Biochimica et Biophysica Acta, 658 (1981) 169—173

BBA Report

BBA 61391

THE EFFECT OF TRIS (HYDROXYMETHYL) AMINOMETHANE ON MEMBRANE BOUND 5′-NUCLEOTIDASE FROM SWINE AORTIC SMOOTH MUSCLE

SAMUEL J. GOLDMAN and LINDA L. SLAKEY

Department of Biochemistry, University of Massachusetts, Amherst, MA 01003 (U.S.A.)

(Received December 1st, 1980)

Key words: 5′-Nucleotidase assay; Tris interference

Summary

5′-Nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) activity was stimulated by increasing the concentration of Tris over the range of 1—100 mM. Significant differences were found when the kinetic parameters of the enzyme determined in Tris were compared to those determined in glycine. These data suggest that Tris interacts with 5′-nucleotidase and is therefore unsuitable for use in the assay of the enzyme.

Tris has become the buffer of choice for the in vitro assay of 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) [1—12]. The assumption that Tris is appropriate for use in the assay of phosphatases is undermined by evidence obtained with alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) that shows Tris modulates enzyme activity [12—17]. We have therefore examined the effect of Tris on a preparation of membrane bound 5′-nucleotidase from porcine aortic smooth muscle. The results of our work show a complex relationship between Tris concentration and enzyme activity.

Segments of hog aorta 10—20 cm in length were obtained from freshly slaughtered hogs. The medial layer was dissected away, rinsed in ice-cold 0.25 M sucrose, minced with scissors and weighed. The minced tissue was suspended in 8—10 vol, by weight, of ice-cold 0.25 M sucrose. The tissue

Abbreviations: EPPS, (4-(2-hydroxyethyl)-1-piperazine propane sulfonic acid; AOPCP, α,β-methylene adenosine diphosphonate.

was homogenized for 20 s at 30 000 rev./min in a Virtis blender. The homogenate was centrifuged for 12 000 $\times g \cdot$ min in a SS34 Sorvall rotor. The supernatant was decanted and the pellet resuspended in 0.25 M sucrose and centrifuged as above. The post nuclear pellet was obtained by centrifuging the combined post nuclear supernatants for $6.9 \cdot 10^6 \times g \cdot$ min in a Beckman 50 Ti or TY 65 rotor. The post nuclear supernatants were resuspended in 0.25 M sucrose/1 mM EPPS, pH 7.85. This suspension was centrifuged for 12 000 $\times g \cdot$ min, the supernatant decanted, and 0.02% sodium azide (final concentration) added as preservative. Typical yield of this preparation was 30—40% of the total homogenate 5'-nucleotidase activity.

The membrane bound 5'-nucleotidase activity obtained was judged to be due to a single activity by the following criteria. (1) 5'-AMP, 5'-GMP, 5'-UMP, 5'-IMP, and 5'-CMP were hydrolyzed while hydrolysis of 2'- and 3'-AMP was undetectable. (2) For each substrate, the dependence of rate on substrate concentration showed a Hill coefficienct equal to one, and each mononucleotide inhibited the hydrolysis of 5'-AMP with a K_i proportional to its $K_{0.5}$ as substrate. (3) The enzyme activity is completely inhibited by the ADP analog AOPCP.

Two methods of enzyme assay were used. Incubation mixtures contained 100 mM glycine, pH 7.85/0.25 M sucrose/100 μM $MnCl_2$/0.75—1.5 μg protein (uniformly suspended in buffer with a glass pestle douce homogenizer)/ 20—500 μM 5'-AMP (or other mononucleotide) in a final volume of 0.400 ml. Incubations were carried out in duplicate at 37°C for 7—15 min. Reactions were stopped by addition of 0.080 ml 0.25 M $ZnSO_4$, pH 2.5. An aliquot was taken and assayed for inorganic phosphate by the method of Penny and Bolger [18]. In experiments involving Tris, the appropriate concentrations of Tris (1—100 mM) at pH 7.85 were substituted for 100 mM glycine. At all concentrations of buffer employed, the pH of the reaction mixture did not change during the time course of the assay.

The second method is a modification of the technique of Glastris and Pfeiffer [19]. Incubation mixtures contained 100 mM glycine, pH 7.85/0.25 M sucrose/100 μM $MnCl_2$/0.75—1.5 μg protein/20—500 μM 5'-AMP/tracer amounts of 5'-[^{3}H]AMP in a final volume of 0.400 ml. Incubations were carried out in duplicate at 37°C for 7—15 min. Reactions were stopped by addition of 0.200 ml of a stirring suspension of Dowex 1 X4. The reaction vials were immediately vortexed for 20 s and the Dowex was removed by centrifugation for 35 000 $\times g \cdot$ min in an Eppendorf Model 3200/30 table-top centrifuge. An aliquot was then counted in 3.5 ml Biofluor. [^{3}H]Toluene was added as an internal standard when needed. Increasing the concentration of Tris above 20 mM altered the binding properties of the Dowex in the assay so that measurements on higher concentrations of Tris were impractical.

