Methods for Site-selective Protein Modification

in post-genome era...

DNA → RNA → protein (primary structure) → well-known

folding
higher-order architecture
posttranslational modification

Is site-selective protein modification possible?

Following conditions are necessary.
- mild condition (temp=rt, pH=neutral)
- H₂O, salt compatible
- functional group specific (OH, NH₂, SH etc.)


contents
1. Site-specific modification of artificial protein
2. Site-selective modification of "natural" protein

1. Site-specific Modification of Artificial Protein
1.1 Unnatural amino acid containing protein

"Expanding the Genetic Code"

transition metal catalyzed C-C bond-forming reaction

Regioselective Carbon–Carbon Bond Formation in Proteins with Palladium Catalysis: New Protein Chemistry by Organometallic Chemistry
Koichiro Kodama,† Seketsu Fukuzawa,‡ Hiroshi Nakayama,† Takazumi Kijima,† Kenzou Sakamoto,‡ Takashi Yabuki,‡ Natsumi Masuda,‡ Mikako Shirouzu,‡ Koji Takao,‡ Kazuo Tachibana,∗ and Shigeyuki Yokoyama∗,†,‡

ChemBioChem 2006, 7, 134

< Mizorogi-Heck reaction >

Pd, 12% DMSO in H₂O buffer (pH=8.3)

R	

I

(WT: Y32) IF-Ras protein

R

< 5 °C, 50 hr>

P(C)_3

5 °C, 50 hr

TPPTS (water soluble ligand)

(TM(biotinylated)) y. 2% byproduct (Ar-H) y. 28%

and SM recovery
Site-Specific Functionalization of Proteins by Organopalladium Reactions**

Keiichi Kodama, Seiichi Kise, Hiroshi Naito, Kenjiro Sakamoto, Takayoshi Higashiyama, Takashi Yubuki, Nobuko Matuda, Mikako Shinzumi, Koji Takio, Shigeyuki Yokoyama, and Kazuo Tachibana


azide chemistry

Staudinger Reaction

Incorporation of azides into recombinant proteins for chemoselective modification by the Staudinger ligation

Kris L. Klick*, Elaine Sizer†, David A. Thirstrup‡, and Carolyn R. Bertozzi

PNAS | January 8, 2002 | Vol. 99 | No. 1 | 19-24

Scheme 1. The Staudinger ligation between a protein containing azide-functionalized amino acid side chains and a phosphine reagent.


Fig. 6. Western blot analysis of the products of Staudinger ligation. (a) Purified protein (mDHFR-3 or mDHFR-Met) was used in the ligation, and the blot was labeled with anti-FLAG M2 mAb followed by HRP-conjugated anti-mouse IgG (1:50). Similar to a band indicated with asterisk-like symbol, (b) Challenged lysate (containing either mDHFR-3 or mDHFR-Met) was used in the ligation, and the blot was labeled with anti-FLAG M2 mAb followed by HRP-conjugated anti-mouse IgG1. (c) Similar to (b) but...
Cu(I)-catalyzed (3+2) cycloaddition


Cu(II) → Cu(I)

- In situ reduction with sodium ascorbate/ascorbic acid
- Cu(II) can be also used, less satisfactorily

Cu, CuOTf/1/2benzene, [Cu(NCCH$_3$)$_2$][PF$_6$]

Proposed catalytic cycle

Stepwise mechanism was proposed (12-15 kcal favor than B-direct/concerted)

Selective Dye-Labeling of Newly Synthesized Proteins in Bacterial Cells

Kimberly E. Beatty,$^{1}$ Fang Xin,$^{2}$ Qian Wang,$^{1}$ and David A. Tirrell$^{*1}$

Department of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125, and Department of Chemistry and Biochemistry, University of South Carolina, 631 Sumter Street, Columbia, South Carolina 29208.

J. AM. CHEM. SOC., 2005, 127, 14150 - 14151

Hpg: homopropargylglycine
Eth: ethynylaniline

Met → Hpg
Phe → Eth

Recombinant protein (barstar) was prepared.

