Fluorescent Sensors

DOI: 10.1002/anie.201208464

Monitoring of Protein Arginine Deiminase Activity by Using Fluorescence Quenching: Multicolor Visualization of Citrullination**

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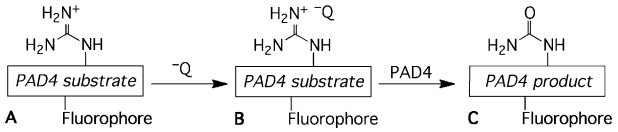
Epigenetics is driven by posttranslational modifications, such as methylation and acetylation of Arg and Lys residues. A less well publicized modification, namely conversion of Arg to citrulline (Cit), began to receive attention in the 1990s, when it was discovered that autoantibodies targeting citrulline-embedded epitopes are present in patients with rheumatoid arthritis.[1] The Arg-to-Cit conversion in histones has now been correlated with the regulation of gene expression.[2] PAD4, a member of the protein arginine deiminase (PAD) enzyme family that catalyze this transformation, is overexpressed in rheumatoid arthritis, cancer, multiple sclerosis, and glaucoma.[3] Only a few assays have been described for the PAD enzyme family and include colorimetric,[4] coupled,[5] SDS-PAGE gel,[6] affinity probe fluorescent polarization,[7] and protein array[8] approaches. We describe herein a PAD4 activity fluorescence-based sensing strategy that can be employed throughout the visible spectrum and into the near infrared. The flexibility inherent within this strategy offers the ability to simultaneously monitor the activity of PAD4 along with other enzymes that modify histones.

A particularly appealing biosensor design strategy employs fluorescence-quenched substrates that experience relief from quenching upon conversion to product. For example, protease sensors have been designed that possess a fluorophore on one side of the scissile bond and a fluorescence quencher on the other.[9] Strategies developed for other enzymes employ fluorescence quencher molecules that are stripped away from the fluorophore-containing product.[10] It occurred to us that the PAD4-catalyzed conversion of a positively charged Arg residue (compound **A**) to a neutral Cit (compound **C**) should lead to the direct release of a noncovalently associated, negatively charged quencher molecule (Q^-) from a fluorescence-quenched complex (compound **B**, Scheme 1). Furthermore, this strategy struck us as potentially applicable to a wide variety of fluorophores, thereby offering the flexibility to simultaneously visualize the action of PAD4 and other enzymes by using sensors operating at distinct wavelengths.[11]

We prepared the following analogues of Ac-Ser-Gly-Arg-Gly-Ala, which is an efficient PAD4 substrate,[12] by: a) introducing a Gly-for-Ser substitution to preclude the possibility of phosphorylation when monitoring PAD4 activity in the presence of protein kinases (see below); b) preparing multimerized versions of $(Gly\text{-}Arg\text{-}Gly\text{-}Ala)_n$ to enhance the formation of the fluorescence-quenched complex **B** (Scheme 1); c) attaching the rhodamine derivative TAMRA at the N terminus and subsequently, upon identification of an optimized *TAMRA*-$(Gly\text{-}Arg\text{-}Gly\text{-}Ala)_n$ substrate, other fluorophores.



Scheme 1. The fluorescent PAD4 substrate **A** forms a nonfluorescent noncovalent complex **B** with a negatively charged quencher dye molecule. Upon citrullination of the PAD4 substrate, the now neutral product **C** loses its affinity for the dye, and a fluorescence response is observed.

A library of 47 commercially available dyes was screened against *TAMRA*-Gly-Arg-Gly-Ala and *TAMRA*-$(Gly\text{-}Arg\text{-}Gly\text{-}Ala)_3$ (**2**, Scheme 2) to identify fluorescence quenchers (see the Supporting Information). Although *TAMRA*-Gly-Arg-Gly-Ala, with only a single Arg residue, is recalcitrant to significant fluorescence quenching, the fluorescence of the corresponding trimer is dramatically quenched by a variety of dyes. Several of the latter were examined for their ability to quench the fluorescence of *TAMRA*-$(Gly\text{-}Arg\text{-}Gly\text{-}Ala)_3$ and generate a fluorescence enhancement upon exposure to PAD4 (see the Supporting Information). The use of our lead, Acid Green 27 (**1**), in combination with the peptide trimer, results in an impressive PAD4-catalyzed (57 ± 2)-fold increase in fluorescence (Table 1). Furthermore, the tetramer, *TAMRA*-$(Gly\text{-}Arg\text{-}Gly\text{-}Ala)_4$, exhibits a (166 ± 10)-fold increase in fluorescence and an analogue, $(Gly\text{-}Arg\text{-}Gly\text{-}Ala)_2$-Lys(*TAMRA*)-$(Gly\text{-}Arg\text{-}Gly\text{-}Ala)_2$, displays a (138 ± 7)-fold enhancement in fluorescence upon citrullination.

