes. Our discovery of the circadian oscillation of PrxlIII–SO₂H followed the finding of John S. O’Neill and colleagues (48) that the hyperoxidation of Prx serves as a transcription-independent circadian biomarker in a green alga. O’Neill and Reddy (49) also uncovered a self-sustained circadian oscillation of Prx–SO₂H abundance in human red blood cells (RBCs), which lack both a nucleus and mitochondria. Circadian fluctuation of Prx–SO₂H was subsequently detected in various organisms, including a fungus, worm, fly, and mouse, suggesting that Prx hyperoxidation cycles represent a conserved, ancestral circadian mechanism. Given that all of these organisms express multiple Prx isoforms, the identity of the isoforms undergoing hyperoxidation and the mechanisms underlying the oscillation remained to be determined. We found that among the four Prx isoforms present in RBCs, PrxlII is the isoform that undergoes hyperoxidation and that this reaction is driven by H₂O₂ produced during hemoglobin-dependent O₂ transport (50). Unexpectedly, the decay phase of PrxlII–SO₂H oscillation was found to be due to selective degradation of PrxlII–SO₂H by the 20S proteasome (50), in contrast to the Srx-dependent decay phase
of PrxIII–SO₂H during its circadian oscillation in mouse adrenal gland. About 1% of total PrxII in RBCs undergoes daily oscillation, resulting in a gradual loss of PrxII during the life span of these cells (50).

**Multiple functions of Prx enzymes and future research directions**

Hyeroxidation was also found to confer a new function on 2-Cys Prxs, namely that of a protein chaperone. Hyeroxidation of 2-Cys Prxs results in a structural transition from a dimer to a decamer that can undergo further aggregation, and the decameric and aggregated forms of the enzyme gain protein chaperone function. The first evidence for this surprising gain of function came from studies on yeast by Sang Yeol Lee (51), who earlier did postdoctoral training in my lab at NHLBI. Chaperone activity was also subsequently demonstrated for hyperoxidized human PrxII.

Several additional functions of Prx enzymes emerged after they were identified as binding partners of many proteins through co-immunoprecipitation analysis or with the yeast two-hybrid assay (27). Although the outcome of Prx binding to these partner proteins has not been fully revealed in all cases, the function of each binding protein appears to be regulated by Prx. Modulation of the peroxidase activity of Prx by binding partners has also been demonstrated (27). As shown by the association of PrxI with lipid rafts and centrosomes, Prx isoforms can become localized to specific cellular compartments. Some Prx-binding proteins might thus serve as anchors for Prx compartmentalization.

A notable finding from the studies of Prx-interacting proteins is that certain proteins with H₂O₂-sensitive cysteine residues are not directly oxidized by H₂O₂, with their oxidation instead being mediated by Prx (42). In this scenario, Prx is oxidized by H₂O₂ and then transfers its oxidation state to its binding partner, thus serving as both a sensor and transducer of H₂O₂ signaling. Examples of such redox-regulated proteins include the kinase ASK1 (52) and the transcription factor STAT3 (53). Prx-catalyzed oxidation appears to be an economic means by which to convey the H₂O₂ message with spatiotemporal precision and target specificity. Many more signaling proteins are likely to be oxidized indirectly via the disulfide transferase activity of Prx. In this regard, it is noteworthy that examination of the relation between the occurrence of cysteine in proteins and the complexity of organisms has revealed that an increase in cysteine content has co-evolved with O₂ sensing and cellular signaling (54).

It is clear that Prxs are more than just simple peroxide-eliminating enzymes. They are localized to various subcellular compartments and function as antioxidant enzymes, regulators of local H₂O₂ levels, protein chaperones, and disulfide transferases for sensing and transducing the H₂O₂ signal. These multiple functions performed at specific locations are regulated via various post-translational modifications, including hyperoxidation as well as tyrosine and threonine phosphorylation. Given that only a small fraction of a given Prx isoform is expected to be modified, identification of the sites of such modification within the cell is critical for understanding the biological relevance of the modification. For this purpose, the development of biochemical tools—in particular, specific antibodies for monitoring the modified Prx enzyme—is paramount. Similarly, with regard to Prx-catalyzed oxidation, the identification of target proteins will be difficult because only a small fraction of the total pool of a given Prx and target protein is likely to interact with each other, and such interaction is likely transient. Tools to selectively detect the oxidized form of H₂O₂ target proteins are thus also needed.

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