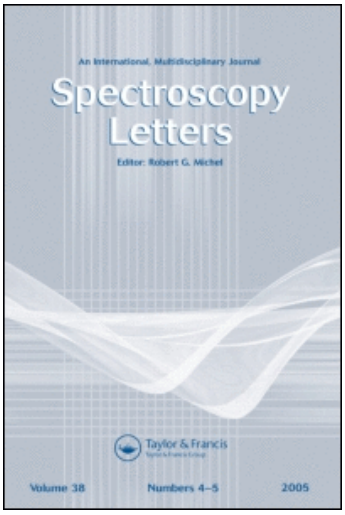

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ANION BINDING TO PORCINE PANCREATIC α-AMYLASE AS PROBED BY BROMINE-81 NUCLEAR MAGNETIC RESONANCE SPECTROMETRY

Keywords: Bromine-81 NMR; porcine pancreatic α-amylase; chloride-binding site; anion-binding site

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ABSTRACT

A bromine-81 NMR study was conducted to characterize the Cl^--binding site of porcine pancreatic α-amylase. Only a single signal was observed in the spectrum in the presence of an equimolar concentration of the enzyme and NaBr. The signal was assigned to free Br^- ions, which are in very slow exchange with the protein-bound Br^- on an NMR time scale. The presence of a common anion-binding site was demonstrated from the competition of Br^- with F^-, Cl^-, NO_3^-, and ClO_4^- for the site.

INTRODUCTION

α-Amylases (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) catalyze the hydrolysis of internal α(1,4)-glycosidic bonds in starch and amylose. The enzymes are widely distributed in various living organisms. Several α-amylases, which include the enzymes from porcine pancreas[1)], sea urchins [2)], and the antarctic bacterium, *Alteromonas haloplanctis*, [3)] are activated

by Cl^- ions. Binding of Cl^- to porcine pancreatic α-amylase (PPA) increases the amylolytic activity by a factor of 30 while leaving the affinity for the substrate unchanged.[1] Proteinligands of the Cl^--binding site are assigned to Arg-195, Asn-298, and Arg-337 in PPA by x-ray crystallography.[4] The Cl^- ion resides approximately 1.1 Å from the center of the starch binding cleft.[4] Alignment of amino-acid sequences of the α-amylases reveals that Arg-195 and Asn-298 (PPA numbering) are conserved in both the Cl^--dependent and the Cl^--independent enzymes. Arg-337 (PPA numbering) is conserved or replaced by Lys in the Cl^--dependent enzymes, whereas no conservation is found in the Cl^--independent enzymes.[5] In addition, mutation of Lys-337 to Gln-337 in the α-amylase from *A. haloplanctis* results in a loss of Cl^--binding ability with a reduced hydrolase activity.[5] These results indicate the basic residue Arg- or Lys-337 is essential for Cl^- binding.

Monovalent anions other than Cl^- can activate the Cl^--dependent enzymes.[1),2),5)] PPA can be activated by Br^- at a level similar to Cl^- and to a lesser extent by I^- and NO_3^-. It is most likely that the Cl^--binding site is also shared with monovalent anions other than Cl^-. However, there has so far been no experimental evidence to support this. In this paper, the binding of monovalent anions to the Cl^--binding site in PPA is shown.

MATERIALS AND METHODS

Preparation of the Enzyme

Two isozymes (PPA I and PPA II) are known for PPA.[6] Both isomers are similarly activated by Cl^-, although the activation is slightly higher for PPA I than for PPA II for Cl^- concentrations higher than 0.1 M.[7] The PPA II used in this study was purified by a slight modification of the method of Sakano et al.[7] Porcine pancreas was homogenized in 0.8 volume (ml/g of pancreas) of a solution containing 3% *n*-butyl alcohol and 10 mM $CaCl_2$. After acetone fractionation (40-67%) and salting-out with 40% saturation of ammonium sulfate, the precipitate was dissolved in a small volume of 1 mM $CaCl_2$ solution and dialyzed against 1 mM $CaCl_2$ for 18 h. The dialysate was loaded onto a column of TSK-gel DEAE Toyopearl 650S (Tosoh Corporation, Tokyo, Japan) equilibrated with buffer A (a 50 mM Tris/HCl buffer solution that had a pH of 8.3 and was 1 mM with respect to $CaCl_2$) and eluted with a stepwise gradient from 0 to 0.2 M NaCl in buffer A. The fraction eluted at 0.2 M NaCl was collected and applied on a column of Sephacryl S-300HR (Pharmacia Biotech, Uppsala, Sweden). The enzyme was concentrated using a Centriprep-30 (Amicon), and the Cl^--free enzyme was obtained by Sephadex G-25 (Pharmacia Biotech, Uppsala, Sweden) column chromatography using 1 mM $Ca(NO_3)_2$ as an eluant.

