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Gunsalus and Stanier set the stage for selection of cold-sensitive mutants apparently impaired in movement of FAD within 4-hydroxybenzoate hydroxylase

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A dynamic duo in microbiology

Gunny and Roger Stanier were very close, but it was possible to discriminate between their scientific interests at the level of the cell membrane. Roger liked it intact and fully energized in a living cell. Gunny's appreciation of cells was profound, but he felt that you had to be able to take them apart in order to understand how they work. Their interests converged in the study of bacterial adaptations to potential growth substrates and, together and separately, they studied mechanisms underlying the extraordinary nutritional versatility of *Pseudomonas*. Implicit in this work was the biological activation of oxygen by the various aerobic bacteria clustered within this genus.

Removal of oxygen by respiration was the tool Roger used to analyze patterns of induction governing catabolism of aromatic acids. It was known that a wide range of the compounds supported the growth of pseudomonads, and it was reasonable to inquire if each compound required a separate catabolic pathway or if different growth substrates were metabolized convergently to common intermediates. It must be remembered that at that time the pathways for aromatic catabolism were largely unknown. Roger reasoned that cells adapted to respiration of a primary growth substrate would respire that compound and also respire intermediates formed in metabolism of that compound. This idea, simultaneous adaptation [1], led to the discovery that *Pseudomonas* cultures grown with mandelate would respire, in addition to mandelate, benzoate and catechol (Fig. 1). An adaptive lag was required for the cells to respire 4-hydroxybenzoate and protocatechuate, potential metabolites that proved not to be intermediates in growth of the cells with mandelate. The results of similar experiments supported the view that peripheral metabolic sequences convert complex compounds to common intermediates, notably catechol and protocatechuate. Thus the enzymes that act upon the latter compounds participate in metabolism of different primary growth substrates.

The specificity of observed induction patterns led Roger to the hypothesis of sequential induction, the proposal that each intermediate in a catabolic pathway was the inducer of the enzyme that acted upon it [2]. In this strict form, the hypothesis was consistent with his observations, and it could not be fully tested before it became possible to measure induced and uninduced levels in cell extracts. The obstacles to analysis by respiration pattern were permeability barriers that impeded transport of multiply charged intermediates such as *cis*,*cis*-muconate and β-ketoadipate across the cell membrane.

Roger crossed the membrane barrier during a profitable visit in Gunny's lab where they were able to exploit Gunny's newly developed procedures using cell extracts to open *Pseudomonas* metabolism to enzymological analysis [3,4]. In later years, Roger referred to his manometric measurements of oxygen consumption as

