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A small fluorophore reporter of protein conformation and redox state†

Graham J. Pound, Alexandre A. Pletnev, Xiaomin Fang and Ekaterina V. Pletneva*

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A new thiol-specific reagent introduces a small bis(methylamino)-terephthalic acid fluorophore into proteins. The noninvasive probe with distinct spectroscopic properties offers many advantages for protein labeling, purification, and mechanistic work promising to serve as a powerful tool in studies of protein folding and heme redox reactions.

There is a strong interest in site-specific protein labeling reagents that introduce small fluorophore probes.[1,2] In studies of protein folding and electron transfer (ET), small proteins, which are amenable to both theory and experiments, are the prime subjects of detailed mechanistic investigations. For them, interprobe distances derived from large chemical dyes and even larger fluorescent proteins become ambiguous. Moreover, perturbation of both the protein structure and the delicate nature of functional conformational changes upon dye labeling is a valid concern.[3,4]

Anthranilic (*o*-aminobenzoic) acid, Ant, which carries a single aromatic ring, is one of the smallest fluorophores available.[5] An intramolecular NH···O=C hydrogen bond between its amino and carboxyl groups is important for the Ant photophysical properties. This probe and its *N*-methyl derivative are popular for labeling of nucleotides[6] and peptides[7,8] but in the absence of efficient labeling reagents their applications to proteins so far have been rare and rather specialized.[9,10] Two *N*-methyl anthraniloyl-derived thiol-labeling reagents (*N*-[2-[(bromoacetyl)amino]ethyl]-2-(methylamino)-benzamide[10] and 2-[(iodoacetyl)-methyl-amino]-6-(methylamino)-benzoic acid methyl ester)[11] have been reported but they have not found widespread use in protein chemistry possibly owing to thermal degradation[10] and limited solubility in aqueous solutions. Analysis of their structures and reported properties, as well as our previous work with dye-labeled proteins,[4] suggested that an additional ionizable group at the aromatic ring could not only improve the reagent's solubility in aqueous solutions but also allow for easy chromatographic separation of labeled and unlabeled proteins. This seemingly simple strategy led us to a new water-soluble thiol-reactive reagent, Atpt iodoacetamide, **1** (Fig. 1a), based on the Ant analog, 2,6-bis(methylamino)terephthalic acid.

Department of Chemistry, Dartmouth College, Hanover, NH 03755, USA. E-mail: ekaterina.pletneva@dartmouth.edu; Fax: +1 603 646 3946; Tel: +1 603 646 0933

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The small size, ease of purification of labeled proteins, and distinct spectroscopic properties of the Atpt fluorescent probe offer a number of advantages for protein modification and mechanistic work. We show that this minimally invasive label provides multiple options for monitoring protein conformational dynamics through fluorescence resonance energy transfer (FRET) interactions with two different intrinsic chromophores, both as a donor (**D**) and an acceptor (**A**), as well as the probe quenching in folded proteins. Furthermore, owing to uncommon difference in the spectral overlap of the dye emission with absorption of the reduced and oxidized hemes (Fig. S1, ESI†), the small probe also acts as a convenient fluorescent reporter of heme redox-state changes.

Scheme S1 (ESI†) outlines a developed synthetic route to **1** starting with commercially available 3,5-dinitro-*p*-toluic acid. Reaction of **1** with *N*-acetyl-L-cysteine yielded a model compound, Atpt-Cys. Atpt-Cys shows bright blue fluorescence with λ_{max} at 459 nm in an aqueous solution, shifted to the red compared to its 2,6-bis(methylamino)benzoate analog.[11] The fluorescence

