Phosphate Ion Binding to α-Chymotrypsin

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Synopsis. The spin-lattice relaxation times (T_1) of ^{31}P in α-chymotrypsin phosphate buffer solutions were measured as a function of the buffer concentration. The absorption isotherm of phosphate ions was obtained from the concentration dependence of $1/T_1$. The value of the equilibrium constant and the number of saturate sites were 66 and 42.

Both the physico-chemical properties and functions of proteins are affected by interactions between the proteins and ions.[1] Hanabusa[2] reported that a phosphate buffer shows a protective effect against freeze denaturation. In this work we report on the binding of phosphate ions to α-chymotrypsin (α-CT) by measurements of the spin-lattice relaxation time (T_1) of ^{31}P nuclei.

Experimental

α-CT (from bovine pancreas, type-II) was purchased from Sigma. A protein solution was dialyzed against a solvent below 4°C. The salts used were of special reagent grade and were used without further purification. Phosphate buffer (pH 7) was prepared at a mixing ratio of 1:2 for NaH_2PO_4 to Na_2HPO_4 in order to keep the composition of dihydrogenphosphate and hydrogenphosphate ions constant.

The concentration of the protein solution was determined by absorption spectra measurements using the value of the extinction coefficient (20 dL g^{-1} cm^{-1}) at 280 nm, 0.1%. All of the experiments were carried out within the concentration range of α-CT (1%).

The spin-lattice relaxation time (T_1) of nuclei in both ^{31}P phosphate buffers and protein solutions was measured as a function of the buffer concentration using a Varian XL-200 NMR spectrometer. All of the measurements were carried out at room temperature. We used the inversion recovery method to measure the values of T_1. Proton homodecoupling was used.

Results and Discussion

Figure 1 gives the values of the relaxation rate ($1/T_1$) of ^{31}P nuclei of both phosphate buffer solutions and protein solutions, plotted against the ionic strength. Though there were both dihydrophosphate ions ($H_2PO_4^-$) and hydrophosphate ions (HPO_4^{2-}) in the buffer solution, only one singlet signal was observed. We thus call these ions phosphate ions together. As can be seen in Fig. 1, although $1/T_1$ of ^{31}P nuclei in the phosphate buffer solution increased linearly with increasing ionic strength, but that in the α-CT solution showed a curve having a minimum point. We obtained the following experimental equations:

For the phosphate buffer solution:

$$1/T_{1,\mathrm{buf}} = 0.0708 + 0.386I. \quad (1)$$

For the protein solution:

$$1/T_{1,\mathrm{sol}} = 0.425 - 2.08I + 4.75I^2. \quad (2)$$

Here, I is the ionic strength of the solvent. Equations 1 and 2 are illustrated by lines in Fig. 1.

For nuclei having a spin quantum number of 1/2, such as ^{31}P, the relaxation rate comprises of an intramolecular part ($(1/T_1)_{\mathrm{intra}}$) and an intermolecule part ($(1/T_1)_{\mathrm{inter}}$). Since $(1/T_1)_{\mathrm{inter}}=0$ at $I=0$,[3] we obtain

$$\left(\frac{1}{T_1}\right)_{\mathrm{intra}} = \lim_{I\to 0}\frac{1}{T_{1,\mathrm{buf}}}. \quad (3)$$

The rotational correlation time (τ_c) of ^{31}P in the buffer solution is calculated based on the following relation:[4]

$$\tau_c = \left(\frac{1}{T_1}\right)_{\mathrm{intra}} \times \frac{2(4\pi)^2R^6}{3\mu_0h^2\gamma_i^4} = 4.27\times10^{-11} \quad (\mathrm{s}), \quad (4)$$

where R is the radii of molecules, μ_0 is the magnetic permeability in a vacuum, h is Plank's constant and γ_i is the gyromagnetic ratio.

This is in agreement with the value (1.1×10^{-11} s) which McCain and Markeley obtained for τ_c of $H_3{}^{31}PO_4$ in 20% D_2O.[5]

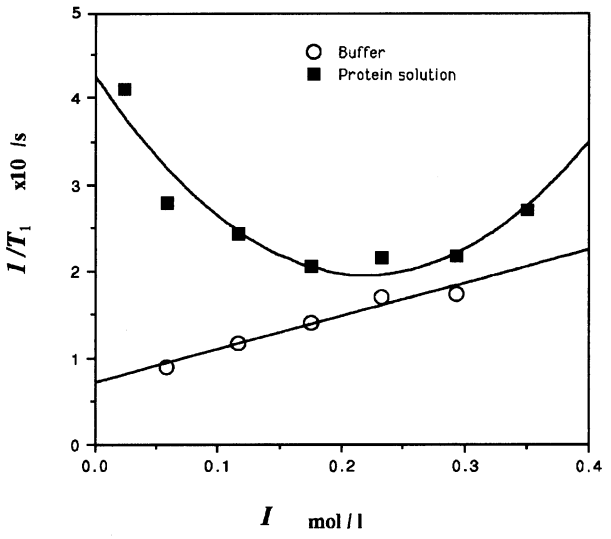

Fig. 1. The ionic strength dependence of the relaxational rate, $1/T_1$.

As can be inferred from Fig. 1, the extrapolated value of $1/T_1$ to $I=0$ for the α-CT solution was six times as large as that for the solvent series. Since the concentration of protein was low, we could neglect the effect of viscosity on the $1/T_1$ value of the protein solutions. These results suggest that the phosphate ions are bound to α-CT.

Assuming that the phosphate ions are in both free and bound states, we can estimate the binding parameters of the phosphate ion to α-CT.

Norne et al.[1] designated the absorption equation using the relaxation time measured by NMR in the following form:

$$\Delta\frac{1}{T_1}=\frac{1}{T_{1,\mathrm{sol}}}-\frac{1}{T_{1,\mathrm{buf}}}=\frac{nKC_\mathrm{p}\,(1/T_{1,\mathrm{bound}})}{1+KC_\mathrm{s}}, \quad (5)$$

where

$$\frac{1}{T_{1,\mathrm{bound}}}=\lim_{I\to 0}\frac{1}{T_{1,\mathrm{sol}}}. \quad (6)$$

Here, n is the number of saturated absorption sites per α-CT molecule, K the equilibrium constant of the absorption reaction, C_p the protein concentration and C_s the molar concentration of the total phosphate ions. Since a 1 mol phosphate buffer contains 1/3 mol of NaH_2PO_4 and 2/3 mol of Na_2HPO_4, I equals (7/3) C_s. We obtained $K=66$ and $n=42$ from Eqs. 1, 2, 5, and 6.

In addition to the electrostatic binding of phosphate ions to positive charged groups, phosphate ions can be bound to the amide groups by hydrogen bonding. According to an X-ray analysis,[6] α-CT has 20 positive charged groups (3 Arg, 2 His, 14 Lys, N-terminal group) and 22 amide groups (13 Asn, 9 Gln) exposed on the protein surface. Since the value of pH of the protein solution (7) is below the isoelectric point (8.1—8.6), the maximum number of binding sites for the phosphate ion is 42, this value is in agreement with $n=42$. Binding of the phosphate ion to the surface of α-CT is weak, since the value of K is relatively small.

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