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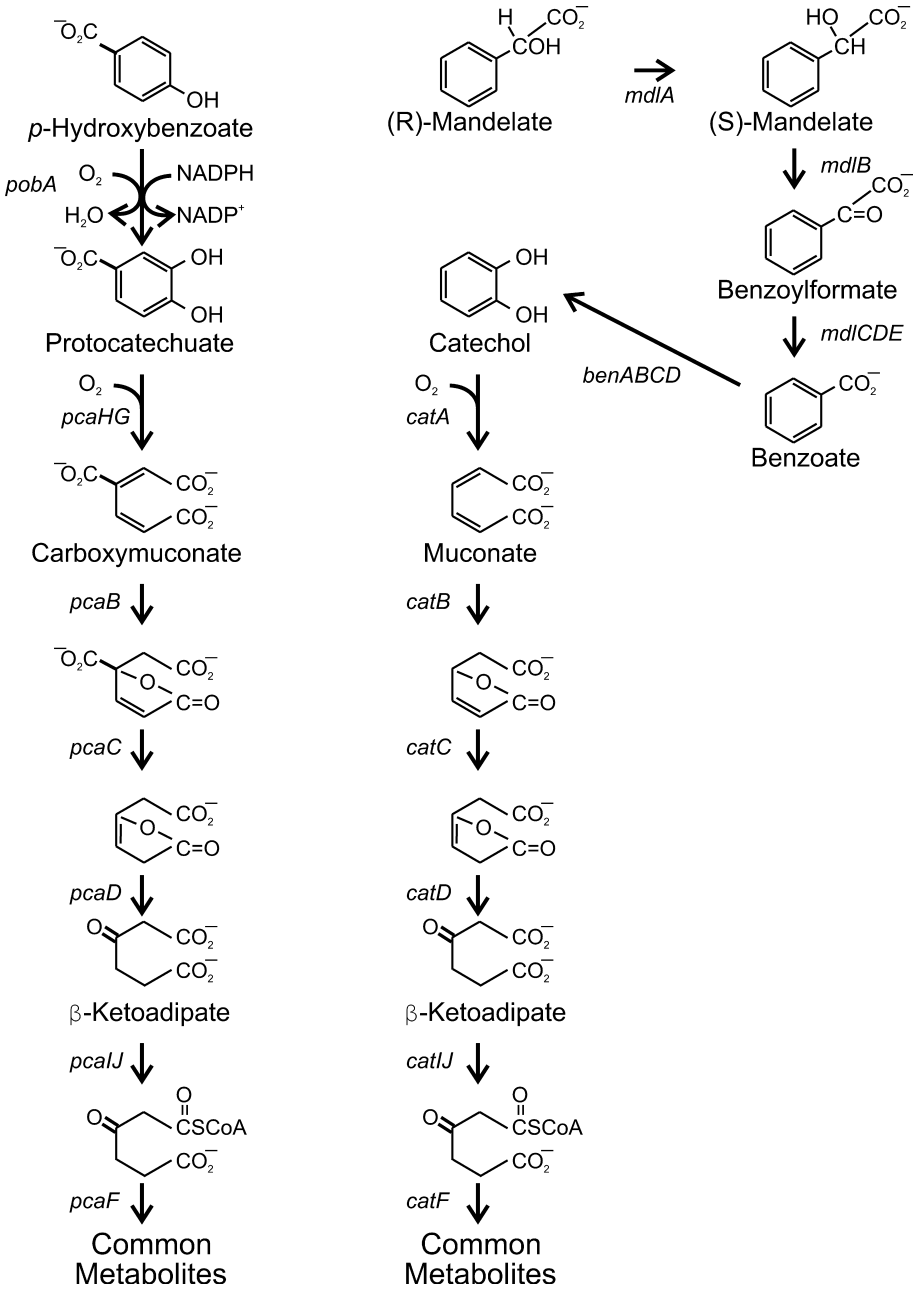

Fig. 1. Metabolic pathways for utilization of aromatic growth substrates by bacteria. Arrows indicating enzyme reactions are accompanied by current gene designations. Stanier's studies of simultaneous adaptation with whole cells elucidated the steps for metabolism of aromatic intermediates. Characterization of subsequent reactions required studies with cell extracts. The seeming parallelism of the *pcaB–pcaC* and *catB–catC* encoded metabolic steps is illusory because the respective reactions have different stereochemistry and are catalyzed by enzymes with separate evolutionary ancestry. In contrast, the enzymes encoded by *mdlA* and *catB* have common ancestry and mediate chemically similar reactions.

"biochemistry without tears" and the analysis of shattered cells as "biochemistry with tears" [5]. Anyone who has greased a manometer cup would have to appreciate Roger's dedication to whole cells!

A significant product of Roger's visit was the demonstration that mandelate dehydrogenase is physically associated with the cell membrane [6]. (Half a century later, it should be noted that genetic engineering has been used to obtain mutants in which mandelate dehydrogenase, a flavoprotein, is produced in soluble form [7,8].) Building upon his experience with Gunny, Roger was able to contribute to growing knowledge that metabolic cleavage of aromatic rings was achieved by enzymes that directly incorporate molecular oxygen into their substrates. Thus one dioxygenase (CatA, Fig. 1)) opens the aromatic ring of catechol to form muconate [9], and another dioxygenase (PcaHG, Fig. 1) cleaves protocatechuate giving rise to carboxymuconate [10].

Other kinds of oxygenases were termed monooxygenases because they assimilate one atom of O_2 into their substrates while reducing the other oxygen atom to water. An example of the latter, studied in Roger's laboratory, is 4-hydroxybenzoate hydroxylase, a flavoprotein that converts its substrate to protocatechuate [11]. As part of this study, evidence for physical association of the flavin with the enzyme was presented by demonstration that denaturation of the enzyme caused a shift in the absorption spectrum of the flavin. This flavin has come a long way. It is now known that FAD shifts a full 7 Å [12] as part of the catalytic cycle requiring "password" recognition of the substrate by the hydroxylase [13].

