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Nuclear magnetic resonance studies of exchange reaction of α-hydrogen of glutamic acid catalyzed by aspartate aminotransferase

The enzymatic reaction catalyzed by aspartate aminotransferase has been believed to start with the ionization of α-hydrogen of glutamic acid in the active substrate enzyme complex[1-3].

Therefore, it seems useful to apply proton magnetic resonance technique for observing the above phenomenon.

The aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) preparation was obtained from pig heart and purified by the method of JENKINS *et al.*[4] or was generously donated by Dr. Y. Nishii, Chugai Pharmaceutical Co. These enzyme preparations had a single sedimentation pattern when investigated with a Hitachi UCA-1-type ultracentrifuge. Apo-aspartate aminotransferase was prepared by the method of WADA AND SNELL[5] from the holo-aspartate aminotransferase obtained as described above. These preparations were lyophilized, and the lyophilization was repeated by treating the preparation with ^2H_2O to eliminate the large amount of NMR signal due to exchangeable protons. All spectra were measured on JNM-4H-100 spectrometer (Japan Electron Optics Laboratory Co.) or Varian A-60 spectrometer. Sodium 2,2-dimethyl-1,2-silapentane-5-sulfonate was used as an internal reference, and all chemical shifts were given in ppm downfield from this origin. The 100 Mcycles/sec spectrum of solution of monosodium glutamate in ^2H_2O is shown in Fig. 1A. The measurement of glutamate at 100 Mcycles/sec, rather than at 60 Mcycles/sec, is more suitable to this study because the signal peaks due to β- and γ-protons are obviously split at 100 Mcycles/sec, whereas the complicated spectrum is obtained at 60 Mcycles/sec. The signals of glutamate are assigned as follows: the signals around 3.7 ppm are due to α-proton and the multiplet around 2.1 ppm is assigned to the β-protons of glutamate because only the multiplet peaks around 2.1 ppm partly disappeared when the signal due to α-proton was irradiated (Fig. 1B). This phenomenon suggests that the signals at 3.7 and 2.1 ppm are without doubt coupled with each other and, consequently, the multiplet peaks of 2.3 ppm are assigned to the γ-protons.

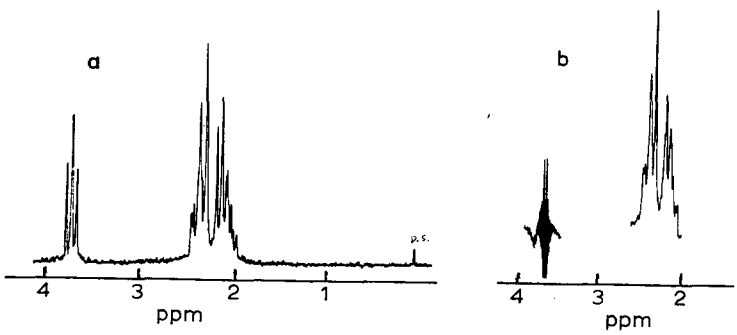

Fig. 1. A. NMR spectrum of monosodium glutamate in ^2H_2O; sodium 2,2-dimethyl-1,2-silapentane-5-sulfonate used as inner reference. B. The spectrum which was obtained by irradiating the α-proton of A.

When 20 mg of aspartate aminotransferase were added to a solution of 50 mg glutamate (without concomitant addition of keto acid) adjusted to pH 8.0 with NaO^2H, the yellow color of the enzyme and the NMR signals due to the α-proton disappeared immediately, while the signals due to the γ-protons remained unexchanged, and the muliplet peaks due to β-protons partly disappeared at the same time (Fig. 2). (The spectral pattern around 2.1 ppm illustrated in Fig. 2 was very similar to that of the glutamate shown in Fig. 1B.) This suggests that only α-proton of glutamate was completely exchanged for ^2H in medium ^2H_2O subjected to aspartate aminotransferase action immediately after addition of aspartate aminotransferase. Therefore the exchange of α-hydrogen of the glutamate subjected to the aspartate aminotransferase action seems fairly rapid. With the enzyme inactivated by boiling for 10 min, an α-^1H–^2H exchange reaction was not observed. Consequently, the dissociation of α-hydrogen is a thermolabile function of the enzyme (*cf.* ref. 6).

