



# Synthesis of modified proteins via functionalization of dehydroalanine

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Dehydroalanine has emerged in recent years as a non-proteinogenic residue with strong chemical utility in proteins for the study of biology. In this review we cover the several methods now available for its flexible and site-selective incorporation via a variety of complementary chemical and biological techniques and examine its reactivity, allowing both creation of modified protein side-chains through a variety of bond-forming methods (C–S, C–N, C–Se, C–C) and as an activity-based probe in its own right. We illustrate its utility with selected examples of biological and technological discovery and application.

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## Background and motivation

Modern chemical biology relies increasingly on protein chemistry, which (ideally) allows precise positioning of labels, cargoes and post-translational modifications (PTMs) in the contexts of complex protein structures [1–3]. The resulting modified proteins prove useful in therapeutic applications, the probing and modulating of function, as well as their tracking and (un)caging in cells [4–6].

Various methods have been developed for the convergent construction of site-selectively modified proteins [6,7]. Traditionally, the non-site-selective chemical modification of proteins has relied on the nucleophilicity of the side-chains of natural amino acid residues lysine (Lys) and cysteine (Cys), as well as protein N-terminus through direct acylation, alkylation and arylation with a wide array of electrophiles [6–8]. However, while these techniques have been extensively used, for instance, for antibody–drug conjugate (ADC) manufacturing, their lack of

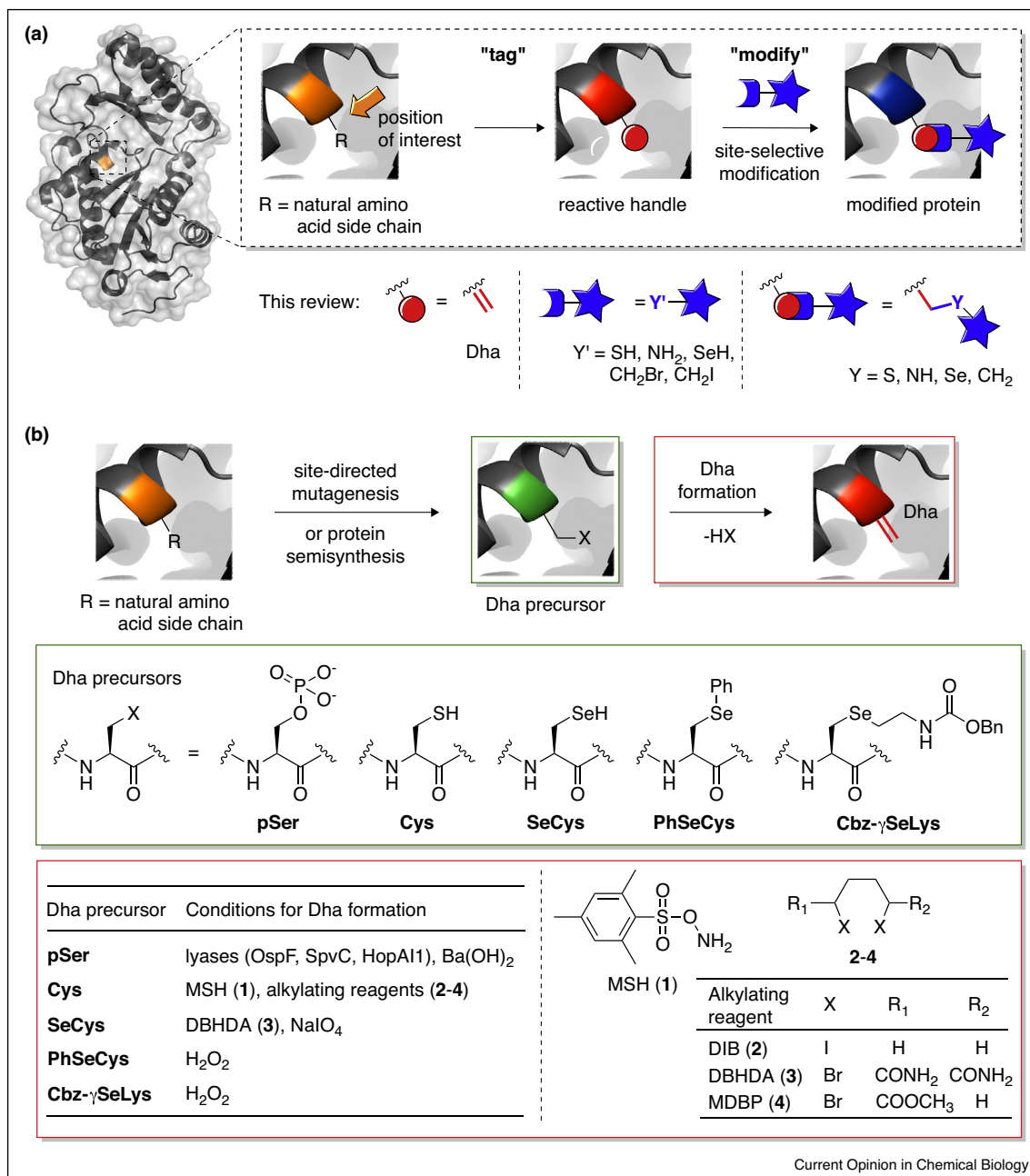
selectivity is a major limitation in applications requiring greater homogeneity and precision. While some site-selectivity can be achieved using the natural rarity of Cys (~1% of Cys on average in proteins [9]), a ‘tag-and-modify’ [10] approach can be generally used as a site-selective protein labelling method that exploits positioning of pre-determined functional groups, ‘tags’ (Figure 1a). It relies on the selective introduction of a ‘tag’ as a reactive handle to each site of interest followed by chemoselective reaction to ‘modify’/graft on to that site the function of interest. In the past decades, the range of non-proteinogenic, reactive tags (e.g. azide, alkyne, tetrazine) has been greatly expanded by biochemical and cellular methods (auxotrophic replacement, nonsense codon suppression [11–13]). Many such bioconjugation methods now enable effective site-selective labelling. However, for the attachment linkage leaves a ligation ‘scar’ in the protein, often larger than the amino residue itself, precluding precise or subtle functional study or biomimicry [14,7].

