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Chemical mutagenesis: selective post-expression interconversion of protein amino acid residues

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The ability to alter protein structure by site-directed mutagenesis has revolutionized biochemical research. Controlled mutations at the DNA level, before protein translation, are now routine. These techniques allow specific, high fidelity interconversion largely between 20 natural, proteinogenic amino acids. Nonetheless, there is a need to incorporate other amino acids, both natural and unnatural, that are not accessible using standard site-directed mutagenesis and expression systems. Post-translational chemistry offers access to these side chains. Nearly half a century ago, the idea of a 'chemical mutation' was proposed and the interconversion between amino acid side chains was demonstrated on select proteins. In these isolated examples, a powerful proof-of-concept was demonstrated. Here, we revive the idea of chemical mutagenesis and discuss the prospect of its general application in protein science. In particular, we consider amino acids that are chemical precursors to a functional set of other side chains. Among these, dehydroalanine has much potential. There are multiple methods available for dehydroalanine incorporation into proteins and this residue is an acceptor for a variety of nucleophiles. When used in conjunction with standard genetic techniques, chemical mutagenesis may allow access to natural, modified, and unnatural amino residues on translated, folded proteins.

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Introduction

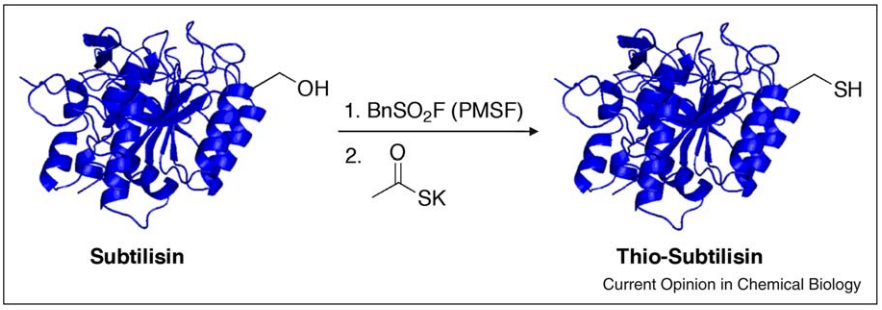
The development of site-directed mutagenesis has revolutionized protein science [1,2]. Specific, high fidelity mutations at the DNA level are now routine and recombinant expression systems enable site-specific incorporation and variation of amino acid residues in the final protein. Standard site-directed mutagenesis, however, is largely limited to 20 natural, proteinogenic amino acid residues. As biochemical endeavors and goals have advanced, a need to incorporate non-natural or modified amino acid side chains has emerged. Unnatural amino acids can be targeted for further labeling as well as protein tracking and analysis [3,4]. Amino acid side chains are also naturally modified — phosphorylation, methylation, acylation, glycosylation are a few such examples — and access to these side chains is highly desirable if we are to explore their roles more precisely [5]. Amber codon suppression and reprogrammed genetic codes are two strategies for incorporating some unnatural or modified amino acids not accessible through normal translation [6–9]. Semi-synthesis by native chemical ligation is another alternative and through this method the total synthesis of proteins has become reality [10]. Another strategy is to use chemistry on an expressed and folded protein to install a desired side chain at a desired site. It is this final strategy that is the focus of this review. Our intention is not to provide a comprehensive review of protein labeling methods. Rather, we wish to explore — and indeed revive — the concept of 'chemical mutagenesis' put forth nearly half a century ago by Daniel E. Koshland, Jr. [11••]. Visionary contributions from the Koshland [11••] and Bender [12••] laboratories described, for the first time, the chemical conversion of one amino acid side chain to another — a chemical mutation. As genetic technology, expression systems, and aqueous chemistry have developed, it is worthwhile to revisit the idea of a chemical mutation. We first trace the inception of this concept and then consider its place in contemporary chemistry and biology as a general method to alter protein structure precisely.

Chemical mutagenesis: a seminal concept in protein science

In 1966, the laboratories of Daniel E. Koshland, Jr. and Myron L. Bender independently reported the first point mutation of an enzyme [11••,12••]. In both reports, the serine protease subtilisin was chemically converted to a cysteine protease. The transformation is depicted in Figure 1. The active site serine was first selectively converted into a leaving group by treatment with phenylmethanesulfonyl fluoride (PMSF) and then displaced by the attack of thioacetate. The resulting thioacyl-enzyme was hydrolyzed through the innate activity of the protease, providing the thiol-subtilisin product.

