

Discoveries in Bacterial Nucleotide Metabolism

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My decision to become a biochemist has its roots in youthful indecision. I grew up on a dairy farm in northwestern Illinois surrounded by domestic and wild animal and plant life and came to love the world of biology. This interest was strongly encouraged by my mother, who had been an excellent student of biology in college but was thwarted by the Great Depression and her family's poverty from pursuing an opportunity for graduate study. Her books, preserved specimens, and microscope slides became my playthings. I set up a home "lab" using commercial chemistry sets from Gilbert and ChemCraft, items that are regrettably deemed unsafe for sale today, but I did not gain a good understanding of chemical principles until high school. There I became enamored of the ability of chemistry to explain the properties of matter, including living matter, in rigorous and elegant atomic and molecular terms. How to compromise between these two fascinating fields of science? I decided in high school to seek a mixture, biochemistry, even though I had no real understanding of what that discipline involved. It proved to be an excellent decision!

As the state's land grant university, the University of Illinois at Urbana was a natural (and the only affordable) choice for my undergraduate education, especially after I won a scholarship by competitive examination that paid tuition and fees for all four years (1957–1961). Only gradually did I discover my good fortune: the University of Illinois had one of the most distinguished chemistry departments in the United States, and I received an excellent, intensive education. Biochemistry was deemed a graduate specialty at the time, so I majored in chemistry but took biochemistry and microbiology lecture and lab courses as soon as I could. Senior research under the direction of Carl Vestling, who left me largely on my own, introduced me to the confusions and ultimate satisfactions of research.

I had the good fortune to win a National Science Foundation fellowship for graduate study, and I enrolled at the University of California at Berkeley, which was recognized as having one of the nation's finest departments of biochemistry. I had no idea of the specialty I wished to pursue, so I was attracted to the breadth and depth of research topics pursued by Berkeley's outstanding faculty. My graduate school years from 1961 to 1966 were wonderfully exciting years for a young biochemist! The genetic code was being cracked. The groundbreaking papers by Jacob and Monod on regulatory genes were the subject of intense discussion: could such powerful biochemical conclusions be based on genetics alone? The concept of the subunit structure of proteins was being clarified, and the first high-resolution x-ray structures of proteins were emerging. I chose to pursue Ph.D. thesis research under H. A. Barker because I was fascinated with his recent discovery of the coenzyme form of vitamin B₁₂ and the fact that no one understood the chemical basis for its role in catalysis. Barker was a modest, gentle man who emphasized the importance of rigorous, critical experimentation. Initially, he observed my work carefully, but gradually he granted me freedom to design and interpret experiments quite independently. We were able to make substantial contributions to the enzymology of the coenzyme B₁₂-dependent glutamate mutase system (1), but the most important contemporary contribution to the mechanism of action of the coenzyme came from the work of Perry Frey and Robert Abeles, who demonstrated the transfer of hydrogen atoms



FIGURE 1. The Stadtman laboratory (officially called the Laboratory of Biochemistry of the National Heart Institute) in 1966. Terry and Earl Stadtman are second and third from the left, respectively, in the second row; I am second from the left in the back row.

between substrates and the 5'-methylene of the [Co]-deoxyadenosyl moiety of the coenzyme (2). We later showed that the same remarkable chemistry occurred in the glutamate mutase reaction (3). From Barker, I learned an important lesson: investigation of an experimental system of seemingly narrow or specialized interest, such as anaerobic fermentations in the clostridia, could lead, if pursued carefully, thoughtfully, and with an alert eye for the unexpected, to discoveries of very great general importance. Herb Weissbach made this same point in his recent Reflections article (4). With respect to my independent research career, one might have thought that the biochemistry of nucleotide biosynthesis was well understood when I began my studies of it, but we were richly rewarded with novel findings.

With Barker's encouragement, I applied in 1966 to Earl Stadtman for postdoctoral studies at the National Institutes of Health (NIH). Again, I had the good fortune to win a fellowship, this time from the NIH, for support of my research training. By doing research with Earl Stadtman (and with Terry Stadtman, who worked in the same laboratory and was an excellent source of intellectual and methodological advice), I was staying in Barker's scientific family; both Stadtman's had trained with Barker and emphasized his style of critical, evidence-driven research, typically with bacterial systems. My work with Barker

emphasized metabolic pathways and enzymology, which were central to the biochemistry of the time, but Stadtman opened my eyes to the regulation of metabolism. An array of clever mechanisms selected through evolution for regulation of enzymes was already known, and new discoveries frequently appeared. My fascination with the regulation of enzyme activity and gene expression has continued to yield significant discoveries for 40 years.

