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Bacterial cupredoxin azurin and its interactions with the tumor suppressor protein p53

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Early days with Gunny

I (A.M.C.) joined the Gunsalus laboratory in September 1965. This event was a fateful combination of his inquiring mind, his determination to go to the bottom of a problem, and his love for science and the young practitioners of science. Right before I obtained my Ph.D from Calcutta University in India, I published a couple of papers in the *Biochemical Journal* (London) on the nature of the pigments elaborated by *Pseudomonas fluorescens* (*putida*) and some of its metabolic activities. Gunny (as he was known among his students and colleagues) was curious about some of these findings and wrote a letter to my mentor, Professor S.C. Roy, asking for some details. As I provided him with the information, and indicated to him my interest in genetics as well as my progress towards my Ph.D, he extended an invitation to me to join his laboratory to set up a genetics system for his camphor-degrading pseudomonads. Thus began a long association between a naive foreign student from India and a renowned scholar, administrator (he was the head of the Biochemistry Division at the University of Illinois at Urbana-Champaign at that time), and educator in the United States. I have written [1] about Gunny's tremendous influence on my career, my humble beginning to the life of a researcher [2], and his illustrious career as an example to follow, albeit without much success. My early research involved isolating transducing phages and setting up a transduction system in the camphor-degrading strain of *P. putida*. Later I also developed a conjugation system and, using conjugational and transductional mapping, I, along with many colleagues such as Irving Crawford, Carol Gunsalus, Jim Rheinwald, and others, demonstrated the lack of clustering of the tryptophan biosynthetic pathway genes and the plasmid-borne nature of the genes specifying the degradation of camphor and octane [1]. The CAM and OCT plasmids were incompatible and while CAM was found to be a transmissible plasmid, OCT was shown to be transmissible through recombination of the OCT plasmid with CAM.

Life after Gunsalus

I left Gunny's laboratory for the General Electric Research & Development Center in Schenectady, New York, in 1971, but Gunny's influence never left me. Both Gunny and I were intrigued by the fact that plasmids could encode the major parts of a degradative pathway in *P. putida*. At the GE R&D center, I began to explore if other pseudomonads would harbor other degradative pathways encoded by plasmid-borne genes. During my 8 years there, I demonstrated the plasmid nature of the salicylate pathway and constructed a multi-plasmid pseudomonad that could consume several hydrocarbons simultaneously, thereby acquiring the ability to grow with crude oil much faster than strains harboring single degradative plasmids. This organism was the subject of a controversy related to the "patenting of life" and was eventually resolved by a decision from the U.S. Supreme Court [3,4].

In 1979, I came back to the University of Illinois, but this time I joined the faculty at the College of Medicine of the Chicago campus. Gunny used to visit me at Chicago and we would have lengthy discussions on what constitutes the high nutritional versatility of the fluorescent pseudomonads. We were particularly interested in cytochrome $P450_{cam}$ and other redox proteins. It was also the time in the mid-1980s when I was involved in

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setting up the International Centre for Genetic Engineering and Biotechnology (ICGEB) based in Trieste, Italy, and New Delhi, India. Gunny became the first Director of ICGEB and as a member of the Panel of Scientific Advisors of ICGEB, I maintained close contact with him. Our focus of discussion no longer centered solely on pseudomonads or nutritional versatility but ranged from global health threats to hunger and famine in developing countries and how to address such global issues from an international perspective.

Gunny always believed strongly that only science could provide answers to the various threats plaguing humans. Thus while he and I mostly discussed our favorite subject, viz., pseudomonads, cytochrome P450, and biodegradation of exotic and toxic compounds, we increasingly became concerned about the menace of infectious diseases, particularly those where pseudomonads like *P. aeruginosa* or its cousin *Burkholderia cepacia* would be implicated. Both these organisms were known to infect the lungs of the cystic fibrosis (CF) patients, and my interest had switched to the interesting but essentially unknown ways of how these pathogens caused chronic, and for *B. cepacia* often acute and fatal, infections in the lungs of CF patients. In particular, because CF patients are immuno-competent, I became interested in understanding how *P. aeruginosa* evaded the host immune system and protected itself from phagocytosis during its colonization of the CF lung.