The amino acid dehydroalanine (Dha), as a biocompatible ‘tag’ in proteins, shows intriguing and varied reactivity with typically minimal (e.g. single  $\beta,\gamma$ -C–X bonds) attachment marks/‘scars’ that therefore allows striking flexibility in, for example, the installation of natural PTMs (or mimics) and chemical mutagenesis to a broad variety of natural and unnatural amino acids. The insertion of Dha tag into proteins proceeds under mild conditions via various complementary methods and can now be robustly scaled up to milligram protein quantities. In this review, we aim to provide an overview of approaches to modify proteins via dehydroalanine. We describe methods of Dha incorporation into a wide range of proteins and illustrate that Dha functionalization is driven by its chemical properties. Finally, we discuss applications of Dha chemistry as a broadly applicable tool by highlighting recent achievements ranging from creating modified nucleosomes to preparing better therapeutics.

## Introduction of dehydroalanine to proteins

Dehydroalanine is a naturally occurring amino acid, which is formed by serine (Ser) dehydration or phosphoserine (pSer) elimination in peptides [15] and proteins [16]. Its formation is observed during lanthipeptide natural product biosynthesis in prokaryotes [17]. Excretion of phosphothreonine lyases (OspF, SpvC and HopA11) in pathogenic bacteria (*Shigella*, *Salmonella* and *Pseudomonas syringae*, respectively) converts pSer to Dha in activation loops of host mitogen-activated protein kinases (MAPK)

Figure 1



**(a)** Site-selective covalent protein modification by a two-step 'tag-and-modify' approach. **(b)** Methods to introduce dehydroalanine (Dha) as a 'tag' to proteins. The position of interest is typically activated by conversion to a Dha precursor followed by its elimination to Dha. Protein taken from PDB: 1N2E [89].

[16]. Finally, Dha is formed by spontaneous non-enzymatic elimination of pSer as a consequence of protein aging in human cells [18,19], a process which in some proteins may be accelerated chemically [20].

Ser and other natural amino acids Cys and selenocysteine (SeCys or Sec) can be used as controllable Dha precursors by protein chemists. These residues, inserted at the

position of interest, are first transformed into leaving groups, which upon elimination, yield Dha (Figure 1b). Historically, the first attempts to form Dha on peptides and proteins relied on Ser sulfonylation followed by elimination under conditions typically too harsh for most proteins and in a manner that is applicable only to activated (e.g. catalytic triad) Ser [21]. Following phosphorylation, *in vitro* treatment of the resulting pSer with barium hydroxide at

ambient temperature can yield Dha [20]. However, despite progress in amber codon suppression technology and semi-synthetic methods, fully selective incorporation of pSer into larger proteins [22–26] remains a challenge and may not always provide a flexible precursor.

Therefore, methods to transform more rare (allowing generality) and more reactive (allowing milder conditions) amino acid Cys to Dha were developed to achieve compatibility with sensitive protein structures as well as selectivity [27\*,28\*]. Contrary to pSer, SeCys and its derivatives (see below), Cys may be introduced to a target protein quite simply by site-directed mutagenesis. Although early pioneering work on Dha formation from Cys was complicated by associated protein cleavage [29], in 2008 an oxidative amidation/Cope-type elimination protocol using *O*-mesitylenesulfonylhydroxylamine (MSH, **1**) was reported on model protein subtilisin [27\*]. Whilst applicable to many proteins without side-reaction, low level undesired reactivity of MSH as an oxidative reagent with nucleophilic amino acids (Met, Lys, His, Asp and Glu) under certain conditions was observed [28\*]. This led to development of a series of milder and more selective reagents (Figure 2b, 2–4) that convert Cys to Dha via bis-alkylation/elimination, inspired [28\*] by the inferred formation of Dha in murine and human metabolic products upon treatment with 1,4-dihalobutanes or the drug Busulfan<sup>TM</sup> (the bis-mesylate of 1,4-butanediol) [30]. Thus, even commercial 1,4-diiodobutane (**2**) can be used, although its broad application in protein chemistry is precluded, in part, by a very low solubility in water. The reagent that was therefore found to have broadest utility, 2,5-dibromohexanediamide (DBHDA, **3**) is more water-soluble, stable, simple-to-prepare, easy-to-handle and is now commercially available (Kerafast: URL: <http://kerafast.com/product/1877>; and Sigma–Aldrich: Cat. No. 900607). More recently, methyl 2,5-dibromopentanoate (MDBP, **4**) — originally used in the creation of multiple Dha in peptides [31]) — has been found to be the reagent of choice for sensitive proteins [32,33] by allowing an apparently rate-limiting second alkylation step.