It would be difficult to imagine a laboratory more exciting than the Stadtman lab during the years I worked there (1966–1968) (Fig. 1). Kingdon and Shapiro discovered the regulation of glutamine synthetase by adenylation; Ron Kaback was studying the mechanism of active transport; and others were investigating the mechanism of coenzyme B₁₂ action, the biochemistry of methane formation, and other pathways of bacterial metabolism. An atmosphere of excitement and intense scientific debate prevailed. The "Stadtman way" of mentoring the development of scientists has been deservedly recognized (5). Good research ideas came readily to Earl, and he shared them unselfishly with his postdoctoral students, encouraging them to develop independent research themes from them; often, as he did with me, he declined even to add his name to the resulting publications. By today's standards, a postdoctoral research period of only two years is too short to qualify one for an independent faculty position, but I had the

good fortune in 1968 to be offered an assistant professorship in biochemistry at my alma mater, Illinois. I jumped at the opportunity and have spent my entire career there.

Phosphoribosylpyrophosphate Synthetase and Regulation of Highly Branched Metabolic Pathways

I often joke that I have made a biochemical feast from crumbs fallen from Arthur Kornberg's table. The first of these "crumbs" was phosphoribosylpyrophosphate (PRPP) synthetase, which was characterized by Kornberg and colleagues in the 1950s (6, 7). PRPP was known to serve as an essential precursor in the biosynthesis of purine and pyrimidine nucleotides, both by the *de novo* pathways and when they are formed from nucleobase precursors. PRPP is also essential for the biosynthesis of histidine, tryptophan, and the pyridine-nucleotide coenzymes. Woolfolk and Stadtman had discovered the remarkable feedback inhibition of *Escherichia coli* glutamine synthetase by no fewer than eight metabolic end products derived from glutamine when I arrived at the NIH in 1966 (8), so Earl suggested that I determine whether PRPP synthetase displayed a similarly complex and sophisticated regulatory pattern. The enzyme was also interesting from a mechanistic viewpoint because it was a rare example of a pyrophosphokinase, *i.e.* it catalyzes the direct transfer of an intact pyrophosphoryl group from ATP to ribose 5-phosphate (6). How did it differ from the far more common phosphokinases and nucleotidyltransferases?

I studied PRPP synthetase for two years in Stadtman's laboratory and continued to study it (often in collaboration with other scientists) for nearly 30 years. The enzyme yielded many surprises. Metabolic regulation proved to be remarkably simple: PRPP synthetase is activated by P_i and inhibited at an allosteric site by ADP and, in many species, GDP. These properties presumably indicate adequate regulation of PRPP synthesis by a combination of "energy charge" (ATP/ADP ratio) and end product inhibition by purine nucleotides. The same pattern is observed with PRPP synthetases from such diverse sources as enteric bacteria, Gram-positive bacteria, and humans. Demonstration of a discrete allosteric site for ADP/GDP, distinct from inhibition at the ATP-binding active site, required careful detective work. The first evidence for this came from analysis of the complex kinetics of inhibition by ADP (9). A separate allosteric site for nucleotides was later documented by direct measurements of nucleotide binding using equilibrium dialysis (10); discrete allosteric sites for nucleotides and P_i were demonstrated directly by the determination of the x-ray crystallographic structure of PRPP synthetase (11).

Gradually, the center of PRPP synthetase research shifted from Urbana to Copenhagen, where my frequent collaborator Bjarne Hove-Jensen isolated the first bacterial mutants with defects in the *prs* gene (12), an objective that had eluded my lab because of the many metabolic functions of PRPP and the difficulty of supplying its end products via PRPP-independent pathways. The isolation of *prs* mutants led Hove-Jensen and his collaborators to cloning and sequencing of the gene and to convenient means of overproduction and purification of the recombinant protein by procedures that quickly supplanted the laborious purification procedure developed in my lab (13). Our studies concentrated on PRPP synthetase from *Salmonella* and *E. coli*, but physical studies of the purified enzyme from these sources were difficult because of its strong tendency to aggregate in solution. Kirsten Arnvig and I collaborated with Hove-Jensen to characterize *Bacillus subtilis* PRPP synthetase and proposed that it would be an excellent candidate for crystallographic analysis (14). Unfortunately, a conservative NIH Study Section refused to fund our attempt to determine the x-ray structure of *B. subtilis* PRPP synthetase because we did not yet have diffraction-quality crystals in hand, so this important objective was later accomplished in Copenhagen by the Hove-Jensen and Larsen groups (11). Another surprise emerged from these studies: two copies of the fundamental structural domain of the commonest type of phosphoribosyltransferase, the so-called Type I phosphoribosyltransferase domain, are fused together to form the PRPP synthetase structure. These domains have evolved so that one of them binds the ribose 5-phosphate substrate, and the ATP-binding pyrophosphoryltransferase catalytic site is formed at the interface between the two domains. The use of these structurally similar domains to catalyze reactions quite different from the phosphoribosyltransferase reactions catalyzed by Type I phosphoribosyltransferases suggests a remarkable instance of the co-evolution of the enzyme that synthesizes PRPP with the enzymes that utilize it in biosynthetic reactions.

