ACTA CHEMICA SCANDINAVICA 15 (1961) 477–482

A Thiamine Diphosphatase from Rat Liver

KARIN TILANDER and KARL-HEINZ KIESSLING

Institute of Zoophysiology, University of Uppsala, Sweden

An enzyme hydrolyzing thiamine diphosphate (cocarboxylase) to thiamine monophosphate has been partly purified from rat liver. The pH optimum is 9.3, and it requires the presence of Mg or Mn ions. Of inhibitors tested only fluoride strongly inhibits enzyme activity.

In previous work [1–2] the content of supernatant TDP * in liver has been shown to be lower in rats treated with CCl_4 as compared with untreated animals. The same was found also in the liver from rats given alcohol for a long time [3]. The low TDP content might be explained by an increase in the dephosphorylation rate of TDP found in liver homogenate. In a later investigation the thiamine diphosphatase (TDPase) activity was determined in the nuclear, supernatant, and mitochondrial fractions from normal and from CCl_4-treated rat liver [4]. TDP was found to be more rapidly dephosphorylated in the supernatant and mitochondrial fractions from CCl_4-treated rats than from normal. The pH-optimum of the TDPase was 9.3.

The present communication describes pH-optimum, activators, and inhibitors of the TDPase for rat liver, roughly purified by fractionation with ammonium sulphate.

EXPERIMENTAL

Materials. TDP and TMP were obtained from the Sigma Chemical Company and $^{32}P_i$ from the Radiochemical Centre, Amersham. TD ^{32}P and TM ^{32}P were synthesized according to Viscontini *et al.*[6] with the addition that also $^{32}P_i$ was present during the synthesis and paper chromatographically isolated in two subsequent solvents (*iso*butyric acid-versene-NH_3[7] followed by propanol-HAc-H_2O[8]).

Preparation and purification of enzyme. White rats, weighing about 200 g, were killed by decapitation, and the liver immediately removed and weighed in 0.02 M $KHCO_3$ at + 3°C. The liver was then homogenized in a Waring blendor for 2 min in 5 vol. 0.02 M $KHCO_3$ and incubated for 15 min with stirring. All operations were carried out at + 3°C. The homogenate was centrifuged for 10 min at 10 000 *g*, and the precipitate

* Abbreviations: TDP thiamine diphosphate, TMP thiamine monophosphate, ATP adenosine triphosphate, ADP adenosine diphosphate, AMP adenosine monophosphate, IDP inosine diphosphate, P_i inorganic phosphate, TDPase thiamine diphosphatase, ATPase adenosine triphosphatase.

Table 1. Fractionation of rat liver extract. Details about incubation conditions are given in the experimental part. Enzyme activity is given as units. One unit is that amount of enzyme which causes hydrolysis of 0.1 μmole of TDP to TMP in 45 min per ml of reaction mixture.

Enzyme preparation	Volume ml	Total protein, mg	Total activity units	Spec. activity, units/mg protein $\times 10^2$
Original extract	29	1 030	79.3	7.7
0 – 15 % saturation	10	62	39.7	64
15 – 25 % saturation	10	74	41.3	56
25 – 30 % saturation	10	45	48.5	108
Supernatant	33	614	9.8	3

was discarded. To the supernatant saturated $(NH_4)_2SO_4$ solution was added with stirring to 15 % saturation. After standing for 15 min the precipitate was centrifuged 10 min at 10 000 *g*. The process was repeated, and the fractions at 15 – 25 and 25 – 30 % saturation were collected. The precipitates were dissolved in 0.02 M $KHCO_3$, and dialyzed against 0.02 M $KHCO_3$ for 18 h. The purification procedure is summarized in Table 1.

Enzyme test. Phosphatase activity was determined in a system containing 1.0 ml 2×10^{-3} M TD ^{31}P and traces of TD ^{32}P, 1.0 ml 0.1 M glycine buffer pH 9.3, 0.1 ml 0.1 M $MgCl_2$, and enzyme solution. Water was added to make a final volume of 2.5 ml. This system was incubated at 37°C for 45 min, and the reaction was stopped by the addition of 0.3 ml 5 M H_2SO_4.

