CrAsH: a biarsenical multi-use affinity probe with low non-specific fluorescence†

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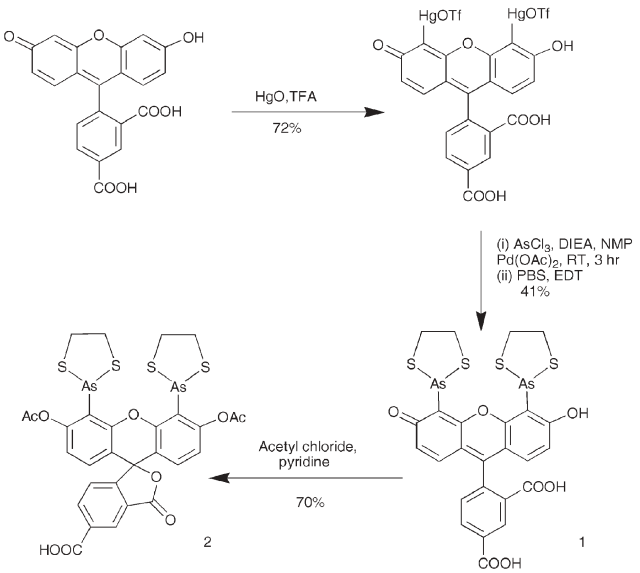
CrAsH is a tetracysteine-binding probe which has improved properties in terms of signal-to-noise ratio and pH dependence of fluorescence compared to the parent compound.

Great interest in the identification and validation of protein–protein interactions *in vitro* and *in vivo* has arisen, as it is becoming apparent that cellular information is being generated not only genetically, but also through protein localization and interactions.[1] In order to find protein interaction networks in a high-throughput fashion, a versatile tag has to be genetically added at one end of the protein, so that all proteins in the organism or pathway of interest can be treated in the same way.[2] Several multi-use affinity probe (MAP)/genetically-encoded tag platforms have been introduced.[3–5] Smaller tags have been shown to be less likely to disrupt protein function in the living cell.[6] One such small tag is the Cys-Cys-X-X-Cys-Cys (tetracysteine; Cys4) tag pioneered by Roger Tsien and coworkers,[7,8] which covalently binds biarsenical MAPs, *i.e.*, fluorescein and other dyes with the xanthene backbone derivatized with two As(III) moieties. These probes, Fluorescein Arsenical Helix binder (FlAsH) and Resorufin Arsenical Helix binder (ReAsH), have been tested for many uses, *e.g.*, *in vivo* fluorescence imaging,[8–10] protein purification,[11] protein complex identification,[12] fluorescence polarization[13] and FRET measurements.[14]

Biarsenical probes are synthesized as the ethanedithiol-capped arsenic derivatives (henceforth referred to as probe, or free probe, as opposed to probe-EDT_2) and it is their great advantage that they have low fluorescence in this form. Upon ligand exchange to a tetracysteine peptide genetically engineered onto the protein of interest (probe–Cys4-peptide complex), the fluorescence of the probe is increased. Unfortunately, in the case of FlAsH, the fluorescence is also increased upon binding to membranes and hydrophobic pockets of proteins, resulting in high background fluorescence which can be reduced by prebinding these pockets with other dyes.[15] Alternatively, a modification which renders fluorescein less hydrophobic should also reduce this non-specific binding. We chose to add a carboxy group to fluorescein to verify this hypothesis.

Careful review of the literature shows that carboxy-FlAsH (CrAsH-EDT_2, or CrAsH) had previously been synthesized as an intermediate for an affinity resin.[7] Because the increased polarity of this compound could interfere with its ability to cross the cell membrane, we also synthesized the diacetylated form acetylCrAsH



Scheme 1 Synthesis of CrAsH (**1**) and acetylCrAsH (**2**). FlAsH lacks the 5-carboxy group.

(Scheme 1). In short, CrAsH was synthesized essentially as described previously.[7] Then, acetyl chloride was used as an acetylating reagent and added into the CrAsH pyridine solution at room temperature. The reaction came to completion quickly and generated acetylCrAsH as white precipitate.