Hove-Jensen's and other laboratories have gone on to illuminate the comparative biochemistry of PRPP synthetases from such diverse sources as plants (15), archaea (16), yeast (17), and mammals (18, 19). PRPP synthetases are found in three classes, and most eukaryotes elaborate multiple isozymes, whose specific functions are not yet clear. A question that deserves further study is the physiological role of the so-called PRPP synthetase-associated proteins, which are inactive PRPP synthetase homologues that form heterologous complexes with active subunits in mammalian species (20, 21).

A rewarding collaboration had its roots in the friendship I formed with Michael Becker when we were both postdoctoral students at the NIH. Becker developed a research program on inherited diseases of purine metabolism during his studies with J. Edwin Seegmiller and discovered that inherited defects in PRPP synthetase led to hyperuricemia and neurological defects in humans (22). Later, we collaborated on the characterization of recombinant human PRPP synthetase isozymes (19) and mutant human PRPP synthetases, all of which had defects in allosteric regulation rather than in catalysis (23). The studies of Frank Nygaard and Sine Larsen on x-ray structures and kinetic properties of mutant *B. subtilis* PRPP synthetases, which have unfortunately not been published except in Nygaard's Ph.D. thesis (24), suggest near identity between the mechanisms of allosteric regulation of human and bacterial enzymes.

Inactivation and Degradation of Enzymes in *B. subtilis*

The second crumb to fall from Arthur Kornberg's table, which had an even greater impact on my research career than PRPP synthetase did, appeared in a 1968 paper by Deutscher and Kornberg describing biochemical changes that occur during sporulation of *B. subtilis* cells (25). The observation that caught my eye was the report that aspartate transcarbamylase (ATCase) activity disappeared rapidly from the cells upon entry into the stationary phase of growth. I had been working in close proximity with Kingdon and Shapiro in the Stadtman lab when they discovered the inactivation of *E. coli* glutamine synthetase by adenylation (8), and I was stimulated by the possibility that ATCase inactivation in sporulating *B. subtilis* cells might result from a novel covalent inactivation. Louise Waindle began an investigation of the problem, ruled out a large number of trivial explanations for ATCase inactivation, and demonstrated that the generation of metabolic energy (presumably, but not definitely, in the form of ATP) was required for intracellular inactivation of the enzyme (26). John Brabson purified and characterized *B. subtilis* ATCase and found another surprise: the enzyme was a simple catalytic trimer, contained no regulatory subunits like its famous cousin from *E. coli*, and was not subject to allosteric regulation (27). Tom Paulus later investigated the two carbamyl-phosphate synthetase isozymes from *B. subtilis* and showed that the pyrimidine-repressible isozyme was the locus of allosteric regulation by pyrimidine and purine nucleotides and PRPP (28). Using the laborious immunochemical methods of the time, Michael Maurizi demonstrated that ATCase undergoes energy-dependent intracellular degradation to an immunochemi-

cally undetectable form in stationary cells (29). Extensive efforts to trap intermediates or to reconstruct the inactivation *in vitro* were unsuccessful; ATCase was stable as a rock in cell-free extracts but disappeared like a dust mote down the hall during inactivation in starving cells. In subsequent studies, we were able to define the metabolic signals for ATCase degradation; these include both carbon and nitrogen starvation. The stability of ATCase is regulated by specific amino acid mixtures in the growth medium and involves, at least under some conditions, elements of the stringent response (30, 31). Ping Hu and I found suggestive evidence that binding of the carbamyl phosphate substrate regulates the stability of the enzyme (32). However, an adequate biochemical description of ATCase inactivation is still lacking. It is not known whether covalent modification precedes and signals degradation, nor are all of the elements that regulate inactivation and their mode of action defined. It is my hope that a curious biochemist will solve these problems some day.

