RECONSTITUTION OF D-AMINO ACID AMINOTRANSFERASE

Kazuo YONAHA, Haruo MISONO and Kenji SODA

Laboratory of Microbial Biochemistry, Institute for Chemical Research Kyoto University, Uji, Kyoto-Fu 611, Japan

Received 12 May 1975

1. Introduction

Although the role of pyridoxal 5′-phosphate (pyridoxal 5′-P) in a catalytic action of aminotransferases has been extensively studied [1–3], the mechanism of reconstitution of the enzymes is still obscure. Based on the observation of binding of aspartate aminotransferase with pyridoxal 5′-P and pyridoxamine 5′-phosphate (pyridoxamine 5′-P), it was proposed that at least two steps are involved in the reconstitution, which may require one hour or even longer [4–6]. No information about the mode of binding of D-amino acid aminotransferase with the coenzymes is available. Recently, we purified a bacterial D-amino acid aminotransferase to homogeneity and crystallized it to elucidate some enzymological properties [7]. Studies on reconstitution of the enzyme with pyridoxal 5′-P and reactivity of the sulfhydryl groups are presented here.

2. Materials and methods

5, 5′-Dithio-bis (2-nitrobenzoic acid) (DTNB) was obtained from Nakarai Chemicals, Kyoto. Pyridoxal 5′-P and pyridoxamine 5′-P, which were chromatographically purified by the method of Peterson and Sober [8], were products of Kyowa Hakko Kogyo, Tokyo. Sephadex G-25 was obtained from Pharmacia, Uppsala. All other chemicals were analytical grade reagents.

The crystalline D-amino acid aminotransferase was prepared from the extract of *Bacillus sphaericus* IFO 3525 as described previously [7]. Enzyme concentration was estimated with the absorption coefficient at 280 nm ($E\,\frac{1\%}{1\,cm}$ = 12.6).

3. Results and discussion

The holoenzyme, which contains 2 moles of pyridoxal 5′-P per mole [7], was resolved into a catalytically inactive form as follows. The enzyme was incubated with 5 mM phenylhydrazine (pH 7.4) at 37°C for 1 hr, and applied to a Sephadex G-25 column (1.5 × 50 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.4), followed by elution with the same buffer. The enzyme thus treated contains still one mole of pyridoxal 5′-P, but is catalytically inactive and reactivated almost fully with pyridoxal 5′-P or pyridoxamine 5′-P [9, 9a]. Therefore, this form is regarded as a semiapoenzyme as reported for L-lysine-α-ketoglutarate ε-aminotransferase [10].

D-Amino acid aminotransferase was markedly inhibited by DTNB, ρ-chloromercuribenzoate, $HgCl_2$ and *N*-ethylmaleimide, suggesting that a sulfhydryl group or groups play an essential role in a catalytic process. Four sulfhydryl groups were titrated with DTNB in the holoenzyme, whereas only the two groups were reactive in the semiapoform. The semiapoenzyme treated with DTNB was reactivated almost fully by pyridoxal 5′-P. Four sulfhydryl groups per mole of enzyme were again titrable with DTNB in the reconstituted holoenzyme. Mercuric chloride inactivated the semiapoenzyme. Sulfhydryl compounds, e.g. 2-mercaptoethanol and dithiothreitol, showed no effect on the reactivation of semiapoenzyme. These findings suggest that a rearrangement of the protein structure

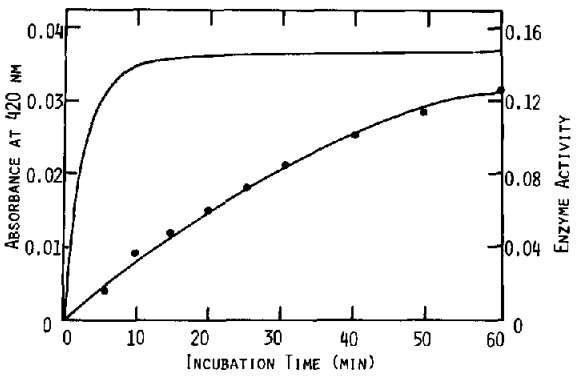

Fig.1. Changes in the absorbance at 420 nm and the enzyme activity caused by formation of Schiff base and active enzyme. (——) Changes in the absorbance at 420 nm. After pyridoxal 5′-P (60 nmol) was added to semiapoenzyme (15 nmol) in 1.0 ml of 0.05 M potassium phosphate buffer (pH 8.0), a Schiff base formation was followed by measuring the absorbance at 420 nm with a Hitachi 356 two-wavelength, double beam spectrophotometer. (—•—) Changes in the enzyme activity. The enzyme activity was expressed as μmoles of pyruvate formed per min.

occurs to eliminate reversibly the reactivity of the two sulfhydryl groups participating in catalysis with DTNB in the process of resolution of the enzyme.

