Biosensors

International Edition: DOI: 10.1002/anie.201509457
German Edition: DOI: 10.1002/ange.201509457

Design of a Hyperpolarized Molecular Probe for Detection of Aminopeptidase N Activity

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Abstract: *Aminopeptidase N (APN) is an important enzyme that is involved in tumor angiogenesis. Detection of APN activity can thus lead to early diagnosis and elucidation of tumor development. Although some molecular probes for APN have been developed, the detection of APN activity in opaque biological samples remains a challenge. To this end, we designed a hyperpolarized NMR probe [1-^{13}C]Ala-NH_2 which satisfies the prerequisites for APN detection, namely, sufficient retention of the hyperpolarized state, a high reactivity to APN, and an APN-induced chemical shift change. The [1-^{13}C]Ala-NH_2 probe allowed sensitive detection of APN activity using ^{13}C NMR spectroscopy.*

Aminopeptidase N (APN) is an enzyme that catalyzes the hydrolysis of peptides or proteins and releases N-terminal hydrophobic amino acids such as alanine.[1] APN plays a number of essential roles in terms of physiological function. APN is also expected to be a tumor biomarker because the function of APN is associated with tumor invasion, metastasis, and angiogenesis.[2] Therefore, the development of a molecular probe that can be used to detect APN activity has been in high demand.

APN-binding probes that are ligands labeled with a fluorophore or radioisotope have been used to image APN expressed within the cell and in vivo.[3–5] However, such probes require washing or excretion of unbound probes, which prevents their use in real-time analysis. Furthermore, the sensing function of these probes does not directly reflect the enzymatic activity of APN. The enzymatic activity has potential to be a reliable parameter in pathological analysis.[6,7] To this end, a molecular probe that can be used to analyze the enzymatic activity of APN is required.

For this purpose, enzymatic-reaction-based probes, which produce optical readouts upon reaction with APN, have been developed.[8,9] However, the detection of APN activity in biological samples, such as tissues, their homogenates, and living animals, remains challenging because visible light is absorbed or scattered by biological material.

NMR molecular probes have good potential for use in the detection of enzymatic activity in such opaque biological samples.[10] Hyperpolarized NMR probes, the NMR sensitivities of which are dramatically enhanced by a nuclear polarization process, have emerged as a particularly promising tool.[11] A representative probe is hyperpolarized [1-^{13}C]pyruvic acid, which allows even in vivo sensing of lactate dehydrogenase activity by ^{13}C chemical shift analysis.[12] Despite its potential use, the number of hyperpolarized NMR probes that have been developed so far remains limited.[13–15] This is because of the stringent chemical and physical requirement for hyperpolarized NMR probes (see below). Herein, we report the design and synthesis of the first hyperpolarized NMR probe that can be used to analyze APN activity.

APN catalyzes the hydrolysis of peptides at an N-terminal hydrophobic amino acid residue, mainly alanine, methionine, and leucine. When designing the hyperpolarized NMR probe for use in assessing APN activity, a straightforward approach is to use a peptide structure such as dipeptide [1-^{13}C]Ala-Xaa (Figure 1, top). This dipeptide appeared to be ideal because it reacts with APN and would work as a ^{13}C NMR chemical-shift-switching probe. However, the use of such dipeptide-type probes presents at least two potential problems, the first of which being selectivity. Given the range of peptidases that are present in the body, the probe peptides could be a substrate for other peptidases in addition to APN. In the case of dipeptides, carboxypeptidases (CPs) and dipeptidases (DPs) are also assumed to react, resulting in low APN selectivity and non-negligible levels of false positive signals. The second potential problem presented by the use of dipeptide-type probes is the hyperpolarization lifetime. Peptide structures tend to have a short hyperpolarization lifetime.[16] The lifetime is directly related to spin–lattice relaxation time (T_1). Therefore, the designed NMR probe should have a long T_1 value that is sufficient for a delivery to the target site, an enzymatic reaction, and NMR measurements.

To address these problems, we designed [1-^{13}C]Ala-NH_2 (Figure 1, bottom) as a probe. To avoid reaction with endogenous peptidases except APN, we focused on the

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201509457.

