Functional Proteomics

Affinity-Based Tagging of Protein Families with Reversible Inhibitors: A Concept for Functional Proteomics**

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Dedicated to Professor Hans-Dieter Jakubke on the occasion of his 70th birthday

Proteomics, one of the most powerful techniques of the postgenomic era, relies on the two-dimensional separation of proteins.[1] However, as more than 10000 different proteins are usually expressed by a cell at the same time, resolution often remains incomplete. Sensitive detection and quantification represent further problems, because conventional staining methods are nonselective and sometimes difficult to reproduce. Detection methods with fluorescent dyes are highly sensitive, but quantification is problematic, because the dye is covalently linked to the proteins in an unselective manner prior to the separation step. Uniform labeling is not possible, and extensive labeling may lead to fluorescence quenching and solubility problems.[2] Consequently, selective labeling with reporter groups (e.g. fluorescent labels, radioactive tags, or biotin) on a mechanistic basis is desirable.

Selective labeling is possible when the reporter groups are conjugated to irreversible enzyme inhibitors and, hence, form covalent links to enzymes in the course of the enzyme reaction. Activity-based profiling has proven to be amenable for serine proteases with fluorophosphonates[3] and cysteine proteases with epoxides[4] as the irreversible inhibitors (Figure 1a). This intriguing concept suffers from the fact that irreversible enzyme inhibitors are required, which severely limits its applicability as irreversibly binding ligands are not known for many classes of proteins. As separation of protein mixtures by 2D-PAGE (2D polyacrylamide gel electrophoresis) usually involves denaturing conditions, the vast majority of protein families can only be addressed by substantial modification of this approach.

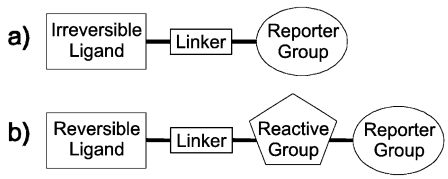

Figure 1. Mechanism-based tagging of proteins with a) irreversible and b) reversible inhibitors.

We have rendered this strategy generally applicable for protein profiling in proteomics. As a covalent bond between the proteins and the reporter group is necessary to prevent dissociation under the conditions of 2D electrophoresis, an additional chemical step is required. Only a few chemical reactions are appropriate for this kind of protein modification; among them are photoreactions, which are well-known in biochemistry and widely used for photoaffinity labeling.[5] An engineered chemical probe was designed on this basis, comprising a (semi-)specific, reversibly binding protein ligand (inhibitor) linked to a reporter group and a reactive group (photoaffinity label) (Figure 1b). This concept is suited for many different classes of proteins and may facilitate the discovery of new members of a protein family. Any method capable of decreasing the amount of data and detecting mechanistically related proteins in 1D- or 2D-PAGE will be helpful for the retrieval of hitherto unknown proteins and for the future development of proteome research. To prove the principle of our concept, the class of isoquinolinesulfonamides of the H series (H-8, H-9, etc.)[6] was envisaged as the first model inhibitors.

Isoquinolinesulfonamides competitively inhibit a broad range of kinases, including protein kinases, by occupying the ATP-binding sites. Kinases play a major role in many regulatory mechanisms of living cells. Phosphorylation/dephosphorylation reactions trigger important metabolic or pathologic pathways and participate in internal and external adaptation mechanisms. Recent advances in protein kinase research rely on systematic analyses of completely sequenced genomes, but the correlation between genome and proteome remains difficult.