The presumed resistance of the Cit-containing product(s) to fluorescence quenching mediated by Acid Green 27 was confirmed by preparing authentic samples containing one, two, and three Cit residues. The fluorescence of Cit-containing analogues of *TAMRA*-Gly*Xaa*GlyAlaGly*Xaa*GlyAla-Gly*Xaa*GlyAla as a function of Acid Green 27 concentration is shown in Figure 1, where *Xaa* = Arg or Cit. Fluorescence quenching by Acid Green 27 is most pronounced as follows: $(GRGA)_3$ > monosubstituted Cit > disubstituted Cit > trisubstituted Cit, thus supporting our hypothesis that the conversion of positively charged Arg to neutral Cit residues disrupts dye binding (see the Supporting Information).

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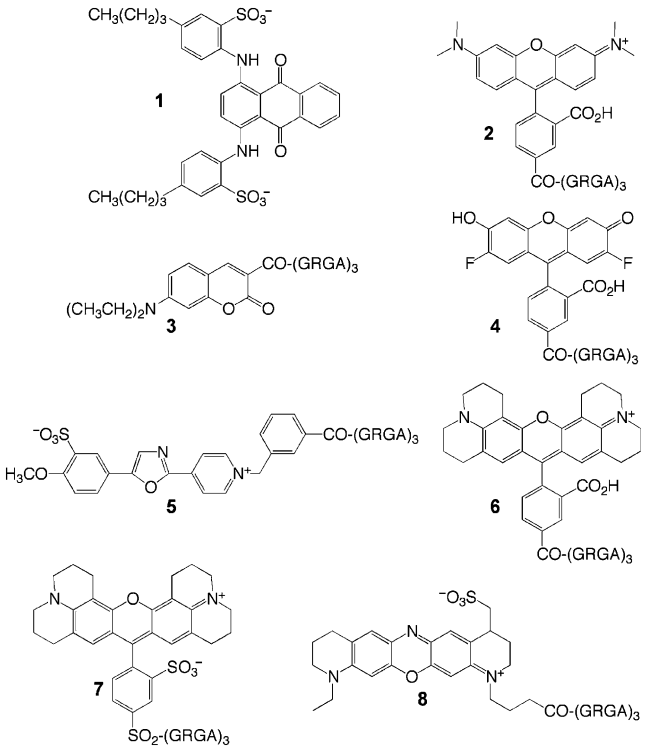
[**] We thank the NIH (CA159189) for financial support.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201208464.



Scheme 2. Structures of Acid Green 27 (**1**) and fluorophore-$(GRGA)_3$ PAD4 substrates **2**–**8**.

Table 1: Fluorescence fold change (ΔFl) and initial rate (μmol min^{-1} mg^{-1}) of PAD4-catalyzed citrullination of TAMRA substrates (λ_{em} = 585 nm)[a]

Substrate	ΔFl	Initial rate
TAMRA-GRGA	1.5 ± 0.1	N.D.
TAMRA-$(GRGA)_3$ (**2**)	57 ± 2	0.12 ± 0.01
TAMRA-$(GRGA)_4$	166 ± 10	0.021 ± 0.002
Ac$(GRGA)_2$K(*TAMRA*)$(GRGA)_2$	138 ± 7	0.033 ± 0.005

[a] 20 μM Acid Green 27, 5 μM substrate peptide, 30 nM PAD4.