A further outgrowth of enzymological analysis was the opportunity to tidy up understanding of the β-ketoadipate pathway, a major mechanism used by microorganisms for growth with aromatic compounds [14]. As assays for enzymes were developed [15,16], it became possible to explore the specificity of enzyme induction in greater detail, and the inference of strict sequential induction was modified. In a number of cases, enzymes are induced by their products, sometimes several enzymatic steps removed. For example, the three *Pseudomonas* enzymes that catalyze consecutive steps in carboxymuconate metabolism are induced by their product, β-ketoadipate [17]. Such observations gain significance in an era in which evidence is eagerly gathered from microarray analysis of gene expression. Expression of a gene does not necessarily mean that its function has been called upon.

Nutritional diversity of pseudomonas

As appreciation of the metabolic diversity of *Pseudomonas* increased, Roger and Gunny turned their attention to how this diversity was achieved. Roger's primary contribution, made in partnership with Norberto Palleroni and Mike Doudoroff, was an expansive nutritional survey of the heterogeneous collection of bacteria then grouped as members of the genus *Pseudomonas* [18]. Sharp divisions in nutritional properties emerged, and recognition of these divides was buttressed by molecular evidence leading to separation of genera now known as *Burkholderia* and *Comomonas* from the original group [19]. Constellations of common traits served to define species among the strains remaining within the genus *Pseudomonas*, but in many instances the distinctions were blurred.

The basis for some of the blurs achieved clarity as a result of Gunny's demand for understanding mechanisms of variation that produced and maintained nutritional versatility. There had to be mechanisms for moving sets of genes that encoded such functions around the bacterial world. In Gunny's laboratory, such mechanisms were identified and developed [20,21]. An abundant and still growing literature can be traced to Gunny's insight.

In retrospect, it is intriguing to note how different yet intertwined intellects can be productive. Roger's sense of biological order led to a greatly increased appreciation of divisions that define bacterial biota, whereas Gunny's intuition opened to analysis mechanisms that can carry genes across these divides. The insights of these two investigators stand together. Contributions of horizontal transfer to evolution now are widely appreciated, yet when all is said and done, bacterial taxonomy works: by and large, one set of characteristics can be used to predict an additional set of characteristics. The success of horizontal transfer can be modulated by the predisposition of the recipient strain to incorporate functions of the acquired genes into its established repertoire.

Gene organization, reorganization, and evolution

In the years since proclamation of a principle attributed to Francois Jacob ("Whatever you are doing, you are better off if you have a mutant"), genetic and biochemical investigations have become more frequently inseparable. It has been said that Gunny's original interest in camphor metabolism [22] emerged from his desire to see "how that little ball of grease is metabolized." Yet it was the Cam plasmid, encoding genes for camphor catabolism, that helped to open the field of catabolic plasmids to genetic investigation [23]. Among the *cam* genes is one encoding a monooxygenase in what became the P_{450} family, proteins with functions cutting a broad swath through biology. As it turned out, the $P450_{cam}$ was particularly amenable to analysis and its investigation was at the forefront in developing understanding about how these proteins activate and utilize O_2 [24,25].

Mutants were essential for unravelling details of the β-ketoadipate pathway, and they opened the door for discovery of another phenomenon, clustering in the *Pseudomonas* chromosome of catabolic genes with physiologically related functions [26]. Many of these findings [27,28] were fostered by a transduction system developed in Gunny's laboratory [21]. The biological basis for such clustering still has not been established but, as described below, it has been observed in other bacteria.

Mutations also facilitate the study of evolution. One approach is to knock out an existing gene and to determine if another gene from the same organism can be recruited to replace the missing function. A project initiated by one of us (L.N.O.) in Gunny's lab was the selection and mapping of *Pseudomonas putida* strains containing deletions in *catB*, the structural gene for muconate cycloisomerase (Fig. 1). The idea was that the

deleted function might be replaced by mutant forms of *pcaB*, the gene encoding the seemingly analogous carboxymuconate cycloisomerase (Fig. 1). Expression of *pcaB* would not have been a problem because this gene is expressed gratuitously in *P. putida* cells metabolizing muconate [17]. It was known that spontaneous mutations tend to be deletions, so the first aim of the project was to obtain spontaneous mutants unable to metabolize muconate. This was easily achieved by adapting earlier procedures for penicillin counter-selection into a sequential counter-selection method leading to populations in which spontaneous mutants predominated [29]. Using the transduction procedure developed by Gunny, it was possible to show that a large fraction of the mutants contained the predicted *catB* deletions [30].

