Post-Translational Site-selective Protein α-Deuteration: Protein Backbone Modification and Use as a Tool for Protein Mechanism

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Abstract *214 words*

Isotopic replacement (e.g. $^{1}H \rightarrow {}^{2}H/D$) is a long-proven, vital tool in *small* molecules: ranging from use in mechanistic analysis to alteration of metabolic properties. However, application in proteins has, to date, been largely limited to biosynthetic strategies (with partial incorporation and/or at multiple sites and/or in a manner that cannot be applied after protein expression) or at exchangeable (e.g. N–H/D) labile sites only (too labile for many studies). The development of methodology for the post-biosynthetic, isotopic $C–{}^{1}H \rightarrow C–{}^{2}H/D$ replacement in proteins would enable new understanding of mechanisms and other uses in biology and medicine. Here, we describe a novel and readily-applied chemical method for selective protein α-carbon-deuteration, the first example of $^{1}H \rightarrow {}^{2}H/D$ at a non-exchangeable backbone site. Its utility is demonstrated here to elucidate mechanisms of model reactions used in protein bio-conjugation. Strikingly, it reveals, together with *ab initio* calculations, stepwise deprotonations via on-protein carbanions and unexpected sulfonium ($S^{+}–C^{-}$) ylids in the conversion of Cys to dehydroalanine (Dha), consistent with an unprecedented 'carba-Swern' mechanism. The facilitating of such precise mechanistic observations coupled with its ready application & exploitation on existing, intact protein constructs (without specialized culture or genetic methods) suggests this new, non-exchangeable C–D labelling strategy as a more general tool for protein mechanism and even protein structure, biotechnology and medicine.

Keywords

protein modification; protein chemistry; protein labelling; deuteration; isotopic replacement; protein mechanism; backbone modification

Introduction 325 words

Despite their clear utility in small molecules,[1-3] methods for site-selective replacement of non-exchangeable hydrogen isotopes (e.g. $^{1}H \rightarrow {}^{2}H = D$) into *intact proteins* are essentially unknown. Since hydrogen isotopes can be used as possible probes of structure and dynamics (e.g. by NMR[4] or vibrational spectroscopy[5,6]) as well as reactivity this could therefore not only broadly enhance study of protein mechanism, structure and function but enable the detailed investigation of chemical mechanism in proteins – detailed studies of the latter are rare.

Current possible strategies for isotope introduction typically require isotope incorporation *prior* to assembly. One approach to building labeled proteins is the segmental isotopic labeling strategy[7,8] using, for example, expressed protein ligation and *trans*-splicing starting from synthetically prepared peptides into which isotopically-labeled amino acid has been incorporated through traditional peptide synthesis.[9-11] Such linear assembly approaches are complemented by incorporation during biosynthetic assembly[5,9,12-17] that leads to global, and hence non-site-selective, labeling of an amino acid type or requires bespoke expression systems.

Notably, direct (convergent), site-selective incorporation of deuterium into proteins post-assembly through non-heteroatom C–D bond formation (i.e. at typically non-exchangeable sites) has not, to our knowledge, been described.[18,19,20] Such a complementary method could be readily applied to intact proteins and hence more directly exploited by the non-expert using existing protein samples. Here, we describe a method that can be readily applied to create a direct and site-selective C–H → C–D isotope replacement in the backbone of intact proteins. We show the utility of this method in the ready study of a model chemical protein reaction where isotope replacement suggests striking and previously unobserved mechanistic intermediates and details.

Results ~2100 words

Development of a Method for Site-Selective Protein Deuteration

We postulated that a general site-selective backbone α-C labelling might be possible via the creation and electrophilic trapping of a suitable α-C-enolate *C*-nucleophile (**Figure 1a**). This, in turn, might be generated via conjugate addition of an appropriate nucleophile to an α,β-unsaturated peptidic amide. In principle, this strategy could be applied not only to small peptides but directly to intact proteins, if α,β-unsaturated protein amides could be accessed. We also reasoned that ideally, if such α,β-unsaturated protein amides could be both derived from *and* used to form the same amino acid residue then the overall process could be performed in an essentially 'traceless' manner (**Figure 1b**).

Dehydroalanine (Dha) is a potentially useful amino acid residue that can be found in proteins or peptides naturally,[21] following metabolism,[22] via amber-codon suppression methods[23] or derived synthetically from Cys.[24] Thus, in one proof-of-principle guise of this general approach (which could in principle be applied to other electrophiles also), α-C H→D replacement could be envisaged at Cys (and Cys derivatives) (**Figure 1c**). Indeed, the possibility of such a mechanism has been noted by others too.[25] The use of "D+" as a small electrophile for enolate trapping might also take advantage of concerted solvent reorganization during Michael-type addition as well as related known[26,27] 'relayed' D-exchange mechanisms for enhanced accessibility. Conversion first of Cys to Dha could then be followed by thia-Michael addition of a suitable sulfur nucleophile (ideally with ready deprotection, if needed) in the presence of deuterium oxide, as a high-concentration source of deuteron electrophile.

Whilst a range of sulfur nucleophiles have been successfully demonstrated with Dha[23,24,28-38] in proteins, these typically generate alkylated Cys variants that cannot be readily converted to Cys itself; these would not therefore readily allow traceless α-C H→D replacement. We therefore tested a range of putative sulfur nucleophiles that might also readily generate Cys (see SI). Whilst use of the thioacetate (R = Ac) proved successful and proceeded with excellent conversion (>95%, see SI), subsequent deprotection proved unsuccessful. The use of unprotected *S*-nucleophile source Na_2S also proceeded well in some systems (see SI); however, in some systems concomitant dimerization via thioether formation led to formation of unwanted byproducts. Finally, use of sodium thiophosphate (R = PO_3^{2-}) proved successful in both incorporation and deprotection steps. Importantly, high deuterium incorporation levels were

achieved, enabled by careful use of fully deuterated reagents, buffer components and protein solutions and/or use of anhydrous reagents[39] (see **Supplementary Information**).

