Protein Chemistry

DOI: 10.1002/ange.201100840

One-Pot Dual-Labeling of a Protein by Two Chemoselective Reactions**

Long Yi, Hongyan Sun, Aymelt Itzen, Gemma Triola, Herbert Waldmann, Roger S. Goody, and Yao-Wen Wu**

Multicolor labeling is a valuable technique for the characterization of proteins with respect to their structure, folding, and interactions both as single molecules and in cellular investigations. The key technique for such studies is based on fluorescence resonance energy transfer (FRET).[1] FRET applications require the attachment of donor (D) and acceptor (A) molecules to specific sites of a given protein or proteins. Such labeling is typically achieved through conjugation at cysteine residues or amino groups or by genetic fusion to different fluorescent proteins.[2–5] Recent advancements in chemical methods have substantially expanded the tools that are available for site-specific modification of proteins.[6] However, site-specific incorporation of multiple fluorophores into a single protein remains a considerable challenge. Dual labeling of a single protein has been achieved using multistep reactions. For example, sortases with different substrate specificity were used for site-specific C- and N-terminal labeling of a single protein.[7] Muir and Cotton reported a method for producing a dual-labeled protein through a multistep expressed protein ligation approach.[8] Recently, Yang and Yang used a three-step strategy based on split inteins for site-specific two-color protein labeling.[9] Herein, we report a facile and efficient method for dual-labeling of proteins based on chemoselective reactions.

Frequently used chemoselective reactions include native chemical ligation (NCL), Staudinger ligation, Huisgen 1,3-dipolar cycloaddition (click chemistry), oxime ligation, strain-promoted cycloaddition, and Diels–Alder ligation.[10] We reasoned that by employing two chemoselective reactions for protein labeling, it should be possible to obtain two-color labeled proteins in a one-pot reaction in a straightforward fashion.

Recently, we reported a method for intein-mediated incorporation of a (bis)oxyamine moiety into the C terminus of proteins, making them amenable to efficient conjugation with a keto fluorophore under mild conditions.[11] For N-terminal labeling, a protein containing an N-terminal cysteine can undergo NCL with thioester probes.[12] The exposure of an N-terminal cysteine can be achieved by TEV (tobacco etch virus) protease cleavage. Hence, we speculated that both NCL and oxime ligation could be employed for one-pot two-color labeling of a given protein.[13] Herein we present a strategy for constructing a dual-labeled Rab7 GTPase in a one-pot reaction and illustrate the use of the method for studying protein refolding and protein–protein interactions.

To generate Rab7 with an N-terminal cysteine, we fused a peptide sequence that provides a TEV cleavage site (ENLYFQ⁝C; the dotted line indicates the cleavage site) to the N terminus of the Rab7Δ3 protein. Rab7Δ3 fused N-terminally to an engineered *Mxe* GyrA intein domain can undergo initial N→S acyl transfer and be subsequently cleaved by thiol reagents (such as 2-mercapoethanesulfonic acid) by an intermolecular transthioesterification reaction, releasing a α-thioester-tagged protein.[14] Subsequently, the Rab7Δ3-thioester (10 mg mL^{-1}, 400 μM) was treated with 500 mM (bis)oxyamine at pH 7.5 to produce the oxyamine protein derivative. TEV protease was then added to cleave the N-terminal protection sequence (Scheme 1). This led to a doubly functionalized Rab7 protein with an N-terminal cysteine and a C-terminal oxyamine, N-Cys-Rab7Δ3-ONH_2, for chemoselective reactions.