After removal of the precipitate by centrifugation $^{32}P_i$ was determined in a 2 ml aliquot of the supernatant, as described elsewhere [9], and corrected for a blanc containing the same components as above, except enzyme, and incubated for the same time.

Protein content was estimated according to Warburg and Christian [10] by measuring light absorption at 260 and 280 mμ.

RESULTS

In the following experiments the fraction obtained between 25 and 30 % saturation with ammonium sulphate (Table 1) has been used. Deviations from the standard incubation as described in Methods are accounted for in detail for every special case.

Requirement for metal ions. The enzyme, like other phosphatases, needs the presence of divalent metal ions (Table 2).

As seen Mn^{2+} and Mg^{2+} were most active. At the rather high pH used in the experiments, Mn^{2+} was, however, partly oxidized and eliminated as a precipitation. For this reason we preferred Mg^{2+} in the following experiments.

Table 2. The influence of divalent metal ions on enzyme activity. Incubation was performed under standard conditions as described in Experimental, with addition of metal ions as seen in the table. The concentration of the metals was 4×10^{-3} M. 0.3 mg of enzyme protein was added.

Additions	% TDP hydrolyzed to TMP
None	2
Mg^{2+}	25
Mn^{2+}	35
Ni^{2+}	8

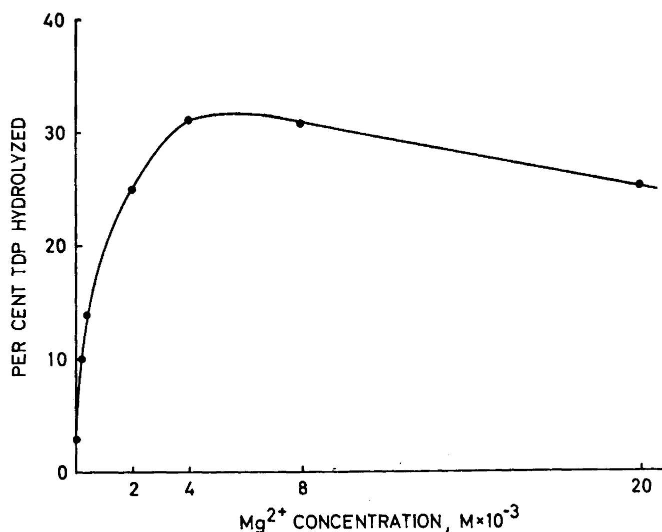

Fig. 1. The effect of Mg^{2+} on the enzymatic dephosphorylation of TDP. Incubation was performed under standard conditions for 45 min with Mg^{2+} additions as indicated. Enzyme fraction obtained between 25 and 30 % saturation with ammonium sulphate was used, corresponding to 0.3 mg protein.

Caffrey *et al.*[11], working with an adenosine triphosphatase from erythrocytes, found the metal ions to inhibit the reactions when concentrations of Mg^{2+} greater than that of the substrate were used. Fig. 1 shows the effect of

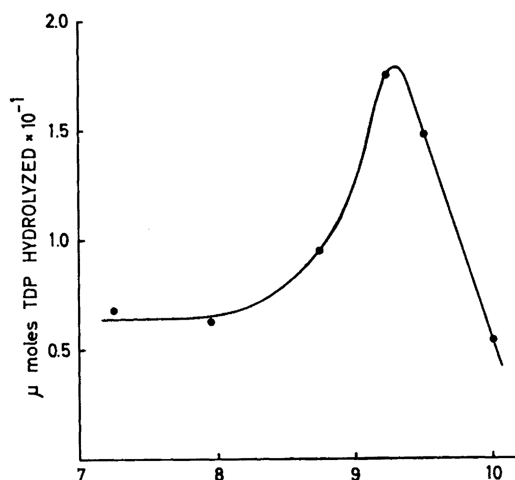

Fig. 2. Relation between pH and enzyme activity. Incubation was performed under standard conditions for 45 min. 0.2 ml Mg^{2+} 0.1 M was added.

