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A fluorescent hydrazone exchange probe of pyridoxal phosphate for the assessment of vitamin B6 status†

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Abstract

Abnormal vitamin B6 status, marked by deficient intracellular concentrations of pyridoxal phosphate (PLP), is classified as a direct biomarker based on its biomedical significance. However, there exist no direct methods for measuring vitamin B6 status in intact cells. Here we describe the development of a fluorogenic probe, RAB6, which shows remarkable selectivity for PLP among the B6 vitamers and other cellular aldehydes.

Pyridoxal-5'-phosphate (PLP), recognized as the most active form of vitamin B6, serves as a cofactor for more than 140 different PLP-dependent enzyme activities in cells (~4% of all classified activities).¹ PLP-dependent activities are essential for amino acid biosynthesis and catabolism, and the cofactor also contributes to fatty acid biosynthesis, breakdown of certain storage compounds, synthesis of neurotransmitters, and the quenching of reactive oxygen species (ROS).^{2,3}

Unlike plants, fungi, and some microorganisms which can synthesize vitamin B6 *de novo*, humans do not encode a biosynthetic pathway for this cofactor; hence they rely on external supplies from their diet.⁴⁻⁷ Vitamin B6 is a term that refers to a group of six interconvertible vitamers consisting of 2-methyl-3-hydroxypyridine structure with variable substituents at C4 and C5 positions, including pyridoxine (PN), pyridoxamine (PM), pyridoxal (PL), and their phosphorylated derivatives (Fig. 1a).⁸ In the salvage pathway, extracellular phosphorylated B6 vitamers, which are impermeable to cells, are first converted to non-phosphorylated forms by pyridoxal phosphatases (PDXP) prior to cellular uptake (Fig. 1b). Consequently, it is essential for these organisms to re-convert the non-phosphorylated variants into PLP by intracellular pyridoxal kinase (PDXK) and pyridoxine phosphate oxidase (PNPO).

Given the many biochemical pathways that vitamin B6 supports, It is not surprising that abnormal levels of this cofactor can have a strong influence on human health. The assessment of vitamin B6 status in cells, classified as a direct biomarker, depends on the intracellular concentration of PLP, resulting from phosphatase/kinase and other interconversion processes.⁸ The most widely used method for the assessment of vitamin B6 status is to measure the plasma PLP concentration, based on the assumption that it reflects

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Conflicts of interest

There are no conflicts to declare.

cellular/tissue stores.⁹ This biochemical test has been exploited to reveal the correlation between the plasma PLP levels and many diseases such as rheumatoid arthritis, cardiovascular disease, diabetes, hypophosphatasia, epilepsy, and cancer.^{8,10}

This method is not suitable for measurement of PLP in intact cells. Direct measurement of intracellular PLP levels is desirable for a number of reasons: First, the concentration of plasma PLP in an individual significantly varies with vitamin B6 intake, while the PLP level in cells and tissues remains relatively constant.¹¹⁻¹³ Second, vitamin B6 deficiency can result from mutations in PDXK, PDXP, or PNPO, in which case plasma PLP concentration no longer reflects the intracellular PLP level.¹⁴ Lastly, direct detection of cellular PLP in intact tissue/cell samples would have high utility in the study of the many pathways that are dependent on this cofactor.

Given the chemical similarities of the vitamers and the presence of other potentially interfering aldehydes in the cell, design of a selective probe is challenging. To date there has been no report of a chemical reaction that selectively detects PLP over other vitamers. Although one might exploit reactivity with the aldehyde group of PLP, interference by the other intracellular aldehydes and ketones presents an additional challenge (Fig. 1a).¹⁵

Herein, we describe the development of a fluorescent probe that overcomes these challenges, selectively yielding a fluorescence signal for PLP. Our initial probe design incorporates the reactivity of acyl-hydrazides with aldehydes, along with the precedent of intramolecular cyclization to generate a fluorescent rhodamine. Ring-opening reactions in fluorescent rhodamine derivatives to sense metal ions have been reported by Ahn (Fig. 2a and b).^{16,17} Based on this general concept, we adopted an initial probe architecture that was designed to react with PLP *via* a two-step process: (i) hydrazone formation with the aldehyde carbonyl, and (ii) a cyclization reaction with nucleophilic attack of the carbonyl oxygen on the hydrazone (Fig. 2c). With generic aldehydes, the lactam ring of rhodamine might be expected to stay closed and remain non-fluorescent. In contrast, with PLP, the corresponding hydrazone is potentially activated by the phosphate,^{18,19} which could assist cyclization, yielding the fluorescent dye chromophore.