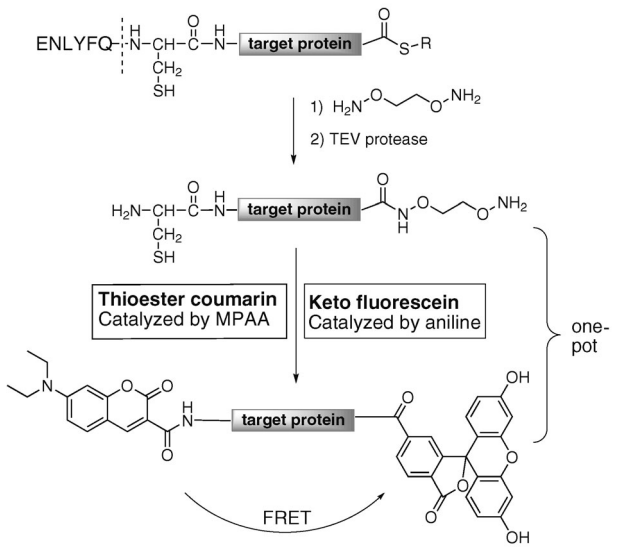
Herein, we chose coumarin thioester (quantum yield of 0.27) as FRET donor and keto fluorescein (quantum yield of 0.97) as FRET acceptor, both of which can be easily prepared from commercially available reagents (for details, see the Supporting Information). R_0 for the coumarin and fluorescein pair is 47 Å. Our first goal was to achieve quantitative conversion for both reactions. The NCL reaction for N-terminal modification was not complete after incubation for three days with 2-mercaptoethanesulfonate (MESNA) as a thiol cofactor (data not shown). The addition of 100–200 mM (4-carboxymethyl)thiophenol (MPAA) significantly accelerated the reaction,[15] and the reaction of N-Cys-Rab7Δ3-ONH_2 (1 mg mL^{-1}, 43 μM) with 0.7 mM coumarin thioester was complete in 2–3 h at pH 7.0 at room temperature or 12 h on ice with quantitative conversion (see the Supporting Information). The C-terminal oxime ligation of N-Cys-

[*] L. Yi,[+] Dr. A. Itzen, Prof. R. S. Goody, Dr. Y. W. Wu
Department of Physical Biochemistry
Max-Planck-Institut für molekulare Physiologie
Otto-Hahn-Strasse 11, 44227 Dortmund (Germany)
E-mail: yaowen.wu@mpi-dortmund.mpg.de
roger.goody@mpi-dortmund.mpg.de
L. Yi,[+] Dr. H. Sun,[+] Dr. G. Triola, Prof. H. Waldmann
Department of Chemical Biology
Max-Planck-Institut für molekulare Physiologie
Otto-Hahn-Strasse 11, 44227 Dortmund (Germany)
and
Chemische Biologie, Fachbereich Chemie
Technische Universität Dortmund
44227 Dortmund (Germany)

[+] These authors contributed equally to this work.

[**] We thank Nathalie Bleimling for excellent technical help. We thank Matthias Machner for the gift of the LidA plasmid. L.Y. acknowledges a Ph.D. fellowship from the International Max Planck Research School (IMPRS) in Chemical Biology. H.S. thanks the Humboldt Foundation for a Humboldt fellowship.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201100840.



Scheme 1. Strategy for the preparation of a two-color coumarin–fluorescein protein by one-pot chemoselective reactions.

Rab7Δ3-ONH_2 (0.5–1.0 mg mL^{-1}, 22–43 μM) with ketone–fluorescein (0.5–1 mM) can be efficiently achieved at pH 7.0 on ice in the presence of 100 mM aniline or under weakly acidic conditions.[11,16] Moreover, the cross reactions of coumarin thioester with oxyamine tagged proteins and keto fluorescein with N-Cys-Rab7 were not observed under the same conditions used for the NCL and oxime ligation reactions, respectively (Supporting Information, Figure S5, S6). Therefore, the tandem NCL and oxime ligation reactions could be used for producing two-color labeled proteins.

Encouraged by these results, we next tested whether the orthogonality of the transformations suffices for one-pot dual-labeling of proteins. We found that the oxime ligation was not affected by MPAA and coumarin thioester. Further experiments showed that one-pot dual-labeling could be achieved simply by incubation of both 0.5 mM thioester and 0.5 mM ketone molecules with 43 μM protein N-Cys-Rab7Δ3-ONH_2 on ice for one day in the presence of the catalysts 200 mM MPAA and 100 mM aniline. Quantitative conversion of N-Cys-Rab7Δ3-ONH_2 to the dual-labeled N-coumarin-Rab7Δ3-fluorescein protein was observed, as shown by ESI-MS (Figure 1a) and fluorescent SDS-PAGE. High yields of fluorescent labeled proteins are very important, because poor labeling efficiency reduces the quality of FRET measurements or requires additional purification steps. After removing excess small molecules on a desalting column, the obtained dual-labeled protein was further purified by gel filtration.