Specifically, model protein histone H3 was readily H→D exchanged, site-selectively first at site 10 (**Figure 2**). H3-Cys10 was converted to H3-Dha10 using the *bis*-alkylation-elimination reagent DBHDA[24] then H3-Dha10 was converted to H3-[d1]Cys10–S– PO_3^{2-} before deprotection to H3-[d1]Cys10 was achieved readily and flexibly in either of two ways: either through use of the phosphatase enzyme PP1, which proved capable of hydrolyzing the P–S bond to give Cys in a range of environments, or through hydrolysis at acidic pH (**Figure 2b** and **Supplementary Information**). Exhaustive dialysis in non-deuterated buffer then allowed full protonation of all *exchangeable* protons, without any loss of the site-selectively installed α-deuteron at the α-carbon of Cys10. Characterization by high resolution MS and tryptic-digest-MS confirmed the level and site of incorporation (**Figure 2c**).

Next, to test breadth and application to other sites, an essentially similar process was applied to site 26 in H3 histone (see **Supplementary Information**). This also proceeded successfully using the same method thereby demonstrating breadth of application. These reactions demonstrated, to the best of our knowledge, the first examples of a traceless, convergent, site-selective chemical protein modification that can be used for selective incorporation / replacement (H→D) of a deuterium atom into the α-position of an amino acid residue (here Cys) on a ~~native~~ protein.

Using H→D Replacement Proteins to Study Detailed Protein Chemistry

Dehydroalanine has proven in recent years to be a reactively versatile and useful residue in polypeptides. It occurs in peptide natural products[40] and in the biosynthetic precursors[41] for some proteins. Selective reactions with Dha have also, for example, allowed the creation of mimics of post-translational modifications (such as peptidylation, phosphorylation, lipidation and glycosylation) of key residues of a number of proteins, including kinases and histones.[23,24,28-38,42] More recently, it has enabled a form of protein editing through the creation of native side chains via C–C bond formation.[42]

Inspired by previous studies on busulfan metabolism and selective protein cleavage,[22,43,44] our group has developed the use of *bis*-alkylating agents to chemically introduce Dha residues into proteins.[24] The design of the water-soluble reagent dibromohexanediamide (DBHDA) allowed ready Dha formation (**Figure 3**) from cysteine (Cys) that is unusually rapid, efficient and highly selective, when compared to typical elimination reactions under the same conditions.[24] This chemical protein reaction is therefore one of

potentially strong, broad interest in that has enabled (and could enable) diverse protein chemistries, yet, its mechanism remains unclear. We therefore chose this seemingly unusual reaction as one that could be studied in detail readily using the traceless deuteration strategy described above.

The postulated[24] mechanism (**Figure 3a**) involves a double (*bis*) alkylation (inter- then intra-molecular) of the γ-S of the Cys residue leading to the formation of an unstable cyclic sulfonium intermediate that then gives Dha (**Figure 3**). In nearly all cases, the sulfonium ion appears to undergo direct elimination readily to yield Dha with good conversion (**Figure 3a**) and is unobservable. Interestingly, however, some of us have observed the formation of a *stable*, directly observable sulfonium intermediate upon treatment of a single variant of the green fluorescent protein (GFP) GFP-Cys147 (GFP-S147C) with DBHDA (**Figure 4a**).[33,45] In addition, the efficiency of the Cys→Dha transformation is strikingly dependent on the nature of the putative sulfonium intermediate formed: reagents that would lead to 5-membered cyclic tetrahydrothiopheniums prove far more efficient than those that would give simple acyclic or even 6-membered counterparts.[24] The detailed basis of the privileged and peculiar reactivity of tetrahydrothiopheniums is therefore unclear.

Together these observations – the implied nature of the privileged sulfonium and the relative inaccessibility to solvent of the α-position of GFP residue 147 – suggested a possibly highly unusual elimination mechanism. In principle, variations on four potential mechanisms of elimination of a sulfonium ion could be considered (**Figure 3b**).[46,47] Various early studies on some small molecule sulfoniums have proposed both E_2-elimination and α'-β elimination mechanisms;[48-52] these and related prior observations are inconsistent with any one dominant pathway, suggesting altered manifolds dependent on e.g. sulfonium type. Moreover, no reactions of this type (or similar) have been mechanistically evaluated previously on-protein, highlighting a potentially powerful influence of substrate (i.e. the protein) upon reaction. Prompted by these intriguing observations of selectivity and putative intermediates, we set out to probe this mechanism in detail as an example of an apparently complex and mechanistically unclear chemical reaction using the deuterium-replacement method.

Exploring the Chemistry of a Stable Protein Sulfonium

First, before use of this stable-deuteration strategy, we set out to further confirm and explore the reactivity of the proposed sulfonium intermediate under potentially *exchangeable C-H* (i.e. basic) conditions. GFP-S147C is the only system in which a stable sulfonium has been observed (**Figure 4a**): GFP-S147Sul, resulting from reaction with DBHDA. Its stability can be

attributed to local microenvironment of the protein; in GFP the structured protein β-barrel appears to shield the α-proton of residue 147, thus rendering it inaccessible and preventing elimination to dehydroalanine.[33] Although this sulfonium species is stable at low temperatures (*ca.* 4 °C) over a protracted period, at 37 ˚C at pH 8 we observed evidence of decomposition (see **Supplementary Information**). In the absence of a pathway for the formation of Dha, and consistent with both the mass differences observed and prior observations in small molecules[51,53] apparently direct fragmentation[54] was observed that appeared to proceed through [3+2] cycloreversion (**Figure 4a**) to form a corresponding protein vinyl sulfide with concomitant elimination of acrylamide. Such cycloreversion implied the formation of a sulfonium ylid from sulfonium GFP-S147Sul **(Figure 4a**).