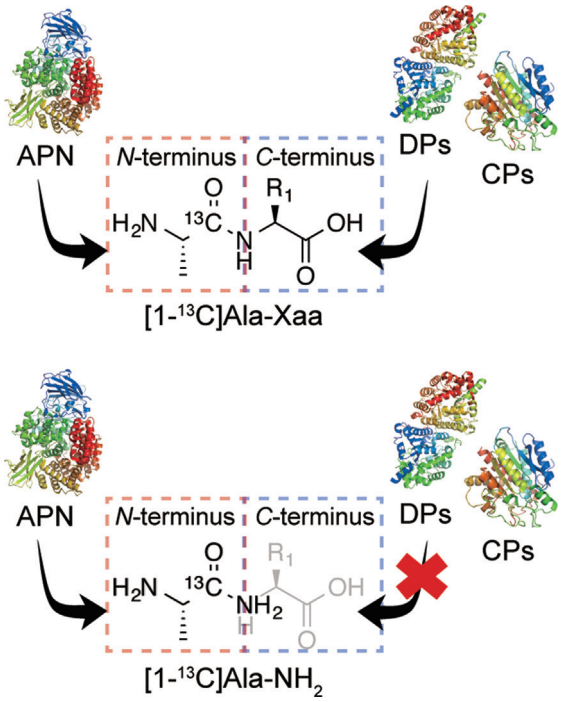
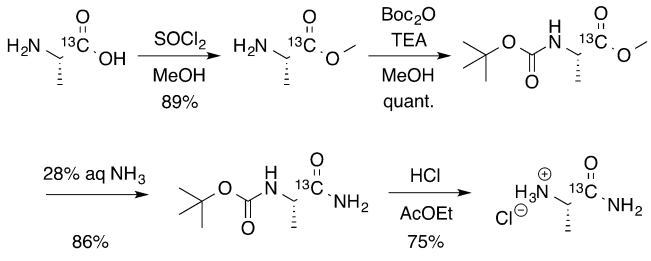


Figure 1. Design of APN-selective probe [1-^{13}C]Ala-NH_2. Model structures of APN, DPs, and CPs were from PDB 3B34, 1ITU, and 2PCU.

carboxylic acid of the dipeptide structure. CPs recognize the C-terminal carboxylic acid in substrates, as its name implies.[17] Some reports suggest that DPs also require the C-terminal carboxylic acid moiety to perform hydrolysis.[18] Based on these reports, we focused on [1-^{13}C]Ala-NH_2 having a primary amide group because this potential probe has no C-terminal carboxylic acid, and we anticipated that it would show a high selectivity to APN over CPs and DPs. Additionally, a long hyperpolarization lifetime for this compound was expected because of its small size compared with dipeptides.

[1-^{13}C]Ala-NH_2 was synthesized from [1-^{13}C]Ala in four steps (Scheme 1). The carboxylic acid of [1-^{13}C]Ala was converted into the methyl ester, followed by protection of the amino group with the *tert*-butyloxycarbonyl (Boc) group. The methyl ester was then converted into a primary amide by treatment with ammonium hydroxide. The Boc group was deprotected with hydrochloric acid to give [1-^{13}C]Ala-NH_2.

First, we determined the T_1 value and APN reactivity of Ala-NH_2. L-Alanyl-glycine (Ala-Gly) and L-alanyl-L-alanine (Ala-Ala), the smallest and second smallest dipeptides having



Scheme 1. Synthesis of [1-^{13}C]Ala-NH_2.

alanine as the N-terminal amino acid, were used as comparable probes.

As expected, Ala-NH_2 displayed a longer ^{13}C T_1 value than both dipeptides. The T_1 values of ^{13}C-labeled substrates, [1-^{13}C]Ala-NH_2, [1-^{13}C]Ala-Gly, and [1-^{13}C]Ala-Ala, were determined by using the inversion recovery method to be 24.8 ± 0.9, 16.6 ± 0.2, and 13.6 ± 0.4 s (9.4 T, 37 °C), respectively (Table 1). The T_1 value of [1-^{13}C]Ala-NH_2 was 1.5–1.8 times larger than that of the dipeptides and was within the T_1 range of probes that have been successfully applied for in vivo analysis.[13–15]

Table 1: T_1 values and kinetic parameters for Ala-NH_2, Ala-Gly, and Ala-Ala.