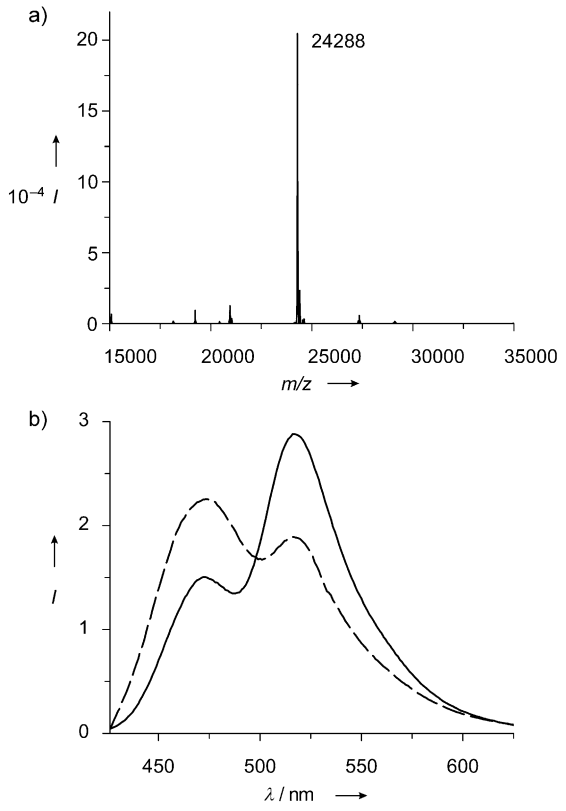


Figure 1. a) ESI-MS spectrum of coumarin-Rab7-fluorescein (M_{calcd} = 24 287). b) Emission spectra of N-coumarin-Rab7Δ3-fluorescein before (——) and after subtilisin treatment (-----). Excitation was at 400 nm.

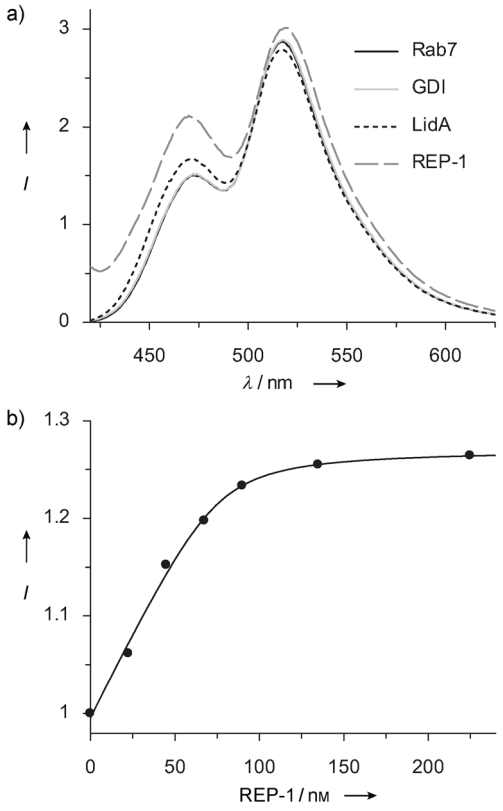


Figure 2. Binding of dual-labeled Rab7 with its effectors. a) Change of emission (excitation, 400 nm) of coumarin-Rab7-fluorescein (——) in the presence of 10 times GDI-1 (——), LidA (-----) and REP-1 (– – –). Excitation was at 400 nm. b) Titration of REP-1 to 80 nM N-coumarin-Rab7-rhodamine. The solid line shows a quadratic fit, giving a value of 3.8 nM for the K_d (excitation was at 400 nm, emission was collected at 464 nm).

