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Genetic Suppressors and Recovery of Repressed Biochemical Memory

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Many of the Reflections articles in this journal have focused on particular biological problems that the authors have devoted their careers to or on particular techniques that have facilitated their biological studies. Although I began my career as a biochemist, one of the most common threads to my research has been the exploitation of a genetic approach, the analysis of suppressor mutations. Particularly in the last 20 years, the success of this approach has led me into biological problems that required a remembering or relearning of my early training in biochemistry. This article will describe the voyage from chemistry and biochemistry to genetics and then to a recovery of remnants of my biochemical memory.

As a graduate student working under Lowell Hager at Harvard in the late 1950s, my thesis research was chemical and biochemical, as I performed the chemical synthesis and studied the biosynthesis of the chlorinated mold product caldariomycin (2,2-dichloro-1,3-cyclopentanediol) (1–3). With his postdoctoral fellow Paul Shaw, Lowell had discovered an enzyme, chloroperoxidase, that was capable of utilizing chloride ion to chlorinate organic compounds (4). Part of my work was to determine how this enzyme might be involved in the biosynthesis of caldariomycin. Apparently, the work became somewhat well known. This I learned when, toward the end of my Ph.D. work, I attended a FASEB meeting in Atlantic City to give a short presentation. As I walked into the conference center on the first day, a scientist passing by me stopped to look at my badge, hesitated a moment, and then commented "Beckwith . . . hmmm, oh yes, I remember . . . chlorination!" Despite this really quite limited chemical/biochemical notoriety, I became more and more fascinated by bacterial genetics in graduate school. I proceeded to do postdoctoral work in several bacterial genetics laboratories.

Suppressors, *lac* Operon Regulation, and Translation Termination

I had not studied any genetics (not even high school biology) until I audited an introductory genetics course late in my graduate career. I am not sure I had even heard of the word "suppressor" when, unwittingly, I happened to work with strains that contained suppressor mutations in the course of a postdoctoral research project. In 1961, Arthur Pardee, my first postdoctoral mentor, gave me a project he had started that was to lead me to an appreciation of the power of suppressor analysis. Art had recently returned from a sabbatical at the Institut Pasteur in Paris, where along with François Jacob and Jacques Monod, he provided the crucial argument for repressor control of gene expression (5). After Art's lab moved from Berkeley to Princeton University, he came up with an idea that was inspired by the Pasteur group's characterization of the *lac* operator. As initially conceived by Jacob and Monod, the operator, located at the beginning of the operon, served both as the site where transcription of the *lac* operon began and the site where the repressor acted to block this initiation (6). This model was based on two classes of mutations: operator-constitutive

mutations (O^c) that were resistant to repression and O^o mutations that mapped early in the operon and eliminated the activity of both genes of the operon, *lacZ* and *lacY*. Because the two classes of mutations appeared to map in the same general region, the Pasteur group proposed that both types of mutations were in the same site, the *lac* operator. The operator was both the start site for transcription of the operon and the binding site for the repressor. This explanation presented a mechanism for gene regulation that was quite satisfying, probably because of its parsimony, as repression could be readily explained by direct competition for access to the operator between the repressor and the transcription machinery (RNA polymerase). Although these studies obviously included the concept that there was a start site for gene transcription, the term "promoter" had not even been coined at this point.

Art, assuming that this characterization of the *lac* operator was correct (as did I), initiated a project that was, to my mind, ahead of its time in thinking about an issue related to gene expression. Proteins are found in cells in widely different amounts, some as high as 100,000 molecules/cell or as low as only a few molecules/cell. Art asked what determines each gene's level of expression, particularly constitutive genes, for which there is no regulation. He postulated that the DNA sequence of the "operators" for different constitutive genes could vary, leading to different potentials for expression for each gene (still accepting the notion that operators included transcription initiation sites). Art thought to obtain evidence for his hypothesis by selecting for revertants of the *lacO*^{o} mutants that restored *lac* operon expression and determining whether they exhibited different levels of expression. He had already begun the project when he invited me to help him out with it. He found exactly the mutants he predicted; a set of *lacO*^{o} revertants selected for growth on lactose each expressed different levels of β-galactosidase. We imagined that we had generated an array of mutant operators (think promoters) with different potentials for expression (7). Art gave me the project, and I followed it up by setting out to define the locations of the mutations that restored the varying expression levels.