The reconstitution of enzyme was followed by spectrophotometry and determination of the enzyme activity. The enzyme was assayed by determining the amount of pyruvate formed as follows. To a mixture of 25 μmol of D-alanine, 25 μmol of α-ketoglutarate, and 80 μmol of potassium phosphate buffer (pH 8.0), were added a mixture of 0.32 nmol of semiapoenzyme and 1.0 nmol of pyridoxal 5′-P in a final vol. of 1.0 ml. After incubation at 37°C for 10 min, pyruvate was determined with salicylaldehyde [11]. Semiapoenzyme was replaced by water in a blank. The formation of a Schiff base between pyridoxal 5′-P and protein, which is characterized by a 420 nm absorption peak, occurred in the early stages of the reconstitution as shown in fig. 1. Semilogarithmic plots of the changes in absorbance at 420 nm, and in enzyme activity against incubation times (12), give straight lines (fig. 2). Thus, both the Schiff base formation and the reconstitution of enzyme follow first order kinetics with half-times of about 2 and 20 min, respectively. these results suggest that the reconstitution of D-amino acid aminotransferase with pyridoxal 5′-P proceeds in at least three steps; a reversible association of semiapoenzyme with pyridoxal 5′-P, a Schiff base formation and a slow conformational change as follows.

$$\text{Semiapoform} \overset{\text{PLP}}{\rightleftharpoons} \text{Complex I} \longrightarrow \text{Complex II (Schiff base)} \longrightarrow \text{Active holoform (PLP, pyridoxal 5′-P)}$$

It was reported that a conformational change follows a Schiff base formation in a reconstitution of aspartate aminotransferase [4] as well, whereas the reverse is the case with glutamate decarboxylase of *E. coli*

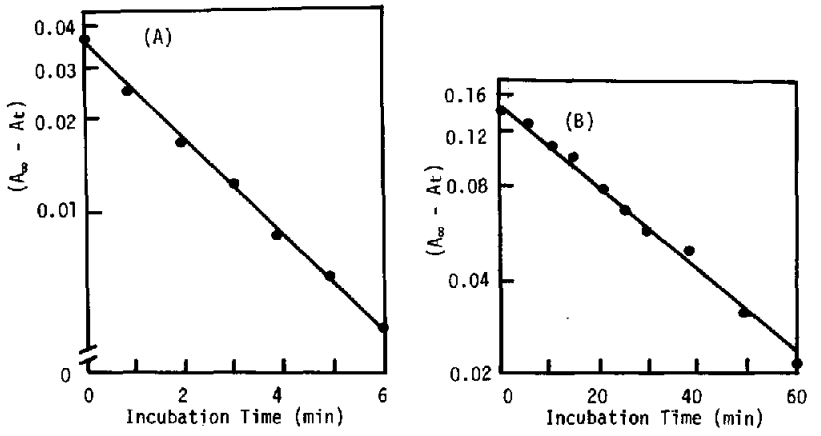

Fig.2. Semilogarithmic plot of changes in the absorbance at 420 nm (A) and the enzyme activity (B) against incubation time. A_∞ and A_t are defined as the values at the end point and the indicated time, respectively.

[13]. A Schiff base formation is not concurrently accompanied with a reappearance of reactivity of the sulfhydryl groups participating in catalysis with DTNB, because only two sulfhydryl groups were titrated immediately after a Schiff base formation. The sulfhydryl groups become susceptible to DTNB in the last step, the rearrangement of enzyme protein from Complex II to the active holoform. The first step of the reconstitution process is now under investigation.

Acknowledgements

We thank Dr K. Asada and Dr T. Yamamoto for their helpful advice.

References

[1] Snell, E. E., Braunstein, A. E., Severin, E. S., and Torchinsky, Y. M. (1966) Pyridoxal Catalysis: Enzymes and Model Systems, Interscience, New York.
[2] Snell, E. E. and DiMari, S. J. (1970) in: The Enzymes (Boyer, P. D. ed.) Vol. 2, pp 335–370, Academic Press, New York.
[3] Braunstein, A. E. (1973) in: The Enzymes (Boyer, P. D. ed.) Vol. 9, pp 379–481, Academic Press, New York.
[4] Arrio-Dupont, M. (1969) Biochem. Biophys. Res. Commun, 36, 306–311.
[5] Churchich, J. E. and Farrelly, J. G. (1968) Biochem. Biophys. Res. Commun. 31, 316–321.
[6] Churchich, J. E. and Farrelly, J. G. (1969) J. Biol. Chem. 244, 3685–3690.
[7] Soda, K., Yonaha, K. Misono, H. and Osugi (1974) FEBS Lett. 46, 359–363.
[8] Peterson, E. A. and Sober, H. A. (1954) J. Amer. Chem. Soc. 76, 169–175.
[9] Yonaha, K., Misono, H., Yamamoto, T. and Soda, K. (1974) Seikagaku 46, 404.
[9a] Yonaha, K., Misono, H., Yamamoto, T. and Soda, K. (1975) J. Biol. Chem. 250, in press.
[10] Soda, K. and Misono, H. (1968) Biochemistry 7, 4110–4119.
[11] Berntsson, S. (1955) Anal. Chem. 27, 1659–1660.
[12] Jencks, W. P. (1969) Catalysis in Chemistry and Enzymology pp 555–565, McGraw-Hill, Inc., New York.
[13] O'Leary, M. H. and Malik, J. M. (1972) J. Biol. Chem. 247, 7097–7105.