We considered that this intermediate might be sufficiently long-lived to allow 'wash in' of label from solvent that could be observed in fragmented acrylamide (see **Supplementary Information and Supplementary Figure S6**). Incubation of GFP-S147Sul in deuterated buffer released unambiguously released β-deutero-acylamide, which was unambiguously identified by MS and MS/MS, consistent with wash-in into GFP-S147Sul followed by fragmentation. Next, we explored 'wash in' in systems that lead to *productive* elimination to Dha might carry such a 'washed-in' label in their eliminated THT leaving groups (as well as in any long-lived carbene in the protein product). Therefore two proteins that, unlike GFP-S147C, are known to productively produce Dha, subtilisin SBL-S156C and histone H3-S10C, were therefore chosen as models for this 'wash-in' strategy (**Figure 4b-d**). In both cases, reaction via treatment with DBHDA in deuterated buffer gave by-product tetrahydrothiophene carbodiamide (THTCD) that showed deuterium incorporation (**Figure 4b,c**). MSMS analysis (see **Supplementary Information**) unequivocally located the deuteration at the Cα′′ carbon of THTCD, not on exchangeable protons. Thus, product ion spectra were collected with a normalised collision energy for each *m/z* corresponding to d_n-THTCD; the precursor isolation window for MSMS scans was 0.4 Th, sufficient to isolate each isobar. Control experiments showed that neither DBHDA nor THTCD were themselves directly deuterated under the reaction conditions (see **Supplementary Information**), eliminating the possibility of direct H-D side-exchange of either during reaction or analysis.

Together these data confirmed the formation of a sulfonium ylid and a site of deprotonation consistent with intermediacy during elimination (Figure 3d). Moreover, the detection of doubly-deuterated THTCD-d2 suggested that the equilibrium between ylid and sulfonium is faster than any subsequent elimination process. Furthermore, no deuteration was observed in Dha product from any of these reactions (both via LCMS as well as tryptic digest-

MSMS; see **Supplementary Information**) leading us to tentatively discard α-elimination *via* long-lived carbene formation as a potential mechanism.

Next, having used such 'wash-in' experiments, we next chose to probe the mechanism more deeply using the protein variants bearing *pre-installed* deuterium labels at predetermined non-exchangeable sites by exploiting the α-C-deuteration chemistry developed above for this purpose.

Use of Protein and Peptide Deuteration to Probe Dha Formation

With stable Cα-Cys-deuterium-labelled proteins in hand (**Figure 2**), we tested whether internal deuteron transfer (e.g. from Cα to ylid) might be observed during formation of Dha from Cys (Figure 4). Deuteration of THTCD was not observed for either H3-dC10 (Figure 4d) or H3-dC26 (see **Supplementary Information**). Together these data suggested that if the sulfonium ylid plays the role of an intramolecular base (**Figure 3b**) then any abstraction is rapid on the timescale of elimination, leading to solvent exchange ('wash-out'). To test this hypothesis, we set out to restrict solvent exchange effects through the use of aprotic solvents of similar polarity. Unfortunately, none of the protein substrates proved sufficiently soluble to allow unambiguous experiments. However, application of the Cα-deuteration methodology (which was readily extended to small molecules) allowed preparation of small molecule models containing Cys-residues with sufficient solubility in DMSO. Consistent with reactivity on proteins, formation of α-deuterated AcNHCysOMe (AcNHCysOMe-d1) (**Figure 5a**) was best achieved in two steps by reaction of the corresponding Dha-residue (AcNHDhaOMe) with Na_3SPO_3 in D_2O followed by treatment with HCl (aq.) up to pH 3 to afforded the desired AcNHCysOMe-d1 with >95% deuterium incorporation in good yield. Use of KSAc in D_2O followed by deacetylation using K_2CO_3 in MeOH also proved possible; whilst the overall recovered yield for this alternative two-step conversion was not outstanding (38%), it also allowed high levels of label incorporation (>95%). Upon treatment with DBHDA, the thus-labelled Dha-model AcNHCysOMe-d1 behaved essentially identically to protein systems (**Figure 5b,c**) in 'protic' (aqueous buffer or D_2O) solvents, thereby suggesting its validity as a model in aprotic systems.

Thus, to test the mechanistic feasibility of intramolecular proton transfer in the absence of solvent exchange, AcNHCysOMe-d1 was treated with DBHDA in the presence of base (K_2CO_3) in non-exchangeable solvent DMSO-d6. Both LC-MS and NMR revealed the formation of THTCD-d1 (**Figure 6a**). Its formation can be explained by the formation of a sulfonium ylid that acts as an intramolecular base to abstract the α-deuterium of the residue in AcNHCysOMe-d1. To further probe the intra- *vs* inter- molecular nature of this abstraction a series of dilution

experiments (**Supplementary Figure S46**) were conducted that confirmed that at higher concentrations (beyond those that may be seen in proteins) deuterium incorporation in THTCD-d1 is lost, consistent with a intra-molecular mechanism via pre-equilibrating ylid formation at lower concentration. It should be noted that such inter-molecular (and hence protein-to-protein) abstraction is much less likely in proteins given the likely more crowded nature as compared with small molecule model AcNHCysOMe.

Computational Analysis of Dha Formation Mechanism

Together, these results on both proteins and amino acid models are consistent with the formation of a sulfonium ylid through deprotonation of sulfonium at Cα′. The lifetime of this ylid appears sufficient to allow 'wash-in' from exchangeable solvent. This ylid is also capable of relayed deprotonation of the Cα' position of the Cys residue resulting in formation of site-specifically deuterated THT in non-exchangeable solvent; concentration dependency implicates intra-molecular deprotonation and this deuteration appears to be 'washed-out' in exchangeable solvent (**Figure 6a**). This implicated E1cB elimination with 'wash-out/in' at rates greater than those for elimination. Density functional theory (DFT) calculations[55] (**Figure 6b**) were used to characterize pathways for a concerted E2 elimination and intramolecular deprotonation via the ylid as internal base: this latter mechanism was computed to be kinetically more favourable by 10 kcal/mol. Ylid formation was computed to be rapid and reversible, lying uphill by 3.5 kcal/mol, consistent with the rapid equilibration observed in the labeling experiments. The subsequent transition state is thermally accessible, demonstrating the potential for a Swern-like intramolecular deprotonation event in the elimination.