I very quickly discovered to my surprise that the mutations that caused the restoration of *lac* operon function did not map to the operator site (8). They did not even map to the *lac* region but instead were located at different loci around the *Escherichia coli* chromosome. They were extragenic suppressor mutations. We had not even thought of that possibility because we had not envisioned that any kind of *trans*-effect could restore gene transcription. Today, the idea that promoters could vary in their activity (the concept behind this project) may seem self-evident and the error we had made a result of lack of rigor, but in those early days of molecular biology, molecular biologists had little sense of the kinds of complexity that we would meet as we began to explore issues of gene regulation more deeply.

I continued this project in a second postdoctoral position in London in the laboratory of Bill Hayes at Hammersmith Hospital. While there, I was contacted by Sydney Brenner, who had followed my work. He suspected that the suppressors of the *lacO*^{o} mutation I had isolated might define a new class of protein chain-terminating mutations. He invited me to spend some time in his laboratory in Cambridge, and our results led to a paper showing that the *lacO*^{o} suppressors restored translation to what Sydney named the *ochre* chain-terminating codon (9), later shown to be UAA (10). This finding, along with other work with O^o mutations I had started in Pardee's lab and continued as a postdoctoral student in Hayes' lab, strongly suggested that these mutations were not in the operator (or promoter, if you will) (11). Instead, they were chain-terminating or frameshift mutants early in the *lacZ* gene with strong polar effects on the downstream *lacY* gene.

While still in Pardee's lab and at the suggestion of a student, John Gerhart, I had begun to look for other kinds of suppressor mutations of the O^o mutations. Because it was possible to select for restoration of the *lacY* gene by asking for growth on melibiose as carbon source, I could seek revertants that might eliminate the polarity effects of the mutation but not restore β-galactosidase. Among these revertants, I found one set of mutations that mapped to yet another locus far from the *lac* region (8). Later, these were shown to be suppressor mutations in the gene for Rho, the transcription termination factor of *E. coli* (12). Thus, although Art and I had started with incorrect assumptions about the O^o mutations, our selection of suppressors of the mutations had led to 1) the identification of a second chain-terminating codon and new tRNA suppressors, 2) the identification of the major protein required for transcription termination, and 3) results that contradicted the evidence used to argue for a single site encompassing both the repressor target and transcription initiation site on the DNA. One final result of this fruitful project was that François Jacob offered me a postdoctoral position (my last), which I unhesitatingly accepted.

Once I had started my own laboratory at Harvard Medical School in 1965, the conclusions I had drawn from the work on O^o mutants led me to focus on specifying the actual site of the transcription initiation region, the promoter. With John Scaife, who came from Hayes' lab to do

postdoctoral work with me, we were able to obtain transcription-down mutations of the *lac* operon (13). We later showed that these mutations were in the binding site for CRP, the transcriptional activator cAMP-binding protein (14). Encouraged by the fruitfulness of the earlier characterization of O^o suppressors, we tried to obtain suppressors of our new transcription-down mutations. However, these turned out to be quite weak suppressors and difficult to study. In the years that followed, the category of projects that we were pursuing did not seem to call for the use of suppressors.

Suppressors and Protein Export

It was not until I began to change my research direction in the early 1970s from gene regulation to protein secretion that suppressor analysis was again to play a central role in my research. One of the approaches we took to understand how proteins can cross the cytoplasmic membrane of bacteria was the use of gene fusions. Our idea was to generate fusions in which the cytoplasmic enzyme β-galactosidase was fused at its N terminus to an N-terminal fragment of an exported protein. We asked how much of the exported protein was necessary to promote export of β-galactosidase to define the region of the protein that was necessary to promote that export. This work was begun before the discovery of signal sequences. We initiated this gene fusion work with cell envelope proteins involved in the transport of maltose and maltodextrins into the *E. coli* cytoplasm, a system that had been developed by Maxime Schwartz at the Institut Pasteur. Before coming to my lab as a postdoctoral fellow, Tom Silhavy had spent time in Maxime's laboratory and brought knowledge of the maltose system back to my laboratory. We exploited several proteins of this system, including the periplasmic maltose-binding protein (MBP) and the outer membrane protein LamB (15, 16). (LamB served both as a receptor for bacteriophage λ and as an outer membrane porin for maltodextrins.)