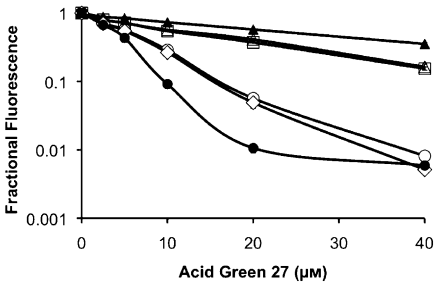


Figure 1. Fractional fluorescence of Cit-containing *TAMRA*-Gly*Xaa*Gly-AlaGly*Xaa*GlyAlaGly*Xaa*GlyAla derivatives as a function of Acid Green 27 concentration. Fractional fluorescence = fluorescence in the presence versus the absence of Acid Green 27. *Xaa* in *TAMRA*-Gly*Xaa*Gly-AlaGly*Xaa*GlyAlaGly*Xaa*GlyAla: Arg···Arg···Arg (●); Arg···Arg···Cit (◇); Cit···Arg···Arg (○); Cit···Cit···Arg (□); Arg···Cit···Cit (△); Cit···Cit···Cit (▲).

Simultaneous visualization of the activity of multiple enzymes offers the possibility of concurrently monitoring the response of several biochemical pathways to environmental stimuli. However, the response of the sensor associated with each enzymatic activity must be wavelength distinct. This lead us to explore the range of fluorophores that respond to the strategy shown in Scheme 1, which offers an assessment of the flexibility of the PAD4 assay to work in concert with reporters of other enzyme-catalyzed reactions. We prepared Fl-$(GRGA)_3$ derivatives where Fl = DEAC (**3**; λ_{em} = 480 nm), Oregon Green 488 (**4**; λ_{em} = 525 nm), Cascade Yellow (**5**; λ_{em} = 545 nm), Rox (**6**; λ_{em} = 591 nm), Texas Red (**7**; λ_{em} = 615 nm), and Atto 655 (**8**; λ_{em} = 680 nm).

Each Fl-$(GRGA)_3$ derivative was screened with the quencher library (see the Supporting Information). With the exception of Oregon Green 488, all substrates are strongly susceptible to quenching, most notably by Acid Green 27: DEAC (49 ± 1-fold at 480 nm), Cascade Yellow (55 ± 2-fold at 545 nm), Rox (133 ± 9-fold at 610 nm), Texas Red (156 ± 10-fold at 610 nm), and Atto 655 (48 ± 5-fold at 680 nm).

The large fluorescence changes associated with the PAD4 substrates are easily visualized without sophisticated instrumentation. The DEAC, Cascade Yellow, TAMRA, and Atto 655 derivatives have very different emission wavelengths and good conversion rates, and thus were selected for side-by-side photoimaging studies. With the exception of *DEAC*-$(GRGA)_3$, for which a Xe flash lamp and proper filters were employed to eliminate UV light interference with the camera, fluorophore excitation was performed with a hand-held UV lamp, and emission was photographed with a Vis-IR camera (Figure 2). In an analogous fashion, we captured color images directly from the spectrofluorimeter by integrating the Vis-IR camera with the sample chamber (see the Supporting Information).

The diversity of fluorophores compatible with PAD4 catalysis furnishes the flexibility to assemble paired fluorescent enzyme sensors that enable simultaneous monitoring of multiple enzymatic activities. For example, histones are known to suffer a wide variety of enzyme-catalyzed modifications which, in turn, influence gene expression. The cyclic adenosine-3′,5′-monophosphate (cAMP)-dependent protein kinase (PKA) is the first enzyme shown to phosphorylate histones.[13] We paired the PAD4 *TAMRA*-$(GRGA)_3$ sensor with a previously described PKA peptide-based sensor, Ac-GRTGRRDap(pyrene)SYP-amide, where Dap(pyrene) is 1-pyreneacetoyl attached to the 3-amino moiety of 2,3-diaminopropionic acid.[5,6] The fluorescence changes of PAD4 and PKA substrates were monitored at 590 and 400 nm, respectively. An increase in PKA substrate fluorescence is only

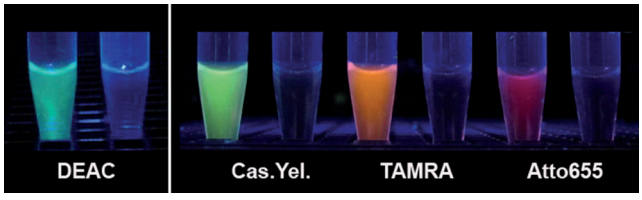


Figure 2. Visualization of fluorophore-$(GRGA)_3$ PAD4 substrates (with Acid Green 27 quencher). The *DEAC* peptide was excited with a Xe Flash lamp and all others with an Hg arc lamp. Each sample set was incubated with (left) and without (right) PAD4 for 60 min prior to image capture.

observed in the presence of PKA and, similarly, an increase in PAD4 substrate fluorescence is only observed in the presence of PAD4 (Figure 3). The activity of both enzymes can be simultaneously monitored, and the observed activity under combined conditions for each enzyme is the same as that of the enzymes alone.