The mutant enzyme was characterized both chemically (the cysteine protease contains a single, easily detected cysteine) and through kinetic analysis of protease activity.

Figure 1



The first point mutation of an enzyme was reported independently by Koshland and Bender in 1966 [11••,12••]. The serine-to-cysteine mutation was accomplished chemically. The more reactive serine of the active site of subtilisin is selectively converted into a leaving group using a sulfonyl fluoride ($BnSO_2F$). Displacement by thioacetate provides a thioacyl-enzyme intermediate that is hydrolyzed to the free thiol of cysteine.

The Ser to Cys mutation provided a near-isosteric mutant for study of the protease active-site and mechanism. It should be pointed out that at the time of these discoveries, no methods yet existed for site-specific mutagenesis at the DNA level and the total chemical synthesis of proteins was not yet feasible. Remarkably, Koshland and Bender both anticipated the use of genetic modifications to specifically control protein structure but until this technology was available, they proposed a more immediately accessible method of mutation performed on the translated protein through the use of chemistry — such modifications were referred to as 'chemical mutations' by Koshland [11••] and 'simulated mutations' by Bender [13].

The nomenclature for these transformations can be a matter of contention and confusion, so a few points regarding the concept of 'chemical mutation' are perhaps in order. In this review, we refer to a 'chemical mutation' as a process that converts an amino acid residue on a translated protein to another amino acid residue. Chemical mutation in this context does not refer to chemical alteration of DNA and it does not refer to covalent labeling of proteins with synthetic probes, cofactors, and other biochemical tools. To avoid any confusion with DNA modification, we propose the term 'post-expression mutagenesis' for chemical alteration of the translated, folded protein [14]. Since in this review we consider only mutations on proteins, we will use 'chemical mutation' and 'post-expression mutation' interchangeably.

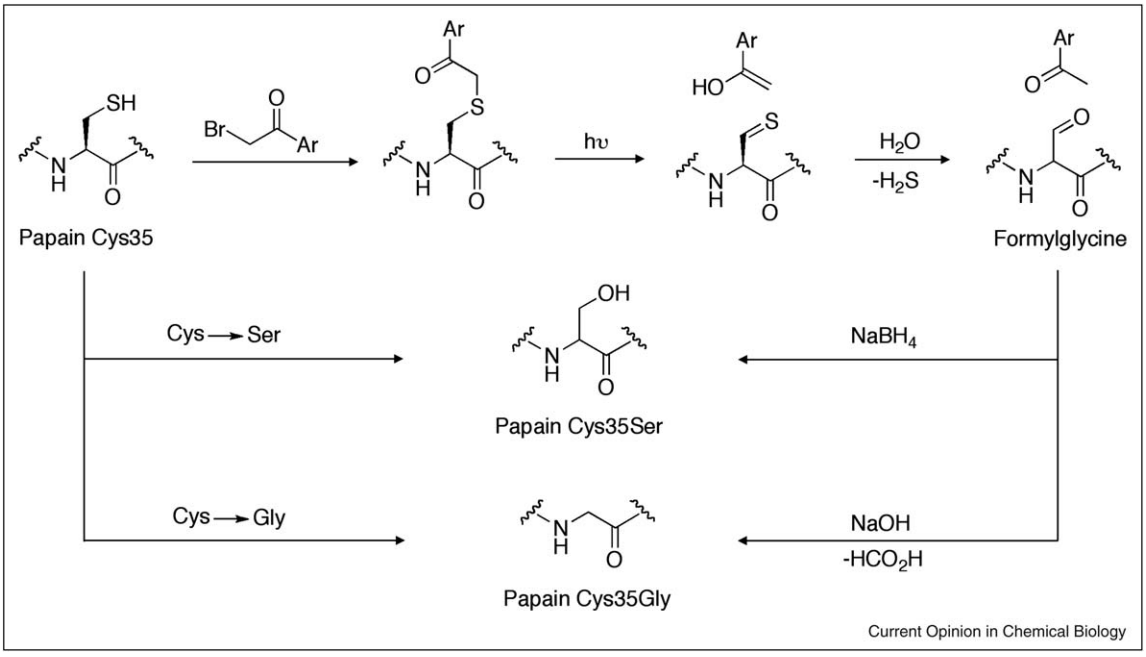
The proof-of-principle set forth by Koshland and Bender was a landmark in protein science. The conversion of active site serines to cysteines has been applied to other protease substrates such as trypsin [15] and related protocols for conversion to selenocysteine [16–19] and even tellurocysteine [20] have been reported. Despite these advances, the serine to cysteine mutation was only possible in these cases because of the unique chemical reactivity of active-site serine.

