Why protein chemist chose phosphorus for protein bioconjugation?

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Since the introduction of Staudinger Ligation, phosphine-mediated chemoselective ligation reactions are accepted as an efficient way of *in vivo* labeling of biomolecules.[1] Nevertheless, phosphines usually direct non-canonical amino acids (e.g. azides) as their reaction partners, which on one hand enables them to react in a bioorthogonal manner. On the other hand, installation of non-canonical amino acids into protein sequences limits the scope of these reactions. Thus, chemical approaches targeting canonical amino acids for residue-specific conjugation are very desirable. In the last three decades, protein conjugation experts developed several established methods using canonical amino acids as selective conjugation sites.[2] For example, cysteine residues are usually chosen for residue-specific introduction of different molecules also due to the fact that cysteine is the second least abundant amino acid and usually exist in oxidized form (disulfide bridges). Furthermore, residues that are rarely modified or are not of great structural importance, e.g. histidine, can be targeted for such conjugation.

To date, only a few or no approaches were described to target sulfhydryls or imidazole functional groups for protein conjugation with phosphorus containing agents. In this contribution, I will comment on two recent approaches using phosphorus chemistry enabling residue-specific bioconjugation on histidine as well as cysteine.[3, 4]

HISTIDINE CONJUGATION: MIMICKING NATURE

Histidine is an essential amino acid with an imidazole group on its side-chain (Figure 1, **1**). The imidazole plays a critical role as an acid-base catalyst in catalytical triades and in the coordination of metal ions (zinc, etc.). This properties stem from its amphoteric character as a good nucleophile and leaving group, whereas this also limits its use with electrophilic agents. One possible solution to this issue might be via mimicking a natural process; histidine phosphorylation (Figure 1, **2**). Inspired by this post-translational modification, Chang and coworkers developed a method to conjugate P(V) compounds, namely thiophosphorodichloridates (Figure 1) site-specifically to histidine residues in proteins (Figure 1, **3**).[3]

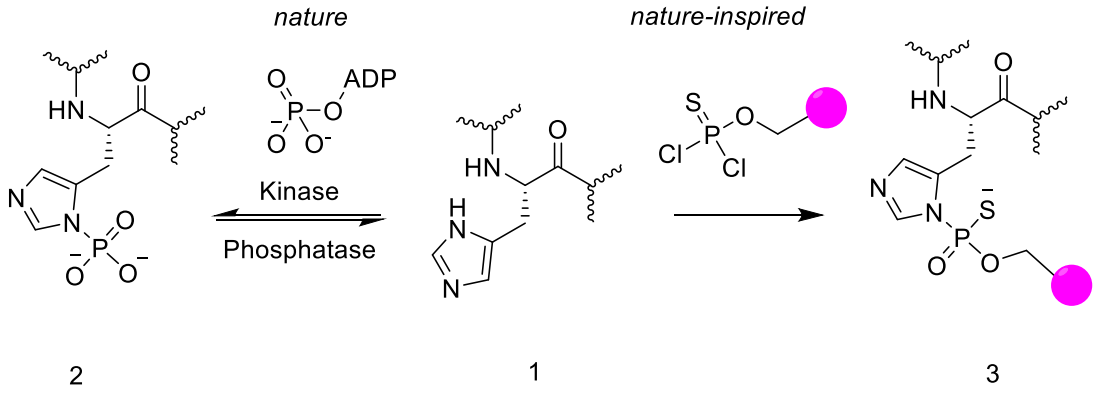

Fig. 1. Native Histidine phosphorylation vs TPAC conjugation

The researchers synthesized a variety of phosphorus-based electrophilic reagents based on the well-known phosphoramidate reaction. After several rounds of optimization, Chang and colleagues made following modifications: i) introduction of thiophosphoamidate backbone for enhanced water solubility and ii) chloride substitution as a better leaving group compared to phosphoesters. Dichloride substitution was of importance, since with phosphoesters hydrolysis to unmodified His was obtained after 11 h.