Back to cytochromes and redox proteins

With the CAM system, the emphasis in the Gunsalus laboratory, as well as in the laboratories of his colleagues, has been on cytochrome P450. Cytochromes are well known for shuttling electrons during oxidation–reduction (redox) reactions but no other functions, particularly in pathogenesis, are known for these redox proteins. During our investigation on how mucoid cells of *P. aeruginosa*, isolated from the lungs of CF patients, evade phagocytosis, we noticed that such mucoid cells secreted enzymes that are normally involved in ATP metabolism and adenine nucleotide interconversion, enzymes such as ATPase, nucleoside diphosphate kinase, adenylate kinase, and 5′-nucleotidase [5]. My postdoctoral colleagues in the laboratory demonstrated that such secreted enzymes modulated the levels of ATP, effluxed from macrophages, and other adenine nucleotides in such a way that allowed activation of a group of macrophage surface receptors called P2Z or $P2X_7$ receptors, which in turn triggered pore formation on macrophage plasma membranes leading to their cell death. Thus secretion of these so-called ATP-utilizing enzymes by mucoid *P. aeruginosa* constituted a major weapon in the arsenal of this organism to fight off and kill phagocytic cells.

The secreted ATP-utilizing enzymes were only effective when ATP was present in the macrophage extracellular fluid, presumably effluxed from the macrophages themselves. During column chromatographic fractionation of the secreted proteins, Zaborina et al. [5] reported the isolation of a column flow-through fraction that appeared to induce macrophage cell death even in the absence of ATP. On further examination of the proteins secreted by a highly virulent nonmucoid clinical isolate of *P. aeruginosa*, we isolated a fraction enriched with two redox proteins, azurin and cytochrome c_{551}, that induced macrophage apoptosis through activation of the caspase cascade. Indeed, when J774 cell-line-derived or mouse peritoneal macrophages or mast cells were incubated overnight with the column (Q-Sepharose) flow-through fraction or with a mixture of purified azurin and cytochrome c_{551}, they underwent extensive cell death due to induction of apoptosis [6].

Redox proteins as inducers of mammalian cell apoptosis

The demonstration that purified azurin and cytochrome c_{551} induced apoptotic death in macrophages represented a hitherto-unknown function of these bacterial redox proteins. It is, however, known that release of a mammalian redox protein such as mitochondrial cytochrome *c* to the mammalian cell cytosol is a major event in the induction of apoptosis [7]. To investigate the mode of action of azurin in macrophage cell apoptosis, Yamada et al. [8] incubated purified wild type (wt) and several site-directed mutant azurin proteins with J774 cell-line-derived macrophages and followed their subcellular localization or their ability to induce apoptosis in such macrophages. Wt azurin was shown to enter macrophages and localize in the cytosol and the nuclear fractions, inducing apoptosis. Two redox-negative mutant azurin proteins had less cytotoxicity than wt azurin. Azurin was shown to form a complex with the tumor suppressor protein p53, thereby stabilizing it and raising its intracellular level. p53 is a known inducer of mammalian cell apoptosis as well as cellular growth arrest through positive (or sometimes negative) transcriptional activation of a set of genes involved in these processes [9]. As a transcriptional regulator, p53 is a major player in an intricate networking system that controls cell growth, death, and cellular regulation [9]. Wt azurin was shown to interact with p53 in such a way that allowed mobilization of Bax to the mitochondria, release of mitochondrial cytochrome *c* to the cytosol, and the resultant activation of the caspase cascade and induction of apoptosis [8].