A different approach was reported not long after Koshland and Bender's disclosures. Laskowski described an 'enzymatic mutation' on soybean trypsin inhibitor that relied on trypsin and caboxypeptidase B to exise an amino acid from the protein and re-ligate another amino acid at the site of mutation [21]. The mutation is carried out on the native protein, post-expression, in the same spirit as the chemical mutation described by Koshland and Bender, but Laskowski's method differs conceptually since the protein backbone is altered, rather than side chain. Moreover, Laskowski himself conceded that this method relies on 'a bit of luck' that all the necessary enzymes can co-exist under the reaction conditions without deleterious proteolysis [21]. Nevertheless, this concept was further developed by Tschesche, who described sequential amino acid excision, chemical coupling, and elastase ligation as a method to mutate soybean trypsin inhibitor [22,23]. Tschesche considered these amino acid exchanges a form of 'chemical mutation' [23]. Importantly, these examples illustrate that some alterations can be accomplished enzymatically and that such a mutation does not necessarily rely solely on selective small-molecule chemistry. Collectively, the mutations described by Koshland, Bender, and Laskowski were the most precise manipulations of protein structure that preceded the genetic and biotechnological revolution.

Chemical mutagenesis: expanding scope for natural residues

A decade after the first examples of chemical mutagenesis were put forth by Koshland and Bender, Peter Clark and Gordon Lowe reported the use of cysteine as a precursor to multiple amino acid side chains. Their 'chemical mutations of papain' can be considered more general than previous efforts in chemical mutagenesis since the cysteine residue need not be in a protease, cysteine residue need not be activated by its presence in an active site, though in this case the residue was indeed the nucleophile of the cysteine protease papain [24••,25•]. In these reports, cysteine was alkylated with a phenacyl bromide (Figure 2). Photolysis of this intermediate led to

Figure 2



Clark and Lowe's 'Chemical Mutations of Papain.' Cysteine was used as a common precursor to multiple amino acids: formylglycine, glycine, and serine [24••,25•].

irreversible Norrish type II cleavage, providing the thioaldehyde dehydrocysteine. Hydration of the thiocarbonyl and loss of H_2S results in the formation of formylglycine. Formylglycine was in turn reduced with $NaBH_4$ to provide serine. In this way, the overall transformation is a mutation from cysteine to serine. Moreover, formylglycine can also be converted to glycine after prolonged incubation at pH 9.0 (Figure 2). Lowe's extension of the concept of chemical mutagenesis was therefore unique in that it could provide multiple mutations from a single, common precursor and, in principle, could be applied to any free cysteine.

Since Lowe's report, there have been notably few publications concerned with chemical mutations. Genetic and recombinant technology was moving forward at a striking rate and amino acid interconversion using post-translational chemistry may have seemed obsolete. Some illustrative reports of chemical mutations have nonetheless appeared that indicate strategic advantages of chemical mutations. For instance, Venkatesh showed that glutamine and asparagine residues can be hydrolyzed with acid to 'chemically mutate' the side chain to glutamic and aspartic acid, respectively. It was shown that these mutations influenced the kinetics of folding in RNase A [26]. In the reverse mutation, Imoto showed that amidation of glutamate and aspartate residues could be used in the study of these residues as catalytic side chains [27]. The mutation in this case is from glutamate and aspartate to glutamine and asparagine, respectively. In these reports, multi-site mutations are accomplished. In the case of amidation, these mutations are carried out on the folded protein. It is conceivable that if these mutations were introduced at the genetic level the protein would not fold properly and so this example highlights a strategic advantage of post-expression mutagenesis where differences in activity between the protein mutants can be attributed to the mutation and decoupled from misfolding during expression.