As expected, the optical properties of the dual-labeled protein N-coumarin-Rab7Δ3-fluorescein are dominated by the acceptor chromophore (Figure 1b), indicating that the two fluorescent dyes located at the N- and C-terminus of Rab7 are close enough to produce an efficient FRET effect, which is consistent with the crystal structure.[17] Digestion of Rab7 by subtilisin protease led to loss of the FRET signal. The ratio of fluorescein to coumarin emission intensities (517 nm/471 nm) upon excitation at 400 nm changes from 1.92 to 0.84 on loss of energy transfer after proteolytic digestion (Figure 1b).

To demonstrate the potential use of dual-labeled proteins for protein–protein interaction studies, we examined the interaction of the dual-labeled Rab protein with the Rab binding proteins GDI (GDP dissociation inhibitor) and REP-1 (Rab escort protein-1). As expected, addition of GDI-1 does not perturb the FRET signal (Figure 2a), as GDI binds unprenylated Rab proteins only with micromolar or even lower affinity.[18] Addition of the tightly binding REP-1 to the labeled Rab led to an increase in the fluorescence intensities of both the donor and acceptor, while the ratio of acceptor to donor intensities decreased. From a previous structural analysis,[19] it was envisaged that the C-terminus of Rab7 interacts with REP-1 and becomes stretched away from the globular GTPase domain upon binding to REP-1. This would be expected to lead to an increase in the distance between the N- and C-termini of Rab7, in accordance with the decrease in FRET efficiency in the dual-labeled Rab7. The increase in fluorescence intensity may result from the fluorescence enhancement of the environmentally sensitive coumarin when it binds REP-1. This was confirmed by using a single-labeled N-coumarin-Rab7 (data not shown). When LidA, a Rab effector from *Legionella pneumophila*,[20] was added to N-coumarin-Rab7Δ3-fluorescein, the interaction led to a change of the emission ratio (517 nm/471 nm) from 1.92 to 1.67, suggesting a slight conformational change when Rab7 binds to LidA.[21]

Another dual-labeled protein, N-coumarin-Rab7Δ3-rhodamine, was prepared using the one-pot method. As there is not much overlap between the absorption spectrum of rhodamine (acceptor) and the fluorescence emission spectrum of coumarin (donor), almost no intramolecular FRET was observed for this protein, which is therefore suitable to study Rab-REP interactions based solely on the change in fluorescence intensity of coumarin (Supporting Information, Figure S1, S9). Titration data could be fitted using a quadratic equation describing the binding curve, giving a K_d of (8.0 ± 3.6) nM from three independent measurements (one is shown in Figure 2b). This is close to the result determined previously using Rab7-dansyl.[11] Our results demonstrate that the dual-labeled Rab protein retains its activity for interaction with its regulator/effector, suggesting that the labeling strategy is mild enough for protein modification.

In further experiments, we tested the use of N-coumarin-Rab7Δ3-fluorescein for protein folding studies. On addition of guanidine hydrochloride (G·HCl), the intramolecular FRET signal decreased with increasing denaturant concentrations from 0 to 8M (Figure 3a). We observed a time-dependent decrease in the FRET signal in the presence of 2M G·HCl (Figure 3b). Refolding was carried out by diluting the denatured protein into the refolding buffer (25 mM Hepes, pH 7.2, 50 mM NaCl, 2 mM $MgCl_2$, and 5 mM DTE) in the presence of guanine nucleotides. The protein was refolded with observed refolding rate constants of 0.16 and 0.24 min^{-1} in the presence of 50 μM GDP and GppNHp, respectively (Figure 3c,d), suggesting similar stabilizing effects of the two