The overall project did not work out. As it happened, muconate cycloisomerase and carboxymuconate cycloisomerase have separate evolutionary origins [31], and the stereochemistry of the two enzyme reactions is different [32]. But the project was not without overall benefit. The physical structure of muconate cycloisomerase [33], determined out of a desire to compare it with the yet undetermined carboxymuconate cycloisomerase structure, turned out to be quite similar to the structure of mandelate racemase. Muconate cycloisomerase and mandelate racemase proved to be evolutionarily homologous [34], and elucidation of unity of mechanism in the two seemingly dissimilar enzyme reactions brought attention to mechanism as a target of selection in enzyme evolution [35–37].

Enter *Acinetobacter*: gene arrangements and rearrangements

As the *P. putida* cycloisomerase project wound down, an extraordinary opportunity for bacterial genetics was opened by Elliot Juni's discovery of a highly expressed system for natural transformation in an *Acinetobacter* isolate subsequently designated strain ADP1[38]. Representatives of the nonflagellated coccobacilli *Acinetobacter* group, readily distinguished from motile members of the genus *Pseudomonas*, appeared frequently among strains contributed to the Stanier laboratory in preparation for the landmark taxonomic survey of *Pseudomonas* [18]. A parallel investigation, by then-graduate-student Paul Baumann, showed that members of the genus *Acinetobacter* (designated *Moraxella* at that time) form a highly heterogeneous group of bacteria [39] including many that are nutritionally versatile and abundant in terrestrial environments [40].

The genera *Acinetobacter* and *Pseudomonas* present a paradox. As judged by their 16S ribosomal DNA sequence, the organisms are closely clustered members of the γ-subdivision of proteobacter. However, the organisms differ greatly in morphology and, quite strikingly, the G+C contents of their DNA differ by more than 10%. Sometime during the divergence of these bacteria, evolutionary clocks did not run on time! Comparison of genes with similar functions in the two genera almost always reveals a close homology reflected in an amino acid sequence identity of about 50%. What is striking about the mechanism of divergence of these genes is not their sequence but their arrangement. Clustering frequently is conserved, but the order of genes sometimes has changed much as cards in a deck are reordered after a few shuffles [41]. Such rearrangement of homologous genes now has been documented in many bacterial genera [42–44], and these genetic migrations clearly mark evolutionary punctuation points [45].

Occurrence of the rearrangements raises questions about mechanisms underlying their origin and selective advantages that they might confer. Insight into possible mechanisms of gene rearrangement emerged from discovery that regions of the *Acinetobacter* chromosome containing catabolic gene clusters undergo frequent mutations causing multiple tandem duplication [46]. Expansion and contraction of the chromosome could provide opportunities for misaligned recombination giving rise to gene rearrangements. In addition, duplication mutants may help to answer a central problem in evolution: how to maintain a gene with a valued function while a copy of the gene is adapted for a novel purpose.

The contribution of recombination to evolution is widely recognized. Less attention is paid to another powerful force, selection for avoidance of homeologous recombination which could produce defective hybrid genes. Enzymes of DNA metabolism provide barriers of defense against such recombination and additional protection may also be provided by rearrangements that have altered the position of genes within a cluster [41,45,47].

Clues about natural selection from differences in transcriptional controls

Gene rearrangement also allows formation of varied transcriptional responses to metabolites that may serve as inducers, and different bacteria may call upon distinctive regulatory responses. This is illustrated by the response of *Acinetobacter*, *Pseudomonas*, and *Bradyrhizobium* to β-ketoadipate, a metabolite likely to be formed transiently in the environment during the turnover of plant materials. β-Ketoadipate does not induce enzymes required for its dissimilation in *Acinetobacter*. In these bacteria, synthesis of the necessary enzymes is elicited by precursors that are converted to β-ketoadipate during their metabolism [48]. It can reasonably be concluded that extracellular β-ketoadipate does not play a significant nutritional role in the niche of *Acinetobacter*.

In contrast to *Acinetobacter*, members of fluorescent *Pseudomonas* species assign a cardinal regulatory role to β-ketoadipate, using it as an inducer of enzymes that give rise to it and enzymes that remove it during intracellular metabolism [17]. In addition, *Pseudomonas* strains are distinguished from *Acinetobacter* by the presence of *pcaT*, which is expressed as part of the *pcaRKFTBDC* cluster in response to β-ketoadipate [43]. PcaT is a transporter that brings extracellular β-ketoadipate into starved *Pseudomonas* cells [49]. Thus the ecological role of β-ketoadipate in *Pseudomonas* biology can be seen to be a regulatory trigger signaling metabolic opportunities for utilization of substrates giving rise to the compound in the environment [50,51].