Our laboratory showed that ATCase inactivation is part of a much more general pattern of inactivation of enzymes of *de novo* nucleotide and amino acid biosynthesis in starving *B. subtilis* cells. Apparently, this phenomenon commits nutrient-limited cells to metabolism based on synthesis of new cell material from turnover of existing polymers instead of *de novo* biosynthesis. This view is supported by studies of Peter Setlow, who showed that germinating *Bacillus* spores lack numerous enzymes of biosynthesis and acquire them by new synthesis during spore outgrowth (33). Among the enzymes we found to be inactivated were carbamyl-phosphate synthetase and ATCase (the first two enzymes of *de novo* pyrimidine nucleotide biosynthesis) (26, 34), glutamine-PRPP amidotransferase (the first enzyme of purine nucleotide biosynthesis) (35), aspartokinase II (36), and ornithine transcarbamylase (37). Immunochemical evidence was obtained that PRPP amidotransferase, aspartokinase II, and ornithine transcarbamylase are degraded.

Although our laboratory obtained a good general description of the phenomenon of the intracellular degradation of enzymes in *B. subtilis*, we failed in our ultimate goal of obtaining a complete biochemical characterization of the proteolytic apparatus involved and explaining why degradation depends on metabolic energy. Fortunately, Michael Maurizi, who had studied ATCase degradation in my lab, retained his intense interest in the problem. He later teamed with Susan Gottesman to discover the Clp protease system in *E. coli* (38) and has become a leading investigator of Clp proteases and their multiple roles in the physiology of many species. The *clp* genes specify the proteins of a complex apparatus devoted to regulated intra-

cellular proteolysis: the ClpP protease and several ATP-dependent accessory subunits (ClpA, ClpC, ClpX, and others depending on the species) that control selectivity of degradation (39, 40). Construction of a collection of well characterized *clp* mutants allowed Michael Hecker and colleagues to investigate the roles of Clp-dependent proteolysis in *B. subtilis*. Years after I had stopped research on intracellular proteolysis, Ulf Gerth and co-workers in the Hecker lab used the *clp* mutants and our old antibody preparations to demonstrate that the degradation of ATCase and PRPP amidotransferase specifically involves ClpP and ClpC and is, as we had suggested previously, part of a pattern of extensive intracellular proteolysis in glucose-starved *B. subtilis* cells (41). It is a source of satisfaction and pride to me that my former Ph.D. student Michael Maurizi played such a crucial role in providing a biochemical explanation for the energy-dependent degradation of ATCase that he studied in my lab and that his discoveries had great impact on the entire field of research on intracellular proteolysis.

Unexpected Iron-Sulfur Cluster in *B. subtilis* Glutamine-PRPP Amidotransferase

Chuck Turnbough and I reasoned that if pyrimidine nucleotide biosynthesis was inactivated by loss of ATCase activity in starving *B. subtilis* cells, purine nucleotide biosynthesis was also likely to be shut down under the same conditions. Turnbough demonstrated that, as predicted, glutamine-PRPP amidotransferase (hereafter called "PRPP amidotransferase") was rapidly inactivated in stationary cells, but tests for dependence of the inactivation on metabolic energy led to a surprise: the inactivation required oxygen specifically (35, 42). There was a report from Wyn-gaarden's group that PRPP amidotransferase from pigeon liver contained iron and that *B. subtilis* amidotransferase was specifically inhibited by iron-chelating agents (43). What was the nature of the iron center, and was it the site of oxygen reaction during inactivation? Building on Turnbough's foundation, Joseph Wong purified *B. subtilis* PRPP amidotransferase. Fortunately, the enzyme could be adequately protected against oxygen with oxygen-free buffers containing the allosteric inhibitor AMP. The purified enzyme was yellow-brown in color and contained about 3 atoms of iron/subunit. Inactivation by oxygen led to bleaching of the chromophore and loss of iron (44). The next surprise came from Wong's attempt to reconstitute PRPP amidotransferase activity to the inactivated enzyme by adding inorganic ferric ions to it: a black precipitate consistently appeared. I recalled from freshman chemistry that ferric sulfide is black. During my graduate school days in Berkeley, Jesse Rabinowitz's group was adja-