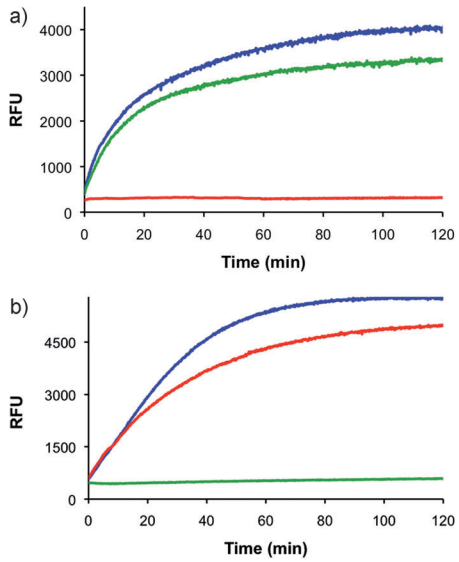


Figure 3. Multicolor monitoring of PKA and PAD4 activity. a) Observed change in relative fluorescence units ($\lambda_{ex}=340$ nm; $\lambda_{em}=400$ nm) of the PKA substrate Ac-GRTGRRDap(pyrene)SYP-amide in the presence of PAD4 (red), PKA (green), and both enzymes (blue). b) Observed change in relative fluorescence units ($\lambda_{ex}=550$ nm; $\lambda_{em}=590$ nm) of the PAD4 substrate *TAMRA*-$(GRGA)_3$ in the presence of PAD4 (red), PKA (green), and both enzymes (blue). PAD4, PKA, and their respective substrates including the quencher **1** were combined as a single mixture. Separation of results based on wavelength is provided for ease of presentation.

Finally, we examined the ability of the molecular construct **2** to detect endogenous PAD4 activity in lysates from a human promyelocytic leukemia cell line (HL-60). Since PAD4 is only found in the nucleus (confirmed by Western blot analysis; Figure 4b), we compared PAD4 activities in nuclear and cytoplasmic extracts (Figure 4a). As expected, only the nuclear extract displays PAD4 activity. Furthermore, the observed deiminase activity is Ca^{2+}-dependent and blocked by the PAD4 inhibitor minocycline; this result is both consistent with and reflective of PAD4 action on sensor **2.**

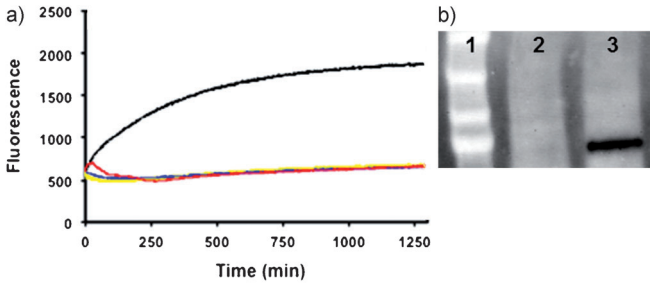


Figure 4. a) Fluorescence change of PAD4 substrate **2**/quencher **1** pair upon exposure to HL-60 nuclear extract in the presence of Ca^{2+} (black); HL-60 cytoplasmic extract in the presence of Ca^{2+} (yellow); HL-60 nuclear extract in the absence of Ca^{2+} (blue); HL-60 nuclear extract in the presence of Ca^{2+} and 2 mM minocycline (red). b) Molecular weight markers (lane 1); cytoplasmic (lane 2) and nuclear extract (lane 3) probed with PAD4 antibody.

In summary, we have created a series of PAD4 sensors that furnish a robust fluorescence response across the visible spectrum and into the near infrared. Given the demonstrated biological and biomedical significance of multiplexing nucleic acid and protein content from cells, sensors that are tunable to specific windows throughout the visible and near IR potentially enable the simultaneous monitoring of multiple enzymatic activities.

Received: October 20, 2012
Revised: December 24, 2012
Published online: January 21, 2013

Keywords: biosensors · citrullination · epigenetics · enzymes · fluorescent probes

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