With the right compound in their hands, the researchers have drawn their attention to bioconjugation reactions under physiological (or physiologically tolerated) conditions. Hereby, they termed their conjugation reaction TPAC after the initial compound thiophosphoro alkyne dichloridate, whereas the alkyne moiety should serve for bioorthogonal conjugation with azide containing payloads.[5, 6] *In vitro* bioconjugation reactions with distinct proteins such as RNAse A, lysozyme, calmodulin and myoglobin gave rise to varying labeling efficiencies, whereas up to 60% mono and/or di-histidine labeling could be achieved. Undesired side reactions with lysine and tyrosine could be prevented via changing the labeling conditions from slightly basic (pH 8.5) to neutral (pH 7.5). Moreover, the rather acidic nature of TPAC modified Histidines allowed a cation exchange mediated separation of treated material from non-treated thus allowing a recycling of starting material.

Further attempts were made to label cell lysates and were shown to be successful as demonstrated via in-gel fluorescence experiments. As a crowning experiment, the researchers equipped a N-terminally Deca-His modified green fluorescent protein GFP (His_{10}-GFP) with TPAC and subsequently conjugated a Arg9 cell penetrating peptide, which resulted in delivery of this GFP derivative into HeLa cells, whereas non TPAC treated His_{10}-GFP $_{10}$ did not penetrated the cells.
To summarize, Chang and colleagues reported an elegant method for residue-specific labeling of proteins using thiophosphorodichloridates and demonstrated that their method is compatible with the state-of-art methods such as in-gel fluorescence as well as confocal microscopy for tracking proteins in live cells.

CYSTEINE CONJUGATION: PHOSPHORUS REVISITED

As described earlier, cysteines are desirable targets for residue-specific targeting in proteins. Indeed, maleimide mediated conjugation is a reliable and efficient method to introduce handles into proteins in a site-specific manner. However, the Retro-Michael type non-specific addition of internal thiols is a major drawback, which can be circumvented via self-hydrolyzing maleimide derivatives. In order to overcome this and other limitations, Hackenberger and coworkers expanded the scope of their Staudinger phosphonite ligation approach [7] to enable sulfhydryl-specific conjugation on proteins.[4] Hence, the researchers used an ethnylphosphonite (Figure 2, **4**) and an azide containing probe to give rise to ethnylphosphonamidate derivatives (Figure 2, **5**). The triple bond on ethynlphosphoamidates adopts a more electron-poor character than in ethnylphosphonites allowing the addition of a sulfhyrdyl group to it (Figure 2, **6**).

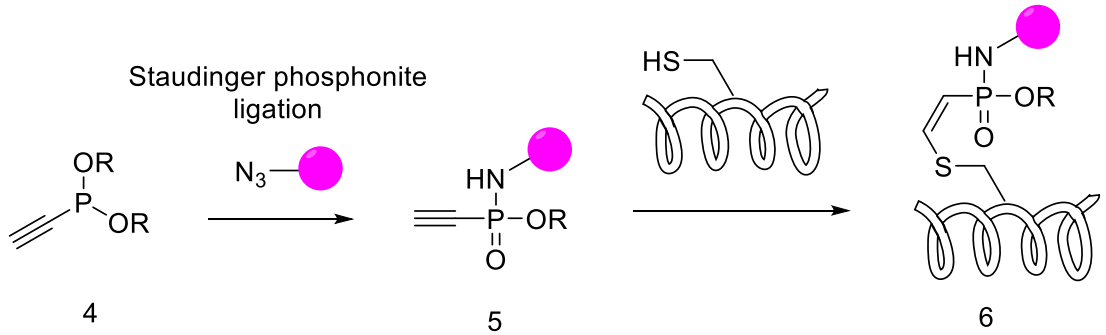

Fig. 2. Staudinger phophonite reaction and its use is cysteine labeling

Based on this hypothesis, Hackenberger and coworkers performed the first proof-of-concept reaction with glutathione under (almost) aqueous conditions to determine the pH dependency of the reaction. The optimal pH was found to be 8.5, at which also the great physical stability (several days at room temperature and months at 4°C) was not affected. With that in hand, the researchers compared the efficiency of their method to commonly used maleimide strategy exemplified by conjugation of biotin to an antibody. Hereby, maleimide showed non-specific labeling of the antibody under non-reducing conditions (without DTT), whereas the ethnylphosphoamidate did not.