To our surprise, most of the fusions of β-galactosidase to MBP and LamB resulted in inactivation of β-galactosidase. As our studies went on, it became clear that the N-terminal signal sequence of the exported protein was initiating export of β-galactosidase across the membrane but that complete export was not achieved. The result was that a portion of the protein was left in the cytoplasm and a portion in the cell envelope, thus preventing β-galactosidase from assuming an active conformation in either compartment. As a result, the cells were incapable of growing on lactose minimal medium. Because the Lac^- phenotype was due to the effort of the cell to export β-galactosidase, we reasoned that a screen for Lac^+ revertants might yield extragenic suppressor mutations that generated defects in components of the bacterial export machinery. The defects in protein export would allow some of the β-galactosidase to remain in the cytoplasm, where it was enzymatically active and able to support growth on lactose. Don Oliver, a postdoctoral fellow, and I obtained such mutants, and they allowed us to define the gene *secA* and its protein product, one of the important factors in bacterial protein secretion (17). Notably, the mutants were only partially defective, as null mutations in genes essential for protein export would have been lethal. SecA was later shown to be a protein that uses ATP to promote translocation through the bacterial translocon in the cytoplasmic membrane.

Preceding our work, Tom Silhavy, who had started his own laboratory, used a different suppressor approach to define a gene central to the protein export process. With his student Scott Emr, he sought suppressors of a signal sequence mutation of the *lamB* gene that made cells unable to utilize maltodextrins as a carbon source (18). Mutations that restored export of the LamB mutant in its signal sequence, suppressing the maltodextrin growth defect, mapped to a gene they termed *prlA*. The mutations altered a membrane protein, later to be called SecY, which is the key component of the SecYEG translocation machinery in the bacterial membrane and is a homolog of the analogous protein in eukaryotic cells. Together, our two laboratories' search for suppressor mutations defined most of the components of the protein translocation machinery (19–22). We had initiated the genetic definition and analysis of the bacterial protein export machinery that would be complemented by *in vitro* studies, pioneered by Bill Wickner's group (23).

Misleading Suppressors

Suppressors of mutations in a particular gene are often sought to provide keys to understanding the function of that gene's product or aspects of the process in which that product is involved; but this is not always the result of suppressor analysis, and the exceptions provide caution in interpreting the identification of suppressor genes. We learned this lesson when we began to extend our analysis of protein export by looking for suppressors of mutations in the *secA* gene. Starting with a *secA* temperature-sensitive mutation, we selected for growth at high temperature, hoping to find mutations in genes encoding proteins that interacted with SecA. The first mutation we characterized was in the gene (*rpsO*) for a ribosomal protein. This led us to hypothesize that the SecA protein interacted specifically with the RpsO protein of the ribosome, an interpretation that seemed reasonable given the relationship

between protein synthesis and protein export observed in eukaryotic cells (24). However, subsequent extension of the search for suppressors in my and other laboratories revealed that mutations in many different genes involved in protein synthesis and even the addition of low levels of chloramphenicol, an inhibitor of protein synthesis, restored growth at high temperature to *sec* temperature-sensitive mutants (25, 26). It appeared that simply slowing down protein synthesis reduced the load on the secretion apparatus, thus allowing the cells to grow. We had learned an important lesson. Suppressor mutations may act to compensate for the mutational defect indirectly by interfering with some central biochemical process or perhaps even the growth rate. One way of avoiding this pitfall is to obtain enough suppressor mutations of a particular mutation to know that there are not multiple genes in which suppressor mutations can occur.

Suppressors and Protein Disulfide Bond Formation (and Membrane Protein Assembly)

In the late 1980s, we extended the β-galactosidase approach for studying how proteins are exported *across* membranes to analysis of how proteins are *integrated into* the cytoplasmic membrane. Accordingly, we constructed a fusion of β-galactosidase to the periplasmic domain of a cytoplasmic membrane protein, MalF, also involved in maltose transport. In strikingly parallel fashion to the fusions with the periplasmic protein MBP, the MalF-β-galactosidase fusion exhibited no β-galactosidase activity. This parallelism led us to assume that we now had a selection for mutants defective in the cellular machinery for inserting proteins into the cytoplasmic membrane. We thought that selection for Lac^+ revertants of the strain would yield mutations that reduced the activity of those cellular components that promote membrane protein insertion, thus causing some of the fusion protein to remain in the cytoplasm, where the β-galactosidase could be active. Karen McGovern, a graduate student, isolated and mapped four such suppressor mutations that restored the Lac^+ phenotype and located them to a *single region* on the chromosome. However, extensive analysis indicated that these mutations were not affecting membrane protein insertion.