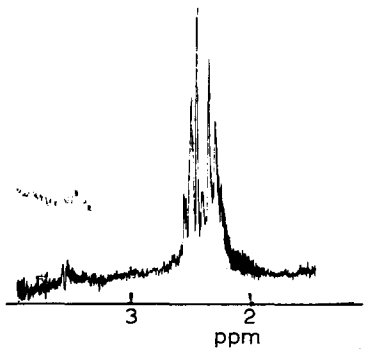

Fig. 2. The spectrum of glutamate in the presence of 2% aspartate aminotransferase.

Under our experimental conditions with aspartate aminotransferase, the ^1H–^2H exchange reaction at the β-position of the amino acid was not seen, even on addition of a ketoacid such as oxaloacetate.

Furthermore, when L-aspartate instead of L-glutamate was added in the same reaction system, the same phenomenon as that of L-glutamate was observed. Since two of the β-protons of L-aspartate are not equivalent nuclear magnetically to each other, the signal due to α-proton appears in 3.90 ppm in quartet and the signals due to β-protons are located at 2.57 and 2.89 ppm. When aspartate aminotransferase was added, the quartet around 3.90 ppm disappeared, while signals at 2.57 and 2.89 ppm joined in one peak. The following amino acids were tested in place of L-glutamate, but no exchange reaction was found: D-glutamate, DL-β-methylaspartate, L-serine, L-cysteine, *erythro*-γ-hydroxyglutamate and L-alanine. When apo-aspartate aminotransferase was added instead of holo-aspartate aminotransferase to L-glutamate or L-aspartate solution, the signals of the α-proton remained unexchanged.

We are now attempting to measure hydrogen-exchange rate in this enzymatic reaction under various conditions to obtain some physicochemical parameters such as ΔH, *etc.*

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1 E. E. SNELL, *Vitamins Hormones*, 16 (1958) 77.
2 A. E. BRAUNSTEIN, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 2, Academic Press, New York, 1960, p. 113.
3 A. M. PERAULT, B. PULLMAN AND L. VALDEMORO, *Biochim. Biophys. Acta*, 46 (1961) 555.
4 W. T. JENKINS, D. A. YPHANTIS AND I. W. SIZER, *J. Biol. Chem.*, 234 (1959) 51.
5 H. WADA AND E. E. SNELL, *J. Biol. Chem.*, 237 (1962) 127.
6 A. S. KONIKOVA, N. N. DOBBERT AND A. E. BRAUNSTEIN, *Nature*, 159 (1947) 67.

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Acid and alkaline ribonucleases of plasma

A study of the properties of plasma ribonuclease was undertaken to differentiate the activity present in lysates of erythroid cells from that of the plasma ribonuclease[1]. Two earlier studies on the ribonuclease activity of mammalian plasma have shown that plasma has an ribonuclease with an optimum pH of 7.4 and is devoid of "acid" ribonuclease[2,3]. Both studies suggested that the "alkaline" ribonuclease is due to circulating pancreatic ribonuclease. Our findings indicate that most of the pH 7.4 ribonuclease of mouse plasma has an apparent molecular weight in excess of 200 000 and cannot, therefore, be pancreatic ribonuclease. We also report that there is a potent "acid" ribonuclease in mouse plasma with a pH optimum of 4.0. The pH 4.0 enzyme is activated by certain divalent cations especially Ni^{2+}.

Blood was obtained by cardiac puncture of mice of the C_3H strain into heparinized syringes. After removal of the cells by centrifugation ribonuclease activity was determined at pH 7.4 in a 0.04 M Tris buffer, 0.15 M NaCl, and 0.64 mg/ml tRNA. The solution was brought to 37° and mouse plasma was added to a concentration of 1–2 mg (plasma protein) per ml. Aliquots of 0.50 ml were removed at zero time and at intervals thereafter and transferred to tubes containing 0.70 ml of 1.0 M $HClO_4$ at 0°. The tubes were kept at 0° for 10 min. They were then centrifuged at $2000 \times g$, for each tube the supernatant was decanted, and its absorbance at 260 mμ was determined with a Beckman DU spectrophotometer. The zero-time value was subtracted. A control without enzyme was run simultaneously. The conditions for assaying plasma ribonuclease at pH 4.0 were identical to those described above except that 0.04 M acetate buffer was used. One unit of enzyme activity is defined as that amount of enzyme