Discussion ~500 words

In summary, we have developed a ready, direct, site-selective method to replace hydrogen with deuterium at the α−position of a Cys residue in both amino acid models and ~~native~~ proteins. Such α−deuterated protein substrates now prove to be useful mechanistic tools to elucidate detailed protein chemistry. Here, strikingly, they suggest that an unusual sulfonium ylid intermediate is involved in the formation of Dha from Cys (formed using bis-alkylating reagent DBHDA) and that this ylid acts as an internal base to abstract a proton from the protein backbone, which in turn leads to elimination. This is consistent with the lower pK_as of α-protons of sulfoniums as compared to those of backbone αCH-protons of amino acid residues.[56-58] Observation of both 'wash-out' and 'wash-in' suggests that the resulting Cα carbanion is sufficiently long-lived, this in turn suggests a step-wise E1cB (perhaps $E1cB_{anion}$) mechanism (**Supplementary Figure S48**). Ylid intermediacy is also supported by the fragmentation of a longer-lived sulfonium in GFP and *ab initio* calculations. Taken together this mechanism thus appears to exploit the relaying of increasingly effective kinetic basicity, using a sulfonium as an intramolecular base akin to the widely-known and used classical "Swern reaction" oxidative elimination that forms carbonyls from alcohols,[59] although other mechanisms cannot be discounted. Such a reaction has never before been implicated in a biological system.

The development of site-selective protein labelling (here D) and MS^n methodology for tracking that label has therefore proven here to be a powerful analytical combination for unpicking complex, previously unknown, protein chemistry. These have not only allowed study of a useful chemical protein modification reaction, they also reveal a more general approach to studying complex mechanistic analyses in protein chemistry. It is notable that whilst hydrogen isotope methods are very common in small molecule chemistry, they have not been applied to protein chemistry previously. Thus these methods may, in turn, may allow better understanding of parameters that determine selectivities and efficiencies of many other key protein reactions.

Given the continuing popularity of Cys as a handle,[60] the targeting of Cys (**Figure 1c**) is a useful first proof-of-principle of a potentially more general method (**Figure 1a,b**), which could in principle be applied to other electrophiles also and combined with either further modification or alteration to other amino acids.[60,61] Notably, although in this mechanistic study we have largely focused here on the use of this novel, site-selective deuteration method for the creation of alpha-deutero-Cys residues in proteins, we were also able to demonstrate in proof-of-principle experiments (see **Supplementary Information**) that a variety of protected-Cys derivatives could be installed in proteins via parallel thia-Michael reactions. These included, for example

photo-caged (and releasable) variants, thereby highlighting potential strategies for use of mechanistic probes with temporal control (e.g. through photo-'uncaging').

It is important to note that the method we use here generates epimers at the alpha position that may limit some applications.[62] Therefore, to be clear, the formation of some D-epimer renders this process traceless only with regard to the constitution of the bonds formed and not completely traceless in configuration. However, such deutero-epimers, as we show here, can be usefully used to probe mechanism. It can also be anticipated that the greatly reduced radius of gyration that arises from backbone modification as compared to strategies based on more mobile side-chain or heteroatom-D labels will allow structural interrogation with potentially reduced ambiguity in distance constraints, even despite the presence of epimers, and in the context of a low-background spectral window allowing good sensitivity.[63,64] and/or 'isotopic editing'.[65]

Finally, other speculative applications can also be envisaged. Alpha-C-deuterated amino acids such as Merck drug candidate MK0641 *Fludalanine*[66,67] have shown clear benefits and there is a recent resurgence in the use of such deuteration in drug candidates.[68,69] Through the methods we describe here, directly analogous alpha-C-deuterated deuterated *proteins* as candidate 'biologics' can now be considered and potentially readily explored.

Author Contributions

SRGG, WLN, VC conducted chemical experiments; RAS, RSP conducted computational experiments; SRGG, JRW conducted mass-spectrometric experiments; SRGG, JRW, RSP, SC, VC, BGD designed the experiments and analysed the data; SRGG, VC, BGD wrote the paper; all authors read and commented on the paper.