cent to Barker's, and I was aware of the experiments that led to their discovery of novel iron-inorganic sulfide complexes in bacterial ferredoxins. I suggested to Wong that he acidify a sample of native PRPP amidotransferase and determine whether the odor of hydrogen sulfide was emitted. It was! Analysis indicated the presence of between 2 and 3 atoms of sulfide/subunit (44). I sent Wong to talk to I. C. Gunsalus, one of my most prominent colleagues at Illinois, who had characterized putidaredoxin, a ferredoxin involved in the hydroxylation of camphor by a cytochrome P-450. Wong returned crestfallen. Iron-sulfur proteins are always involved in oxidation-reduction reactions, and they contain 2 or 4 atoms of iron, he was told. He was admonished that his PRPP amidotransferase was grossly contaminated with an iron-sulfur protein. It was an attitude of disbelief that we frequently encountered in the early days of our studies of *B. subtilis* PRPP amidotransferase: an enzyme with no redox element in its catalysis would not contain an iron-sulfur center. The fact that our early preparations contained 3 atoms each of iron and sulfide instead of the 2 or 4 atoms found in known iron-sulfur cluster types added to the confusion. Furthermore, PRPP amidotransferase purified from *E. coli* by Howard Zalkin's group contained no iron or sulfide. Gradually, we clarified the picture. *B. subtilis* PRPP amidotransferase was shown by Mössbauer spectroscopy and several other means to contain a [4Fe-4S] cluster, even though the purified enzyme always contained from 3 to 3.5 atoms of each/subunit (45). Thorough biophysical characterization of the iron-sulfur cluster (46) and proof that its oxidative dissolution resulted in loss of PRPP amidotransferase activity (47) followed by energy-dependent intracellular degradation, like ATCase (48), firmly established the presence of the cluster in the enzyme. If any doubt remained, it was removed by the beautiful solution of the enzyme's structure by x-ray crystallography in Janet Smith's laboratory (49).

Today, the presence of iron-sulfur clusters in enzymes that do not participate in redox reactions is well established. Swiftly following our report was the discovery by Beinert and co-workers of an iron-sulfur cluster in the catalytic center of aconitase, where it is involved in reversible addition of water in the interconversion of isocitrate and aconitate (reviewed in Ref. 50). Several members of the aconitase family of enzymes, in which the iron-sulfur cluster acts as a Lewis acid to participate in addition-elimination reactions, have been described (50). In other cases, iron-sulfur clusters are found in enzymes in which they play no direct role in catalysis but are essential for maintenance of native protein structure, as with PRPP amido-

transferase. Examples of this class include *E. coli* endonuclease III (51) and the Rad3 family of helicases (52).

Our studies support the hypothesis that the initiating event in PRPP amidotransferase degradation by the Clp protease system in starving *B. subtilis* cells is the oxidative destruction of the enzyme's iron-sulfur cluster, but it is difficult to establish this definitively. Oxidation of the cluster leads to extensive loss of native protein structure *in vitro* and presumably *in vivo*, which renders the protein a good substrate for degradation. In collaboration with Howard Zalkin's group, Jerry Grandoni and I studied the rates of degradation *in vivo* of several mutant PRPP amidotransferases with increased susceptibility to inactivation by oxygen *in vitro*. There was a significant correlation between relative rates of oxidative inactivation and relative rates of degradation of PRPP amidotransferase protein *in vivo* (53). Inactive PRPP amidotransferase accumulates in *relA* cells or in cells in which degradation is inhibited by chloramphenicol and can be isolated by immunoprecipitation; this material has lost iron, suggesting that it had undergone prior oxidation (54). Regulation of the oxidative inactivation *in vivo* is also unclear. PRPP amidotransferase is protected against oxygen by the allosteric inhibitor AMP and more weakly by PRPP, but David Bernlohr's thorough studies of substrate and nucleotide pools in *B. subtilis* cells did not support a correlation between levels of these metabolites and PRPP amidotransferase stability (55). My hypothesis is that PRPP amidotransferase stability is a direct function of intracellular oxygen tension. In adequately nourished cells, oxygen is rapidly consumed at the cell membrane, whereas in starving cells, oxygen is able to diffuse into the cell and react with the enzyme's iron-sulfur center.

PRPP amidotransferase was an excellent experimental object for other areas of investigation, some of which we studied in collaboration with Janet Smith's and Howard Zalkin's groups at Purdue University. Like a number of biosynthetic enzymes leading to multiple end products, PRPP amidotransferase is subject to synergistic allosteric inhibition; the end products AMP and GMP (or GDP) are much more effective inhibitors in combination than they are individually (56). Through a combination of x-ray crystallography and characterization of mutant enzymes constructed *in vitro*, it has been possible to map the sites of nucleotide binding to *B. subtilis* PRPP amidotransferase quite precisely and to identify the interactions between allosteric and catalytic sites that account for synergistic inhibition (57). I am not aware of any other instance of synergistic inhibition for which this has been accomplished.