Probe	K_m [mM][a]	k_{cat} [s^{-1}][a]	T_1 [s][b]
Ala-NH_2	9.7 ± 1.7	210 ± 10	24.8 ± 0.9
Ala-Gly	3.6 ± 0.4	74 ± 8	16.6 ± 0.2
Ala-Ala	2.9 ± 0.6	150 ± 10	13.6 ± 0.4

[a] Nonlabeled substrates were used to evaluate kinetic parameters. Conditions: 100 mM phosphate buffer pH 7.4 (37 °C). [b] Isotopically labeled [1-^{13}C]Ala-NH_2, [1-^{13}C]Ala-Gly, and [1-^{13}C]Ala-Ala were used. Conditions: 10 mM ^{13}C-labeled compounds in 90 mM sodium phosphate buffer (pH 7.4) containing 10% D_2O (9.4 T, 37 °C).

Kinetic parameters for APN, k_{cat} and K_m, were determined by using the non-isotopically labeled substrates Ala-NH_2, Ala-Gly, and Ala-Ala (Table 1). The K_m values revealed that the affinity of Ala-NH_2 for rat APN is slightly less than the affinity of the dipeptides. On the other hand, interestingly, the k_{cat} value of Ala-NH_2 was larger than those of the dipeptides. In hyperpolarized NMR experiments, a large k_{cat} value is required to afford detectable amounts of reaction products within a limited hyperpolarization lifetime. The detected large k_{cat} value ($210\ s^{-1}$) would satisfy this requirement.

Next, the enzyme selectivity was evaluated by using ^{13}C NMR spectroscopic analysis (Figure 2). [1-^{13}C]Ala-NH_2, [1-^{13}C]Ala-Gly, and [1-^{13}C]Ala-Ala afforded ^{13}C NMR signals at $\delta = 175.2$, 172.0, and 171.2 ppm, respectively (Figure 2, top line). These substrates were each mixed with a homogenate of mouse kidney, which is known to contain APN and other peptidases.[19–21] After incubation, the ^{13}C NMR spectra of all three samples showed the same new signal at $\delta = 176.5$ ppm (Figure 2, middle line). This new signal was assigned as [1-^{13}C]Ala, suggesting that peptidases in the kidney homogenate hydrolyzed all of these substrates to release [1-^{13}C]Ala. However, when the same reactions were conducted in the presence of phebestin (10 μM), which is a selective inhibitor of APN ($IC_{50} = 0.41$ μM),[22] the reaction of [1-^{13}C]Ala-NH_2 was inhibited almost completely (Figure 2, bottom), whereas [1-^{13}C]Ala-Ala and [1-^{13}C]Ala-Gly still reacted to give [1-^{13}C]Ala. These combined results indicate that [1-^{13}C]Ala-NH_2 reacts selectively with APN, whereas dipeptide-based probes react with a broad range of peptidases or enzymes.

The APN selectivity of Ala-NH_2 was further confirmed by comparison with the known APN-selective chromogenic probe alanine-*p*-nitroanilide (Ala-pNA). The relative reac-