Site-selective Dha formation in proteins containing multiple Cys poses a significant challenge. In early studies, selectivity has been driven by Cys residue accessibility [34,35] or reactivity [36]. Nevertheless, a general method for addressing this challenge is still missing. One strategy is further elaboration of the core structure of **3** in order to fine-tune properties of the alkylating reagent. The right reagent to achieve selective Dha formation can then be chosen with respect to the local environment of the targeted Cys [37].

The unique chemical properties and ultra-low natural abundance of SeCys also make it a possible Dha precursor. SeCys itself can be introduced into proteins [38] directly (under the control of the SECIS RNA element)

[39], by native chemical ligation [38,40\*,41], SeCys-mediated expressed protein ligation [42], or in modified-forms (e.g. phenyl-selenocysteine (PhSeCys) [43] or *seleno*-Lys variants [44,45]) by amber codon suppression. These can be converted to Dha via the corresponding selenoxides (oxidation/Cope-type elimination) using hydrogen peroxide or sodium periodate [43–46]; however, undesired side-oxidations of susceptible amino acids (Met and Cys) are also observed. Therefore, DBHDA **3** can be used with SeCys, which, when coupled with the greater acidity of SeCys *cf* Cys (pKa(SeCys) = 5.2) allows some selectivity over Cys [40\*,42].

Together these techniques have now allowed the selective incorporation of Dha into several sites of many proteins, for example, GFPs [47], ubiquitins [48\*,49–51,52\*\*,53], histones (H2A [54], H2B [54], H3 [55] and H4 [56\*]) antibodies (cAbs [28\*,57] and so-called ‘ThioMabs’ [33]) kinases (Aurora A [34] and p38 $\alpha$  [58]), as well as *N*-acetylneuraminic acid lyase [59], AcrA and annexin V [56\*] and Np $\beta$  [60], pantothenate synthetase [32], protease SBL [27\*], phosphatase PTPa [35], and keratin hydrogels [61].

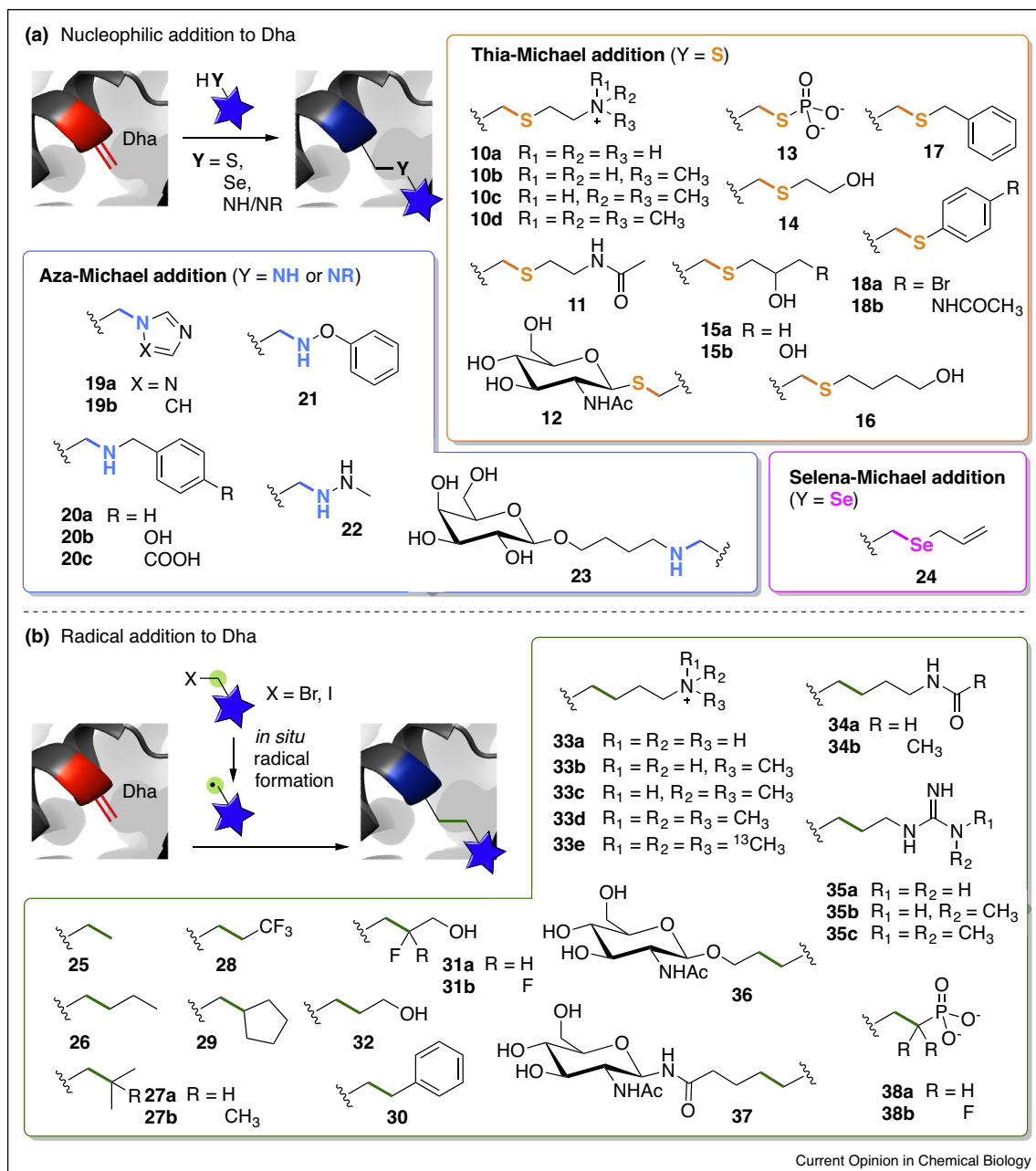
### Protein functionalization at dehydroalanine