Since azurin is a copper-containing redox protein (cupredoxin) involved in electron transfer [10] and since it was previously shown that reactive oxygen species (ROS) were involved in the context of azurin-mediated

cytotoxicity [8], an important question was if the redox activity of azurin was necessary for its cytotoxicity. Using apo-azurin devoid of copper and site-directed redox-negative mutants such as Cys112Asp (C112D) or Met44LysMet64Glu (M44KM64E) azurins, Goto et al. [11] demonstrated that while the wt or the C112D mutant azurin could form complexes with the tumor suppressor protein p53 and generate high levels of ROS, the M44KM64E mutant azurin was defective in complex formation with oligomeric forms of p53 and generated low levels of ROS. Interestingly, the ability to form a complex with p53 and thereby generate high levels of ROS correlated with the presence of cytotoxicity towards macrophages. The conclusion was that complex formation with p53 and ROS generation, rather than azurin redox activity, were important in the cytotoxic action of azurin towards macrophages [11].

If the redox activity of azurin is not important for its cytotoxicity, then how does azurin induce apoptosis in macrophages? Incubation of macrophages with purified azurin allows its entry to the cell cytosol and finally to the nucleus, suggesting the possible presence of receptors for azurin internalization on macrophage cell surfaces. The nature of such receptors, if any, is currently unknown. The presence of azurin in the cytosol, however, allows complex formation with p53, which has nuclear import–export signals and which can traffic to the nucleus. p53 is believed to piggy-back azurin to the nucleus, where its stabilization and relative abundance enhance its transcriptional activity towards proapoptotic genes such as *bax*, whose enhanced expression and subsequent trafficking of the Bax to the mitochondria trigger the apoptotic process. Similar to mitochondrial cytochrome *c*, whose release in the cytosol initiates complex formation with the cytosolic protein Apaf-1 and subsequent apoptosome formation [7], the presence of a redox protein such as azurin is somehow sensed by the mammalian cell as a loss of mitochondrial integrity, even though azurin is not a part of mitochondrial matrix or intermembrane space in higher eukaryotes. This sensing is then believed to lead to complex formation with the cytosolic protein p53, thereby initiating the apoptotic process [12].

Microorganisms, redox proteins, and cancer

The above scenario raised two interesting questions. The J774 macrophages are ascites forms of murine reticulum cell sarcoma with macrophage-like properties of adherence, morphology, receptors for immunoglobulin, and antibody-dependent lysis of target cells [13]. If azurin can induce apoptosis in these cells, can azurin also induce apoptosis in human cancer cells? Also, if complex formation with p53 is important and azurin is cytotoxic against cancer cells, will it still be cytotoxic against cancer cells that lack p53? Will azurin enter such p53-null cells but not be transported to the nucleus if piggy-backing by p53 is important for azurin trafficking to the nucleus? We have previously reported that azurin could enter the human cancer (melanoma UISO-Mel-2) cells and induce apoptosis in cells that harbored a functional p53, but was much less effective in inducing apoptosis in a p53-null mutant cell line (UISO-Mel-6). In UISO-Mel-2 cells, azurin was localized predominantly in the cytosol and the nuclear fractions. In the p53-null UISO-Mel-6 cells, azurin was localized mainly in the cytosol. Most importantly, because of its ability to induce apoptosis in cultured UISO-Mel-2 cells, azurin was shown to allow regression of human UISO-Mel-2 tumors xenotransplanted in nude mice and did not show any obvious toxicity symptoms in such mice. Thus azurin behaved as an anticancer agent in nude mice [14].