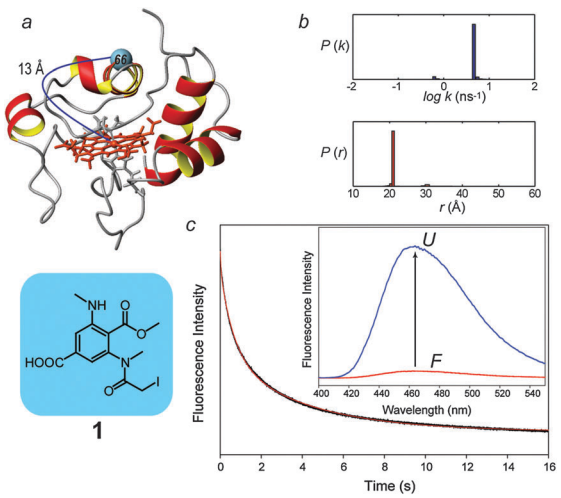

Fig. 1 (a) Atpt iodoacetamide **1** and structure of yeast iso-1 cyt *c* showing the Atpt labeling site. Indicated is the C^{α}(Residue66)–Fe distance from the crystal structure, 1YCC.[12] (b) Distributions of fluorescence decay rates, $P(k)$ and **D**–**A** distances, $P(r)$, from regularization analysis[4] of FRET kinetics for folded Atpt66-cyt *c*. The average lifetime τ_0 = 8.0 ns of the unquenched probe was used to calculate $P(r)$. (c) Refolding of Atpt66-cyt *c* from 2.0 M to 0.2 M GuHCl at pH 7.0. Inset: steady-state fluorescence spectra at pH 7.0 for the protein folded in a 100 mM sodium phosphate buffer (*red*) and unfolded in 5.9 M GuHCl (*blue*).

decay of Atpt-Cys is biexponential in a phosphate buffer at pH 7.4 (with very similar components $\tau_1 = 6.1 \pm 0.2$ ns (23%) and $\tau_2 = 8.6 \pm 0.1$ ns (77%), $\Phi = 0.28$) and monoexponential in 100% ethanol ($\tau = 9.3 \pm 0.1$ ns, $\Phi = 0.51$). Such a solvent dependence and two-component decay in aqueous solutions have been previously reported for other anthraniloyl probes.[10]

We have labeled with Atpt cysteine mutants of two representative proteins, a positively charged heme protein cytochrome *c* (cyt *c*, Fig. 1),[12] and a negatively charged D1 domain of cytoskeletal protein vinculin (Fig. 2).[13] The labeled and unlabeled proteins elute at different concentrations of salt on cation- and anion-exchange columns (Fig. S2, ESI†) providing an easy way to purify reaction products, critical for quantitative FRET work. The proteins were labeled under nondenaturing conditions and the yields of *purified* labeled proteins were above 50%. For both proteins, labeling at solvent-exposed sites did not cause perturbations in the protein secondary structure, stability (Fig. S3, ESI†) and, based on results below, tertiary structure.

Absorption and fluorescence spectra of Atpt overlap with Trp emission and heme absorption spectra, respectively (Fig. S4, ESI†), allowing FRET measurements of distances in these proteins. Analyses of Atpt quenching in cyt *c* (Fig. 1b) and Trp quenching in vinculin (Fig. 2b) yielded Atpt66–heme and Trp258–Atpt121 distances of 21 and 20 Å, respectively. These distances are consistent with crystal structures, if one accounts for the length of the probe linker[4] and conformational freedom of the C-terminal Trp258.[13] Moreover, Atpt lifetime measurements in Atpt66-cyt *c* gave a very similar fluorophore–heme distance to that in the protein derivative labeled with dansyl at the same site.[4] Guanidine hydrochloride enhances Atpt and Trp fluorescence in cyt *c* (Fig. 1) and vinculin (Fig. 2), respectively, reflecting increases in the Atpt66–heme and Trp258–Atpt121 distances upon protein unfolding. Prominent fluorescence signals with Atpt-labeled proteins enable high signal-to-noise kinetics measurements of Atpt–heme and Trp–Atpt approach during refolding.