Finally, comparison of the N-terminal amino acid sequence of purified *B. subtilis* PRPP amidotransferase with the gene sequence revealed yet another surprise (58). The enzyme undergoes post-translational processing to remove 11 N-terminal amino acids, which exposes a new N terminus, cysteine, whose sulfhydryl and amino groups are crucial elements in the glutamine amidohydrolase active site. It appears likely that processing is autocatalytic. *E. coli* PRPP amidotransferase does not undergo post-translational processing other than removal of the initiating formyl-methionine residue, but otherwise the mechanisms of catalysis by the *E. coli* and *B. subtilis* enzymes are surely the same. In elegant independent research, the Smith and Zalkin groups obtained a detailed characterization of the glutamine amidohydrolase and phosphoribosyltransferase active sites of the *E. coli* enzyme, described the conformational changes that occur upon substrate binding, and revealed a remarkable 20 Å channel through which ammonia liberated from glutamine travels to the site where it reacts with PRPP (59, 60). What a beautiful enzyme!

Regulation of *pyr* Genes in *Bacillus*: Expanded View of Transcription Attenuation

Originally, the cloning and sequencing of *B. subtilis pyr* genes in my lab had a rather limited purpose: to determine the amino acid sequence of ATCase from the *pyrB* gene sequence so it would be easier to characterize putative products of intracellular proteolytic degradation. Claude Lerner cloned and sequenced *pyrB* and showed that the gene lay within a cluster of pyrimidine biosynthetic genes (61, 62), as had been suggested by genetic mapping studies of Potvin *et al.* (63). Lerner found that a strong bacterial transcription terminator preceded the *pyrB* gene and speculated that it was involved in regulation of gene expression. It eventually became clear that the *pyr* promoter was not contained on the segments cloned and characterized by Lerner and that the region specifying it could not be cloned in *E. coli* on high-copy plasmids. Cheryl Quinn solved this problem, and she and Barry Stephenson sequenced the entire 10.5-kilobase pair *pyr* gene cluster using the laborious manual methods available at the time (64). Two more transcription terminators were found in front of each of the initial genes of the cluster. What were they doing there? Analysis of the *B. subtilis pyr* cluster yielded considerable interesting comparative biochemical information, but the mechanism of regulation of expression of the genes, known to be coordinately repressed by exogenous pyrimidines (65), remained obscure.

Rob Turner brought an exciting solution to the problem with his demonstration that the first gene of the *pyr* clus-

ter, which we renamed *pyrR*, encoded a protein essential for repression of *pyr* genes (66). Turner also recognized, following insights gained from the studies of Charlie Yanofsky's group on regulation of the *B. subtilis trp* operon, that the internal transcription terminators preceding the first three genes of the *pyr* operon were each, in turn, preceded by sequences that specified antiterminator sequences in the mRNA. Finally, he observed that a conserved segment of RNA sequence in each antiterminator was positioned so that the binding of the PyrR protein to it would prevent the antiterminator RNA hairpin from forming and, as a result, permit a downstream terminator hairpin to form. He postulated that the affinity of PyrR for its site on *pyr* RNA is regulated by uridine nucleotides. Functionally similar RNA sequences, *i.e.* terminator-antiterminator regions, were positioned in front of each of the first three genes of the *pyr* operon. From these observations, Turner *et al.* proposed a model for PyrR-dependent regulation of the *pyr* operon by attenuation that, with only modest refinements, has withstood rigorous testing.

While these studies were in progress, we became aware that Jan Neuhard's group in Copenhagen had sequenced the *pyr* operon from the thermophile *Bacillus caldolyticus* (67). The *B. subtilis* and *B. caldolyticus* sequences are very similar; the arrangements and functions of the genes and regulatory sequences are functionally identical. Sharing information helped both groups interpret their findings: the regulatory function of the first gene of the operon, *pyrR*, was unrecognized by the Danish group, who had, however, demonstrated that it encoded a uracil phosphoribosyltransferase. We had failed to recognize that *pyrR* encoded this activity. Soon, both groups obtained convincing evidence that PyrR is an unusual bifunctional protein; it is both an RNA-binding protein that regulates transcription attenuation of the *pyr* operon and a uracil phosphoribosyltransferase. Ghim and Neuhard (67) also identified the second gene in the operon, *pyrP*, as a uracil permease, an integral membrane protein. This explained our difficulty in cloning the upstream end of the *B. subtilis pyr* operon: overexpression of PyrP is apparently toxic to *E. coli* cells because an excess of the protein disrupts membrane function. The spirit of scientific cooperation between my group and the Danish group is reflected in the fact that we arranged to publish our papers on this topic simultaneously.