Next, the serum stability of ethnylphosphoamidate conjugates were analyzed via an elegant fluorescence quenching method with a dark quencher (DABCYL, conjugated to the phosphoamidate on sulfhydryl) and EDANS fluorophore (on amide functionality). The authors followed the fluorescence signal for 24 h to observe, whether it was increased within this given time. To their delight, no fluorescence could be recorded suggesting high stability in serum. Furthermore, an *in vitro* experiment comparing the ability of the sulfhydryl-probe to migrate to other cysteine containing proteins in the serum (BSA in excess) was utilized. Hereby, the phosphamidate conjugate was compared to a maleimide conjugate, whereas the latter showed a high migration of the probe to BSA. Note that for the phophoamidate conjugate, no such migration was observed.

Encouraged by these advances, further experiments involving cellular systems were utilized. First, the authors analyzed the binding ability an antibody (Trastuzumab) phosphoamidate-fluorphore conjugate to its target Her2 via confocal microscopy. As expected, the antibody showed efficient binding to cells overexpressing Her2 and discriminated cells with low or no Her2 expression on their surface. In a further experiment, GFP was modified with a cyclic cell penetrating peptide (cCPP) via ehtynylphoshphoamidate conjugation allowing it to be taken up by HeLa cells, whereas its non-modified counterpart was not taken up as expected. The uptake efficiency was confirmed via confocal microscopy using a GFP filter.

Taken together, Hackenberger lab reported a new, residue-specific application for their well-established Staudinger phosphonite ligation with a dual labeling strategy for cysteine containing proteins. There are no known cross-reactivities with other side-chains, the conjugation occurs under almost-physiological (pH 8.5) conditions and it is applicable for a wide range of commonly-used methods by the biorthogonal protein-conjugation community.

CONCLUSION

Both publications are dealing with hot topics in biorthogonal protein conjugation field involving following questions: a) How can we target native but specific residues without interfering with their genetic properties and b) can we do it traceless and in a homogeneous manner? Interestingly, the answers to these questions given by both research groups were different, with one exception: their chemical solution was centered around phosphorus chemistry.

Chang lab have chosen histidine as their target, inspired by kinase catalyzed native His-phosphorylation reactions. Due to its important role in catalytical triades, histidine was rarely considered as a target for residue-specific conjugation of molecules into proteins. Chang and coworkers showed that histidines positioned on the active site of enzymes are not targeted for their TPAC ligation. Furthermore, Hackenberger lab demonstrated a powerful approach to obtain non-migrating and stable cysteine conjugates using a modified version of their previously published Staudinger phosphonite reaction. The gimmick to lower the electron density on the alkyne is elegant and allows installation of a variety of molecules to cysteines. Additionally, the compatibility with live cell imaging makes this method very exciting. For both methods, *in cellulo* (overexpression of a protein and subsequent conjugation in petri dish) as well as *in vivo* experiments would add up to their value and should be desirable for future applications.

In summary, one might say that the future of protein bioconjugation will likely be shaped around exploring the possibilities in native residue-specific chemistries and putting the focus on their bioorthogonality.

REFERENCES

1. E. Saxon, C. R. Bertozzi, *Science*, 2000, **287**, 2007-2010. PMID: 10720325.
2. 2. O. Boutureira, G. J. Bernardes, *Chem. Rev.* 2015, **115**, 2174−2195. PMID: 25700113.
3. S. Jia, D. He, C. J. Chang C.J., *J. Am. Chem. Soc.* 2019, **141**, 7294−7301. PMID: 31017395.
4. M.A. Kasper, M. Glanz, A. Stengl, M. Penkert, S. Klenk, T. Sauer, D. Schumacher, J. Helma, E. Krause, M. C. Cardoso, H. Leonhardt, C.P. R. Hackenberge, *Angew. Chem. Int. Ed. Engl.*, 2019, Epub ahead of print. PMID: 30828930.
5. M. R. J. Vallée, P. Majkut, I. Wilkening, C. Weise, G. Müller, C. P. R. Hackenberger, *Org. Lett.* 2011, **13**, 5440–5443. PMID: 21958352.
6. V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angew. Chem. Int. Ed.* 2002, **41**, 2596– 2599, PMID: 12203546.
7. C. W. Tornøe, C. Christensen, and M. Meldal, *J. Org. Chem.* 2002, **67**, 3057– 3064, PMID: 11975567.