The anticancer activity of bacterial cupredoxin azurin, normally involved in electron transfer, raises several questions. Azurin is a member of a family of cupredoxins [10]. May other cupredoxins have similar anticancer activity? Will other non-cupredoxin redox proteins demonstrate cancer-killing property? It has been known for a long time that many live bacteria, when introduced into tumor-bearing mice, will allow tumor regression [15]. Interesting examples are the use of attenuated *Salmonella* that targets the tumor cells preferentially for their replication [16] or attenuated strains of *Clostridium novyi* that can produce dramatic effects on tumor regression in the presence of other chemotherapeutic or antiangiogenic agents [17]. *Mycobacterium bovis* BCG has been and is still used widely in the treatment of bladder cancer [18]. The use of live bacteria in the treatment of cancer, however, is often accompanied by toxicity problems that limit its use [15]. Also, very little is known about the mechanism by which live bacteria target the tumor cells or cause tumor regression. It is generally accepted that facultative or anaerobic bacteria grow at the anaerobic core of the tumors, thereby depriving the tumor cells of their essential nutrients. The introduction of live bacteria in mice or humans also leads to the activation of the immune system and the cytokine network which might, at least in part, be responsible for the regression of the tumor. In some cases, microorganisms may also induce production of antiangiogenic factors that prevent sufficient blood vessel formation for the supply of nutrients to the tumor cells, thereby delaying their growth [19]. Live microorganisms may also be used as vectors for delivering cytotoxic agents preferentially to the tumors. For example, attenuated strains of *Salmonella* engineered for the elaboration of the enzyme cytosine deaminase have been used in the regression of tumors in mice. Cytosine deaminase converts the relatively nontoxic prodrug 5-fluorocytosine to the highly toxic anticancer agent 5-fluorouracil. Thus injection of engineered

Salmonella followed by injections of 5-fluorocytosine to tumor-bearing mice has been reported to lead to tumor shrinkage because of the 5-fluorouracil-induced tumor cell death.

Because of the toxicity and associated side effects, as well as a lack of predictability in some cases [15], the use of live microorganisms in the treatment of cancer has been limited. The idea of using microbial products, if they can be demonstrated to have anticancer activity and a lack of toxicity, thus appears to be attractive. Myxobacteria such as *Sorangium cellulosum* are known to produce secondary metabolites such as macrolides epothilone A and epothilone B with known anticancer activity [20]. These act as microtubule depolymerization inhibitors, similar to Paclitaxel (taxol), a well-known drug isolated from the Pacific yew tree and used for the treatment of breast and other forms of cancer. Epothilones have two major advantages over taxol. They are water-soluble, which minimizes the use of other ingredients in formulations that often have toxicities that add to that of taxol. Epothilones are also active against certain multidrug-resistant forms of tumors for which taxol shows little effect. Thus bacterial epothilones may have the potential to be useful clinical products once their toxicity problems are minimized. A synthetic analogue of epothilone B, called desoxyepothilone B, is reported to have efficacy against a range of cancers with relatively less toxicity than epothilone B [21].

The potenital use of low-molecular-weight, water-soluble azurin or similar redox proteins with anticancer activity and without much demonstrated toxicity in nude mice nevertheless has one significant problem. As a protein, azurin will likely elicit antibody formation and be rendered ineffective during long term treatment. It is thus important to use smaller truncated derivatives of azurin or synthetic peptides that will elicit little immunologic response in the body. Since the protein–protein interaction between azurin and p53 allows p53 stabilization and cancer regression, it is important to develop an understanding regarding the domains of azurin and p53 that are involved in their physical associations. The hope is that such an understanding will allow us to develop small truncated versions of azurin that will enter tumor cells and physically interact with p53, thereby enhancing cancer regression through its stabilization.