Chemically, one facet of Dha is as an  $\alpha,\beta$ -unsaturated carbonyl moiety that can undergo conjugate (Michael-type) addition reactions with various nucleophiles (Figure 2a) under conditions compatible with proteins, that is, in aqueous media at moderate pH and temperatures below 40°C. Such additions to Dha in proteins currently allow various types of  $\beta,\gamma$ -bond formation: thia-Michael, aza-Michael, seleno-Michael (C–S/N/Se, etc.) according to nature of the nucleophile.

The intermolecular reaction of Cys residues with Dha leading to the formation of thioether-linked lanthionine has been implicated for some time in metabolic processes [30] and the intramolecular process is well known in certain biosynthetic pathways in peptides, where it is typically catalyzed by enzymes [17]. Due to relatively high concentrations in cells (mM), adducts of glutathione, as a natural thiol, have been seen from Dha in peptides [30] and proteins [19]. Demonstration of the flexibility of this reaction as a method for protein modification was illustrated by its application to various exogenous thiols, allowing installation of diverse functionality including mimics of various PTMs, such as methylated/acetylated Lys (**10a–d/11**) [27\*,46]; GlcNAcylated Ser (**12**) [27\*] and phosphorylation (**13**) [27\*]. Since then, a wide variety of thiols, from smaller, for example, alkyl polar (**14–16**) [62\*\*] and aromatic-containing (**17, 18**) [63] right up to larger, for example, peptides [53,60] have been successfully introduced, allowing various applications (see below).

Recently, the reactivity of Dha in proteins with *N*-heterocycles [32], amines, hydroxylamines and hydrazines [33] in aza-Michael additions was demonstrated allowing

Figure 2



Selected amino acid and post-translational modification (PTM) mimics introduced to proteins via **(a)** nucleophilic (thia-, aza-, selena-Michael), and **(b)** radical additions to dehydroalanine, listed by type of a newly formed bond. Protein from PDB: 1N2E [89].

creation of histidine (His) analogues (**19a,b**) [32], or conjugation via modified aminoalanine formation [33] using benzylamines (**20a–c**) or other amino  $\alpha$ -nucleophiles (**21**, **22**).

Only one example of selena-Michael addition to Dha has been reported [64<sup>\*</sup>], inspired by the intermediacy of Dha in allowing  $\beta,\gamma$ -C–Se bond formation in SeCys biosynthesis. Se-allyl-selenocysteine **24** was installed by *in situ* generation

of a suitable selena-nucleophile from the precursor allylselenocyanate, and enabled further functionalization of histone proteins by Se-relayed olefin cross-metathesis and then oxidative removal, as a series of reactions that chemically mimic epigenetic ‘write-read-erase’ cycles.

Dha, as well as acting as a competent conjugate-electrophile, has the potential for other modes of reactivity. Its potential as an efficient partner radical acceptor (‘SOMO-ophile’) in

carbon-centred C• radical additions to proteins was recently demonstrated (Figure 2b) [20,56•,65] thereby allowing the first examples of C(sp<sup>3</sup>)–C(sp<sup>3</sup>) bond formation on proteins and demonstrating a proof-of-principle in realising the previously suggested [66,67] potential of ‘post-translational chemical mutagenesis’. Dha provides good radical acceptor reactivity and, hence, chemoselectivity in the typical background of inertness afforded by most natural protein residues to C• radical chemistry [68]. Addition of a C• radical to the Cβ of Dha generates a *capto*-dative stabilized Cα radical, which can be suitably quenched. This allows for its use in aqueous media using mild generation from the corresponding halides (iodides and bromides) using sodium borohydride [56•], or certain metals [20,56•,67,69] and can be applied to even complex protein scaffolds. The compatibility of the method with a wide-range of *unprotected* side-chain precursors, typically from just the corresponding iodide, allows ready, rapid and divergent installation of many side chains. This approach enables post-translational mutagenesis to unmodified, natural hydrophobic aliphatic (25–29) and aromatic (30) as well as polar residues (31 and 32). For PTMs it allows direct introduction into proteins simply through ‘pre-installation’ of the desired modification into the radical precursor side-chain reagent of choice. This allows access to methylated lysines 33b–e, including <sup>13</sup>C-labelled variant 33e useful for protein NMR studies; acetylated and formylated lysines 34; methylated arginines 35b,c; *O*-glycosylated or *N*-glycosylated amino acids (36 and 37) and even functional, phosphatase-resistant carba-pSer analogues (cpSer 38a and cf<sub>2</sub>pSer 38b).

It should be noted that despite the ready structural and functional diversity generated by these constitutionally native transformations, there are some key practical considerations [65] in application. Detailed mechanistic analysis [56•] revealed that metal-mediated conditions can suffer from backbone cleavage and side-reaction necessitating use of a suitable hydrogen atom source for efficient ‘quenching’.