Other examples of chemical mutations have been the products of efforts to generalize native chemical ligation (NCL) and overcome its inherent reliance on cysteine [10]. A number of cysteine desulfurizaton processes have been put forth that allow the use of NCL in the synthesis of peptides and proteins that do not contain cysteine. Dawson has disclosed a method to reduce cysteine to alanine using palladium or Raney nickel [28]. Related transformations have since been reported to provide phenylalanine [29] and valine [30,31] at the site of ligation. Of note is Danishefsky's mild, radical based method for the desulfurization of cysteine [32]. In these examples the application of chemical mutagenesis in native chemical ligation is apparent: a chemical mutation of cysteine to another residue constitutes a ligation, in the formal sense, at the final residue.

Chemical mutation to an amino acid analog

Chemical mutagenesis in its purest form constitutes the conversion of some precursor side chain to a natural

residue. In some cases, however, the natural residue may not be easily obtained by chemical methods. In these instances, a mimic or analog may suffice [33]. Indeed, some of the earliest examples of chemical protein modification were the conversion of lysine to homoarginine and the conversion of cysteine to thialysine. As early as 1949, it was shown that treatment of human serum albumin with *O*-methyl isourea provided homoarginine analogs that facilitated protein crystallization [34]. Such a transformation has also been used to investigate active-site arginine residues [35,36]. In the 1950s, aminoethylation of cysteine was used to install a synthetic site of proteolysis for trypsin, which recognizes lysine residues [37–39]. Although this technique was used mainly for tryptic analysis and sequencing proteins, the underlying enabling technology is the conversion of cysteine to thialysine. Although this pseudo-mutation does not provide a natural residue, the similarity to lysine has since been used many times in the investigation of catalytic lysine residues, highlighting the clear functional utility of these analogs [40–44]. Similar alkylations of cysteine have also provided arginine analogs [45]. Recent interest in cysteine aminoethylation has intensified since access to methylated lysine residues is required, especially on histone proteins [46]. This pseudo-mutation has allowed an increased understanding of the effects of histone modification and the overarching epigenetic code [47–49]. Other pseudo-mutations have allowed access to different post-translational mimics, such as sulfated and phosphorylated tyrosine [3,50]. Although these amino acid analogs have proven useful in a number of contexts, they are still only analogs, indicating perhaps as much about the flexibility of the system that recognizes them. Flexible methods to access the residue, modified or otherwise, in its *natural* state remains an ongoing challenge in chemical mutagenesis and in protein science in general. We now turn to the challenges and recent efforts that have advanced the notion of a chemical mutation.