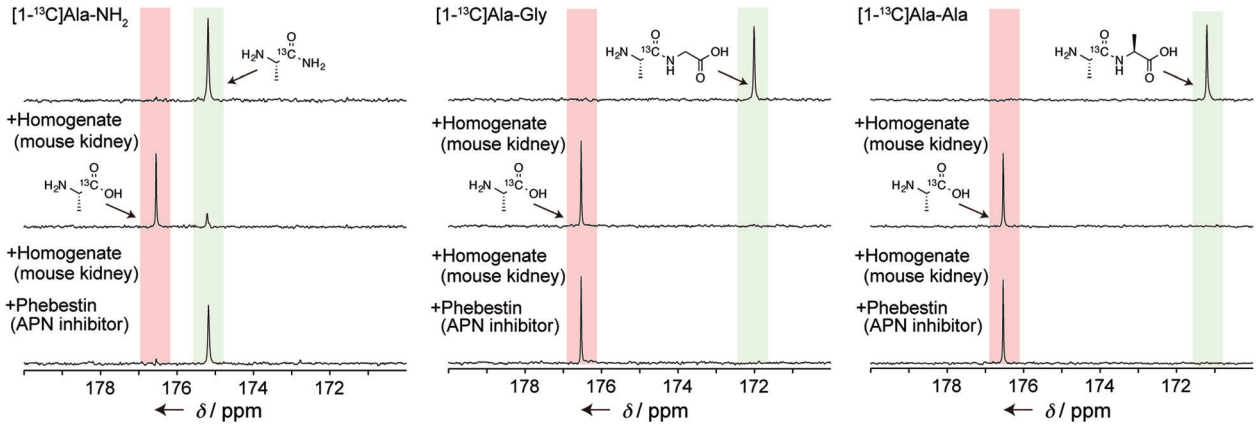


Figure 2. ^{13}C NMR spectra of [1-^{13}C]Ala-NH$_2$, [1-^{13}C]Ala-Gly, and [1-^{13}C]Ala-Ala before incubation (top line) or after incubation for 6 hours with kidney homogenate in the absence (middle line) or the presence (bottom line) of phebestin. Incubation conditions: 10 mM ^{13}C-labeled substrate in 100 mM phosphate buffer (pH 7.4, 500 µL) containing mouse kidney homogenate (10 µL, 39 mg mL^{-1} protein by BCA assay) and 0 or 10 µM phebestin. All solutions were mixed with 60 µL of dioxane/D$_2$O = 15:1 v/v as an internal reference (δ = 67.2 ppm) before ^{13}C NMR measurements (128 scans).

tivity of the Ala-NH$_2$ probe in each of the tissue homogenates (kidney, liver, pancreas, lung, spleen, and heart) was highly correlated with those of Ala-pNA. On the other hand, those of Ala-Ala and Ala-Gly showed different profiles (see Figure S1 in the Supporting Information). These results also support the conclusion that Ala-NH$_2$ is likely to be a selective substrate for APN.

With a potent APN-selective probe in hand, we then performed a proof-of-concept hyperpolarization study. The [1-^{13}C]Ala-NH$_2$ solution containing 15 mM of the trityl radical OX63 (in D$_2$O/[D$_8$]glycerol = 85:15 v/v) was hyperpolarized at 1.4 K, irradiating at 94 GHz, using HyperSense (Oxford Instruments). This sample was dissolved rapidly and subjected to ^{13}C NMR spectroscopic analysis (9.4 T). An enhanced NMR signal was clearly detected after a single scan (Figure 3a, left). Compared with the thermally equilibrated ^{13}C NMR spectrum (Figure 3a, right), the signal was enhanced 6200-fold. The time course of the hyperpolarized ^{13}C NMR spectra is shown in Figure 3b. As a result of the relatively long T_1 value, the hyperpolarized signal could be detected for over 60 seconds by repeated single NMR scans. Curve fitting to the signal decay gave $T_1 = 30.1$ s, which was consistent with the T_1 value obtained for the thermally equilibrated sample ($T_1 = 30.2 \pm 0.6$ s, inversion recovery, 10 mM [1-^{13}C]Ala-NH$_2$, 250 mg L^{-1} EDTA aqueous solution containing 10% D$_2$O, 9.4 T, 37 °C; EDTA = ethylenediaminetetraacetic acid).

Finally, we applied the hyperpolarized probe for the detection of APN activity in kidney homogenate (Figure 3c). When the hyperpolarized [1-^{13}C]Ala-NH$_2$ probe was added to mouse kidney homogenate, the probe produced a new NMR signal corresponding to [1-^{13}C]Ala as a reporting product. In contrast, in the presence of phebestin, the signal of [1-^{13}C]Ala was largely absent. The signal of hyperpolarized [1-^{13}C]Ala seemed to reach a plateau (Figure 3c). This is probably because [1-^{13}C]Ala was gradually produced by the enzymatic reaction during the measurement time. It is highly likely that the sum of the hyperpolarized signal decay and enzymatic production of [1-^{13}C]Ala resulted in the signal reaching a plateau. Thermally equilibrated ^{13}C NMR spectra measured after these hyperpolarization experiments also support that the new resonance signal is actually the signal derived from [1-^{13}C]Ala produced (Figure S2).