Azurin–p53 interactions: using the body's tumor-suppressing mechanisms for cancer treatment

In an effort to understand the nature of the azurin or p53 domains that interact, we have used glycerol gradient centrifugation [8,11,14] and glutathione *S*-transferase (GST) pull-down assays [22] to study such interactions. Azurin is known to possess a hydrophobic patch where such amino acids are known to be involved in protein–protein interactions with azurin's electron transfer partners such as cytochrome c_{551} [10]. When the two hydrophobic Met residues from this region were replaced by two polar amino acids (Met44LysMet64Glu), the resultant mutant azurin was shown to be much less cytotoxic than the wt azurin and did not contribute to p53 stabilization as does the wild type [11]. The mutant protein appeared to interfere with p53 oligomerization [11]. It thus appears that the hydrophobic patch of azurin may also be involved in p53 interaction, and the loss in hydrophobicity may alter the nature of the interaction, interfering in oligomerization and perhaps shifting its transcriptional activation from the target *bax* gene promoter to other promoters and thus reducing the level of Bax-induced apoptosis. Further investigations are under way to define the role of hydrophobic amino acids in azurin in its interaction with p53.

To obtain a better understanding of the domain of p53 that interacts with wt azurin, we hyperexpressed four GST-p53 fusion constructs (Fig. 1A) containing full-length p53 (GST-FLp53), the N-terminal to central domain of p53 from amino acids 1 to 295 (GST-p53-N), the middle region of p53 with amino acids 103 to 295 (GST-p53-M), or the C-terminal part of p53 containing amino acids 319–393 (GST-p53-C). These proteins were hyperexpressed in *E. coli* to produce the respective full-length (FLp53) or truncated p53 derivatives (Fig. 1B). The GST pull-down assays were conducted by overnight incubation of various concentrations of azurin with beads of Sepharose 4B-glutathione (GSH)-conjugated GST-FLp53 fusion protein or GST alone without the fusion protein. Any complexed azurin was then eluted with reduced glutathione and detected by immunoblotting (IB) using anti-azurin or anti-p53 antibodies (Figs. 1C and D). Azurin could be eluted from the beads harboring GST-FLp53 but not GST alone, suggesting that the retention of azurin was due to complex formation with p53 and not with GST (Fig. 1C). When similar GST pull-down assays were conducted with beads of Sepharose 4B-GSH-conjugated GST-FLp53 or GST-p53 truncated derivatives, significant amounts of azurin could be eluted only from beads harboring either the full-length p53 or the N-terminal or middle regions of p53 (p53-N or p53-M) but very little azurin could be detected in the eluant from beads containing GST-p53-C (Fig. 1D). These results suggest that the azurin binding sites are located in the middle portion of p53 and perhaps in the N-terminal region as well, but C-terminal was less efficient in binding azurin.

Epilogue

The tumor suppressor protein p53 is a central player in a coordinated network of cellular growth, differentiation, and regulation [9]. Many proteins modulate p53