Towards a general chemical mutagenesis

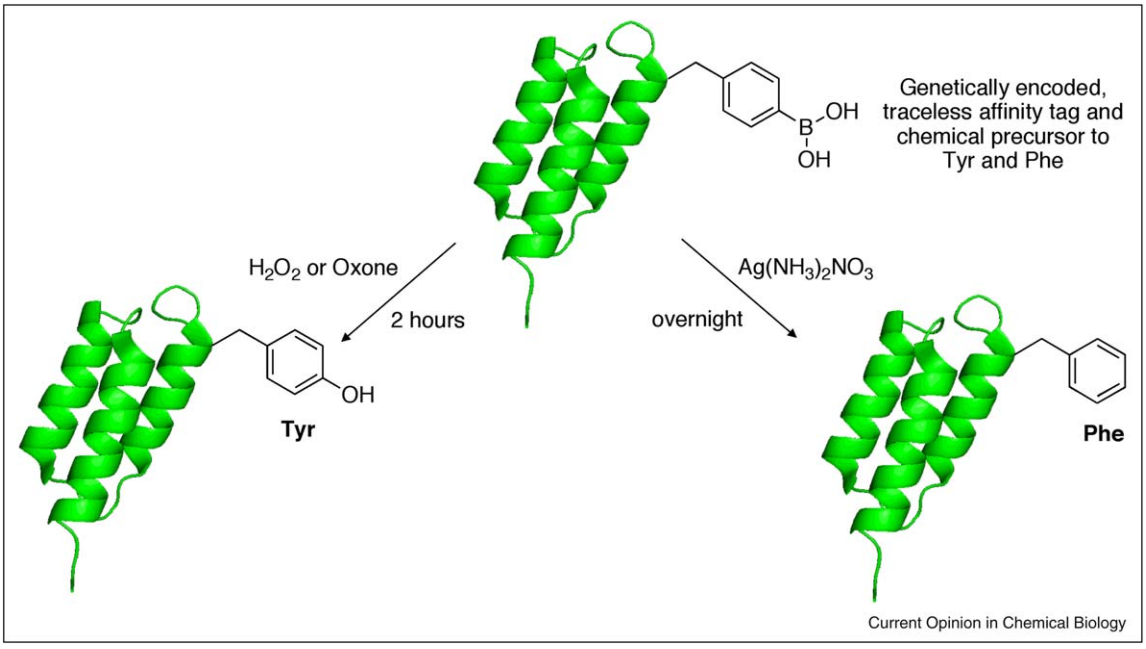
For chemical mutagenesis to be a general strategy for protein amino acid interconversion, it is desirable to use a precursor, natural or unnatural, that can provide access to a functional set of side chains. Ideally, this universal precursor would provide divergent access to the 20 most common natural side chains, along with many unnatural residues. For example, in the work of Koshland [11••] and Bender [12••] such a precursor was the active serine of subtilisin. This residue can be selectively converted into a leaving group and displaced with a nucleophile. In their case the conversion to cysteine was their target, yielding a near isosteric mutant to investigate the catalytic active site of these proteases. Yet one can imagine other nucleophiles that could displace the sulfonylated serine of subtilisin. Such a transformation could provide other chemical mutants, and indeed has since been extended to Se- and Tellurocysteine variants [16,20]. Nonetheless, when such transformations are possible, they rely on the innate reactivity of the protease to render the hydroxyl moiety of the serine a leaving group. The method could not be easily extended to other protein substrates. For general application, a different handle is needed.

A recent example of a precursor to other amino acids was reported by the Schultz group. Although their method was not presented in the context of chemical mutagenesis, the overall transformations could be considered chemical mutations. The incorporation of *p*-boronophenylalanine in response to the amber stop codon created a protein with an unnatural amino acid that can be converted to phenylalanine and tyrosine when reduced or oxidized, respectively (Figure 3) [51•]. To aid this work, the authors elegantly took advantage of the boronic acid as an affinity tag since it binds to polyhydroxylated resin. Elution with oxidant or reductant provided the native protein, free of tag. Full conversions were observed in the oxidation after 2 hours while overnight incubation was required for the reduction. While the main application of this technology is traceless affinity purification, the chemical conversion of a common precursor to other amino acids can be considered a chemical mutation. Moreover, the chemical handle for mutation (the boronic acid) can be incorporated at a predetermined site of the protein in response to a unique codon and does not rely on the innate functionality or sequence of the protein of interest.

The preceding example provides access to phenylalanine and tyrosine from a common precursor. A different precursor is needed for aliphatic side chains. Koshland again provides inspiration for such an amino acid precursor. In a protease system similar to the one where he reported the first point mutation of an enzyme, Koshland showed that the sulfonylated serine of chymotrypsin can be eliminated under basic conditions to give dehydroalanine [52,53•]. This enamide in dehydroalanine can be considered to be an electrophile, and various thiols were added to this residue. Koshland recognized that this residue could, in principle, be a precursor to other amino acid side chains [53•]. Addition of H_2S, for instance, would provide cysteine. Indeed the addition of thiols to dehydroalanine has since provided access to multiple cysteine derivatives and amino acid analogs. These examples have relied largely on the multiple methods that have become available for the incorporation of dehydroalanine into proteins.

For instance, the Schultz group has described the use of phenylselenocysteine as a precursor to dehydroalanine on proteins [54•]. Oxidative elimination using hydrogen peroxide provided dehydroalanine. Addition of glycosyl thiols and aliphatic thiols provided *S*-hexadecylcysteine and *S*-mannosylcysteine. Our own lab has reported a novel chemical method to oxidatively eliminate cysteine directly to dehydroalanine using *O*-mesitylenesulfonylhy-