The effect of various concentrations of Tris on 5'-nucleotidase activity is shown in Fig. 1. When analyzed by assay of inorganic phosphate, the activity at 1 mM Tris was decreased by about 65% relative to the 100 mM glycine reference. As the concentration of Tris increases, the activity also increases, and at concentrations of Tris above 40 mM, there is a relative stimulation of activity compared to 100 mM glycine. Although the analysis by radioisotope assay parallels the phosphate assay, the apparent activity at low Tris concen-

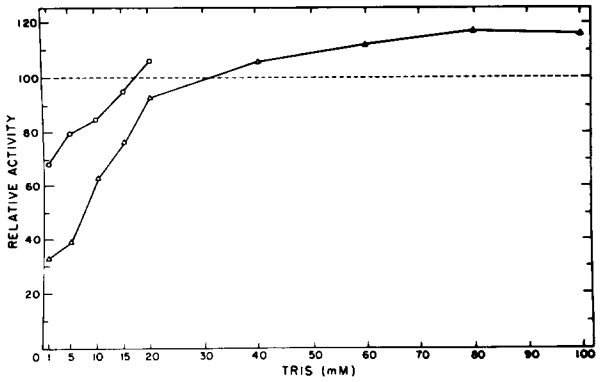

Fig. 1. Initial velocities at 100 μM 5′-AMP were determined by either direct assay of phosphate (△———△) or radioisotope assay (○———○) as described. Each point represents the mean of two determinations. Relative activity is expressed as the percent activity of a 100 mM glycine reference. Control experiments showed that varying the concentration of glycine from 10 to 100 mM had no effect on the enzyme activity.

trations is greater, and the activity increases to equal that of 100 mM glycine at about 15 mM Tris. The magnitude of the difference in activity in 1 mM Tris as compared to 100 mM glycine was dependent on the particular preparation and varied between 20 and 70%; however, the qualitative relationship between the two assay methods was always maintained.

The effect of increasing the concentration of Tris was to stimulate the enzyme activity (Fig. 1). Evidence that this apparent stimulation was not due to appearance of a new activity was 2-fold. First, the activity in the presence of 10 mM Tris did not hydrolyze 2′- or 3′-AMP. Second, the activity in both 10 mM Tris and 100 mM glycine was completely inhibited by 20 μM AOPCP and inhibited approx. 50% by 1 μM AOPCP. The change in activity from low to high concentrations of Tris compared to activity in 100 mM glycine was not due simply to changes in ionic strength. When the reaction mixtures were adjusted to physiological ionic strength with saline and compared to standard reaction mixtures, the ratio of activity in 100 mM glycine to corresponding activity in 10 mM Tris was constant within experimental

TABLE I

EFFECT OF TRIS ON KINETIC PARAMETERS OF 5′-NUCLEOTIDASE

Initial velocities were determined by the method indicated. Values are expressed as means ± S.D. Each experiment included a minimum of eight substrate concentrations. The number of experiments is given in parentheses. *P* is calculated by Student's *t* test for paired variables and denotes the probability that the means for the two buffers differ by chance. $K_{0.5}$ was calculated by the direct linear plot method of Cornish-Bowden and Eisenthal [23]. Hill coefficients were calculated using a least-squares linear regression.

	100 mM glycine	10 mM Tris	*P*	Method of assay
$K_{0.5}$ (μM)	49.9 ± 15.7 (7)	23.8 ± 17.3 (7)	<0.010	Phosphate
Hill coefficient	0.94 ± 0.10 (7)	0.94 ± 0.10 (5)	—	Phosphate
$K_{0.5}$ (M)	48.9 ± 5.0 (4)	27.0 ± 5.0 (4)	<0.025	[^{3}H]AMP
Hill coefficient	0.90 ± 0.11 (4)	0.93 ± 0.05 (4)	—	[^{3}H]AMP

error. Changing the concentration of glycine in the range of 10—100 mM had no effect on the enzyme activity. Concentrations of glycine less than 10 mM were not measured since the buffering capacity is not adequate to maintain constant pH in the reaction mixture.