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Notes

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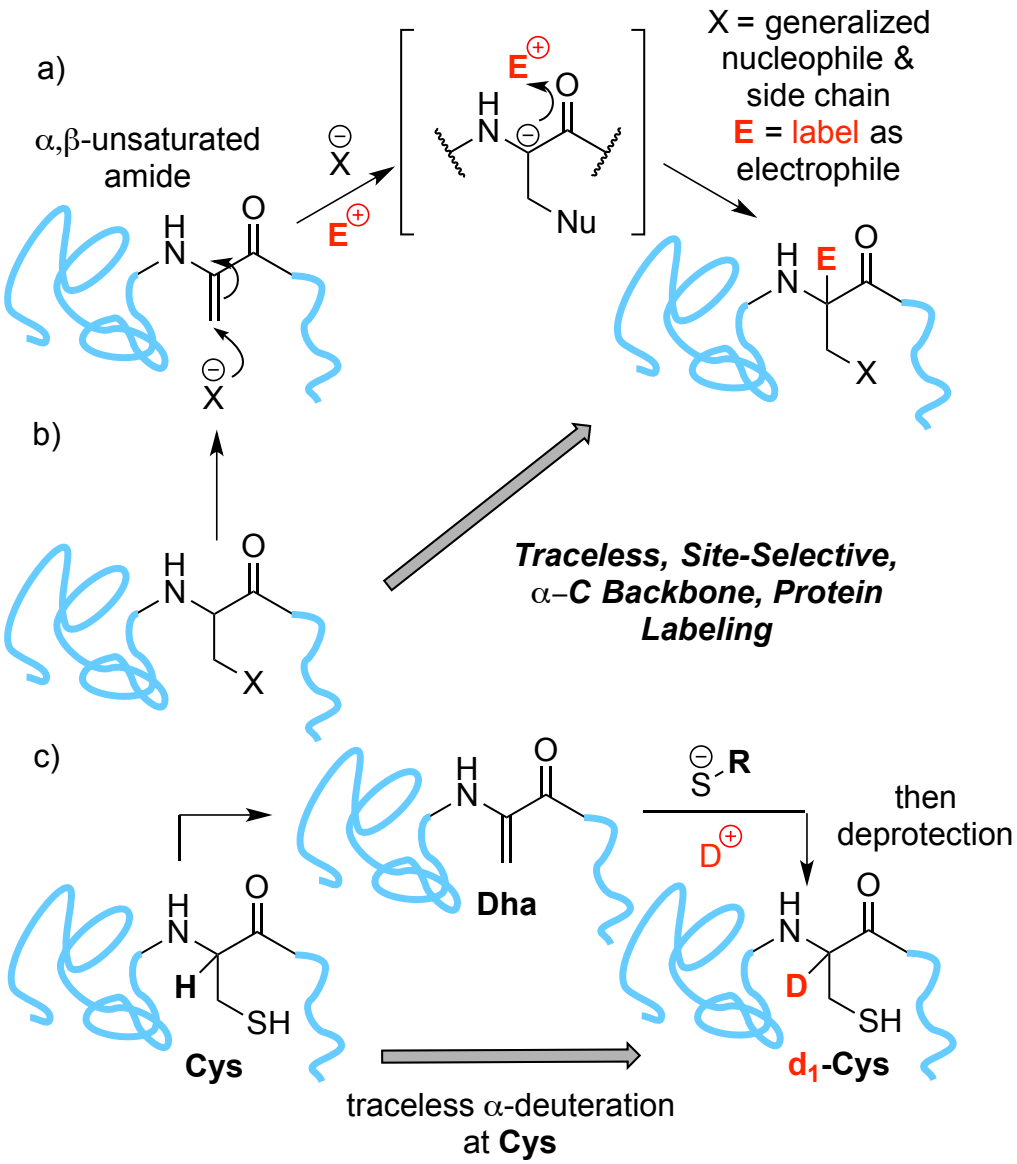

Figure 1. Traceless Post-Translational Site-selective Protein α-Deuteration Strategy (**a**) Strategy for a generalized, site-selective α-C labelling approach with any electrophile E in an intact protein (light blue ribbon cartoon) backbone. (**b**) By combining this with a method of both deriving the key α,β-unsaturated amide intermediate from side-chain X *and* forming the same side-chain X back again then the process can be rendered traceless. (**c**) Proof-of-principle H→D replacement process at the α-carbon of Cys demonstrated in this work that exemplifies the general strategy.