The fact that the mutations fully restored β-galactosidase activity and all mapped to one chromosomal locus (see above) indicated to me that they must be interesting. I was determined to pursue characterization of the mutations. Ultimately, Jim Bardwell, a postdoctoral fellow, demonstrated that the mutations were in a single gene that was essential for the formation of protein disulfide bonds in the *E. coli* periplasm (27). The mutations eliminated the activity of a thioredoxin-like protein that we named DsbA. More extensive selection for suppressors revealed the existence of a second gene essential for this process, *dsbB*, thus defining the two proteins required for disulfide bond formation (28). A cytoplasmic membrane protein with two pairs of redox-active cysteines, DsbB maintains DsbA in the active oxidized, disulfide-bonded state and itself is re-oxidized by quinones. Our findings and those of others led to further elaboration of the details of the pathway and to the discovery of comparable pathways in eukaryotic cells (29–31).

To explain how it could be that *dsb* mutants would act as suppressors of the Lac^- phenotype of the MalF-β-galactosidase fusion strain, we proposed that this phenotype was due to disulfide bonds that were formed in the β-galactosidase exported to the periplasm. Such bonds are not present in the protein when it is located in its normal (reducing) compartment, the cytoplasm. When disulfide bonds did accumulate in β-galactosidase, they resulted in misfolding of the enzyme. The suppressor mutations in the *dsbA* or *dsbB* gene allowed the cysteines in β-galactosidase to remain reduced and permitted the proper folding of β-galactosidase.

Why did we not obtain the suppressor mutations that interfered with membrane protein assembly of the MalF-β-galactosidase fusion, the mutations that we had originally sought? Further characterization of the *dsbA* and *dsbB* mutants eventually led us to realize that the selection for growth on lactose was a very strong selection, a selection requiring that mutations completely inactivate the membrane protein assembly process; these would have been lethal to the bacteria. Once we recognized this, we switched to a suppressor mutation hunt that screened for partial but not complete restoration of β-galactosidase activity. With this approach, we were successful and obtained suppressor mutations in genes for all of the components of the bacterial signal recognition particle (SRP) pathway in addition to new classes of *dsbA* and *dsbB* mutations (32, 33). Alas, our findings came well after these SRP components had been identified in other laboratories, although the mutations proved a useful tool for studies on SRP-dependent export.

As we characterized the *dsb* mutants, we found that, in the absence of DsbA or DsbB, there was still a low level of disulfide bond formation in cells. Although we recognized that this activity might simply be due to air oxidation, we also considered that there might be a weak enzymatic activity in the periplasm that provided an alternative mechanism for disulfide bond formation. On the chance that this background activity was due to another enzyme,

Arne Rietsch, a graduate student, tried to identify this hypothetical enzyme by obtaining suppressor mutations that would restore high levels of disulfide bond formation. Such mutations might increase the amount or activity of the hypothetical enzyme and allow us to identify it. Here, again, the suppressors we obtained were not what we expected. Instead, they were either in the gene (*dsbD*) for a cytoplasmic membrane protein known to be involved in cytochrome *c* biogenesis or in the gene (*trxA*) for a cytoplasmic reductant protein, thioredoxin-1. We proceeded to show that these suppressor mutations resulted in the failure of the strains to maintain the active-site cysteines of the periplasmic protein DsbC in a reduced state. DsbC is itself a thioredoxin-like protein that ordinarily acts as a reductant, breaking disulfide bonds in periplasmic proteins that have been misoxidized. However, in the *dsbD* and *trxA* mutants, DsbC accumulated in the oxidized state and could now partially replace DsbA as an oxidant. Via the suppressor mutations, we had identified an unusual pathway that channeled electrons from cytoplasmic thioredoxin to the membrane protein DsbD and then to DsbC in the periplasm by an extensive disulfide bond reduction cascade (34, 35).