Table 3. Effect of various inhibitors on TDPase activity. Incubations were performed as described in Experimental, but with optimal amounts of Mg^{2+} (0.2 ml 0.1 M). Enzyme was added corresponding to 0.3 mg protein.

Substance	Concentration M	% Inhibition
NaF	1×10^{-3}	17
	1×10^{-2}	66
	1×10^{-1}	92
KCN	1×10^{-3}	13
	1×10^{-2}	5
p-Chloromercuribenzoate	5×10^{-6}	0
	5×10^{-5}	0
	5×10^{-4}	0
N-Ethylmaleimide	1×10^{-4}	7
	1×10^{-3}	11
Iodoacetamide	5×10^{-4}	7
	5×10^{-3}	24

varying Mg^{2+} concentrations on the TDPase. Activation occurs up to a Mg^{2+} concentration ten times that of the substrate. Still higher concentrations are slightly inhibitory.

Relation between pH and enzyme activity. Previous experiments on TDPase activity in liver cell fractions [4] gave 9.3 as an optimal pH. The enzyme preparation obtained between 25 and 30 % saturation with ammonium sulphate (Table 1) was incubated in buffer solutions of the pH values indicated in Fig. 2. The pH optimum was found to be 9.3 also with this partly purified TDPase. Incubation of TDP at different pH without enzyme gave no hydrolysis.

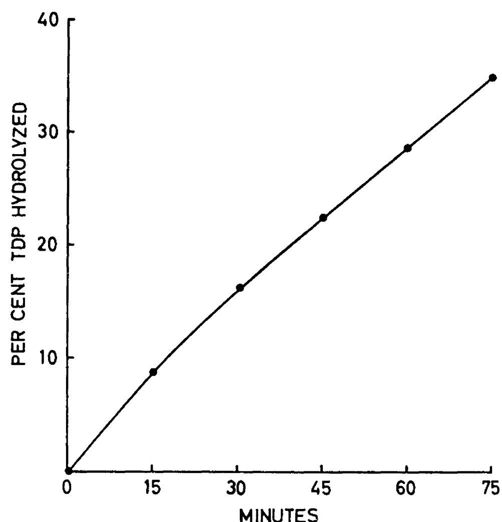

Fig. 3. Time course of hydrolysis of TDP. Incubation as in Fig. 2. Enzyme corresponding to 0.3 mg protein was added.

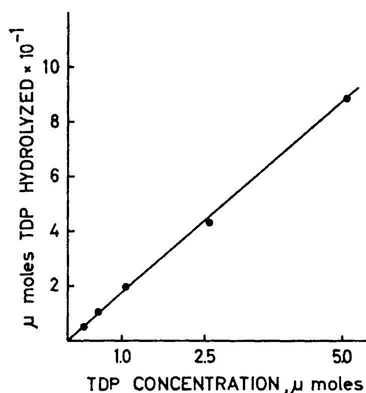

Fig. 4. Relation between substrate concentration and enzyme activity. Incubation as in Fig. 2 except that varying amounts of TDP have been added. The TDP concentration has been expressed as μmole TDP added per sample at the beginning of the incubation.

Inhibitors. The effect of several inhibitors on TDPase is summarized in Table 3. Fluoride is a powerful inhibitor, while cyanide and various SH inhibitors show no or little effect. Also TMP inhibits the hydrolysis of TDP. When added in the same concentration as TDP, it gives an inhibition of about 25 %.

Time course of hydrolysis of TDP. The progress of the hydrolysis of TDP with incubation time is seen in Fig. 3. Sometimes a slight bend on the line could be found after 30 min incubation. This we have ascribed to an inhibitory effect of TMP, formed during the incubation, as will be seen later. It is not seen on time curves at any other pH tested.

Relation between substrate concentration and enzyme activity. The hydrolysis of TDP at initial concentrations from 0.25 to 5 μM and constant concentration of enzyme during 45 min incubation was determined (Fig. 4). The values obtained plotted in the double reciprocal manner gave a K_m for TDP of about 1×10^{-3} M.