As seen in Fig. 1, CrAsH emits at 513 nm in the ethanedithiol- (Fig. 1B) and at 534 nm in the peptide-ligated forms (Fig. 1C).‡ Binding with the model Cys4-peptide Ala-Arg-Glu-Ala-Cys-Cys-Pro-Gly-Cys-Cys-Lys (AREACCPGCCK) increases fluorescence of both CrAsH and FlAsH about 35-fold. Thus, both compounds

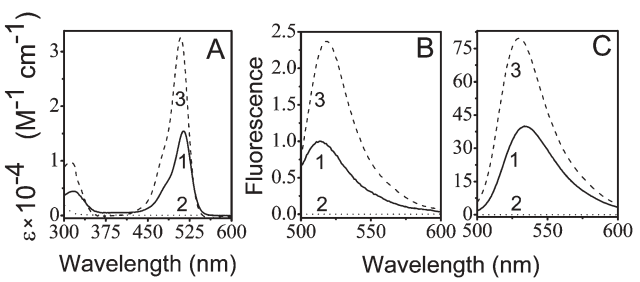


Fig. 1 Absorbance (A) and fluorescence emission spectra (B,C) of ethanedithiol- (A,B) and Cys4-peptide- (C) bound CrAsH (1), acetyl-CrAsH (2) and FlAsH (3). All fluorescence intensities are normalized to CrAsH-EDT_2.

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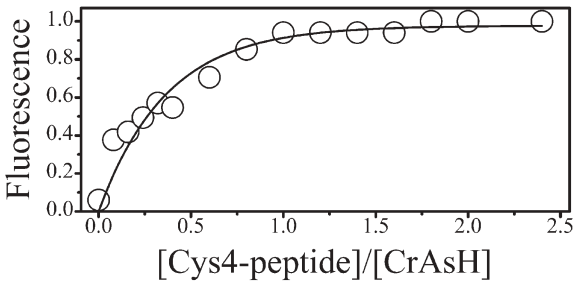

Fig. 2 CrAsH shows 1 : 1 binding with Cys4-peptide.

show the same dynamic range under these aqueous conditions, even though the CrAsH–peptide complex is a little less than half as fluorescent as the FlAsH–peptide complex. AcetylCrAsH exists in the lactone form and has almost no absorbance or fluorescence (Fig. 1A–C). Since *in vivo* acetylCrAsH is expected to be rapidly converted to CrAsH by esterase enzymes, just like acetylfluorescein to fluorescein,[16,17] we have concentrated on the *in vitro* characterization of CrAsH only.

In order to characterize CrAsH binding to the protein tag, we titrated our model Cys4-peptide into a 1 μM solution of CrAsH. As shown in Fig. 2, CrAsH binds to its target binding sequence in a one-to-one binding mode with a K_d of 407 $\pm$ 11 nM in the presence of 1 mM 2-mercaptoethanol (BME) and 1 mM tris(2-carboxyethyl)phosphine (TCEP). This affinity is one order of magnitude weaker than FlAsH under the same conditions, and virtually the same as that of the red analog, ReAsH.

It is desirable that a probe to be used *in vivo* has fluorescence properties that are stable at biological pH (7.1–7.2 in the cytosol, 7.4 extracellular, indicated with dotted lines in Fig. 3). We therefore measured the fluorescence of free and bound CrAsH and FlAsH at different pHs. Both probes have a strong pH dependence (Fig. 3); however, the CrAsH–peptide complex reaches a maximum around pH 7 and is stable above that pH. In contrast, we find that the fluorescence signal associated with the FlAsH–peptide complex is at a saddle point around pH 7. The FlAsH–peptide complex, like fluorescein,[18] has multiple pK_as, two of which we calculate to be 5.6 and 8.1 and assign to the neutral to anion, and anion to dianion transitions (Fig. 3). These results are consistent with prior reports, which found an apparent pK_a of 5.4 for FlAsH.[8] The observed pK_as are larger than those associated with fluorescein, consistent with the notion that the arsenic groups are functioning as electron donors, raising both pK_as by about one unit compared to fluorescein. In the case of CrAsH, the corresponding pK_as would be from anion to dianion, and dianion and trianion. We probably see only one transition because the trianion is energetically more unfavorable than the FlAsH dianion, raising the highest pK_a from pH 8.1 in FlAsH into the phenolic range above pH 10. CrAsH will therefore be the preferred probe for quantitative fluorescence measurements, especially important when labeled proteins move between different cellular environments or stress factors cause pH changes.

In order to test our hypothesis that CrAsH has lower non-specific binding to hydrophobic proteins than FlAsH, we titrated both free dyes with serum albumin. This protein is the most abundant protein in human serum, comprising more than 50% of total serum protein.[19] It is also a component of many cell growth media, and is rapidly taken up by cells in culture through specific receptors.[20] Titration of CrAsH with albumin results in a minimal increase in fluorescence intensity. The fluorescence intensity of CrAsH with 1.0 mM added albumin is similar to that observed for FlAsH in the absence of albumin (Fig. 4A). In contrast, there is an approximately 4-fold increase in the fluorescence intensity of FlAsH at an equal concentration of albumin, and an 11-fold increase at 1 mM albumin. This latter high affinity association between albumin and FlAsH can obscure fluorescence intensity changes associated with binding to peptide tags; indeed, upon peptide association in the presence of albumin one respectively observes a 2.1-fold and 8.5-fold increase in the fluorescence signal of FlAsH and CrAsH (data not shown). Thus, the reduced nonspecific association between CrAsH and albumin results in a dramatic improvement in the signal-to-noise ratio that should enhance the application of cell permeable probes to study protein localization and dynamics.