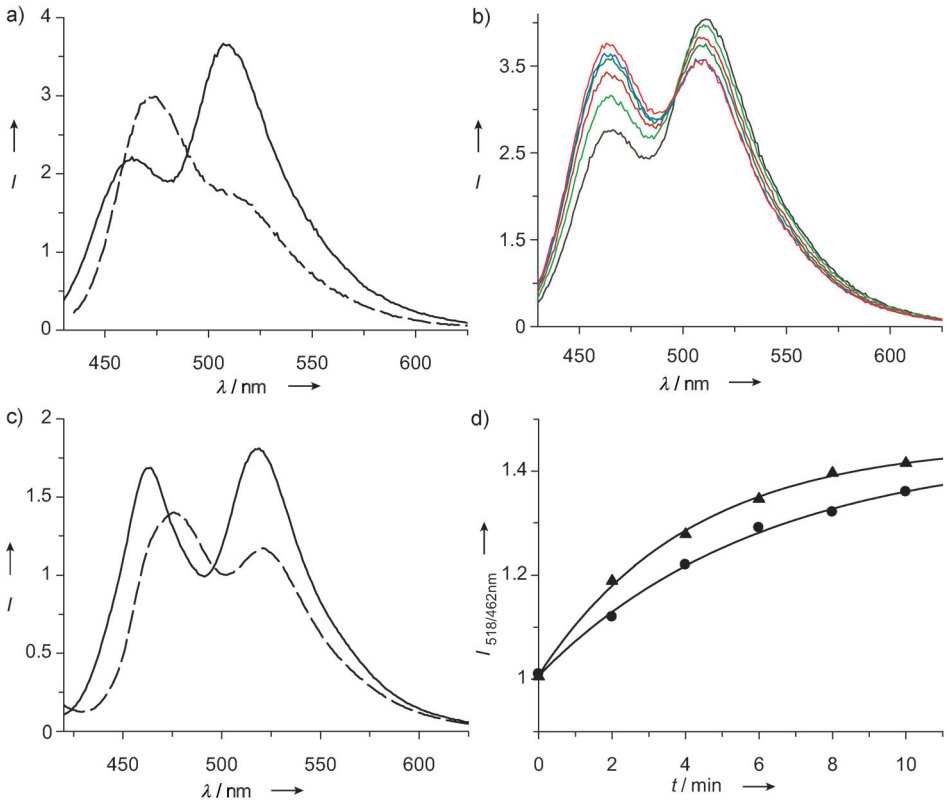


Figure 3. Unfolding and refolding of the dual-labeled protein. a) Fluorescence spectra of N-coumarin-Rab7-fluorescein before (——) and after denaturation in 8M G.HCl for 30 min (– – –). Excitation was at 400 nm. b) Emission spectra of N-coumarin-Rab7-fluorescein in 2M G·HCl. Each spectrum was recorded at 2 min intervals. $T = 0$ min (black), $t = 10$ min (pink line). c) Emission spectra of the denatured N-coumarin-Rab7Δ3-fluorescein in 6M G.HCl (– – –) and after diluting into the refolding buffer (——). Excitation was at 400 nm. d) The emission ratio of 518 nm/462 nm (excitation at 400 nm) as function of time for refolding in the presence of GDP (●) and GppNHp (▲).

nucleotides. This is in keeping with the similar affinities of the nucleotides to Rab7.[22] The refolded GDP-bound Rab7 protein is able to bind REP-1 and displays a similar response in the emission spectra as that of the native Rab7 protein, suggesting that the protein is correctly folded and functional (Supporting Information, Figure S10).

In summary, we have presented the principle of using two chemoselective reactions to site-specifically label a single protein in a one-pot reaction. We have developed a general, facile and efficient strategy for N- and C-terminal two-color labeling exemplified for the Rab7 protein using native chemical ligation and oxime ligation. The reaction conditions are biocompatible and mild enough for protein modifications. The strategy presented herein could be a general method for generating dual-labeled proteins. We have shown how the sensor can be used to study protein refolding and protein–protein interactions by FRET. This method could also have a wide range of applications for studying protein functions at the single molecule and cellular level.