Analysis of d.r. suggests the formation of typically 1:1 D/L-Cα-epimers at the site of mutagenesis. Notably, in some cases the formation of epimeric mixtures by both types of addition reactions described above can either be deconvoluted (e.g. D versus L [55]) in functional assays (see also below) or by refolding or crystallization that favours the native, L-stereoisomer [59,62••]. Moreover, the apparently low level of substrate control upon stereoselectivity in most [53] (but not all [70]) additions to Dha suggests opportunities for others modes of stereocontrol, as suggested by models in amino acids [71] and peptides [72•] (see below).

### Study of PTMs using Dha protein chemistry for installation, mimicry and recapitulation

Deciphering function of individual PTMs in proteins and their mutual interactions has been limited by an inability to

generate pure, homogeneous post-translationally modified proteins from natural sources. This issue can be overcome using protein chemistry to precisely position PTMs (or their mimics) in proteins of interest. One path is modification via Dha (see above) and key examples have seen application in chromatin biology, kinase activation and mechanisms of protein ubiquitination (Figure 3).

In eukaryotic cells, DNA is bound by histone proteins (H1, H2A, H2B, H3, and H4) into nucleosomes, forming the basis of chromatin. Chromatin architecture has been directly implied in gene transcription and is dynamically regulated by a myriad of histone PTMs. To dissect contributions to chromatin function, various approaches to create synthetic nucleosomes with precisely positioned PTMs have been developed and are reviewed elsewhere [73–75]. Dha chemistry has allowed for the study of histone PTMs (Figure 3a).

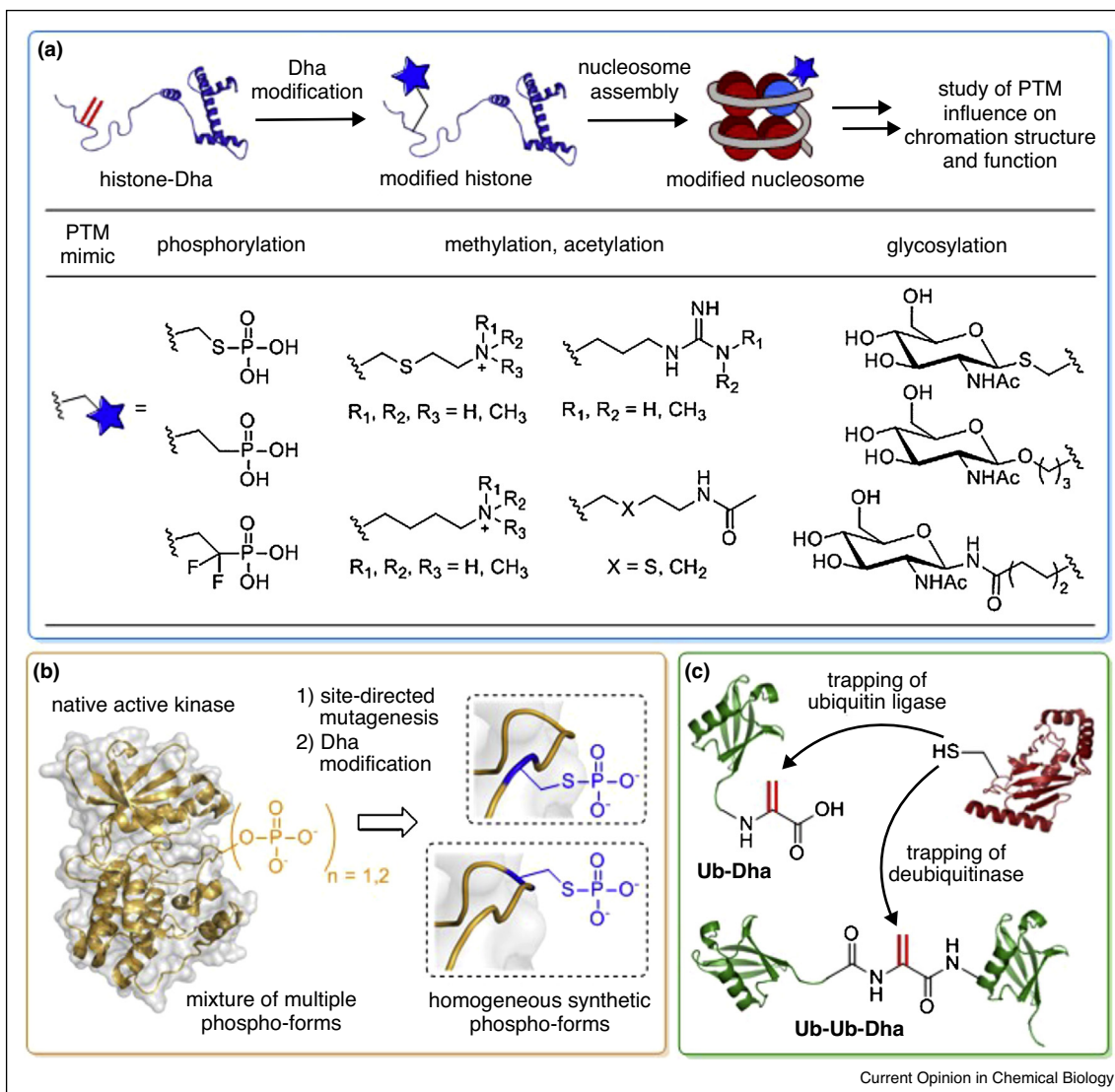
Gene transcription is regulated by the methylation and acetylation of histone lysines [76]. Using Dha, methylated H3 on Lys9 (mono-, di-, trimethyl and <sup>13</sup>C-labeled trimethyl [56•] or *thia*-Lys mimics at Lys9 [45,46,55]) and Lys79 [20] have been generated. Similarly, acetylated histones on various lysine residues have been prepared. These variants are recognized by native anti-LysMe and anti-LysAc antibodies, respectively [20,45,46,55,56•]. Lys9 demethylation by lysine demethylase JMJD2A/KDM4a could be simultaneously determined using protein MS and NMR of a labelled variant H3-[<sup>13</sup>C]Me<sub>3</sub>-Lys9. Methylation in the *thia*-Lys variants of Me<sub>n</sub>-Lys79 were shown to stimulate chromatin transcription [20]. Histone deacetylase (HDAC) assays using *thia*-Lys H3-Ac-Lys9 variants revealed precise activity of different HDACs. Notably, in some instances conversions of 50% were observed, revealing an HDAC selectivity that is sensitive to Cα configuration and consistent with a 1:1 D-/L-mixture at Cα [55]. It should also be noted that use of C–C bond-forming methods may be preferred to install methylated Lys variants, since recent analyses have suggested methylated *thia*-Lys may not be ideal mimics of methylated Lys [77,78].

