THE STABILIZING EFFECT OF ADP AND THE BINDING OF ADP BY THE THREONINE DEHYDRASE OF CLOSTRIDIUM TETANOMORPHUM*

H. R. Whiteley** and Osamu Hayaishi

Department of Medical Chemistry
Faculty of Medicine
Kyoto University
Kyoto, Japan

Received October 25, 1963

Evidence has been presented earlier (Hayaishi, et al., 1963) that ADP stimulates the deamination of threonine to alpha ketobutyrate by threonine dehydrase obtained from extracts of Clostridium tetanomorphum. The effect of ADP is relatively specific and is much more pronounced at low concentrations of threonine than at high concentrations. ADP does not participate directly in the reaction but markedly increases the affinity of the enzyme for its substrate (Hayaishi, et al., 1963), i.e., ADP may be called an "allosteric effector" (Monod and Jacob, 1961; Monod, et al., 1963). However, the mechanism by which ADP exerts its effect is unknown. The present communication describes two additional observations which may lead to an understanding of the mode of action of some allosteric effectors: 1) ADP protects threonine dehydrase activity against inactivation by dilution and heat, and 2) ADP is bound by a partially purified enzyme fraction.

MATERIALS AND METHODS

Enzyme preparation: Cells of C. tetanomorphum, harvested after 14 to 16 hours of growth in glutamate medium (Whiteley, 1957), were subjected to sonic oscillation in a Raytheon 10KC oscillator for 30 minutes and the preparation

*Supported in part by funds from grant number CA-03931 from the USPHS (to H. R. W.) and by grant number C-4222 from the USPHS, the Rockefeller Foundation, the Jane Coffin Childs Memorial Fund for Medical Research, the Squibb Institute for Medical Research and the Scientific Research Fund of the Ministry of Education of Japan (to O. H.).

**Visiting scientist from the Department of Microbiology, School of Medicine, University of Washington, Seattle 5, Washington, U. S. A. (present address).

was centrifuged. The resulting extract was fractionated to yield a 25-fold increase in specific activity by means of the following steps: treatment with protamine sulfate, precipitation with solid ammonium sulfate, adsorption to calcium phosphate gel, precipitation of the gel eluate with a solution of saturated alkaline ammonium sulfate and passage through a column of Sephadex G-25 (details of fractionation will be published elsewhere). All experiments were performed with this fraction.

Assays: One unit of enzyme activity is that amount which produces 1 μmole of alpha ketobutyrate in 20 minutes at 37°C in a reaction mixture containing 100 μmoles each of threonine and tris (hydroxymethylamino) methane buffer (TRIS, pH 8.5), 200 μmoles of β-mercaptoethanol and 10 μgrams of pyridoxal phosphate in a volume of 0.5 ml. Alpha keto acid was determined by a modification of the Friedemann and Haugen method (1943). Protein was estimated according to Lowry, et al. (1951). Specific activity is defined as units of enzyme activity/mg protein/20 minutes.

Radioactivity was assayed with a Packard Tri-Carb scintillation counter as described previously (Hayaishi, et al., 1963).

Materials: C^{14}-labeled nucleotides and C^{14}-labeled threonine were purchased from Schwarz Bio-Research, Inc., and the Radiochemical Center, Amersham, England, respectively, and unlabeled nucleotides came from Sigma Chemical Co.

RESULTS AND DISCUSSION

As shown in Table 1, ADP enhanced the activity of the enzyme (first column) and protected it from inactivation during storage at 0°C in dilute solution (second column). Thus, a fraction containing 0.2 mg protein/ml lost approximately 70% of its activity if stored for 7 hours in water, in 0.1 M TRIS buffer at pH 8.0, or in 0.001 M β-mercaptoethanol, but only 2% of its activity if stored in 2.5×10^{-3} M ADP. Storage in more dilute solutions of ADP gave partial protection. Under the same conditions, AMP, ATP or threonine (2.5×10^{-1} M) did not significantly protect the enzyme.

TABLE 1

The effect of ADP on the activity of a dilute enzyme preparation during storage at 0°C

Enzyme fraction diluted in:	0 hrs storage	7 hrs storage
water, buffer, or mercaptoethanol	49.8-49.5*	14.1-14.5**
2.5×10^{-3} M ADP	69.5	68.0
2.5×10^{-4} M ADP	61.0	54.0
2.5×10^{-5} M ADP	50.0	34.4
2.5×10^{-6} M ADP	49.7	20.8

*Specific activity. **When assayed in the presence of 2.5×10^{-3} M ADP these samples had specific activities of 18.2-18.6. Samples were diluted 20-fold and assayed in the absence of ADP (there was no appreciable loss of activity during the assay).

ADP also protected the enzyme preparation from inactivation by heat (Table 2), whereas AMP, ATP and threonine were not effective. This effect appears to be similar to the protection of certain enzymes against heat inactivation by their feedback inhibitors (Changeux, 1961; Stadtman, et al., 1961).

TABLE 2

The effect of ADP and other compounds on the activity of an enzyme preparation after heat treatment

Enzyme fraction diluted in:	0 min	5 min	10 min
water	49.0*	16.5	10.0
2.5×10^{-3} M ADP	69.0	46.6	33.8
2.5×10^{-3} M AMP	48.2	18.0	11.0
2.5×10^{-3} M ATP	49.8	21.4	14.7
2.5×10^{-1} M threonine**	48.4	15.2	8.6

*Specific activity. **Samples were passed through a short column of Sephadex G-25 prior to assay. Preparations diluted to 1 mg protein/ml as shown were brought to 54°C for the indicated time, cooled to 0°C and assayed immediately without centrifugation.