A striking feature of *Bradyrhizobium* is the high-level expression of activities toward β-ketoadipate in the absence of induction. These bacteria constitutively form a strong chemotactic system allowing them to swim toward β-ketoadipate [52] and also to synthesize constitutively enzymes for β-ketoadipate utilization [53].

In sum, the transcriptional responses of *Acinetobacter*, *Pseudomonas*, and *Bradyrhizobium* to β-ketoadipate indicate that the respective encounters of these bacteria with the compound in the environment are rare, occasional, and frequent. Distinguishing features of these bacteria are expressed in the cell membrane, a location where Gunny's interest coincided with Roger's, as systems associated with transport and chemotaxis.

Using genetics to explore biochemical fluidity: cold-sensitive mutants of 4-hydroxybenzoate hydroxylase

The fluidity of membranes, a basis for the bacterial "plasticity" that so intrigued Roger [54], is based largely on noncovalent interactions between molecules. By their very nature, these properties present barriers to conventional analysis by biochemical dissection but may offer opportunity for genetic investigation. One avenue, championed by John Ingraham, a former student of Roger's, is characterization of cold-sensitive mutants [55]. As a first approximation, heat-sensitive phenotypes probably are a consequence of a breakdown of molecular interactions in response to thermal vibrations at elevated temperatures. In contrast, a cold-sensitive phenotype may be attributed to a loss of fluidity required for function in the restrictive condition. Simply put, cold-sensitive molecules may be ineffective because they are stuck.

The known demand for substantial FAD migration during the catalytic cycle of 4-hydroxybenzoate hydroxylase [12] suggested that the protein might be particularly susceptible to cold-sensitive mutations that "froze" FAD within the enzyme under the restrictive condition. To explore this possibility, the frequency and properties of cold-sensitive mutations arising after PCR-

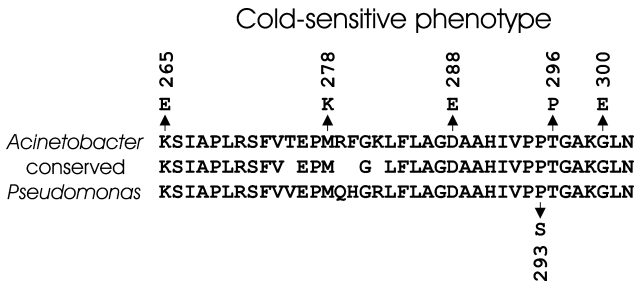

Fig. 2. Locations of mutations in the flavin-binding region of 4-hydroxybenzoate hydroxylase. Arrows indicate amino acid substitutions at the numbered positions in the aligned sequences of the *Acinetobacter* and *P. aeruginosa* enzymes. The five indicated substitutions in the *Acinetobacter* enzyme cause a cold-sensitive phenotype. A similar phenotype is caused by the Ala189Asp substitution not shown here. The site-directed Pro293Ser substitution in the *P. aeruginosa* enzyme increases the flexibility of the peptide backbone [58].

mutagenesis of *Acinetobacter pobA*, the structural gene for 4-hydroxybenzoate hydroxylase (Fig. 1), were determined. As described below and in Fig. 2, the closely similar amino acid sequences of the *Acinetobacter* and *Pseudomonas aeruginosa* hydroxylases in the region of FAD binding allow mutations obtained with the former protein to be interpreted in the context of the latter for which the crystal structure has been established [56].

The general procedure for coupling random PCR mutagenesis with natural transformation of *Acinetobacter* was first demonstrated with *pobR*, which neighbors *pobA* and encodes its transcriptional activator [57]. For the present investigation of *pobA*, different primers, POBRNAR (5′-CTTCACTTGAATGGGGATGTGC-3′) and POBA3 (5′-GGCGGTAAAATGATTGTGGT GC-3′), were used with *Taq* polymerase (Roche Applied Science) according to the manufacturer's directions to amplify a 1852-bp DNA segment containing the 1215-bp *pobA* gene from *Acinetobacter*. After transformation of strain ADP230(Δ*pcaBDK1*) with the PCR-mutagenized DNA, strains able to grow in the presence of 4-hydroxybenzoate were selected as previously described [57]. The mutations acquired in these strains prevented formation of the toxic metabolite carboxymuconate (Fig. 1) from 4-hydroxybenzoate. After replacement of Δ*pcaBDK1* with wild-type DNA [57], the acquired mutations were the sole barriers to growth with 4-hydroxybenzoate. Additional primers used for sequencing these mutations were POBA204 (5′-AAATCGACCGATAC TTGCTC-3′), POBA420 (5′-ATCGGCTTCGCGTTTC TGAG-3′), POBA2 (5′-CTCTGCTTTCCATACACGT TGC-3′), and POBA4 (5′-TATAACGGTACAGGTG ACAGTG-3′).