Barstar binds barnase

its function is to inhibit the ribonuclease activity of its binding partner barnase...

Figure 2. Fluorescence of induced E. coli cells after reaction with 3. Fluorescence was also measured for uninduced cells in media supplemented with tetracycline (tch).

Table 1. Characteristics of Protein Synthesis Inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Ex (nm)</th>
<th>Em (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline</td>
<td>395</td>
<td>470</td>
</tr>
<tr>
<td>TCEP</td>
<td>350</td>
<td>440</td>
</tr>
</tbody>
</table>

Conditions:
- CuBr ligand, PBS buffer pH 7.9, 4C, 15 hr
- CuSO$_4$, ligand, TCEP, PBS buffer pH 7.9, 4C, 15 hr
- TCEP = tris(carboxymethyl)phosphine

Cell Surface Labeling of Escherichia coli via Copper(I)-Catalyzed [3+2] Cycloaddition

A. James Link and David A. Tirrell$^{*}$

J. AM. CHEM. Soc. 2003, 125, 11184 - 11185

Scheme 1. Structure of Azidohomalanine 1 and Biotin-PEO
Propargylamide 2; Biotinylation Reaction of Whole E. coli via [3+2] Cu-Mediated Azide-Alkyne Cycloaddition

Similar strategy mentioned above, but azide was incorporated into outer membrane protein C (OmpC) of E. Coli.
1.2 Tag-Fused Protein

Non-enzymatic Covalent Protein Labeling Using a Reactive Tag

Yoshihito Nonaka, Shinya Takahashi, Akio Ojida, and Itaru Hamachii

Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University,
Katsura Campus, Nishikyo-ku, Kyoto, 615-8510, Japan, and PRESTO (Life Phenomena and Measurement Analysis),
JST, Senceicho, Chiyoda-ku, Tokyo, 102-0072, Japan

JACS asap

**Figure 1.** New covalent protein labeling method reported herein.

**Figure 2.** (a) Time trace of the labeling reaction of 1-2Zn(II) with CA6D4 (●), CA6H4 (●), and SA6D4 peptide (●), (b) Summary of the initial rate (v_i, M·min⁻¹) of the labeling reaction of 1-2Zn(II) with the CA6D4 peptide (n=0, 2, 4, 6, 8), 6(PPi) means the reaction with the CA6D4 peptide in the presence of 3 mM of pyrophosphate (PPi). Reaction conditions: 20 mM 1-2Zn(II), 10 mM tag peptide in 50 mM HEPES, 100 mM NaCl, pH 7.2, 20 °C.

**Figure 3.** Covalent labeling of the CA6D4-tagged EGFP with 2-2Zn(II).
(a) MALDI-TOF mass analysis of the labeling reaction. Reaction conditions: 20 μM 2-2Zn(II), 5 μM CA6D4-EGFP in 50 mM HEPES, 100 mM NaCl, 1 mM DTT, pH 7.2, 20 °C. (The asterisk (*) is the peak of CA6D4-EGFP + matrix) (b) SDS-PAGE analysis of the labeling reaction using Coomassie staining (upper) and in-gel fluorescence visualization (lower). The analysis was performed after Huisgen reaction with coumarin azide 4. Experimental details of the Huisgen reaction are described in Supporting Information.
2. Site-specific Modification of "natural" Protein precedents

- Reaction with Electrophilic Reagents

Most reactions are for transformation of amine or thiol.

- Photoaffinity Labeling

Fig. 1. Photoaffinity labeling: principle and successive steps for the identification of amine acids.

non specific binding quenched by H$_2$O or ...

low yield, although useful method

One way for the site selective modification of protein is...

To target less common residue in the presence of abundant residue (Lys, Cys etc.)