Our initial experiments are directed towards the detection of plant kinases. Despite the progress in understanding detailed functions of plant protein kinases, it is still very difficult to assign the vast amount of recently identified kinase genes by systematic genomics. Serine/threonine kinases can be divided into more than twelve groups based on the sequence relationships and are of special interest in plants. In particular, chloroplasts are reported to contain several serine/threonine kinases that are functionally involved in adaptation mechanisms as biological responses to changes in environmental conditions (e.g. light).[7–9] The sensitivity of protein

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[**] This project was supported by the University of Bielefeld and the Fonds der Chemischen Industrie. The authors thank Professor Dr. J. Mattay for providing access to photochemical equipment, Dr. K. Niehaus and N. Kuepper for MALDI TOF MS analysis, Dr. M. Letzel for ESIMS analysis, and Dr. K. Stembera for helpful discussions regarding SPR analysis.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

 DOI: 10.1002/anie.200352084

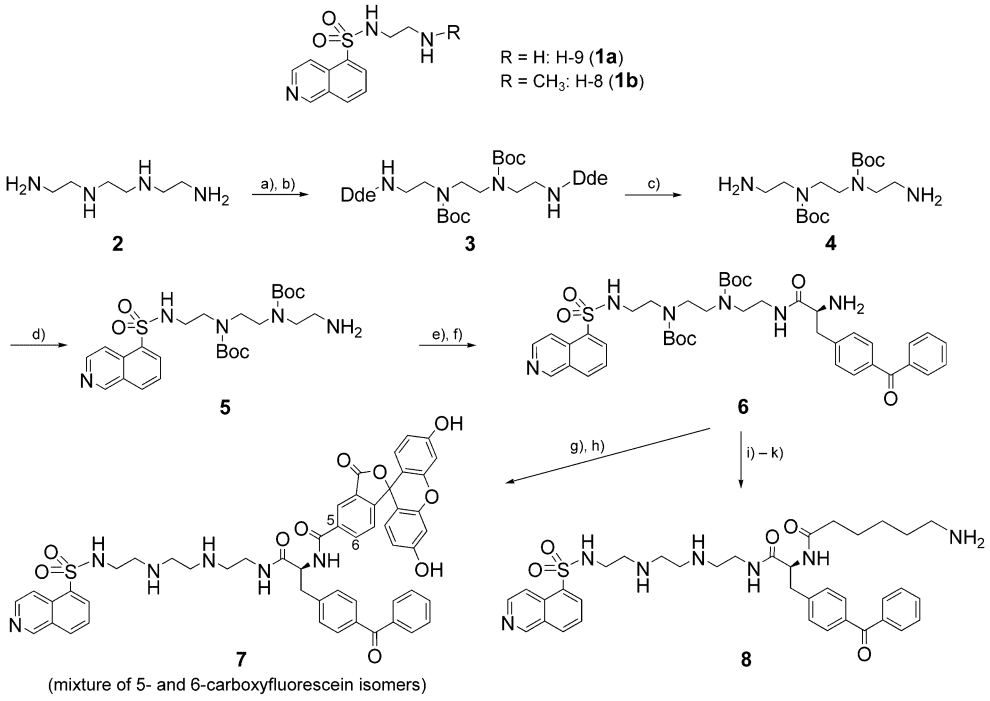
phosphorylation of the light-harvesting proteins (LHC) and photosystem II proteins to inhibitors of kinase C and cAMP-dependent kinases has been described.[10,11] The isoquinolinesulfonamide-type inhibitor H-9 (**1a**)[12] inhibits the phosphorylation of LHCII proteins at concentrations of about 50–100 μM, presumably by interacting with the ATP-binding site of membrane-associated threonine kinases. No unambiguous proof of a kinase in *Chlamydomonas reinhardtii* thylakoid membranes existed before a kinase that is assumed to be involved in LHC phosphorylation was identified only recently by a genome-based approach.[13]

The conjugate **7**, which consists of H-9 with a polar linker, a photoreactive group, and a fluorophore, was designed as an engineered chemical probe to identify kinases after covalent tagging and protein separation by SDS-PAGE (SDS = sodium dodecyl sulfate). The linker attachment was chosen on the basis of the crystal structure of a protein kinase–inhibitor complex (with H-8, **1b**) in such a way that inhibitor binding would only be minimally affected by the conjugation.[14] 4-Benzoylphenylalanine was selected as the photoreactive group, because it can be activated reversibly by excitation–relaxation cycles, and the triplet state exclusively inserts into C–H bonds within a 3.1-Å distance of the oxygen atom of the carbonyl group. It is stable in common protic solvents and leads to efficient single-site modification.[5]