In conclusion, we have presented data on two green multi-use affinity probes (MAPs), which can be used to bind tetracysteine tags genetically engineered into proteins to be studied by fluorescence imaging. AcetylCrAsH-EDT$_2$ is completely non-fluorescent, but turns into the fluorescent CrAsH–peptide complex after cleavage of the acetyl group and ligand exchange. As opposed to the parent compound, FlAsH-EDT$_2$, the fluorescence of the newly characterized MAP reaches its maximum at about pH 7. As predicted, the fluorescence signal associated with CrAsH-EDT$_2$ does not change much due to to hydrophobic associations with albumin in comparison to the parent compound. This results in an

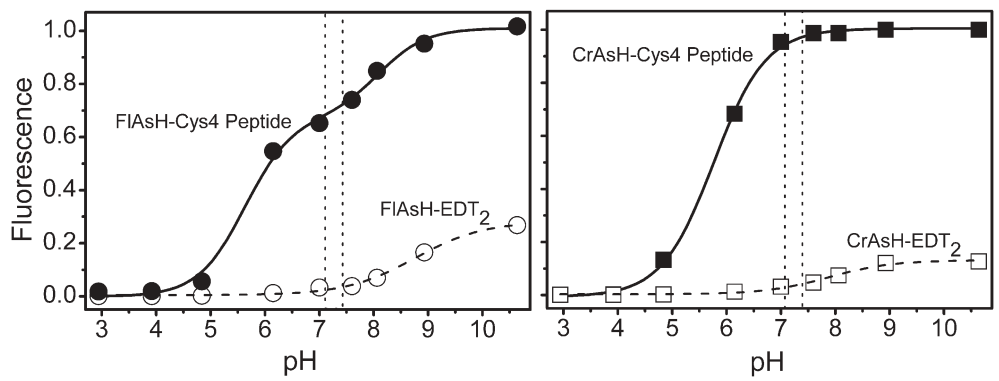

Fig. 3 Comparison of the fluorescence properties of free and peptide-bound FlAsH and CrAsH as a function of pH, using the following buffers: acetate (pH 3–5), phosphate (pH 6–8) and carbonate (pH 9–11).

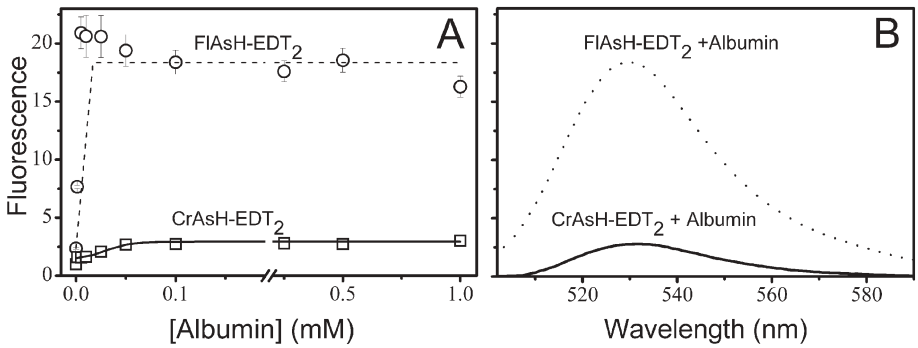

Fig. 4 Albumin-dependent changes in fluorescence intensity of FlAsH (circles) and CrAsH (squares) (A) and emission spectra in the presence of 0.25 mM albumin (B).

enhanced signal-to-noise ratio due to a greater difference between specific and non-specific fluorescence in the presence of hydrophobic moieties.

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Notes and references

‡ All fluorescence measurements were performed in 150 mM HEPES, 140 mM KCl, pH 7.5, 1 mM tris(2-carboxyethyl)phosphine (TCEP), 1 mM 2-mercaptoethanol (BME) and 1 μM ethanedithiol (EDT) with 1 μM FlAsH (λ_{ex} = 495 nm, λ_{em} = 529 nm) or 1 μM CrAsH (λ_{ex} = 495 nm, λ_{em} = 533 nm). A portion of the research was performed in the Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by DOE-OBER and located at PNNL.

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