Transition metal catalyzed reaction

R$_h$ carbenoid (Trp)
reductive amination (Lys)
mannich-type reaction (Tyr)
Tsuji-Trost reaction (Tyr)
oxidative cross-linking (Tyr)
Modification of Trp

Selective Tryptophan Modification with Rhodium Carbeneoids in Aqueous Solution

John M. Antos and Matthew B. Francis*
Department of Chemistry, University of California, Berkeley, California 94720, and
Materials Science Division, Lawrence Berkeley National Lab, Berkeley, California 94720

in aqueous media
possibility of O-H insertion?

protein sequence
many nucleophilic group (amine, thiol, OH) exist

preliminary study using 3-methylindole

\[
\begin{align*}
\text{Ph} & \quad \text{80% H}_{2}\text{O}\text{H} \\
\text{3-40 mM} & \quad \text{4 eq} \\
\text{Me} & \quad \text{1 eq}
\end{align*}
\]

\[
\begin{align*}
3-40 \text{ mM} & \\
1 \text{ eq}
\end{align*}
\]

O-H insertion
pyrazole without carbeneoid

51% combined yield

HONH₃-HCl dramatically enhanced the reactivity of catalyst.

drawback
Ethylene glycol was necessary to increase solubility.
N-alkylated and 2-alkylated products was obtained.
Reaction media of pH is low (pH≈3.5)

applicaiton to protein modification

azide 100 eq.
1 eq.

75 mM HONH₃-HCl
20% Ethylene glycol
7 h at RT

Myoglobin (100 μM)

50% conversion was observed by ESI-MS.

MS/MS analysis of doubly modified protein

y ions

b ions

Parent ion (m/z): 1215

Where the selectivity comes from?
Hydrophobic interaction between aromatic carbeneoid intermediate
and indole ring might facilitate the reaction.
Modification of Lys (-NH₂)

pH-Dependent Chemoselective Synthesis of α-Amino Acids: Reductive Amination of α-Keto Acids with Ammonia Catalyzed by Acid-Stable Iridium Hydride Complexes in Water

Sei Ogo, Keiji Uehara, Tsutomu Abara, and Shunichi Fukuzumi

JACS 2004, 126, 3020.

Reductive Alkylation of Proteins Using Iridium Catalyzed Transfer Hydrogenation

Jesse M. McFarland and Matthew B. Francis

JACS 2005, 127, 13490

**Figure 1.** Modification of lysozyme using reductive alkylation. Under the reaction conditions summarized in (a), a distribution of alkylated products results (b). (c) Control experiments lacking catalyst yielded no reaction products. Spectra shown are reconstructed from charge ladders obtained using ESI-MS analysis.

Site selectivity was confirmed using model substrate (peptide containing Lys).
Modification of Tyr
A Three-Component Mannich-Type Reaction for Selective Tyrosine Bioconjugation
Neel S. Joshi, Learna R. Whitaker, and Matthew B. Francis

Tyrosine Selective Protein Alkylation Using α-Allylpalladium Complexes
S. David Tilley and Matthew B. Francis

Oxidative Protein Cross-linking
Chemistry for the analysis of protein–protein interactions: Rapid and efficient cross-linking triggered by long wavelength light
Crosslinking Photosensitized by a Ruthenium Chelate as a Tool for Labeling and Topographical Studies of G-Protein-Coupled Receptors

Isabelle Duron-Richard,1,2 Philippe Vassault,1,2 Guy Seks,1 Jean-François Guillemin,1 Eric Richard,1 Bernard Messier,1 Claude Berthoin,1 Jacky Busn,1 and Jean-Claude Bonnefous1

Chemistry & Biology, Vol. 12, 15–24, January 2005

About Bradykinin

9-amino acid peptide chain
(Arg - Pro - Pro - Gly - Phe - Ser - Pro - Phe - Arg)
involved in the mechanism of pain