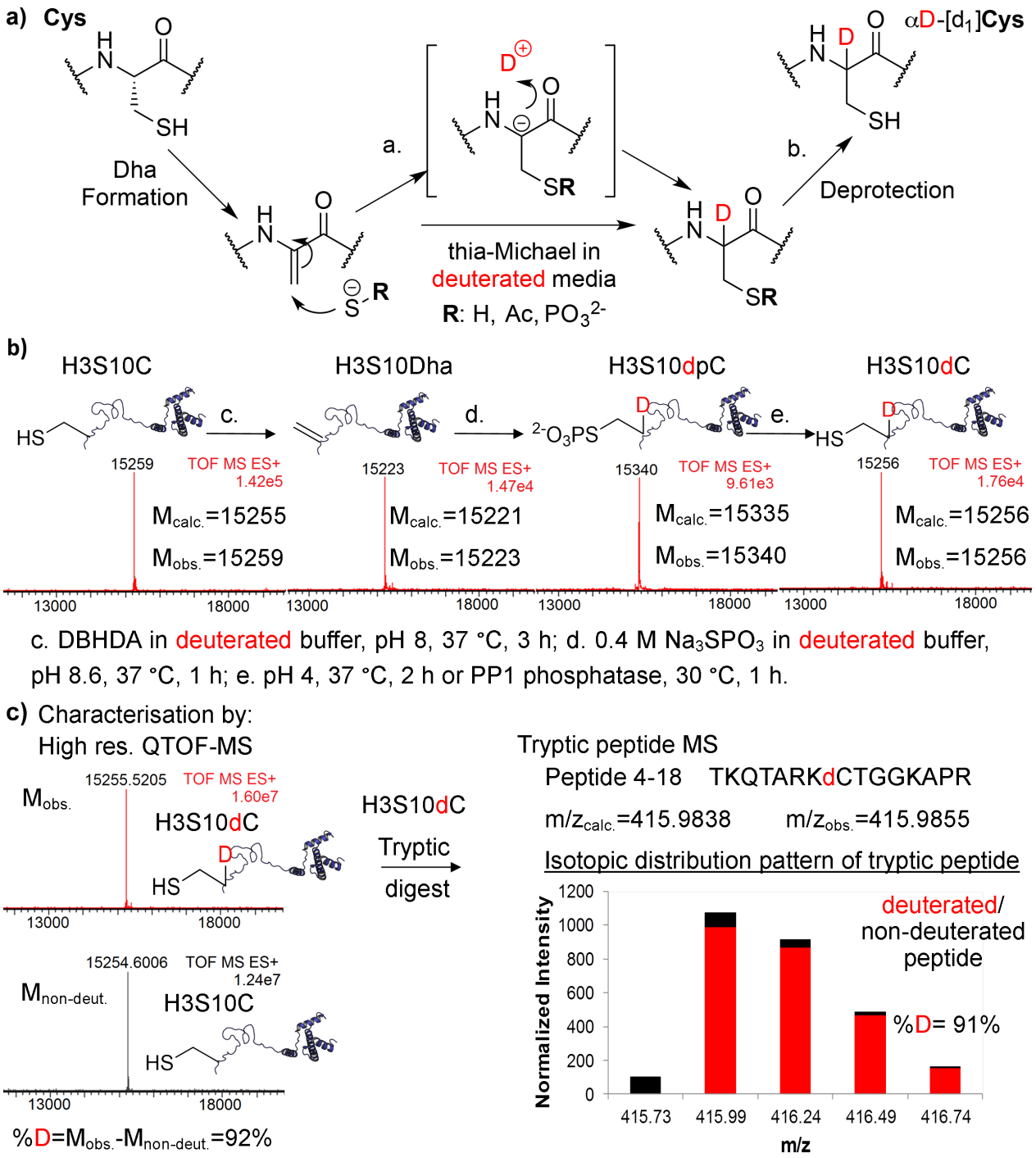

Figure 2. A Method for Site-Selective Deuteration of Intact Proteins (**a**) Formation of α-deuterated cysteine with two key intervening steps: a. thia-Michael reaction with thiol in deuterated solvent followed by b. deprotection / removal of the general 'protecting' group **R**. (**b**) Formation of deutero-H3 protein H3-dC10 (R=PO_3^{2-} shown), monitored with ESI-MS. (**c**) High resolution QTOF-MS and subsequent tryptic digest-MS analyses (as acetamide dC_{CAM}) confirmed D incorporation >90%; shown here for H3-dC10 (see **Supplementary Information** for further details).

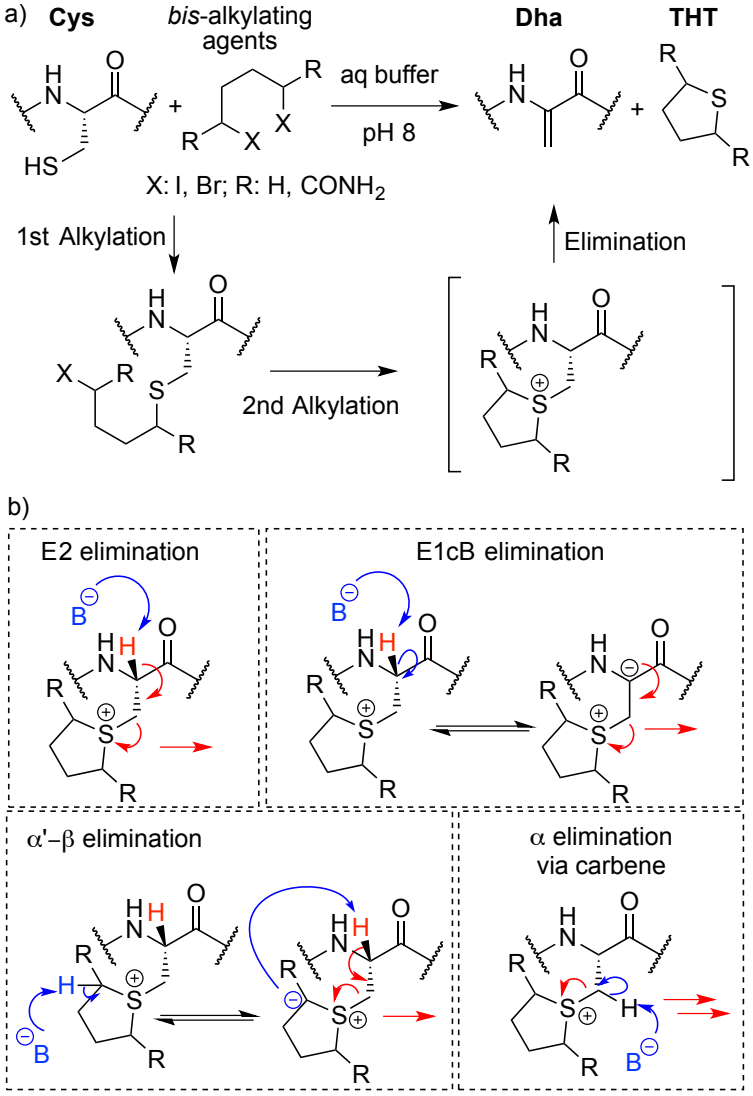

Figure 3. The Cys→Dha Protein Chemistry Reaction to be Mechanistically Probed by Deuteration. (a) Formation of **Dha** from **Cys** via double alkylation & elimination of a sulfonium intermediate and creates tetrahydrothiophene (**THT**) by-products. (b) Four broad mechanisms of elimination were considered in this study: anti-E2-elimination; two-step E1cB elimination via carbanion; α'-β elimination via sulfonium ylid; and α-elimination carbene formation (leading to Dha). B represents a generalized base (intra- or inter-molecular).