The first-generation acylhydrazide PLP probe structure (Fig. 2c), **RAB6**, was prepared as described.²⁰ The responses and selectivity of the probe were initially tested with a range of alkyl and aryl aldehydes at 100 μ M, near the range of their intracellular concentrations.²¹ With PLP, the probe yields a strong response (23-fold light up at 550 nm) over a period of 2 h. Responses were also examined for potentially interfering alkyl and aryl aldehydes (Fig. S5, ESI[†]). As the reactivity of both steps (hydrazone formation and cyclization reaction) changes with pH, the selectivity tests were performed both at pH 7 and 5. The data showed that the **RAB6** probe was exclusively selective toward PLP over these other aldehydes at pH 7, and remarkably even over PL, for which the only difference relative to PLP is the phosphate group (Fig. 3a). We hypothesize that, consistent with recent literature, the phosphate proton can act as a general acid to promote the hydrazone formation reaction,^{19,22} and it also likely increases the electrophilicity of the formed hydrazone to promote

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cyclization.²³ Proton NMR evidence provides support for this proposed cyclization with PLP along with its robustness (Fig. S8, ESI†). Not surprisingly, the reaction of the probe was faster and yielded higher fluorescence signals at pH 5. However, the increased reactivity in acidic conditions was compromised by an increase in nonselective signals from salicylaldehyde and PL (Fig. 3a).

The possible origins of the surprising selectivity of the probe for phosphorylated PLP were investigated with DFT calculations performed with varied background aldehydes as well as PL and PLP (Fig. 3e). The activity of the proposed cyclization reaction was estimated by the partial charges of the nucleophile and electrophile marked as red and blue stars in Fig. 2c, respectively. The results showed that the charges for salicylal and PL, which contain an ortho hydroxy group, are higher than for the adducts of simple aldehydes and pyridinal (Fig. 3c). Further, the addition of phosphate group to PL sharply increases the partial positive charge of the hydrazone. The results suggest that a combination of hydrogen bonding and the general acid/base effects of the phosphate combine to yield the special selectivity of the probe for PLP (Fig. 3f and Fig. S7, ESI†).

To investigate kinetic factors in the response and selectivity of the probe, we carried out measurements of relative rates of fluorescence response for the varied aldehydes. The data show that fluorescence intensity reaches 90% of maximum in 60 min upon the addition of PLP, while the fluorescence does not increase (indeed, it was slightly decreased) upon the addition of the other aldehydes (Fig. 3b). Notably, the fluorescence signal of the probe increases proportionally with the concentration of PLP from 50 nM (the detection limit) up to 20 μ M (Fig. 3g and Fig. S4, ESI†), suggesting its possible use in quantitative measurements. To test this, we examined whether the probe could be employed to follow the course of the biologically relevant phosphorylation of PL to PLP by pyridoxal kinase (PDXK), which transfers the phosphate from ATP in the presence of Mg^{2+} (Fig. 3h).²⁴ The high selectivity of the probe toward PLP over PL resulted in a fluorescence intensity increase over 9 hours, while no such signal was seen in the absence of PDXK (Fig. 3i and j). As this process is one of the major pathways by which intracellular PLP is supplied,²⁵ the results suggest the possibility of applying this probe to measure the endogenous vitamin B6 status in intact cells.

Prior to exploring measurements of intracellular PLP, two potential concerns regarding **RAB6** function were considered: (i) If the probe reacted with general aldehydes prior to PLP, the corresponding hydrazone(s) might not be replaceable by PLP, leading to attenuated signals; and (ii) the oxidation of the probe in cells might cause false-positive signals. First, to validate the replacement of the hydrazones resulting from different aldehydes by PLP, **RAB6** was pre-incubated with varied aldehydes for 30 min, followed by addition of PLP. Significantly, all hydrazone intermediates tested were successfully replaced by PLP at both pH 5 and 7, as judged by TLC and fluorescence intensities (Fig. S1, ESI†). We hypothesize that the protected probe undergoes hydrazone exchange *via* a mechanism observed previously with a hydrazone probe for other aldehydes.²⁶ Secondly, according to published studies,^{27,28} a hydrazide moiety in such a rhodamine scaffold is oxidizable by hypochlorous acid (HOCl), which consequently yields a fluorescent signal. We found that **RAB6** also showed a fluorescence response upon the addition of a hypochlorous acid source (Fig. 4d).

Although the fluorescence intensity upon oxidation was significantly lower than that from PLP at the same concentration, the false-positive signal from the oxidation may not be negligible in some cases depending on relative cellular concentrations (reports suggest that local cellular concentration of HOCl can be as high as 5 mM in the airway of individuals with inflammatory diseases).²⁹

To avoid these potential undesired signals, we adopted a hydrazone exchange strategy, protecting the **RAB6** with a non-signaling aldehyde group, which should hinder reactions with nonspecific aldehydes and suppress the signal upon oxidation as well (Fig. 4a, 4b), while remaining reactive to PLP. The protection of the probe was tested with three different aldehydes, evaluating the undesired (false) positive fluorescence from sodium hypochlorite (Fig. S3, ESI[†]). Among tested aldehydes, the adduct with acetaldehyde showed the lowest false-positive signal. Based on this result, **Ac-RAB6**, the probe protected as the acetaldehyde hydrazone, was synthesized for the cellular experiments. **Ac-RAB6** exhibited negligible false positive signals upon the treatment with NaOCl, while reacting with PLP nearly as rapidly as **RAB6** and maintaining the selectivity (Fig. 4c, d and Fig. S6, ESI[†]).