Biochemical and genetic studies of PyrR and the mechanism by which it regulates *pyr* gene expression were very rewarding research topics for the next 14 years. These studies were recently described in a thorough review (68) and will be only briefly summarized here. The model for

PyrR-dependent attenuation control of the *pyr* operon was tested and refined by extensive genetic and molecular biological experimentation. Regulation of *pyr* transcription by PyrR and uridine nucleotides was reconstituted with *pyr* DNA templates and RNA polymerase *in vitro*. Purified PyrR was characterized, and the structure of PyrR from several species was determined by x-ray crystallography. PyrR was shown to bind selectively to an RNA stem-loop containing a purine-rich internal bulge in the 5'-strand and two segments of highly conserved nucleotide sequence. This structure, called the PyrR-binding loop, is located in the leading strand of antiterminator hairpins; PyrR binding to the binding loop prevents the antiterminator hairpins from forming and allows termination of transcription downstream. Binding of PyrR is stimulated by uridine nucleotides and antagonized by guanosine nucleotides, which explains how *pyr* expression is repressed by pyrimidines and stimulated by guanosine *in vivo*. PyrR-dependent attenuation of *pyr* genes is widely distributed among bacterial species. In some species, PyrR apparently acts as an inhibitor of translation by binding to a PyrR-binding loop located adjacent to the ribosome-binding site for a *pyr* gene. Chris Fields and I have published strong genetic evidence for this translational repression mechanism for regulation of the *pyr* genes of mycobacteria (69).

The *pyrG* gene encodes CTP synthetase, the enzyme that converts UTP to CTP, and is thus part of the pyrimidine biosynthetic pathway. The gene is not part of the *B. subtilis pyr* operon and is not co-regulated with it. How is *pyrG* expression regulated? Qi Meng and I established that *pyrG* expression is specifically regulated by cytidine in *Bacillus*. Deciphering the mechanism of *pyrG* regulation led to the last big surprise of my research career: a transcription attenuation mechanism in which the antiterminator is generated by CTP-sensitive reiterative transcription and no regulatory protein is involved. This work was also recently reviewed in detail (68). In brief, Meng found that a transcription terminator that precedes the *pyrG* gene is essential for regulation but could not identify any antiterminator sequences or regulatory protein. The four nucleotides at the 5'-end of the *pyrG* mRNA (GGGC) are crucial for normal regulation, but their role remained obscure until my former student Chuck Turnbough, who has developed a splendid career discovering novel mechanisms governing transcription of *pyr* and related genes in *E. coli*, suggested a regulatory mechanism in which the sequence of the 5'-end of *pyrG* transcripts is dependent on the intracellular CTP concentration. When CTP levels are low, RNA polymerase pauses when it cannot add a C res-

idue at position 4 (C in the GGGC... sequence). This leads to slippage of the transcript on the template and reiterative addition of extra G residues to the transcript until a C residue is finally inserted and transcription continues. The resulting transcripts with a series of extra G residues are able to cause antitermination by base pairing with the leading strand of the downstream terminator. RNA polymerase then reads through the disrupted terminator, and elevated synthesis of full-length *pyrG* message results. On the other hand, when CTP levels are high, little or no reiterative transcription occurs, and the transcripts cannot cause antitermination. Thus, *pyrG* transcripts are prematurely terminated at high CTP levels, little full-length mRNA is made, and expression of *pyrG* is repressed. Experimental support for this mechanism is convincing (68). Reiterative transcription occurs in cells grown on limiting cytidine and is essential for transcription attenuation *in vivo*. Regulation depends only on the sequence of the *pyrG* initially transcribed region and the properties of *B. subtilis* RNA polymerase; no regulatory protein or "riboswitch" is involved. As the model predicts, all of its essential features (in particular, CTP-sensitive reiterative transcription and regulation of transcription termination by CTP specifically) can be reconstituted *in vivo* with RNA polymerase, a *pyrG* template, and appropriate concentrations of nucleoside triphosphates. It has been a particular source of pleasure to me that this remarkable story was developed in close collaboration with my former student Chuck Turnbough, with whom I also recently published a thorough and critical review of research on the regulation of pyrimidine biosynthetic genes in bacteria (68).