Figure 3



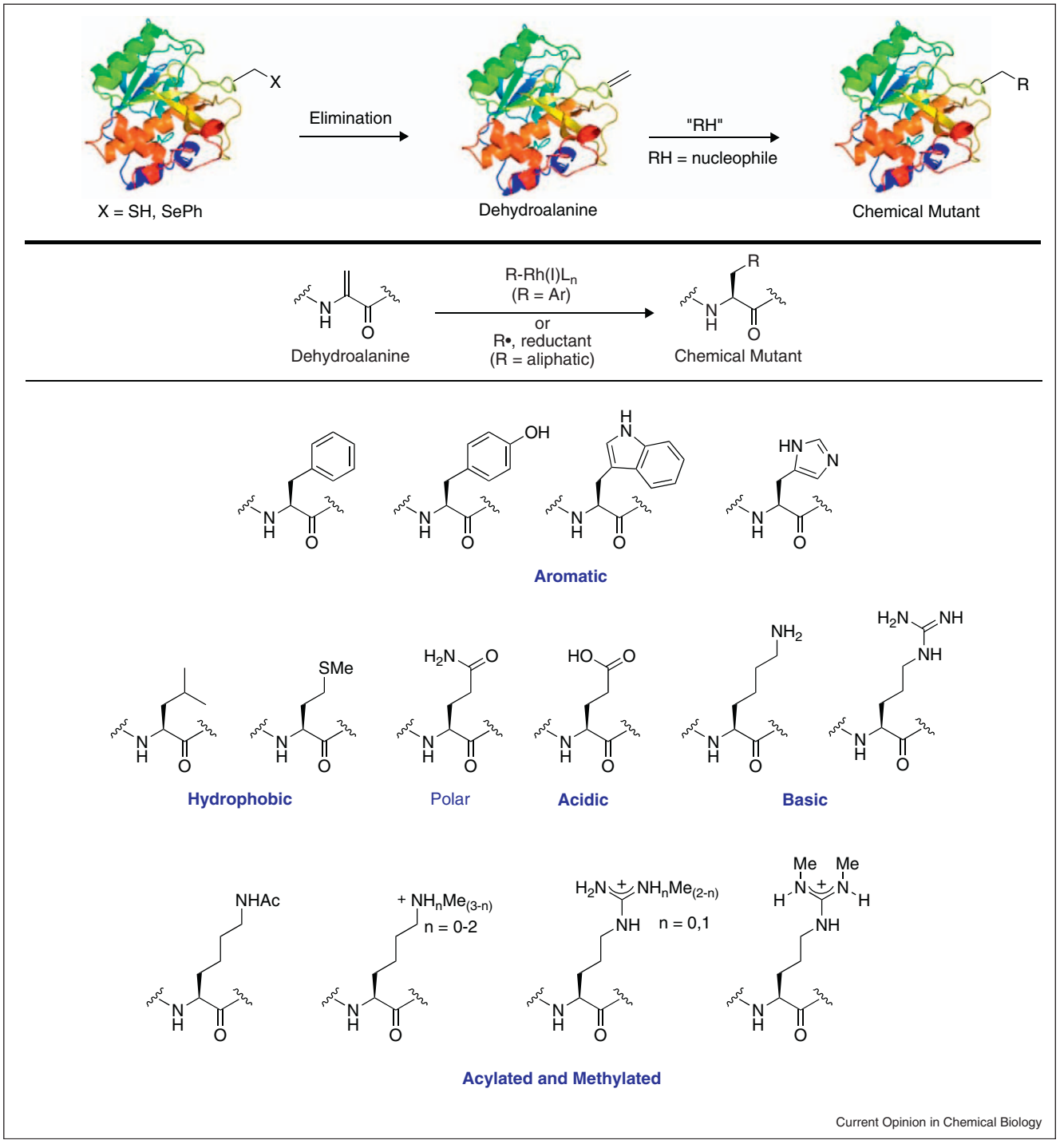
Schultz's boronic acid: a chemical precursor to Phe and Tyr mutant proteins [51•].

droxylamine (MSH) [55•]. In this report it was shown that dehydroalanine is a precursor to glycosyl cysteines, phosphocysteine, and farnesyl cysteine — all residues that are found in nature. We also reported access to methylated lysine analogs; this use of Dha as a precursor to lysine analogs is a complementary method to the more common aminoethylation protocol [46]. Similarly, the Schultz group later used dehydroalanine as a precursor to methyl and acyl lysine analogs on histone H3 [56]. In their report, they demonstrated the first use of *N*-acetyl-thialysine as a substrate for histonedeacetylase enzymes.