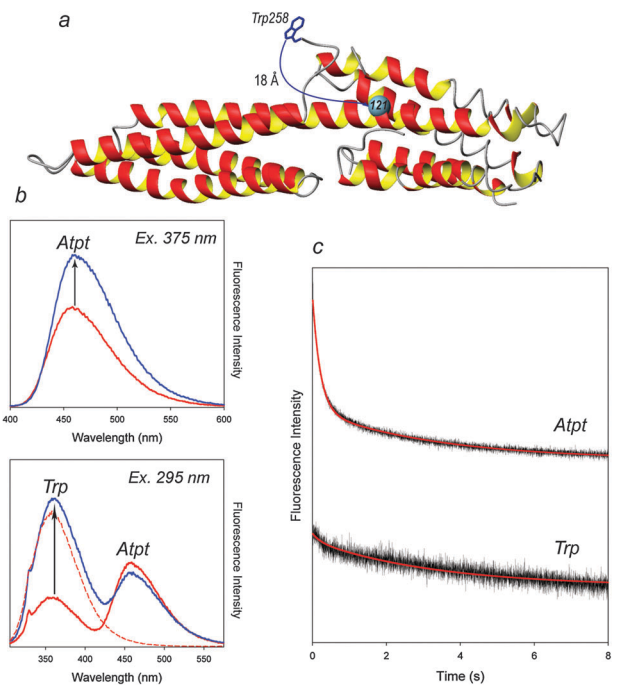

Fig. 2 (a) Structure of vinculin D1 showing the Atpt labeling site and Trp258. Indicated is the C^{α}(Residue121)–C^{α}(Residue258) distance from the crystal structure, 1RKC.[13] (b) Steady-state fluorescence spectra at pH 7.4 for Atpt121–vinculin D1, folded in a 100 mM HEPES buffer (*red*) and unfolded in 6.3 M GuHCl (*blue*). The spectrum of unlabeled folded protein is shown in a *dashed* line. (c) Refolding of Atpt121–vinculin D1 from 4.2 M to 0.4 M GuHCl at pH 7.4 monitored by decrease in Atpt (*top*) and Trp (*bottom*) fluorescence. Global fitting of the kinetics data yielded $k_1 = 4.7\ s^{-1}$ and $k_2 = 0.3\ s^{-1}$.

We find that Atpt fluorescence is considerably quenched in the vinculin D1 folded state (Fig. 2). Previous studies have found drastic reduction of the Ant quantum yield when the probe is coupled to the N^{α}-amino group of Trp compared to other amino acids.[14] High Trp concentrations do increase the rate of the Atpt-Cys fluorescence decay (Fig. S5, ESI†), suggesting that Trp could indeed act as a dynamic quencher of Atpt fluorescence when the Atpt–Trp pair comes into contact. However, the Trp-to-Atpt distance of 20 Å in vinculin is too long to support contact dynamic quenching between these sites. Instead, distinct differences in the steady-state spectra and yet very similar Atpt lifetimes for folded and unfolded Atpt-labeled vinculin D1 (Fig. 2b) suggest that the mechanism of quenching is static. Furthermore, the Atpt quenching is not a unique property of the site 121, as we have also observed a similar decrease in the Atpt fluorescence signal with folded vinculin D1 labeled at site 85.

A couple of scenarios could explain such quenching. Previous studies have indicated that only one of the two Ant rotamers contributes to the observed fluorescence spectrum.[5] Thus, similar to *cis–trans* prolyl imide bond isomerization, protein folding may change the ratio of the two Atpt rotamers, affecting the steady-state fluorescence signal but not necessarily the probe lifetime or anisotropy. Alternatively, the close interactions between Atpt and another group[15] in the folded protein could lead to rapid quenching not detectable by the fluorescence lifetime measurements. Future studies with a variety of model peptides will distinguish between these two possibilities. However, because the Atpt signal is so intense and sensitive to the protein folded state, it is already apparent that it provides another powerful option for monitoring protein folding.