Suppressors and the Reducing Environment of the Cytoplasm

The discovery in my lab of the DsbA/DsbB pathway for disulfide bond formation was not completely accidental. We had been wondering how disulfide bonds were introduced into proteins for some time as a result of our studies on the properties of the periplasmic enzyme alkaline phosphatase (PhoA). This enzyme contains two structural disulfide bonds important for its stability and activity. My student Susan Michaelis had obtained mutants defective in the signal sequence of PhoA that prevented its export to the periplasm and caused it to remain in the cytoplasm. In this unaccustomed location, PhoA was totally devoid of enzymatic activity (36). We then showed that this lack of activity was due to the absence of its disulfide bonds (37). From these results, I became interested in two questions: how is disulfide bond formation achieved in the periplasm, and what prevents disulfide bond formation in cytoplasmic proteins?

To identify the cytoplasmic components that were responsible for keeping protein cysteines from being oxidized into disulfide bonds, we devised an approach for selecting mutations that would allow the cytoplasm to become an oxidizing environment. In particular, we sought suppressor mutations that would restore the enzymatic activity to a cytoplasmically localized (signal sequenceless) PhoA. Because PhoA is a quite promiscuous phosphomonoesterase, we suspected that if PhoA were to be active in the cytoplasm, it might be able to substitute for any of a number of specific phosphatases that participated in important metabolic pathways. To test this idea, Alan Derman, a student in the lab, asked whether we could get an active cytoplasmic PhoA to substitute for two such enzymes, serine-1-phosphate phosphatase, required for serine biosynthesis, and fructose-1,6-bisphosphatase, essential for reverse glycolysis. We constructed strains that contained mutations in the genes (*serA* or *fbp*) encoding one or the other of these two enzymes and that expressed a PhoA deleted for its signal sequence in the cytoplasm of the strains. We then selected for mutants that would restore serine prototrophy to the *serA* mutant or growth on glycerol as the carbon source to the *fbp* mutant. In both cases, we obtained suppressor mutations and found that they all mapped to the *trxB* gene encoding the enzyme thioredoxin reductase. Because the *trxB* mutations now allowed disulfide bond formation in the cytoplasm, the strains expressed high levels of alkaline phosphatase, which could substitute for the missing phosphatases (38).

At the time, if one were to ask why there were no structural disulfide bonds in cytoplasmic proteins, the typical response would have been “because of the reducing environment of the cytoplasm.” At first glance, our finding that the absence of thioredoxin reductase caused such disulfide bonds to accumulate in proteins appeared to be consistent with that explanation. We supposed that, in wild-type strains, thioredoxins with their active-site cysteines were constantly reducing any disulfide bonds that formed; the *trxB* mutations eliminated the activity that maintained thioredoxins in the reduced state, and now disulfide bonds could accumulate. However, further experiments in my lab showed that the answer was not so simple. In fact, our studies showed that the ability to accumulate disulfide bonds in cytoplasmic PhoA (and other proteins) in the *trxB* mutant strains depended not on the elimination of reduced thioredoxins but on the *accumulation of oxidized thioredoxins*. The oxidized thioredoxins actively promoted disulfide bond formation in the cytoplasm, much like DsbA oxidizes proteins in the periplasm (39).

As this work proceeded, one of the directions it took reflected the influence of Dominique Belin, a visiting researcher in the lab from the University of Geneva. Dominique, a phage geneticist, had switched to studies on mammalian proteases, in particular mouse urokinase, a protein with multiple disulfide bonds. He suggested that we remove the signal sequence of urokinase so it would be cytoplasmically localized and see whether the enzyme

would fold into its active disulfide-bonded form in the *trxB* mutant background. We followed this suggestion and found that small amounts of active urokinase could accumulate in the cytoplasm of the *trxB* mutant but not in a wild-type strain (38). Our findings of a strain with an oxidizing cytoplasm aroused a good deal of interest because there were advantages to being able to produce disulfide-bonded proteins in the cytoplasm for both basic research and biotechnological purposes. Encouraged by the properties of the *trxB* strain, we asked whether we could genetically alter *E. coli* to permit even higher levels of cytoplasmic disulfide bond formation.

Our first effort to increase the potency for cytoplasmic disulfide bond formation in *E. coli* was to combine the *trxB* mutation with a glutathione reductase mutation (*gor*), altering the reducing capacity (or enhancing the oxidizing capacity) of the cytoplasm even further. Not surprisingly, the double mutant did not grow, as we had eliminated the two reductive pathways required to maintain the essential enzyme, ribonucleotide reductase (RNR), in the reduced active state. Fortunately, for manipulating these strains, a student, Will Prinz, found that addition of dithiothreitol (DTT) to the medium allowed their growth. When we washed the bacteria free of DTT, they now made amounts of active urokinase in the cytoplasm vastly increased over those seen in the *trxB* mutant background (40).