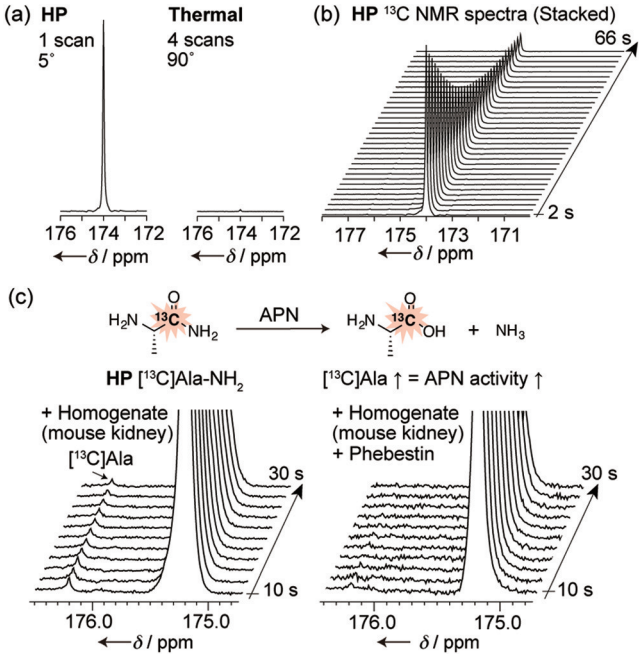


Figure 3. a) Hyperpolarized (HP, left) and thermally equilibrated (thermal, right) ^{13}C NMR signals of [1-^{13}C]Ala-NH$_2$ (7.5 mM in 250 mg L^{-1} EDTA solution). b) Stacked hyperpolarized ^{13}C NMR spectra of [1-^{13}C]Ala-NH$_2$ (7.5 mM in 250 mg L^{-1} EDTA solution), showing spectra recorded from 2–66 s (spectra recorded every 2 s, pulse angle = 5°). c) Detection of APN activity using hyperpolarized [1-^{13}C]Ala-NH$_2$, with ^{13}C NMR spectra recorded every 2 s (pulse angle = 25°). The solutions of hyperpolarized [^{13}C]Ala-NH$_2$ (16 mM, 3 mL) in 100 mM phosphate buffer (pH 7.4) were mixed with mouse kidney homogenate (1 mL) in the absence (left) or in the presence (right) of phebestin (5 µM).

At the moment, the presence of unknown enzymes that hydrolyze [1-^{13}C]Ala-NH_2 cannot be excluded; however, considering its high selectivity for APN, it is reasonable to conclude that hyperpolarized [1-^{13}C]Ala-NH_2 works as a selective probe for detecting APN activity.

In conclusion, we have developed [1-^{13}C]Ala-NH_2 as the first hyperpolarized NMR probe for detecting APN activity. The compound satisfies prerequisites for hyperpolarized NMR analysis: sufficient retention of the hyperpolarized state, a high reactivity to APN, an APN-induced chemical shift change, and low toxicity (Figure S3). In addition, APN is a membrane protein bearing a catalytic domain outside of the cell.[1] Therefore, [1-^{13}C]Ala-NH_2 can react with APN without the need for cellular uptake. This would be also an advantage of this probe in practical applications. Proof-of-concept experiments demonstrated the detection of APN activity in mouse kidney homogenate by using hyperpolarized [1-^{13}C]Ala-NH_2. The [1-^{13}C]Xaa-NH_2 probe concept demonstrated here is not limited to APN, but can be applied in the design of hyperpolarized NMR probes for other aminopeptidases. We expect that probes of the type [1-^{13}C]Xaa-NH_2 will open up new opportunities for the application of hyperpolarized NMR systems. Further work along these lines is under way in our laboratory.

Acknowledgements

This work was supported by CREST, Japan Science and Technology Agency (JST). The use of HyperSense was in part supported by the funding program "Creation of Innovation Centers for Advanced Interdisciplinary Research Areas" from the JST. R.H. was funded by a fellowship from the Japan Society for the Promotion of Science. We thank Ms. Kaori Inoue for measurements of DNP-NMR.

Keywords: aminopeptidases · biosensors · dynamic nuclear polarization · hyperpolarization · NMR spectroscopy

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 1765 – 1768
Angew. Chem. **2016**, *128*, 1797 – 1800

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Received: October 9, 2015
Revised: November 9, 2015
Published online: December 21, 2015