The possibility that the two effects of ADP on the enzyme (enhancement of activity and stabilization, as shown in Tables 1 and 2) might be associated with the binding of the allosteric effector was examined. 6.2 mg of a 25-fold purified enzyme fraction were incubated with 0.1 μC of C^{14}-labeled ADP (specific activity = 25.8 μC/mM) at 0°C for 30 minutes. The mixture was then passed through a column (1.3 x 25 cm) of Sephadex G-50 equilibrated with 0.1 M TRIS

buffer at pH 8.0 containing 0.001 M mercaptoethanol and eluted with the same buffer (Wilcox and Lisowski, 1960). After the first 2.0 ml, representing the internal volume of the column, fractions of 0.1 ml were collected and diluted to 4.0 ml. Aliquots were assayed immediately for enzyme activity, radioactivity and protein.

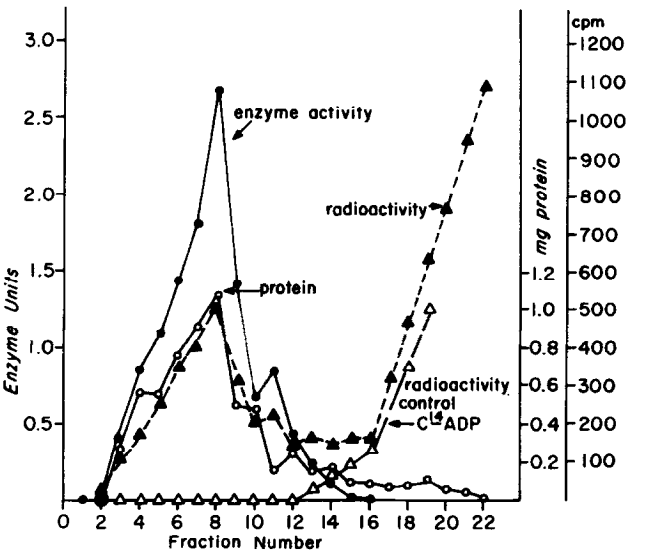

Fig. 1. Elution of threonine dehydrase activity, radioactivity and protein from a column of Sephadex G-50.

All values corrected to the same total volume. ● = enzyme activity; o = protein; ▲ = radioactivity; Δ= radioactivity of control C^{14}-ADP (0.1 μC incubated for 30 minutes at 0°C with 15 mg of serum albumin). The protein for the control, not shown in Fig. 1, was eluted in fractions 2-12.

As seen in Fig. 1, a significant amount of radioactivity (3% of the total) was eluted in the tubes containing the enzymatic activity and the peaks of radioactivity and enzymatic activity coincided. The bulk of the radioactivity was eluted in tubes following fraction 17. Recovery of the enzyme from the column was 90-98%. Preliminary experiments established that the enzyme was eluted between fractions 2 and 16 and that there is no significant difference in the elution pattern in the presence and absence of ADP. Control experiments with an aqueous solution of C^{14}-ADP and with a mixture of 15 mg of serum albumin and C^{14}-ADP (treated as described above) showed no radioactivity in fractions prior to tube 13.

The contents of tubes 2-9 were pooled, deproteinized with perchloric acid, concentrated and chromatographed on paper using an isobutyric acid-ammonia-EDTA solvent system (Magasanik, et al., 1950) and the R_f compared with the R_f values found for C^{14}-labeled ATP (0.420), ADP (0.518) and AMP (0.616) run on the same paper as standards. It should be noted that the preparation of C^{14}-ATP was contaminated with C^{14}-ADP whereas the C^{14}-ADP solution contained C^{14}-AMP. Only one radioactive area was obtained from chromatography of the pooled fraction from the column and this corresponded in R_f to ADP (0.520).

When the pooled fraction was passed through a second column of Sephadex G-50 and eluted as before, approximately 1/3 of the radioactivity was still eluted in the fractions containing the enzyme; 2/3 was eluted as free ADP. These experiments suggest that ADP is rather loosely bound by the fraction and may dissociate under certain conditions of treatment.

When labeled AMP and labeled threonine were tested under the same conditions, no radioactivity could be detected in the fractions eluted from the column containing enzymatic activity. There was no binding of threonine when the labeled substrate was incubated with the enzyme for varying periods of time (30 seconds to 30 minutes) with or without large amounts of unlabeled threonine. However, when C^{14}-ATP was substituted for ADP, a small amount of radioactivity was eluted with the enzyme. These fractions were pooled, deproteinized, concentrated and chromatographed on paper. The pooled fraction contained only labeled ADP; presumably the protein bound the ADP present as a contaminant in the ATP preparation or produced from ATP during the incubation.

REFERENCES

Changeux, J. P., Cold Spring Harbor Symp. Quant. Biol., 26, 313 (1961).

Friedemann, T. E. and G. E. Haugen, J. Biol. Chem., 147, 415 (1943).

Hayaishi, O., M. Gefter and H. Weissbach, J. Biol. Chem., 238, 2040 (1963).

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall, J. Biol. Chem., 193, 265 (1951).

Magasanik, B., E. Visher, K. Doniger, D. Elson and E. Chargaff, J. Biol. Chem., 186, 37 (1950).

Monod, J. and F. Jacob, Cold Spring Harbor Symp. Quant. Biol., 26, 389 (1961).

Monod, J., J. P. Changeux and F. Jacob, J. Mol. Biol., 6, 306 (1963).

Stadtman, E. R., G. N. Cohen, G. LeBras, H. de Robichon-Szulmajster, Cold Spring Harbor Symp. Quant. Biol., 26, 319 (1961).

Whiteley, H. R., J. Bacteriol., 74, 324 (1957).

Wilcox, P. E. and J. Lisowski, Fed. Proc., 19, 333 (1960).