While working with the *trxB gor* strains, we noticed that when the double mutant was plated on growth medium not containing DTT, mutations that restored growth arose at relatively high frequency. These mutations mapped to neither the *trxB* nor *gor* gene; rather, they were all extragenic suppressor mutations located in the gene *ahpC*, which encoded a bacterial peroxidase. (The gene designation *ahpC* is based on the original name for this enzyme, alkyl hydroperoxidase, so named because it destroys alkyl hydroperoxides as well as hydrogen peroxide.) Characterization of the suppressor mutations with our collaborator Leslie Poole showed that they had altered the peroxidase so that it could now reduce glutathionylated glutaredoxins, thus restoring reduced glutathione to cells (41, 42). In effect, we had obtained suppressor mutations that generated a new pathway for channeling electrons to reduce RNR. That the function of AhpC could be changed to that of a reductase of disulfide bonds was not totally surprising, as AhpC ordinarily uses the redox activity of its cysteines to reduce hydrogen peroxide. Finally, we found with our collaborator George Georgiou that a *trxB gor ahpC*^{sup} strain, which exhibited nearly normal growth rates, was quite useful for expressing high levels of eukaryotic disulfide-bonded proteins in the *E. coli* periplasm (43).

We later obtained suppressor mutations in *ahpC* that could even overcome the growth defect of a double mutant (*trxB gshA*) lacking both thioredoxin reductase and the glutathione biosynthetic pathway (44). In these strains completely lacking glutathione, the suppressor mutations appeared to alter the AhpC peroxidase so that it could directly reduce either oxidized glutaredoxins or thioredoxins. The AhpC peroxidase appeared to have remarkably chameleon-like qualities, being able to alter its specificity largely by single amino acid changes.

We had first come upon *ahpC* suppressor mutations by chance: the observation that the *trxB gor* double mutant spread on agar medium, where it could not grow, threw off colonies at high frequency that could grow. However, once I had seen how productive such suppressor analysis could be, it occurred to me that we might be able to exploit suppressor analysis further to identify other alternative pathways for generating reduced RNR. These could be of interest in terms of biological pathways and for biotechnological purposes. We imagined that these novel pathways might be generated in several ways: 1) activation of cryptic thioredoxin-like proteins, many of which have been identified on the *E. coli* genome by a bioinformatics approach (45); 2) generation of new pathways of electron transfer to RNR that did not involve thioredoxin-like proteins; 3) alteration of one of the other RNRs or of another enzyme to substitute for RNR using a different source of electrons; or 4) other ways of channeling electrons into the glutathione or thioredoxin pathways.

To seek different types of suppressors from those obtained with the *trxB gor* double mutant, we constructed strains that were missing the three thioredoxin family proteins, any one of which could suffice to reduce RNR, thioredoxin-1 (*trxA*), thioredoxin-2 (*trxC*), and glutaredoxin-1 (*grxA*). The *trxA trxC grxA* triple mutant could not grow but could be complemented, for example, by a plasmid expressing a regulatable *trxC*. Two postdoctoral fellows, Ron Ortenberg and Stéphanie Gon, isolated and characterized three suppressor mutations. To our immediate surprise, the mutations mapped not to genes involved in cysteine sulfhydryl-based electron transfer processes but instead to genes involved in the initiation of DNA replication, *dnaA* and *dnaN*. However, we quickly realized that a likely link of the suppressors in genes for DNA replication and the process we were restoring was that we were selecting for the activity of RNR, which is essential for generating deoxyribonucleotides for DNA replication. Further studies with the suppressor mutations in *dnaA* and *dnaN* revealed that they mediated the restoration of growth by causing derepression of RNR (46).

Apparently, the increased levels of RNR allowed, by mass action, its reduction by another glutaredoxin, glutaredoxin-3, which was known to have very weak activity for RNR. Furthermore, these studies led to the discovery of a mechanism that coordinates the synthesis of deoxyribonucleotides with the initiation of DNA replication (47).