Radical-mediated, C–C post-translational mutagenesis allowed the first installation of asymmetric dimethylarginine into nucleosomes via Dha [56•]. Affinity proteomic analyses revealed partners consistent with cross-talk between H3-Arg26 and H3-Lys27 methylation in generating a repressive chromatin state.

Generation of synthetic, GlcNAcylated nucleosomes has enabled the study of the effects of histone glycosylation on chromatin stability and interactome. H2A-T101 GlcNAcylation was found to affect chromatin stability by destabilizing the H3/H4 tetramer-H2A/B dimer interface providing a possible model for effects on transcription [79]. By contrast, H2B-S112 GlcNAcylation caused



Figure 3



Installation of post-translational modification (PTM) mimics to proteins via dehydroalanine. **(a)** Selectively modified histones are assembled into nucleosomes to probe PTM effects (phosphorylation, methylation, acetylation, *O*-glycosylation and *N*-glycosylation) on chromatin structure and function. PDB: 1KX5 [90]. **(b)** Synthetic phosphorylation of kinases provides homogeneous synthetic phosphoforms that allow for dissection of the contribution of individual phosphorylation sites to kinase activation. PDB: 1OL7 [90,91]. **(c)** Activity-based systems targeting ubiquitin ligases or deubiquitinases through reaction with their active site cysteines exploit Dha-containing mono-(**Ub-Dha**) or diubiquitin (**Ub-Ub-Dha**) probes, respectively. PDB: 1UBQ [92], 5IFR [48\*].

changes in the nucleosome interactome by promoting binding of the facilitate chromatin transcription (FACT) complex [54].

Synthesis of both *N*-glycosylated and *O*-glycosylated histone variants was enabled by C–C bond forming chemical mutagenesis at Dha sites [56\*]. Enzymatic extension to more complex glycans catalyzed by either glycosyltransferase or endoglycosidase proved possible. Interestingly, whilst certain synthetic *N*-glycans were not cleaved by peptide-*N*-glycosidase (PNGase), a widely used *N*-

glycosidase, synthetic *O*-glycoproteins were readily cleaved by *O*-glycosidases, including the human protein *O*-GlcNAcase (hOGA) enzyme. hOGA has been previously considered to be selective but appears from these experiments to be tolerant of site, side-chain and configuration at GlcNAcylated residues.

Histone H3 phosphorylation occurs on Ser10 during mitosis. The detailed analysis of its effects is challenging due to the difficulty of isolating pure homogeneous H3-pSer10 [80]. To address this issue, pCys and stable,

non-hydrolysable analogues cpSer or cf<sub>2</sub>pSer have all been chemically installed as mimics [45,55,56<sup>•</sup>]. All are recognized by anti-H3-pSer10 antibody and phospho-‘reader’ proteins (14-3-3ξ and MORC3) suggesting good functional similarity to pSer and applicability to further studies of histone phosphorylation in chromatin biology.

The key role played by protein kinases in regulation of intracellular signalling cascades is itself triggered and regulated by phosphorylation at multiple sites in activation loops by upstream kinases. Pure kinase phosphoforms would allow precise study of kinase function but most ‘active’ kinase preparations from biological samples are heterogeneous mixtures of multiple phosphoforms. Reaction of Dha with thiophosphate provides a method [27<sup>•</sup>] (Figure 3b) for site-selective chemical protein phosphorylation [81] that complements recent progress in direct pSer incorporation using amber codon suppression [22,24,25]. When applied to certain sites in kinases it provides a closer functional mimic of pSer than prior approaches of so-called constitutive activation through Glu/Asp [34,58]. Mitogen-activated protein kinase (MAPK) p38α was chemically activated *in vitro* by phosphorylation at native site T180 — monophosphorylation of the activation loop was sufficient to trigger activity. However, chemical phosphorylation of T172 in the loop, which is not enzymatically phosphorylated, led to no activation, revealing that position within the activation loop proves key also [58]. Interestingly, Aurora A kinase activation loop can even be activated by extended chain variants (e.g. phospho-2-hydroxyethylcysteine) towards autophosphorylation as well as substrate phosphorylation [34]. These pure mimic phosphoforms also allow rare, detailed kinetic analyses of the modes of activation and inhibition by current drugs [58].