The *pobA* mutant selection was conducted at 37 °C and at 22 °C; the resulting strains were screened for growth at both temperatures. Of 85 mutant strains selected at 37 °C, none exhibited a heat-sensitive phenotype. In contrast, selection at 22 °C produced 217 strains

Table 1
Cold-sensitive PCR-generated *pobA* mutations

Strain	Mutation	DNA Change	Protein change
ADP7450	*pobA7450*	C575A	A192D
ADP7451	*pobA7451*	A793G	K265E
ADP7452	*pobA7452*	T833A	M278K
ADP7453	*pobA7453*	T864G	D288E
ADP7454	*pobA7454*	A886C	T296P
ADP7455	*pobA7455*	G899A	G300E
ADP7456	*pobOP7456*	ΔG (−62)	Promoter

of which 13% were cold-sensitive. The remarkably high number of cold-sensitive mutants may be an underrepresentation of the frequency of cold-sensitive mutations because sequencing of the *pobA* region in 12 such mutant strains revealed that 5 contained multiple mutations.

One of the singly occurring cold-sensitive mutations was a single bp deletion of a G in the promoter region 62 bp upstream from *pobA*. The six remaining cold-sensitive mutations caused amino acid substitutions in 4-hydroxybenzoate hydroxylase (Table 1). All of these replaced amino acids conserved in the *Acinetobacter* and *Pseudomonas* hydroxylases, and five of the substitutions were clustered in a 36-residue region containing amino acids known to contribute to the binding of FAD (Table 1). Of particular interest are three amino acid substitutions near *Acinetobacter* Pro295 corresponding to Pro293 in the aligned amino acid sequence of *P. aeruginosa* 4-hydroxybenzoate hydroxylase (Fig. 2). Site-directed substitution of Pro293 to Ser293 increases the flexibility of the peptide backbone in the *P. aeruginosa* enzyme [58]. The resulting protein heightens exposure of the flavin hydroxyperoxide intermediate to solvent, causing an increase in formation of hydrogen peroxide and a decrease in the efficiency of the enzyme. The results suggest that charge repulsion at the carbonyl oxygen of Pro293 is moved along the polypeptide through Thr 294, Gly295, and Ala 296 toward Asn 300, which triggers the flavin conformational change [58].

Amino acid substitutions causing a cold-sensitive growth of *Acinetobacter* with 4-hydroxybenzoate include Asp288Glu, seven residues away from Pro295 (Fig. 2). This amino acid substitution is interesting because a very conservative change, addition of a methylene group, changes the phenotype. The Thr296Pro substitution, one residue removed from Pro295 (Pro293 in *P. aeruginosa*), is close to a reversal of the Pro293Ser substitution that increased the flexibility of the *P. aeruginosa* enzyme. One might reasonably infer that the mutant *Acinetobacter* tripeptide Pro294-Pro295-Pro296 has rigidity exceeding that of Pro294-Pro295 in the wild-type enzyme [58]. Finally, the Gly300Glu substitution increases the bulk and changes the charge in the region inferred to transfer movement to Asn302 in the *Acinetobacter* protein [58].

The frequency of cold-sensitive mutations in a region of known fluidity within 4-hydroxybenzoate hydroxylase appears to defy coincidence and reinforces the suggestion that such mutations can be effective probes for analyzing noncovalent interactions in biological macromolecules [55]. The basis for the present investigation with cold-sensitive mutations had been set by site-directed mutagenesis in which the targets were selected by the investigators [12,59–62]. In the study reported here, targets were selected by the organisms that grew after a demand for defective 4-hydroxybenzoate hydroxylase had been imposed. Convergence of results upon the same peptide within 4-hydroxybenzoate hydroxylase (Fig. 2) illustrates the merit of both approaches, the advantage to the mutant selection being that it assumes no prior knowledge of the subject of inquiry. There is room for mutant selection to make a contribution because absence of knowledge impedes understanding of the noncovalent interactions that are the hallmark of cell membranes, the location where Gunsalus and Stanier combined their interests. It is fitting that the example provided here is 4-hydroxybenzoate hydroxylase, an enzyme that harnesses the energy of molecular oxygen to modify chemical structure and another subject of their shared fascination.

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