We also extended our search for suppressors of the *trxB gor* double mutant by selecting for mutations that would restore growth to a triple mutant, *trxB gor ahpC*. We did this to avoid the very frequent *ahpC* suppressor mutations that were overwhelming in a number of other less frequent mutations. With the triple mutant, we obtained six suppressor mutations that restored growth, all of which mapped to the gene for the *oxidizing* enzyme lipoamide dehydrogenase (*lpdA*). Lipoamide is the form of the small molecule lipoic acid that is ligated to lysine residues of protein complexes such as pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. Lipoamide uses the redox activity of its two sulfur atoms in disulfide-bonded form to allow these two enzymes to oxidize their substrates. Lipoamide dehydrogenase regenerates the oxidized lipoamide to maintain the activity of these enzyme complexes. However, lipoic acid is also known to be capable of reducing glutaredoxins *in vitro* (48). Our *in vivo* genetic evidence obtained so far in this very recent project suggests that the defect in LpdA results in the accumulation of the reduced form of lipoamide (or lipoic acid), which can then channel electrons to RNR via glutaredoxin-1 (M. Feeney, S. Gon, M. J. Faulkner, and J. Beckwith, unpublished data). Interestingly, one of the mutations obtained replaces a conserved serine with a tyrosine, the latter residue being that of the wild-type version of the analogous protein in *Mycobacterium tuberculosis*. Some evidence indicates that this latter lipoamide dehydrogenase acts both as an oxidant and a reductant, oxidizing the enzyme complexes described above and reducing a thioredoxin-like molecule in a distinct pathway (49). We have not arrived at the end of this story yet, but it appears that our suppressor mutations have diminished or altered the activity of lipoamide dehydrogenase so that the cells accumulate reduced lipoic acid or lipoamide. Because we found that the suppressors restore growth to a cell that is also missing glutathione, the reduced lipoamide appears to be able to effectively replace glutathione in the pathway that leads to reduction of RNR.

Suppressors and Biochemical Memory

Suppressors have always been a powerful tool for genetic approaches to biological problems. Suppressor mutations that restore a selected phenotype to cells mutated in a particular gene may give information on the function of that gene or properties of the original mutation (*e.g.* the *ochre* suppressors). Using the power of bacterial genetics, one can design strains for selection of suppressor mutations that allow elucidation of important cellular processes (*e.g.* the *prlA* and *sec* mutations). Suppressor hunts that are specifically designed to restore a phenotype may evolve an existing protein to express an enhanced activity, not only giving information on how evolution of new functions can occur but also revealing the function of ill defined proteins (50). Most surprisingly, some of the most fruitful results of suppressor hunts are the unexpected ones. In reviewing our own work, perhaps more often than not, suppressors arose that were completely unexpected: *ochre* codon suppressors of *lacO*° mutations, *dsbA* and *dsbB* mutations in a selection designed to obtain mutations defective in membrane protein assembly, and mutations affecting DNA replication or in a gene for a peroxidase restoring reduction of RNR. Not to be heretical, but it seems that if you select for suppressors of mutations affecting an interesting process, there is a good chance you will find something interesting, even though it may not be what you sought. In some sense, these kinds of findings illustrate thinking that is becoming commonplace today, that the cell is a much more complex network of interacting pathways and processes than we had imagined. Eliminating one pathway or process can affect more processes than we once thought, and therefore, restoring it can occur by ways we might not have considered. The suppressor approach itself and the yield of unexpected but important results obtained provide one of the better rationales for basic science and risk taking.

In recent years, it has been a delight to find my lab studying suppressor mutations that occur in well known biochemical pathways, sometimes revealing unanticipated functions of these pathways or discovering new pathways such as protein disulfide bond formation. The biochemistry and the pathways that we had to learn in graduate school had faded from memory over the years after I had switched my allegiance to genetics, but as we picked up these novel suppressor mutations, bits of the memory began to seep back. I find myself pleased to gather up the shards of buried knowledge; to reconstruct in my mind the biochemical pathways and molecules that I had long ago "abandoned"; and to explain to my students what quinones, lipoic acid, glutathione, the reverse glycolysis pathway, etc. do, knowledge that few students are picking up today.

Recently, I read of the death of Irwin Gunsalus, who had been Lowell Hager's Ph.D. mentor and whom I got to know while finishing my Ph.D. at the University of Illinois. I immediately E-mailed Lowell to tell him I had read an

obituary for “Gunny” and noticed something I had not remembered, that Gunny had discovered lipoic acid, a molecule we were now working on. Lowell quickly E-mailed me back, pointing out that, as a student with Gunny, he had been the one who had discovered the enzyme lipoamide dehydrogenase (51)! Yet another closing of the circle.

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