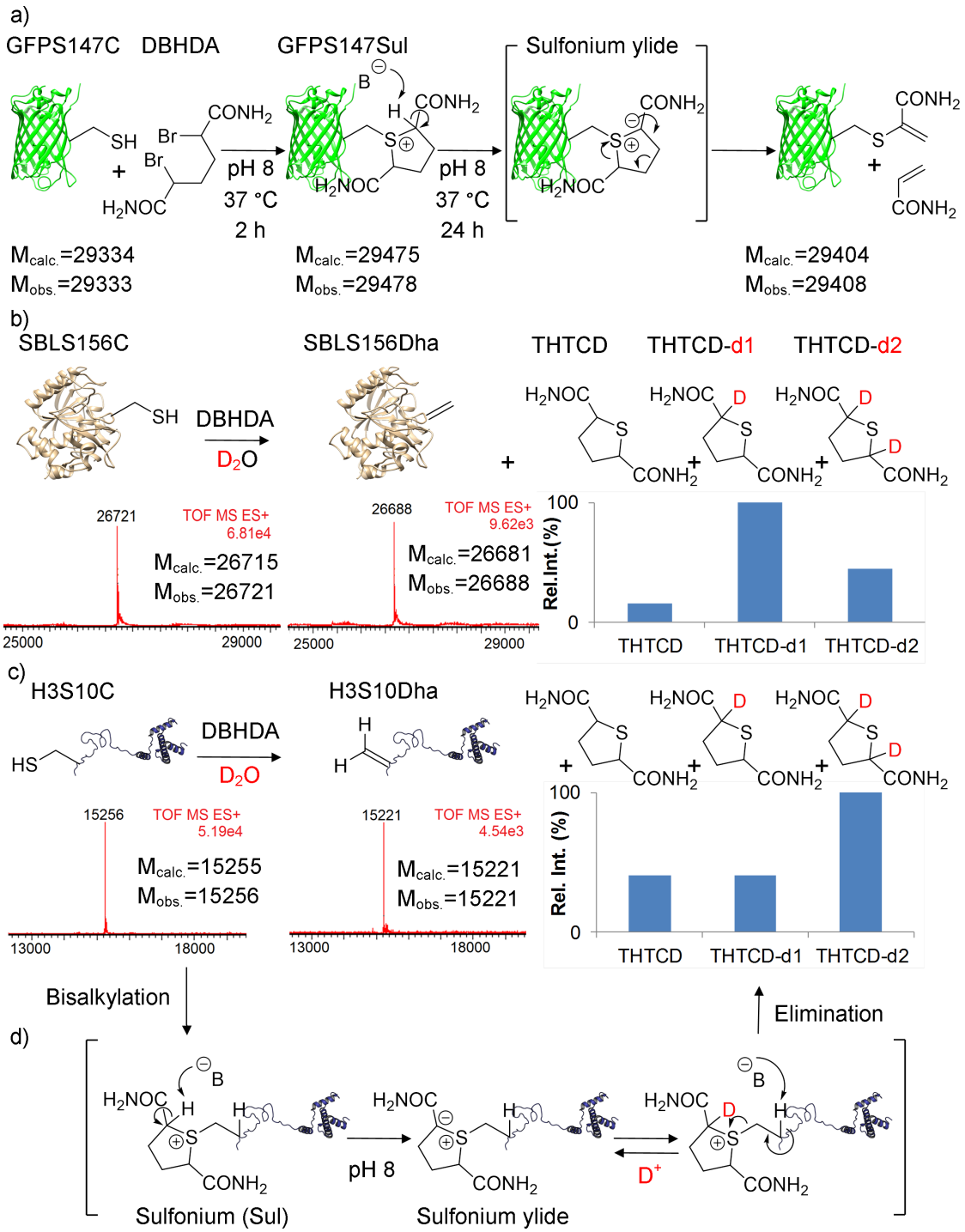

Figure 4. Exploring the Chemistry of Stable and Unstable Protein Sulfoniums with 'Wash-in'. (a) Observable GFP-S147Sul sulfonium undergoes [3+2] cycloreversion to vinyl sulfide. (b-d) Mono- & bis-deuteration of by-product THTCD observed during formation of SBLS156Dha (b) & H3S10Dha (c,d) in deuterated buffer using DBHDA confirmed a sulfonium ylid intermediate. Deuteration was determined by MS & site confirmed by MSMS; see **Supplementary Information**. (d) Proposed mechanism of D incorporation *via* sulfonium ylid.