In this way, dehydroalanine is a useful precursor to cysteine derivatives, including *natural* residues such as glycosyl-, phosphoryl-, and farnesyl cysteine. Thialysine analogs, however, are only mimics of the natural side chain. While mimicking this functionality may suffice in many cases, it is desirable to access native structures. With the development of a host of aqueous and asymmetric transformations, the potential of dehydroalanine as a general handle in chemical mutagenesis is not out of the question and we may speculate on future directions. For instance, aryl side chains can be accessed from dehydroalanine by the Rh(I) catalyzed conjugate addition of aryl boronic acids. This reaction is compatible with water, has several asymmetric variants [57], and its use on peptide substrates has also been investigated [58,59]. For aliphatic side chains, radical additions to unsaturated systems are possible in water [60]. The deployment of such transformations at dehydroalanine can, in principle, provide access to many diverse side chains. Applying a similar retrosynthetic analysis to other side chains reveals that a significant number of residues can be considered accessible from a dehydroalanine precursor. Alanine, for instance can be accessed by catalytic, and perhaps asymmetric, hydrogenation. A similar exercise in retrosynthesis may reveal other transformations that will constitute a 'chemical mutation.' A proposal for several side chains is depicted in Figure 4. It is worth noting the diverse functionality accessible from dehydroalanine. Aromatic, aliphatic, acidic, basic, and post-translationally modified side chains are all accessible, in principle, from this precursor. Figure 4 only depicts natural residues. It is clear that many other complementary unnatural amino acids should be accessible through a similar route.

Dehydroalanine as a handle for chemical mutagenesis is not without potential pitfalls and cautionary notes. The first is the requirement for dehydroalanine. Although several methods for its incorporation into proteins are known, none is entirely general. A simple, selective (or better yet, specific) installation of dehydroalanine is required. Second, the transformations proposed in Figure 4 have not been reported on any protein substrates, much less fragile, pH sensitive protein samples. The challenge of water compatible, chemoselective mutations of Dha to other residues is the second outstanding problem. In addition, many of these proposed transformations may require an asymmetric variant or some understanding of substrate control of the diastereoselectivity. The very analysis of the chemical and stereochemical outcome of the proposed transformation is also no small feat.

Figure 4



A proposal: dehydroalanine as a general chemical mutagenesis handle. Aryl and aliphatic nucleophiles may allow access to a diverse functional set of amino acid side chains.

When these challenges are met using diverse and divergent intermediates such as dehydroalanine, we envision many advantages and applications of chemical mutagenesis. For instance, a single mutant derived from routine site-directed mutagenesis can be a chemical precursor for multiple mutant proteins. Thus, a single round of expression and purification could yield a diverse library of mutant proteins after chemical mutation. Furthermore, the final chemical mutation could be carried out on the *folded* protein, so protein activity can be attributed to the

mutation itself and not misfolding during expression. Another significant application would be access to unnatural amino acids and modified natural amino acids that are not yet accessible in standard expression systems. Ultimately, chemical mutagenesis may resolve supply issues for proteins bearing these post-translational modifications, several examples of which are shown in Figure 4. Finally, efforts in chemical mutagenesis are attractive chemical challenges that may inspire new chemistry that is mild and compatible with biological systems. These challenges and potential applications are guiding our current efforts while we revisit Koshland's idea of chemical mutations.

Conclusions

The concept of chemical mutagenesis dates back to the first point mutation of an enzyme by Koshland and Bender in 1966. However, as DNA and recombinant expression technology developed, the use of chemistry in amino acid conversion was largely replaced. We have revisited the idea of chemical mutagenesis again in an effort to revive its use in protein science. We are not in any way pitting chemistry against biology but rather encourage a dual and reinforcing use of current methods in biosynthetic incorporation of natural and unnatural amino acids and their post-expression 'mutation' using chemistry. It is our hypothesis that mutation of translated, folded proteins using chemistry can allow rapid access to natural, unnatural, and modified natural side chains. The chemistry and biology required for a general 'chemical mutagenesis' is rife with opportunity. Meeting the challenges described above will help build the conceptual and practical foundation for a general method of protein construction free of many of the current limits of purely biological strategies.

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