DISCUSSION

In previous papers we have shown that under certain conditions, as for example after intraperitoneal injection of CCl_4 or after forcing the animals to consume alcohol for a long time, the level of TDP in rat liver, especially in the supernatant fraction, is markedly lowered. In the same time an increase in the ability of the liver cell fractions to hydrolyse TDP to TMP could be shown [2-4]. The supernatant fraction contained most of the enzyme, here named TDPase, the activity of which could be increased more than 100 % by previous injections of CCl_4 into the rat. This increase, however, only implies a liberation or activation of the enzyme already present in the supernatant fraction (including microsomes and lyzosomes), as preparation of acetone extracts from supernatants from CCl_4-treated and normal rat livers gave no difference in the TDPase activity (unpublished data). The fact that an en-

zyme, hydrolyzing TDP, occurs latent, very likely hidden within subcellular particles, was already shown by Dianzani (personal communication) who treated the tissues with deterging agents, and supposed the enzyme to be liberated hereby from lyzosomes.

Enzymes transforming pyrophosphate groups into monophosphates are described among others by Gibson *et al.*[12], Plaut [13], Goldberg and Gilmour [14], Jones and Ernster [15]. In all cases nucleoside diphosphates were used as substrates, and only in the case with the ATPase, investigated by Goldberg and Gilmour, also TDP had been added without, however, being hydrolyzed.

Histochemically Naidoo and Pratt [16] have studied a thiamine pyrophosphatase in nervous tissue of normal and of thiamine deficient chickens. Their enzyme as well as the TDPase from liver, studied by us, was activated by Mg^{2+}, possessed optimal activity at a rather high pH as compared with the nucleoside diphosphates mentioned above, and did only split off one phosphate group from TDP. This indicates that their enzyme from chicken brain may be the same as that from rat liver studied by us.

Whether or not the TDPase examined by us also catalyzes the dephosphorylation of other phosphate esters, and then especially nucleoside diphosphates, has not been investigated in detail. Preliminary results indicate, however, that ADP is hydrolyzed by the same enzyme.

Acknowledgement. This work was supported by grants from the *Swedish Medical Science Research Council.*

REFERENCES

1. Dianzani, M. U. and Dianzani Mor, M. A. *Biochim. et Biophys. Acta* **24** (1957) 564.
2. Kiessling, K.-H. and Tilander, K. *Acta Chem. Scand.* **14** (1960) 841.
3. Kiessling, K.-H. and Tilahder, K. *Exptl. Cell. Research* **19** (1960) 628.
4. Kiessling, K.-H. and Tilander, K. *Biochim. et Biophys. Acta* **43** (1960) 335.
5. Dianzani, M. U. *Biochim. et Biophys. Acta* **14** (1954) 514.
6. Viscontini, M., Bonetti, G. and Karrer, P. *Helv. Chim. Acta* **32** (1949) 1478.
7. Kiessling, K.-H. *Acta Chem. Scand.* **10** (1956) 1356.
8. Siliprandi, D. and Siliprandi, N. *Biochim. et Biophys. Acta* **4** (1952) 479.
9. Kiessling, K.-H. *Biochim. et Biophys. Acta* **39** (1960) 412.
10. Warburg, O. and Christian, W. quoted by Layne in *Methods in Enzymology* II, p. 447.
11. Caffrey, R. W., Tremblay, R., Gabrio, B. W. and Huennekens, F. M. *J. Biol. Chem.* **223** (1956).
12. Gibson, D. M., Ayengar, P. and Sanadi, D. R. *Biochim. et Biophys. Acta* **16** (1955) 536.
13. Plaut, G. W. E. *J. Biol. Chem.* **217** (1955) 235.
14. Goldberg, M. and Gilmour, D. *Arch. Biochem. Biophys.* **51** (1954) 411.
15. Jones, L. C. and Ernster, L. *Acta Chem. Scand.* **14** (1960) 1839.
16. Naidoo, D. and Pratt, O. E. *Biochim. et Biophys. Acta* **16** (1958) 75.

Received October 27, 1960.