The purity was checked by SDS-PAGE, and the protein concentration was determined by UV absorption spectrometry (the absorption at 280 nm for a 1% PPA II solution is 24).[8]

Measurement of ^{81}Br-NMR Spectra

^{81}Br-NMR spectra were obtained using an NMR spectrometer (model α-500, JEOL) operating at 134.95 MHz, with the following parameters: a 10-μs (π/2) pulse length, an acquisition time of 5 ms, and a pulse delay of 40 ms. Each FID was zero-filled to 32k, and an exponential multiplication resulting in a line broadening of 50 Hz was applied prior to Fourier transformation. The chemical shift was referred to a 10 mM NaBr solution as zero ppm. All samples were made up in 10% D_2O and locked internally on the deuterium signal. The line widths at half maximum of the spectra were corrected for the broadening factor (50 Hz).

RESULTS AND DISCUSSION

FIG. 1 compares the ^{81}Br-NMR spectrum (A, B) of a solution containing 0.2 mM NaBr, 0.2 mM PPA II, and 0.9 mM $Ca(NO_3)_2$ with the spectrum (C, D) of a solution containing 0.2 mM NaBr. The ingredient $Ca(NO_3)_2$ was added to stabilize the enzyme. A single peak was observed at a chemical shift of 0 ppm with a half width of 590 Hz and no other signals were observed in the spectrum in the presence of the enzyme. As shown in FIG. 2, the line width of the spectrum was almost constant when the enzyme was titrated by NaBr up to 5 mM. These results indicate that the observed signal can be attributed to free Br^- ions and that protein-bound Br can be undetectable.

A nucleus with a nuclear-spin quantum number greater than 1/2 has an electricquadrupole moment. The interaction of the quadrupole moment with the electric-field gradient across the nucleus induces transverse relaxation effectively, giving rise to a broadened spectrum.[9] The ^{81}Br nucleus has a sufficiently large nuclear quadrupole moment,[9] so that a ^{81}Br-NMR signal from the protein-bound Br could be rendered undetectable due to an excessively large line width.

The observed spectrum was broadened by the presence of PPA II compared with that of the 0.2 mM NaBr solution, which showed a half width of 370 Hz (FIG. 1D). To determine the cause of the signal broadening, ^{81}Br-NMR spectra of a solution containing 0.2 mM NaBr, 0.2 mM PPA II, and 0.9 mM $Ca(NO_3)_2$ were measured at various temperatures. The line width decreased with temperature, as shown in FIG. 3, indicating that free Br^- ions could be undergoing a very slow exchange with the protein-bound Br^-, where the transverse relaxation time of the Br nuclei is much shorter than the lifetime of Br^- in the protein-bound form.[10] A high viscosity for the enzyme solution would increase the correlation time of Br^- resulting in an increased line width. This was also supported by the constancy of the line width in the titration experiment. It was therefore confirmed that free Br^- ion alone contributed to the signal observed at a chemical shift of 0 ppm in the presence of the enzyme.

TABLE 1 shows the effect of monovalent anions on the signal areas of ^{81}Br-NMR spectra. The anions were added as sodium salts. The intensity increased in the presence of F^-, Cl^-, NO_3^-,