The synthesis of **7** starts with protection of the primary amino groups in **2** with Dde (Scheme 1). Protection of the secondary amino functions with a Boc group gives the orthogonally protected linker **3**. Hydrazinolysis of the Dde groups yields **4**, which reacts with 5-isoquinolinesulfonyl chloride to give **5**. The isoquinolinesulfonamide **5** is then coupled to Fmoc-protected L-4-benzoylphenylalanine (Fmoc-Bpa-OH). The free amine **6** is obtained by cleavage of the Fmoc group and then coupled to the isomeric mixture of 5- and 6-carboxyfluorescein. Any other dye or any other reporter group can be used. In the final step, the Boc groups are removed to yield the target molecule **7**.

The kinase-binding ability of modified H-9 **7** was proved by surface plasmon resonance experiments (Figure 2). The conjugate **8**, an immobilizable analogue of **7** (Scheme 1), was synthesized by coupling **6** to Fmoc-6-aminohexanoic acid, followed by complete deprotection.

It was proven that protein kinase A (PKA) and creatine kinase[15] bind in a concentration-dependent manner (Figure 2a). Binding could be reduced or even totally eliminated by preincubation of the corresponding protein with a series of soluble ligands such as H-9 (**1a**; Figure 2b), **7**, and **8**.[15] These experiments clearly prove that conjugation of H-9 (**1a**) with Bpa and reporter groups does not significantly affect binding to the model kinases. The K_D value for the interaction of PKA with immobilized **8**[16] was calculated to be $(2\pm3)\times10^{-7}$ M, which is similar to the K_D value reported for **1a** and PKA (1.9×10^{-6} M).[12] Moreover, unspecific binding of a series of non-kinase proteins (hemoglobin, ovalbumin, bovine serum



Scheme 1. Synthesis of the chemical probe **7**, which comprises the kinase inhibitor moiety H-9 (**1a**), the photoreactive group 4-benzoylphenylalanine, and the fluorescent reporter group 5-/6-carboxyfluorescein. Conjugate **8** is a derivative of **7** that can be immobilized to surfaces, for example, on a surface plasmon resonance sensor chip. Reagents and Conditions: a) 2-acetyldimedone, DMF, 74%; b) Boc_2O, CH_2Cl_2, 89%; c) $N_2H_4\cdot H_2O$, CH_2Cl_2, 45%; d) 5-isoquinolinesulfonyl chloride·HCl, NEt_3, $CHCl_3$, 63%; e) EDC·HCl, NMM, Fmoc-Bpa-OH, $HOBt\cdot H_2O$, CH_2Cl_2, 68%; f) 4-(aminomethyl)piperidine, CH_2Cl_2, 85%; g) EDC·HCl, NMM, 5-/6-carboxyfluorescein (isomeric mixture), $HOBt\cdot H_2O$, CH_2Cl_2/DMF (1:1), 45%; h) TFA (30% in CH_2Cl_2), 99%; i) EDC·HCl, NMM, Fmoc-6-aminohexanoic acid, $HOBt\cdot H_2O$, CH_2Cl_2, 52%; j) 4-(aminomethyl)piperidine, CH_2Cl_2, 92%; k) TFA (30% in CH_2Cl_2), 98%. Dde = 2-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl; DMF = *N*,*N*-dimethylformamide, Boc = *tert*-butoxycarbonyl, EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, NMM = *N*-methylmorpholine, Fmoc = 9-fluorenylmethyloxycarbonyl, HOBt = 1-hydroxy-1*H*-benzotriazole.