Interestingly, the Atpt-labeled cyt *c* shows distinct fluorescence spectra in the ferrous and ferric states (Fig. 3), allowing for differentiation of the heme redox state by Atpt fluorescence in the labeled protein. Furthermore, the progress of heme redox reactions in Atpt-labeled cyt *c* can be conveniently monitored by fluorescence. Importantly, the observed kinetics match those monitored by traditional absorption methods.[16] Fluorescent redox-state sensors have advanced studies of copper metalloproteins,[17,18] however, applications of FRET to heme redox processes so far have been limited to the Cy5-labeled cyt *c*550.[17] Modification with the bulky red and near-IR fluorescent dyes, such as Cy5, is known to perturb the protein structure, stability, and binding interactions,[3,19,20] properties that may critically affect metalloprotein redox behavior.[21,22] Such perturbations are less likely with much smaller dyes that typically have blue or green emission. However, prominent

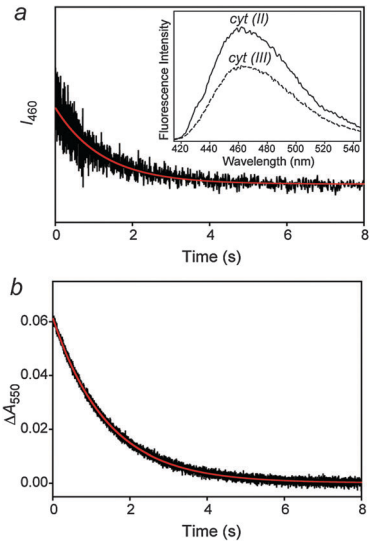

Fig. 3 Kinetics of cyt *c* oxidation by 0.73 mM $Co(phen)_3^{3+}$ in a 50mM sodium phosphate buffer at pH 7.0 monitored by (a) Atpt66 fluorescence (k_{bim} = 1.1 × 10^3 $M^{-1}s^{-1}$) and (b) heme absorption (k_{bim} = 1.0 × 10^3 $M^{-1}s^{-1}$) changes. Inset: steady-state fluorescence spectra for folded ferrous (*solid* line) and ferric (*dashed* line) Atpt66-cyt *c*.

absorption spectra of *both* ferrous and ferric hemes in this spectral region cause small differences in their overlap with emission of many popular small dyes (Fig. S1, ESI†), which makes it difficult to discriminate the two oxidation states by fluorescence. The broad Atpt emission spectrum and its favorable position of λ_{max} at 459 nm allow for the overlap with both the Soret and Q absorption bands of cyt *c* amplifying differences in the integrals $J(\lambda)$ for the reduced and oxidized hemes (Fig. S1 and S4, ESI†).

The kinetic results obtained with a small noninvasive Atpt dye suggest a valuable approach for studying redox reactions of heme proteins, particularly those involving two or more heme centers. ET in cyt *c* oxidase, *bc*1 complex, and between cyt *c* and cyt *c* peroxidase are just a few examples[21] where overlapping heme spectra complicate measurements of the heme redox changes by absorption techniques. Replacement of the heme iron by zinc in one of the heme centers for photoinduced redox reactions is one strategy to solve this problem.[23] However, the metal reconstitution procedure requires protein treatment with hydrogen fluoride, which may lead to large protein losses, and is not easily applicable to multiheme[24–26] proteins. A well-established thiol labeling procedure, which is effective even for more challenging membrane proteins,[27] provides a straightforward alternative to protein modification for studies of the thermal redox reactions with widely-available fluorescence instrumentation. Furthermore, because of the FRET distance dependence, a strategic placement of Atpt in multiheme proteins could enable changes in the redox and potentially also the ligation state of a particular heme center to be measured.

In summary, Atpt iodoacetamide is a new reagent for highly-efficient and minimally invasive fluorescent labeling of proteins. The photophysical properties of the small Atpt probe provide multiple options for monitoring protein folding and conformational changes through FRET interactions with intrinsic as well as extrinsic chromophores and the probe quenching in folded proteins. With the bright Atpt fluorescence, such studies may be potentially expanded to single-molecule work. Differences in overlap of the Atpt emission with absorption spectra of reduced and oxidized hemes afford new applications of fluorescently-labeled proteins for probing heme redox reactions.

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