Received: February 1, 2011
Revised: April 14, 2011
Published online: July 14, 2011

Keywords: chemoselective reactions · fluorescent labeling · native chemical ligation · oxime ligation · site-specific modification

[1] a) B. N. Giepmans, S. R. Adams, M. H. Ellisman, R. Y. Tsien, *Science* **2006**, *312*, 217–224; b) D. W. Piston, G. J. Kremers, *Trends Biochem. Sci.* **2007**, *32*, 407–414; c) R. Roy, S. Hohng, T. Ha, *Nat. Methods* **2008**, *5*, 507–516; d) S. Weiss, *Science* **1999**, *283*, 1676–1683.
[2] S. H. Hong, W. Maret, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 2255–2260.
[3] A. Schleifenbaum, G. Stier, A. Gasch, M. Sattler, C. Schultz, *J. Am. Chem. Soc.* **2004**, *126*, 11786–11787.
[4] A. J. Lee, A. A. Ensign, T. D. Krauss, K. L. Bren, *J. Am. Chem. Soc.* **2010**, *132*, 1752–1753.
[5] G. R. Szilvay, M. A. Blenner, O. Shur, D. M. Cropek, S. Banta, *Biochemistry* **2009**, *48*, 11273–11282.
[6] a) Y. W. Wu, R. S. Goody, *J. Pept. Sci.* **2010**, *16*, 514–523; b) J. Kalia, R. T. Raines, *Curr. Org. Chem.* **2010**, *14*, 138–147; c) B. K. V. Lim, Q. Lin, *Chem. Commun.* **2010**, *46*, 1589–1600.
[7] J. M. Antos, G.-L. Chew, C. P. Guimaraes, N. C. Yoder, G. M. Grotenbreg, M. W.-L. Popp, H. L. Ploegh, *J. Am. Chem. Soc.* **2009**, *131*, 10800–10801.
[8] G. J. Cotton, T. W. Muir, *Chem. Biol.* **2000**, *7*, 253–261.
[9] J. Y. Yang, W. Y. Yang, *J. Am. Chem. Soc.* **2009**, *131*, 11644–11645.
[10] E. M. Sletten, C. R. Bertozzi, *Angew. Chem.* **2009**, *121*, 7108–7133; *Angew. Chem. Int. Ed.* **2009**, *48*, 6974–6998, and the references therein.
[11] L. Yi, H. Sun, Y. W. Wu, G. Triola, H. Waldmann, R. S. Goody, *Angew. Chem.* **2010**, *122*, 9607–9611; *Angew. Chem. Int. Ed.* **2010**, *49*, 9417–9421.
[12] a) P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. Kent, *Science* **1994**, *266*, 776–779; b) P. E. Dawson, S. B. Kent, *Annu. Rev. Biochem.* **2000**, *69*, 923–960.
[13] a) K. Rose, *J. Am. Chem. Soc.* **1994**, *116*, 30–33; b) L. E. Canne, A. R. Ferredamare, S. K. Burley, S. B. H. Kent, *J. Am. Chem. Soc.* **1995**, *117*, 2998–3007.
[14] T. W. Muir, *Annu. Rev. Biochem.* **2003**, *72*, 249–289.
[15] E. C. B. Johnson, S. B. H. Kent, *J. Am. Chem. Soc.* **2006**, *128*, 6640–6646.
[16] A. Dirksen, T. M. Hackeng, P. E. Dawson, *Angew. Chem.* **2006**, *118*, 7743–7746; *Angew. Chem. Int. Ed.* **2006**, *45*, 7581–7584.
[17] A. Rak, O. Pylypenko, A. Niculae, K. Pyatkov, R. S. Goody, K. Alexandrov, *Cell* **2004**, *117*, 749–760.
[18] Y. W. Wu, K. T. Tan, H. Waldmann, R. S. Goody, K. Alexandrov, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 12294–12299.
[19] Y. W. Wu, R. S. Goody, R. Abagyan, K. Alexandrov, *J. Biol. Chem.* **2009**, *284*, 13185–13192.
[20] M. P. Machner, R. R. Isberg, *Dev. Cell* **2006**, *11*, 47–56.
[21] Further biophysical and structural biology studies are needed to investigate the interaction between LidA and Rab GTPases.
[22] I. Simon, M. Zerial, R. S. Goody, *J. Biol. Chem.* **1996**, *271*, 20470–20478.