Post-translational modification by ubiquitin or ubiquitin-like proteins, tightly regulates various cellular processes such as protein degradation, cellular localization and DNA repair [82,83]. Many synthetic strategies have been developed to help understand the dynamics of ubiquitination–deubiquitination system [84,85<sup>•</sup>], including two types of Dha-based ubiquitin activity probes (Figure 3c). In one, when Dha is introduced to the C-terminus of ubiquitin (giving ‘Ub-Dha’), it can be used to covalently trap the catalytic Cys of ubiquitin ligases [48<sup>•</sup>,50] in an activity-based manner. In this way, Ub-Dha enabled sequential targeting of all three types of ubiquitin ligases (E1, E2, E3) involved in a protein ubiquitination cascade, allowing affinity-based proteomic profiling in cancer cell extracts [48<sup>•</sup>]. In another mode, Dha-containing probes have been designed to probe deubiquitination. By installing a reactive Dha between two Ub units (christened ‘Ub-Ub-Dha’) [40<sup>•</sup>,49,52<sup>••</sup>], the catalytic Cys of deubiquitinases can also be trapped. Ingeniously, positioning of Dha differently relative to

the scissile peptide site, enables differentiation amongst deubiquitinases [49,52<sup>••</sup>].

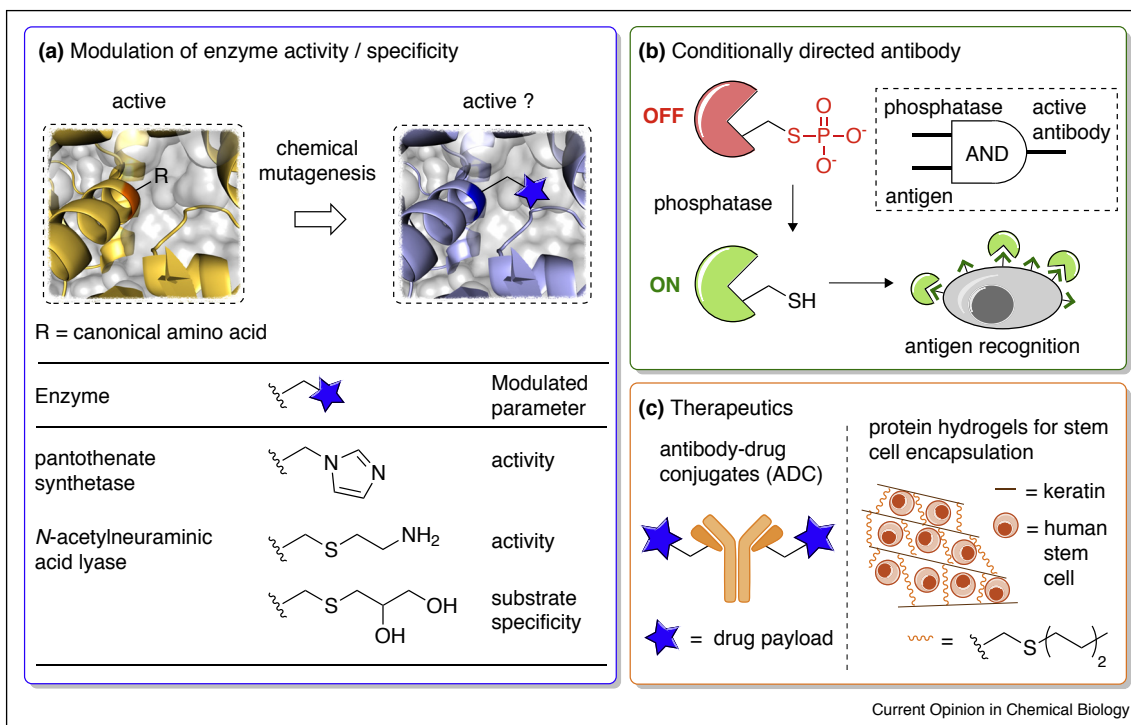
### Application of Dha chemistry in enzymatic and De Novo mechanistic hypotheses

Such precise, site-selective chemical modification of proteins can provide an unique opportunity for precise changes of amino acid structure beyond the limits of traditional biology even down to very subtle, single-atom changes. This in turn allows mechanistic questions to be posed for both existing (e.g. catalytic) or *de novo* (synthetic/programmed) protein function.

Controlled alterations in enzyme active sites can probe mechanism or alter selectivity (Figure 4a). Chemical mutagenesis frees such experiments from the limits of the proteinogenic residues in principle in an almost unlimited way and Dha chemistry can provide a virtually traceless way of accomplishing this. For instance, aza-Michael-type chemistry allowed conversion of key active site histidine residue, His44, to its direct regioisomer *iso*-histidine (linked instead through its pro-Nπ atom rather than C4) in pantothenate synthetase (PanC) from *M. tuberculosis* (*Mtb*), suggesting an essential role as a hydrogen bond donor to ATP during catalysis [32]. Use of thia-Michael-type chemistry on Dha in *N*-acetylneuraminic acid lyase (NAL) from *Staphylococcus aureus* has enabled chemical mutation of key active site lysine Lys165 to γ-thialysine, shifting the enzyme pH optimum from 7.4 to 6.8 [59]. And in the same enzyme (NAL), an ingenious systematic variation of active site residues [62<sup>••</sup>] applied thia-Michael chemistry (with each of thirteen different thiols) to Dha residues introduced to twelve different positions. Thiols were selected to introduce different stereochemistry and functional groups not accessible by other methods and allowed discovery of an NAL ‘mutant’ bearing a dihydroxy side-chain up to ten times more efficient in NAL-catalyzed aldol reaction with erythrose compared to the wild-type enzyme. Use of Dha as a mutation itself can also provide insight; introduction of Dha into *Mtb* protein tyrosine phosphatase PtpA has led to the suggestion that a water-mediated bridge between two cysteines (Cys-H<sub>2</sub>O-Cys) may confer resistance to oxidative conditions in host macrophages [35].