The kinetic properties of 5′-nucleotidase in 100 mM glycine and in 10 mM Tris are summarized in Table I. Whether analyzed by direct assay of inorganic phosphate or by accumulation of radiolabeled adenosine, the results were similar. The $K_{0.5}$ in 100 mM glycine buffer was about 2-fold greater than the $K_{0.5}$ in 10 mM Tris. Values for V were dependent on the particular preparation and varied by as much as 2-fold; however, a statistically significant trend was established. The values for V in 100 mM glycine exceeded those in 10 mM Tris by an average of 1.6-fold ($^*P < 0.01$) measured by phosphate assay and 1.4-fold ($^*P < 0.025$) measured by radioisotope assay. It is apparent from these data that the effect of 10 mM Tris on 5′-nucleotidase kinetic behavior was relatively independent of assay technique. This argues against the possibility that the effect seen is an artifact of assay techniques and suggests that Tris modulates the enzyme activity.

The possible problems associated with the use of amine buffers in biological studies with enzymes were first addressed by H.R. Mahler [20]. He suggested, and presented evidence for, several possible mechanisms of interaction of amine buffers, particularly Tris, with enzymatic systems. These included (1) utilization of Tris as substrate. (2) action as inhibitor of protein activity, (3) actual participation of Tris in reaction sequences, and (4) compound formation of Tris and substrate. Examples of such interactions include use of Tris as substrate for liver alcohol dehydrogenase [20], formation of 5′-AMP ester of Tris by liver plasma membrane [21], chelation of Mn^{2+} by Tris [22], and the various effects of Tris on alkaline phosphatase.

A review of recent literature shows that Tris is still commonly used as buffer for kinetic [8,9,11,12] and clinical [10] studies on 5′-nucleotidase. The evidence presented above shows complex effects of Tris on 5′-nucleotidase activity. The effects appear to be dependent on the concentration of Tris employed as well as the concentration of substrate. Whether these effects are analogous to other 5′-nucleotidases will likely depend on the specific characteristics of those systems. However, it is clear that the assumption that Tris does not interact with system components in the assay of 5′-nucleotidase is not valid.

We wish to thank Scott Stylos for capable technical assistance. A computer program for calculation of kinetic parameters was the kind gift of Dr. Athel Cornish-Bowden. This research was supported by NIH Grant No. HL. 24660-01. L.L.S. is an Established Investigator of the American Heart Association.

References

1 Sullivan, J.M. and Alpers, J.B. (1971) J. Biol. Chem. 246, 3057—3063
2 Nakatsu, K. and Drummond, G.I. (1972) Am. J. Physiol. 223, 1119—1127
3 Edwards, J. and Maguire, M.H. (1970) Mol. Pharmacol. 6, 641—648
4 Burger, R.M. and Lowenstein, J.M. (1975) Biochemistry 14, 2362—2366
5 Newby, A.C., Lazio, J.P. and Hales, C.N. (1975) Biochem. J. 146, 625—633
6 Nakamura, S. (1976) Biochim. Biophys. Acta 426, 339—347

7 Ikehara, Y., Takahashi, K., Mansho, K., Eto, S. and Kato, K. (1977) Biochim. Biophys. Acta 470 202—211
8 Stefanovic, V., Mandel, P. and Rosenberg, A. (1979) Biochemistry 18, 357—361
9 Fox, I.H.a nd Marchant, P.J. (1976) Can. J. Biochem. 54, 1055—1060
10 Edwards, N.L., Magilavy, D.B., Cassidy, J.T. and Fox, I.H. (1978) Science 201, 628—630
11 Dormand, J., Bonnafous, J.-C. and Mani, J.-C. (1978) Eur. J. Biochem. 87, 459—465
12 Doss, R.C., Carothers Carraway, C.A. and Carraway, K.L. (1979) Biochim. Biophys. Acta 570, 96–106
13 Reid, T.W. and Wilson, J.B. (1971) The Enzymes (Boyer, P.D., ed.), pp. 373—415, Academic Press New York
14 McComb, R.B. and Bower, G.N., Jr. (1972) Clin. Chem. 18, 97—104
15 Amidor, E. (1972) Clin. Chem. 18, 94
16 Wilson, J.B., Dayan, J. and Cyr, K. (1964) J. Biol. Chem. 239, 4182—4185
17 Newmann, H., Kezdy, F., Hsu, J. and Rosenberg, I.H. (1975) Biochim. Biophys. Acta 391, 292–300
18 Penny, C.L. and Bolger, G.A. (1978) Anal. Biochem. 89, 297—303
19 Glastris, B. and Pfieffer, S.E. (1974) Methods Enzymol. 32, 124—125
20 Mahler, H.R. (1961) Ann. N.Y. Acad. Sci. 92, 427—439
21 Kiss, Z. (1979) Biochim. Biophys. Acta 568, 485—487
22 Morrison, J.F. (1979) Methods Enzymol. 63, 285
23 Cornish-Bowden, A. and Eisenthal, R. (1978) Biochim. Biophys. Acta 523, 268—272