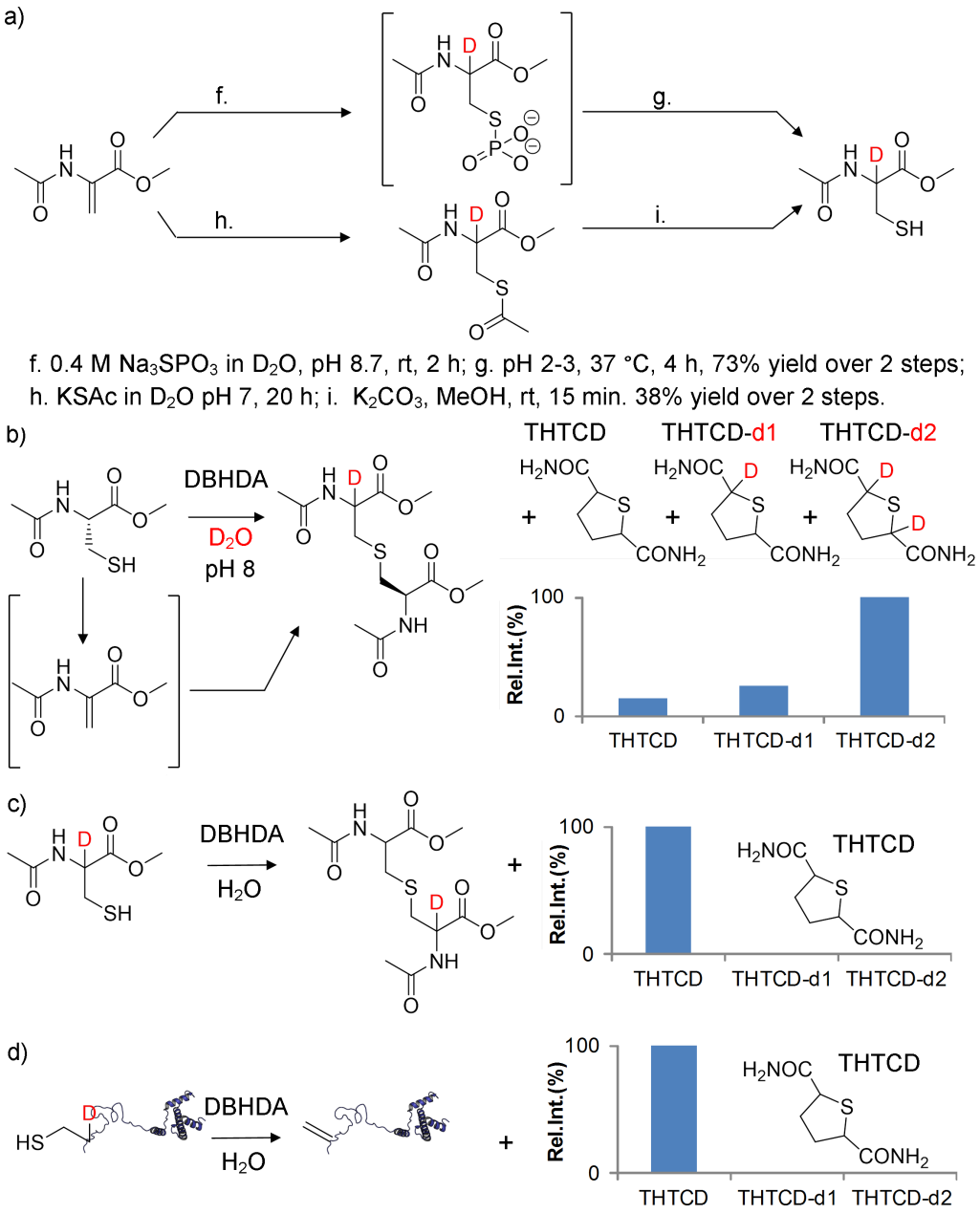

Figure 5. Application of The Post Translational α-C-deuterium Method to Explore the Chemistry of Dha Formation. (a) Alternative labelling routes to dC-containing model. Consistent with reactions on proteins, both wash-in implicating an ylid (b) and no detectable intramolecular D transfer (c) were observed upon treating AcNHCysOMe-d1 with DBHDA. (d) Analysis of eliminated THTCD from Dha formation using DBHDA on labelled H3-dC10.

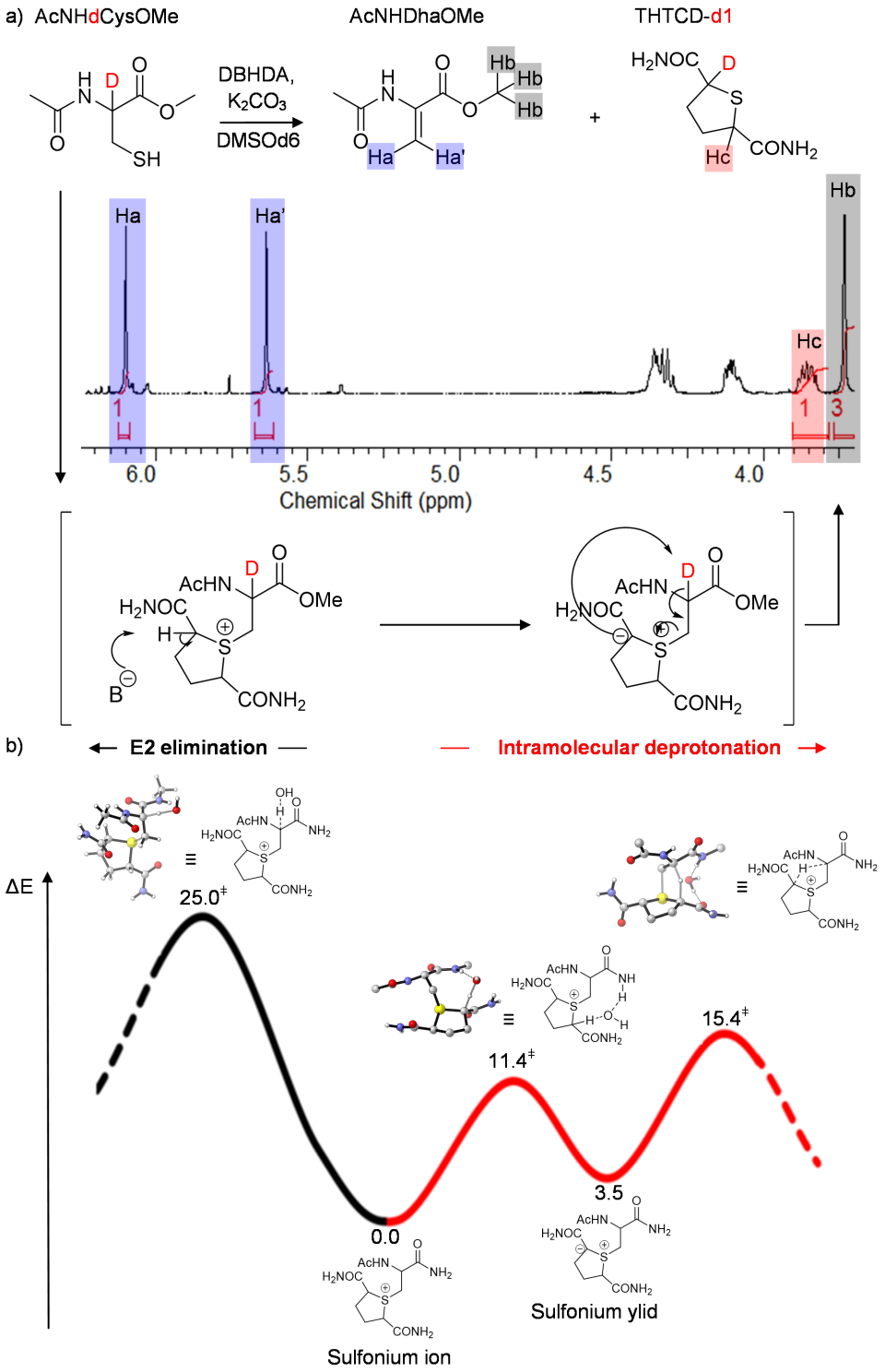

Figure 6. Mechanistic Analysis Reveals the Role of the Sulfur Ylid as an Intramolecular Base in a 'Swern Reaction'. (a) Intramolecular D transfer during formation of Dha from Cys is observed for AcNHCysOMe-d1 in aprotic solvent by both MS and NMR. (b) DFT calculations were used to characterize both E2 and intramolecular pathways; the latter passes through lower activation barriers consistent with the observed D exchange and transfer in both labelled proteins and model systems.