As well as probing of internal enzyme active sites, key functional sites in other proteins can be explored. In one application, the ability to precisely install an unnatural but responsive functional side-chain group to control an active binding site was explored in the CDR of single-domain antibody cAb-Lys3 [57]. Thus, chemical phosphorylation by Michael-type addition of thiophosphate to Dha (see above) allowed ‘gating’ of the CDR and hence the Ab itself. Recognition of cognate antigen lysozyme was hence blocked and restored only in the presence of two inputs: expression of a secreted phosphatase and the antigen. This suggests exploration of concepts of *de novo*

Figure 4



Examples of functional synthetic proteins prepared via modification of dehydroalanine. **(a)** Modulation of enzyme activity and/or specificity by chemical mutagenesis in active sites. PDB: 1N2E [89]. **(b)** Conditionally directed antibodies can be caged with thiophosphate in their recognition domains to create a protein operating under rudimentary ‘AND’ gate logic. Antigen binding is induced by the presence of two inputs, that is, enzyme (phosphatase) AND antigen. **(c)** Protein therapeutics: antibody drug conjugates, keratin hydrogels for stem cells encapsulation.

conditionally functional proteins as, in this case, logic gates (here an ‘AND’) as a largely unexplored realm of synthetic biology (Figure 4b).

The ability to allow ready conjugation via Dha has also seen more biotechnological applications, for example in potential therapeutics (Figure 4c). For instance, stable and chemically defined antibody-drug conjugate (ADC) was prepared by direct conjugation of an IgG using aza-Michael-type reaction of a Dha residue with the piperidine unit present in the anticancer drug Crizotinib<sup>TM</sup>, giving a more homogeneous ADC variant with improved stability in human plasma [33]. Injectable hydrogels based on keratin have been proposed for encapsulation and delivery of stem cells in tissue regeneration [61]. Keratin cysteines were converted to S-allylcysteines via Dha modification followed by human mesenchymal stem cell encapsulation and photocrosslinking of the S-allylcysteines to form a hydrogel.

## Conclusions and outlook

This review has highlighted the versatility of dehydroalanine (Dha) as a ‘tag’ towards late-stage functionalization of proteins. Its selective incorporation into a variety of proteins can now be achieved through a variety of mild, scalable and facile methods. Its chemical reactivity allows

the precise, chemical installation of numerous natural and unnatural residues into proteins including post-translational modifications and their mimics. The function of, for example, methylation, acylation, glycosylation, phosphorylation and ubiquitination of histones, kinases and various proteins can therefore be investigated and new protein functions designed or selected-for following ‘chemical mutagenesis’. In this way Dha methods complement the current ‘protein modification tool box’ by allowing proof-of-principle for broad-ranging, post-translational mutagenesis and ‘chemical editing’ of proteins.

Clear challenges and opportunities remain. Other bond-forming events [50,86,87][M. W. Schombs, B. G. Davis *et al.*, unpublished results] including those based on the flexible reactivity of Dha [88], offer future synthetic potential to expand this ‘chemical mutagenic/editing’ approach.

Whilst stereocontrol arising from the peptide/protein environment has been described this tends to be modest in most native sequences [43,46,53,70,71]. The resulting formation of epimeric (D-/L-) mixtures is therefore a current limitation of the synthetic functionalization of Dha. This will be aided by the development of analytical techniques for the determination of stereoselectivity in



modified proteins [53,56\*]. Biological use will also continue to reveal the impact of configurational mixtures on protein structure and function. Interestingly, this may not be an issue in some functional (see above) or structural studies. For example, in some X-ray crystallography studies only the natural 'L-configuration-protein' crystallizes [59,62\*]. Given the typical lack of substrate control in stereoselectivity, there is also clear potential for reagent or catalyst (chemical or biological) control in this regard. Notably, in this way, stereoselective additions to Dha have been successfully performed on amino acid and peptide models [72\*].

Such reagent/catalyst control is likely to also be a critical additional mode of chemo-selectivity and regio-selectivity for the translation of Dha methodology into more-and-more complex (*cellular/in vivo*) environments.

### Conflicts of interest statement

B.G.D. is the editor-in-chief of *Current Opinion in Chemical Biology*. B.G.D. is a supplier of the DBHDA reagent through the Kerafast platform. B.G.D. is a member of Catalent Biologics Scientific Advisory Board; Catalent holds patent WO 2009103941 (Bernardes, Chalker, Davis, 2009) on use of Dha chemistry in proteins including C–C-bond formation.

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Papers of particular interest, published within the period of review, have been highlighted as

- of special interest
- of outstanding interest

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