Ac-RAB6 was then exploited for detecting endogenous PLP in living cells (Fig. 4f). The fluorescence intensity of the probe rose as the dose of PL increased in HeLa cells, which implies the PL supplied to the cells was converted into PLP by PDXK. As a control experiment, PDXK-attenuated HeLa cells were prepared by using siRNA to suppress the pathway converting PL to PLP (Fig. 4e). The fluorescence intensity of the probe in PDXK knock-down cells was significantly lower than in intact HeLa cells, reflecting lower conversion of PL to PLP in addition to endogenous PLP. PLP levels in PDXK deficient cells were maintained similar to those in intact cells most likely by PNPO which converts PMP and PNP into PLP (Fig. 1b). However, PLP-production level in PDXK knock-down cells upon the addition of exogenous PL was observed to be significantly lower compared to the intact cells (Fig. 4f and g). The results document the capability of **Ac-RAB6** to detect endogenous PLP in intact cells, which enables the direct assessment of vitamin B6 status.

In summary, we have described a hydrazone light-up probe design that can discriminate pyridoxal-5'-phosphate (PLP), the most active form of six different vitamin B6 vitamers. The hydrazone-exchange probe **Ac-RAB6** is selective for PLP among a wide range of aldehydes and avoids background signal from hypochlorite. To our knowledge, this is the first PLP-selective fluorescence probe, and we have shown that it can report on PLP kinase enzymatic activity, as well as on changes in PLP levels in human cells. The most widely used current method for the assessment of vitamin B6 status is to measure the PLP concentration in plasma by HPLC based on the assumption that PLP in plasma reflects the concentration of PLP in cells and tissues, which is known not to be the case in certain diseases.³⁰ The new probe design enables rapid and simple measurement of changes in PLP concentrations both *in vitro* and in cells. Given the close association of vitamin B6 status to a wide range of diseases, the probe is expected to have broad utility in the field.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

(a) Structures of B6-vitamers and different classes of intracellular aldehydes. (b) The salvage pathway of vitamin B6 that generates PLP in human cells.

Fig. 2.

(a and b) Previously reported rhodamine based fluorescent probes for metal ions that utilize a cyclization reaction. (c) Scheme of the proposed chemical reaction of the probe with pyridoxal phosphate, affording a fluorescence signal.

Fig. 3.

(a) Selectivity test with different aldehydes (100 μM) at pH 7 (25 mM Tris buffer) and 5 (25 mM acetate buffer). (b) Fluorescence change variation with aldehydes at pH 5. (c and d) Calculated partial charges of the (c) hydrazone carbon and (d) carbonyl oxygen marked as blue and red stars, respectively, in Fig. 2c. (e) Chemical structures of aldehydes used in calculations. (f) 3D molecular structure of the proposed intermediate of the probe reacting with PLP, showing an H-bond between hydrazone and phosphate. (g) Dose-response titration of fluorescence change. (h) The scheme for PDXK producing PLP by transferring the phosphate from ATP to PL. (i) Fluorescence spectrum of **RAB6** upon the addition of PL (100 μM), MgCl_2 (250 μM), and ATP (1.5 mM) with/without PDXK (0.37 $\text{ng } \mu\text{L}^{-1}$) in Tris buffer (25 mM, pH 7) after 12 h of incubation. (j) Time-dependent fluorescence comparison of (g). Experiments were carried out with 10 μM of probe. Fluorescence signal was measured at 550 nm (525 nm excitation) after 12 h of incubation at 37 $^{\circ}\text{C}$.

Fig. 4.

(a) Scheme showing a potential competing signal pathway from the oxidation of **RAB6**. (b) Scheme for **Ac-RAB6** preventing the false-positive oxidation signal by protecting the probe with aldehydes. (c) Comparison of kinetics between **RAB6** and **Ac-RAB6** for the detection of 100 μ M PLP at pH 7 and 5. (d) Comparison of false-positive signal from the oxidation by NaOCl between the first-generation **RAB6** and the protected **Ac-RAB6**. (e) Western blot of PDXK levels in intact cells and in PDXK knock-down (siPDXK) cells. (f) Epifluorescence images of intact/siPDXK cells upon the addition of exogenous PL. Emission was collected at 520 nm with excitation of 460–500 nm after 12 h of incubation. (g) Fluorescence intensity plot from the imaging experiments in (f).