Teaching and Being Taught: Science as a Social Activity

A theme I have emphasized in these reflections is the great reward and personal pleasure I have received from doing research with other investigators. As a professor of biochemistry at a major university, I was, of course, expected to train advanced undergraduate and graduate students to do research. I was privileged to work with many very capable students, several of whom I have named above. Most of them have gone on to outstanding independent careers in biotech, pharmaceutical, or agricultural science companies, in academic research and/or teaching, and in medical practice. Several have developed research programs that, to speak frankly, have surpassed my own in productivity and breadth of impact. Their contributions to science, far more than my own, constitute my greatest legacy.

I did not anticipate at the outset of my academic career the wealth of knowledge I would gain from my students and postdoctoral students. Their hard work at the bench

made possible a very productive career. Their fresh and original ideas led to our most significant findings. They pushed me to learn powerful new technologies, especially in the realm of molecular genetics. Teaching in the classroom and informally in the laboratory forced me to deepen my understanding of a broad range of biochemical topics and to sharpen my ability to communicate that understanding. My former students tend to give me a lot of credit for their successes, but they gave me as much valuable insight as I gave them.

I should also make clear that I have always sought to collaborate with other independent scientists, to combine their expertise with our own, resulting in not only deeper and broader analyses of our problems but often strong friendships as well. Especially significant were my collaborations with Janet Smith, Howard Zalkin, Bjarne Hove-Jensen, and Michael Becker, but I shared efforts with many others in biophysical characterization of our favorite enzymes. I gained a better mastery of molecular biology at the bench during a six-month sabbatical with Roy Doi at the University of California, Davis. At its best, international collaboration and open sharing of research results serve as a model for the rest of society. I especially value the many collaborations I have had with colleagues and students from Germany, Denmark, and China, but I have had positive interactions with scientists from many other countries, including Australia, Canada, France, Great Britain, Italy, Spain, and Sweden. The opportunity to live and do research for extended periods in Freiburg with Helmut Holzer, when I was a Guggenheim Fellow, and in Copenhagen with the community of researchers at the University of Copenhagen and the Danish Technical University enriched not only my scientific work but my life and the life of my family.

The Role of Good Fortune

The reader may have noted how frequently I have referred in this article to good fortune and to lucky, unexpected findings. Although I would like to claim talent and insight as a researcher, candor requires me to admit that I have benefited again and again from good fortune. I was lucky to spend my entire research career during what must be regarded as the Golden Age of biochemistry, an age when wonderful discoveries were being made every year and when powerful new methods of research were being developed. The new methods of molecular biology made possible the cloning and sequencing of genes, facile isolation of proteins, construction of precisely defined mutant strains, and powerful tests of the physiological functions of genes and regulatory sequences. The now nearly routine determination of protein structures by x-ray crystallogra-

phy added deeply satisfying approaches to understanding the functions of the proteins we studied. How exciting it was to be part of this creative ferment, to know personally many of the fine scientists involved, and to apply their approaches to my own research questions! I also had the good fortune to develop my professional career during the post-Sputnik era of abundant Federal support of science education and research. I was supported by the National Science Foundation for both undergraduate research and my graduate education and by the NIH during my postdoctoral years. My independent research received funding from the NIH, and, for a period, from the National Science Foundation as well, without interruption for 40 years. Science funding is much more competitive and difficult for beginning researchers to obtain today. I was wonderfully lucky in my choice of mentors. Barker and Stadtman set the highest standards, presented fascinating and important research problems, and were strongly supportive of my fledgling research efforts. They were the models for excellence as mentors of scientists-in-training that I strove to emulate as a research director at Illinois. And, yes, the University of Illinois was a most fortuitous choice. I received an excellent undergraduate education at Illinois and was even more fortunate to return to Illinois for my independent academic career. Lowell Hager had just been appointed head of the Biochemistry Department when I was hired, and he provided confident encouragement and support. My colleagues at Illinois set a high standard, and most of the undergraduate and graduate students who came to work with me were bright, motivated, and talented in research. I would have been hard pressed to fail in such an environment. It is no wonder that I never left Illinois and that I was proud to serve as head of the Department for five years.

On the personal side, I must mention one other stroke of great good luck. While at Berkeley, I met Bonnie George; we were married there in 1965. A student of psychology, a teacher, and a talented artist, Bonnie has enriched my life immeasurably. She has been a loyal, loving (but always honest) best friend, she has given me a wonderful family, and she has proudly supported my career as a biochemist and scholar.

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