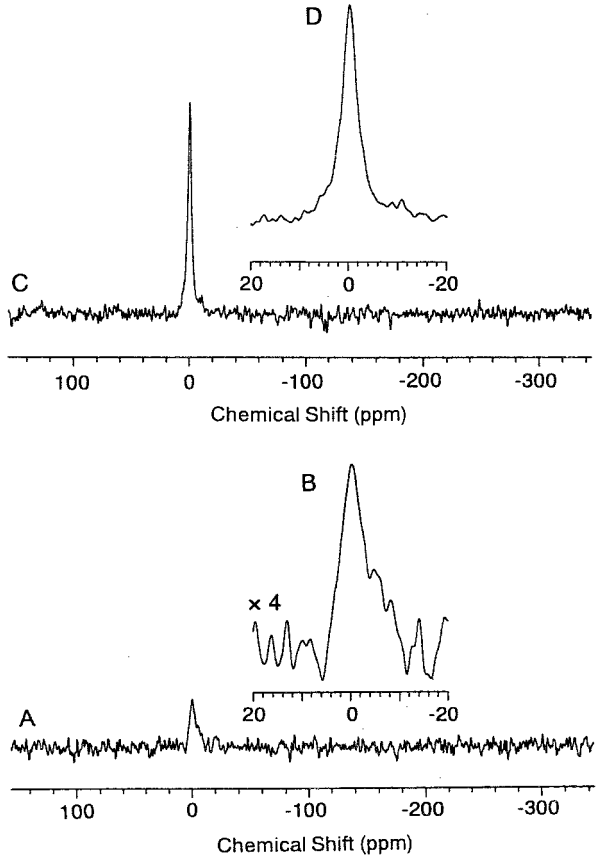

FIG. 1. ^{81}Br-NMR spectra of a solution containing 0.2 mM NaBr, 0.2 mM PPA II, and 0.9 mM $Ca(NO_3)_2$ (A, B) and a solution containing 0.2 mM NaBr (C, D). The peaks around 0 ppm were expanded in abscissa and are shown in B and D. The spectra in A, C and D are shown with the same magnification factor. The spectrum in B was magnified by a factor of four. The spectra were recorded at 25∞C, and 400,000 scans were acquired using a 6.8-kHz sweep width. An exponential window function (50 Hz) was multiplied prior to Fourier transformation.

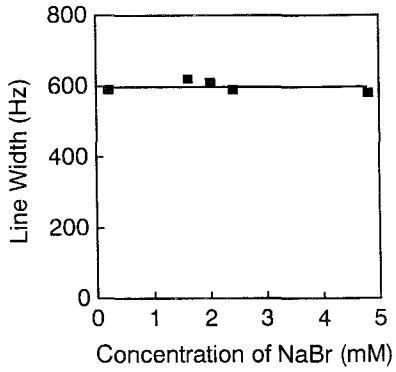

FIG. 2. Effect of Br^- concentration on the line width of ^{81}Br-NMR spectra. The spectra were recorded for a solution containing various concentrations of NaBr, 0.2 mM PPA II, and 0.9 mM $Ca(NO_3)_2$. The spectra were recorded at 25∞C, and 400,000 scans were acquired using a 6.8-kHz sweep width. An exponential window function (50 Hz) was multiplied prior to Fourier transformation. The half width at a half maximum was corrected for the broadening factor (50 Hz) and shown as the line width.

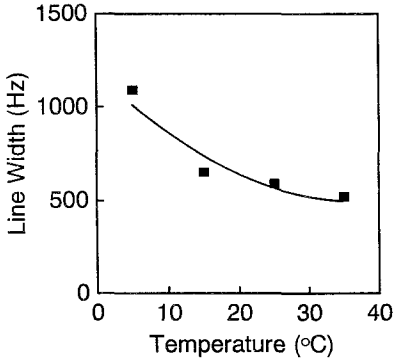

FIG. 3. Effect of temperature on the line width of ^{81}Br-NMR spectra. The spectra were recorded for a solution containing 0.2 mM NaBr, 0.2 mM PPA II, and 0.9 mM $Ca(NO_3)_2$ at various temperatures. 400,000 scans were acquired using a 6.8-kHz sweep width. An exponential window function (50 Hz) was multiplied prior to Fourier transformation. The half width at a half maximum was corrected for the broadening factor (50 Hz) and shown as the line width.

TABLE 1

Effect of Monovalent Anions on the Signal Area of the ^{81}Br-NMR Spectrum

added anion	signal area (%)[1)]
none	41.2
F^-	66.9
Cl^-	77.2
NO_3^-	85.7
ClO_4^-	60.3

^{81}Br-NMR spectra were measured for a solution containing 0.2 mM NaBr, 0.2 mM PPA II and 2.0 mM $Ca(NO_3)_2$ in the presence of 10 mM monovalent anion. The anions were added as sodium salts.

1) Areas relative to that of 0.2-mM NaBr are shown.

and ClO_4^-, indicating the liberation of Br^- from the enzyme. These results provide the first evidence that the Cl^--binding site in PPA II is shared with these anions. A precise determination of the binding constants for these anions is not available because of a relatively high content of NO_3^- ion in the enzyme solution, which also binds to PPA II. However, it can be concluded from the intensities of the spectra that NO_3^- has the highest affinity, followed by that ofCl^-, F^-, and ClO_4^-.

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