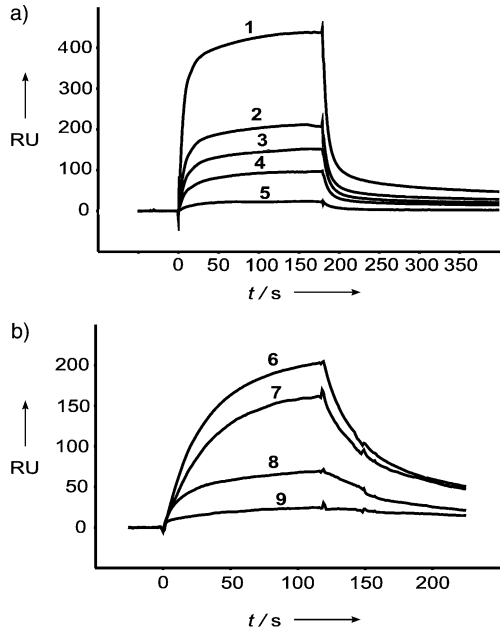


Figure 2. Surface plasmon resonance sensorgrams: a) concentration-dependent binding of cAMP-dependent protein kinase A (PKA) to immobilized **8**: 1) 120 nM, 2) 60 nM, 3) 30 nM, 4) 15 nM, 5) 7.5 nM; $K_D = (2 \pm 3) \times 10^{-7}$ M. b) Competition with H-9 (**1a**): 6) 24 nM PKA, 7) 24 nM PKA+19 nM H-9, 8) 24 nM PKA+184 nM H-9, 9) 24 nM PKA+1.85 μM H-9. RU = Response Unit.

albumin, and α-glucosidase) to immobilized **8** was shown to be negligible.

Excitation–emission spectroscopy (EES) was used to study the effects of potential radiation-free fluorescence resonance energy transfer (FRET) in **7**, particularly between the benzophenone triplet state and the neighboring fluorescein.[15,17] We found the fluorescence quantum yield to be very low for both **1a** and 4-benzoylphenylalanine ($\Phi_{Fl} \approx 10^{-4}$).

By comparing normalized emission spectra of **7** and of the analogous compound without a photoreactive group, no significant FRET could be found at $\lambda_{exc} = 350$ nm ($\Phi_{FRET} \leqslant 10^{-2}$). Energy transfer between H-9 and fluorescein was found to be negligible in all cases ($\Phi_{FRET} \leqslant 10^{-4}$). Therefore, quenching and chemical reactions are the dominant channels of loss after photoactivation at this wavelength.

In the next step, purified proteins were used for tagging experiments with **7**. Four commercially available kinases were compared to prove the specificity of the photoaffinity labeling with the photoreactive and fluorescent inhibitor **7**. Three kinases (Figure 3, lanes 1, 2, and 4) were fluorescently tagged by photoaffinity labeling with **7** to a high extent, whereas the fourth (Figure 3, lane 3), although present in a higher concentration, exhibited a very low binding tendency for **7**. The specificity of photoaffinity labeling of a particular kinase is highlighted by the fact that in the case of the hexokinase and the cAMP-dependent protein kinase A assays, only the catalytic unit is tagged, whereas the regulatory unit remains unlabeled.

To prove the feasibility of our concept for the affinity-based identification of putative kinases, creatine kinase was treated with SDS prior to incubation and photoreaction with **7**

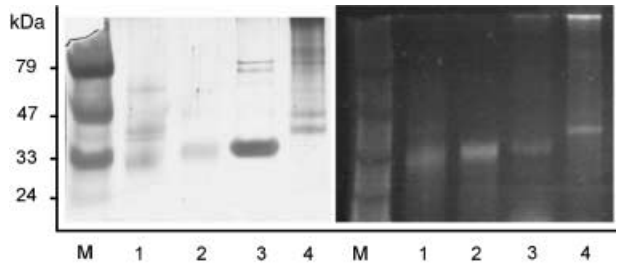


Figure 3. Specific photoaffinity labeling of different kinases with the H-9 derivative **7**. Left: silver stain; right: fluorescence image. M: prestained protein marker; Lane 1: hexokinase; lane 2: creatine kinase; lane 3: 3PGA phosphokinase; lane 4: cAMP-dependent protein kinase A.