Kininogen → bradykinin → kallikrein

target human B2 bradykinin receptor (GPCR) expressed on COS-7 cell surface

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand name</th>
<th>Peptide sequence</th>
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</thead>
<tbody>
<tr>
<td>B2</td>
<td>L1 [125I]HPP-HOE 140</td>
<td>HO-CH2-CO-D-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg</td>
</tr>
<tr>
<td></td>
<td>L2 [125I]Tyr6-BK</td>
<td>Tyr[125I]-Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg</td>
</tr>
<tr>
<td></td>
<td>L3 [125I]Tyr6-BK</td>
<td>Arg-Pro-Gly-Phe-Ser-Pro-Tyr[125I]-Arg</td>
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<tr>
<td></td>
<td>L4 [125I]lys6(Tyr6)-BK</td>
<td>Lys-Arg-Pro-Gly-Phe-Ser-Pro-Tyr[125I]-Arg</td>
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<tr>
<td></td>
<td>L5 [125I]Tyr6-HOE 140</td>
<td>Tyr[125I]-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg</td>
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<tr>
<td></td>
<td>L10 [125I]Tyr6-HOE 140</td>
<td>Gly-Gly-His-Tyr[125I]-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg</td>
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<td>L12 [125I]GGHY-HOE 140</td>
<td>Biotinylated G-His-Ala-Tyr[125I]-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg</td>
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<tr>
<td></td>
<td>L13 [125I]Bio-Tyr6-HOE 140</td>
<td>Functionalized ligand</td>
</tr>
</tbody>
</table>

**A Antagonist cross-linking**

[125I]HPP-HOE 140 (L1)

<table>
<thead>
<tr>
<th>Autoradiography</th>
<th>Staining</th>
<th>Immunoblotting</th>
</tr>
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<tr>
<td>hv(s) 0 10 30 30 30 30 30 30</td>
<td>0 30 30 30 30</td>
<td>0 30 30 30 30</td>
</tr>
<tr>
<td>76 kDa (TM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>partially deglycosylated?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>yield</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-hv APS 18% 25% 30% w/unlabeled no BK2-R ligand</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B Agonist cross-linking**

Tyr positioned at N-terminal

[125I]Tyr6-BK (L2)

Tyr located at internal position

[125I](Tyr6)-BK (L3)

[125I]lys6-(Tyr6)-BK (L4)

<table>
<thead>
<tr>
<th>Autoradiography</th>
<th>Staining</th>
<th>Immunoblotting</th>
</tr>
</thead>
<tbody>
<tr>
<td>hv(s) 0 3 3 3 3 3 3 3</td>
<td>0 30 30 30 30</td>
<td>0 30 30 30 30</td>
</tr>
<tr>
<td>175 Kda</td>
<td></td>
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</tr>
<tr>
<td>137 Kda</td>
<td></td>
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</tr>
<tr>
<td>83 Kda</td>
<td></td>
<td></td>
</tr>
<tr>
<td>62 Kda</td>
<td></td>
<td></td>
</tr>
<tr>
<td>47.5 Kda</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 Kda</td>
<td></td>
<td></td>
</tr>
<tr>
<td>yield</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16% w/unlabeled BK</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The presence of a phenol moiety at the ligand N-terminal ends was required for efficient covalent labeling.
Chemical mechanism is not fully understood. And thus, receptor linking site is not well-determined.

Potential Applications of Oxidative Cross-linking to GPCR Structural and Signalling Mechanism Studies

A  B₂ Receptor

L₁₀  L₁₂  L₁₃

Kda
177
133
113
81
60
47
36
25

(1) Targeted cross-linking
(2) Spatio-temporal detection of receptor-ligand complex

Using Ru/APS crosslinking system
a, c, e: photolabelling for 5s
b, d, f: with unlabeled ligand

functionalized ligand = Gly-Gly-His or biotin-containing ligand

(1) Selective cross-linking
   (increase specificity and efficiency with auto-photo-sensitized process)
(2) Detection and purification of receptor-ligand complex
(3) Stabilization of complex
   (provide topographical information)

Site-selective Modification of Protein Would Open Up a New Field in Pharmaceutical Science

agonist

protein kinase

protein phosphatase

in vivo site-selective modification

agonist

metabolically stable

protein of interest