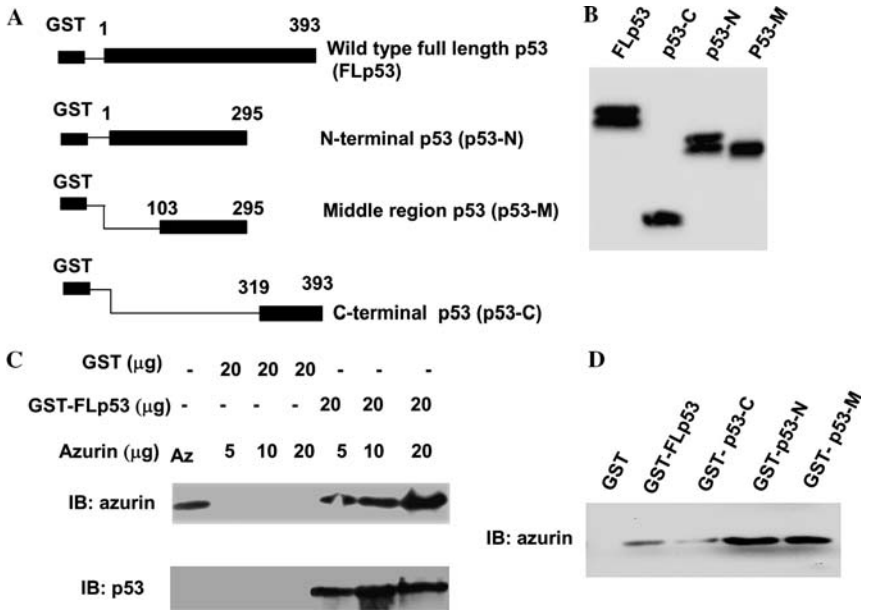

Fig. 1. (A) Genetic constructs for the expression of full-length or truncated p53. (B) Characterization of full-length and truncated p53 proteins hyperproduced in *E. coli*. Recombinant proteins were separated on 10–20% SDS–PAGE and blotted with anti p53 polyclonal antibody. (C) GST (glutathione *S*-transferase) pull-down assay demonstrating complex formation between GST full-length p53 and azurin but not between GST and azurin; Az, wt azurin input; IB, immunoblotted with antibody against the protein specified. (D) GST pull-down assay is also used to demonstrate complex formation between GST-FLp53, GST-p53-N/GST-p53-M, and azurin, but only weak complexing activity between GST-p53-C and azurin.

activity through physical interaction and thereby control cellular functions [23], such as human NAD(P)H:quinone oxidoreductase 1 [24] and poly(ADP-ribose) polymerase-1 [25]. All these are of viral or mammalian cellular origin [9,23]. Azurin, to our knowledge, is the first bacterial protein shown to modulate p53 function through physical association (Fig. 2). It is interesting to note, however, that although wt azurin, in association with p53, promotes ROS generation and apoptosis in eukaryotic cells [8,11,14], M44KM64E mutant azurin is less cytotoxic, is less efficient in complex formation with oligomeric p53 [8], with very little enhancement of Bax production and induction of apoptosis but significant enhancement of inhibition of cell cycle progression (unpublished observations). Thus the p53–bacterial azurin complex may direct the specificity of p53 between apoptosis and cellular growth arrest, somewhat similar to the effects reported for viral proteins such as NS5A of hepatitis C virus [26] or mammalian tumor suppressor protein BRCA1 [27]. Therefore, bacterial redox proteins or synthetic peptides derived from them with specific p53 binding domains may potentially represent an arsenal of weapons that can be used in inducing tumor cell death or growth arrest in the treatment of cancer.

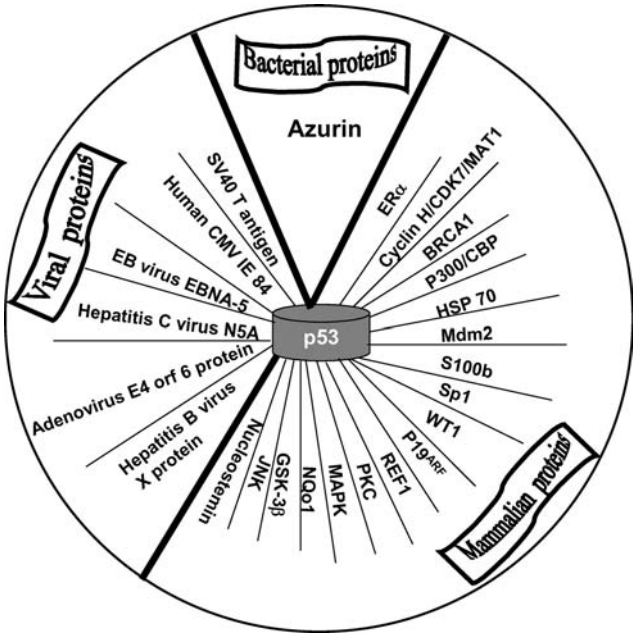

Fig. 2. Proteins that are known to interact physically with p53. A more complete list is given in Prives and Hall [23]. Most of the p53-interacting proteins that modulate p53 stability or activity are of viral or mammalian cellular origin. Azurin is the only bacterial protein that has been reported so far to stabilize p53 through complex formation as reported here.

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