(Figure 4A, lanes 2 and 4). The drastic decrease in fluorescence of this preincubated preparation (Figure 4A, lane 4) relative to the reference sample (Figure 4A, lane 3) clearly demonstrates that photoaffinity labeling with **7** occurs specifically, as the SDS-denatured protein was not tagged.

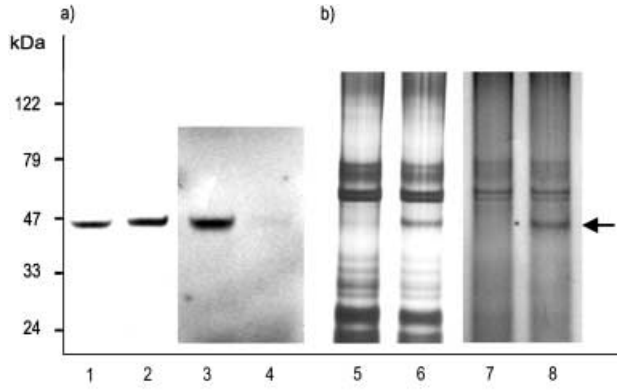


Figure 4. Selective kinase tagging by photoaffinity labeling with derivative **7**. a) Comparison of photoaffinity labeling efficiency of native creatine kinase with labeling of denatured creatine kinase. Lane 1: silver stain, native creatine kinase; Lane 2: silver stain, denatured creatine kinase; Lane 3: fluorescence image, native creatine kinase; Lane 4: fluorescence image, denatured creatine kinase. b) Retrieval of added creatine kinase from thylakoid membrane preparations after photoaffinity labeling. Lane 5: silver stain; Lane 6: silver stain, with additional 150 ng creatine kinase; Lane 7: fluorescence image; Lane 8: fluorescence image, with additional 150 ng creatine kinase (see arrow).

In a second approach creatine kinase was added to isolated thylakoid proteins prior to labeling with **7**. The results demonstrate that the H-9 derivative **7** targeted several thylakoid membrane proteins predominantly in a molecular mass range between 40 and 80 kDa (Figure 4B, lane 7). The creatine kinase that was added to the thylakoid membrane preparation could be found again (Figure 4B, lane 8). The subsequent identification of the labeled protein band at 45 kDa by MALDI TOF MS fingerprint analysis[15] as the supplemented creatine kinase clearly demonstrates the functionality of the tagging system in combination with mass

spectrometry.[18] The identification and characterization of the other labeled proteins is currently underway.

Notably, the selectivity of the labeling process with **7** is also shown by the fact that the LHC proteins (Figure 4B, proteins with molar masses < 33 kDa) are not labeled. To exclude the possibility that other photochemical processes (e.g. photooxidation) prevail over the desired CH insertion of the benzophenone triplet, photoaffinity control experiments with a conjugate that lacks the inhibitor moiety (linker-Bpa-carboxyfluorescein) and with carboxyfluorescein alone were carried out.[15]

In summary, the engineered chemical probe **7**, which comprises the reversibly binding protein ligand H-9, a fluorescent reporter group, and a photoreactive group, allows affinity-based tagging of kinases. Selective detection of kinases in functional proteomics is now possible. The concept is generally applicable to affinity-based tagging of proteins and will be exploited in the first instance for plastidial plant serine/threonine kinases by using new techniques[18] for thylakoid protein separation by 2D gel electrophoresis followed by isolation and further identification. The presence of basic groups in **7** may change the pI value of the protein after the tagging step. This could lead to a decoupling of tagged and untagged proteins in the isoelectric focusing domain of 2D gel electrophoresis. Further experiments to improve this shortcoming are currently in progress. However, the concept may, in its current state, be applied to the discovery of new kinases.

Received: June 10, 2003
Revised: September 15, 2003 [Z52084]

Keywords: fluorescent probes · inhibitors · kinases · photoaffinity labeling · proteomics

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