ON THE MECHANISM OF ENZYMATIC HYDROLYSIS OF CARBAMYL PHOSPHATE

D. DIEDERICH, G. RAMPONI and S. GRISOLIA

Departments of Biochemistry and Medicine, University of Kansas Medical Center, Kansas City, Kansas and Istituto di Chimica, Biologica, Dell' Università Di Firenze, Florenz, Italy

Received 12 February 1971

1. Introduction

The decomposition of carbamyl phosphate in solution proceeds by different paths depending on the pH; these pathways (reactions 1 and 2) have been referred to as acid and base-catalyzed hydrolysis [1].

$$NH_2CO_2PO_3H_2 + H_3O^+ \longrightarrow NH_4^+ + CO_2 + H_3PO_4 \quad (1)$$

$$NH_2CO_2PO_3H_2 + OH^- \longrightarrow NCO^- + HPO_4^{2-} + H_2O \quad (2)$$

As indicated, reaction 1 leads to the simultaneous production of ammonia, CO_2, and inorganic phosphate, while reaction 2 yields cyanate and phosphate. The half-life of cyanate is reported to be ∿7–8 times that of carbamyl phosphate [1]; therefore, reaction 2 results in initial accumulation of cyanate and as the cyanate decomposes, ammonia is produced. Thus at low pH the rates of ammonia and phosphate liberation are equal, while at pH 6 the release of ammonia is ∿15% the rate of phosphate formation [2]. This seemingly paradoxical rapid appearance of inorganic phosphate and delayed appearance of ammonia led to the earlier postulation that a carbamyl phosphate derivative of acetyl glutamate was the intermediate in citrulline synthesis [2].

In spite of the fact that acyl phosphatase (an enzyme which is highly specific for acyl phosphates such as carbamyl phosphate and 1,3-diphosphoglycerate) has been purified extensively from a number of sources [3], no attempts have been carried out to determine whether the enzymatic hydrolysis of carbamyl phosphate involves the production of cyanate. The pH optimum for muscle acyl phosphatase is 5.4–5.6 [3, 4] while for the brain enzyme, the pH optimum is pH 7.0 [5]. Thus in the pH range of acyl phosphatase activity, there could be intermediate formation of cyanate.

2. Methods and materials

Bovine liver glutamate dehydrogenase (Type II, free of ammonium ions, 3.1 μmolar units/mg), α-keto-glutaric acid, urease (Type VI, 83,000 units/g), β-diphosphopyridine nucleotide, reduced form, carbamyl phosphate and hydroxylamine hydrochloride were purchased from Sigma Chemical Company. Ultra-pure urea was obtained from Mann Laboratories and potassium cyanate was purchased from Fisher Chemical Company.

Acyl (carbamyl) phosphatase from pork heart, horse skeletal muscle, beef brain and beef liver were purified as previously described [3–6].

Cyanate and carbamyl phosphate react rapidly with hydroxylamine [2, 7] to form hydroxyurea. The previously described semidine modification of the diacetyl monoxime method [8] for determining carbamyl compounds was recently shown to be useful for the determination of carbamyl phosphate or cyanate after reaction of these compounds with hydroxylamine to form hydroxyurea [9]. Interestingly, our procedure [8] results in color development with hydroxyurea even in the absence of the otherwise required $FeCl_3$. Carbamyl phosphate and cyanate were measured by this assay method [8] after conversion to hydroxyurea.

Ammonia was measured with a large excess of glutamate dehydrogenase as recommended by Rubin [10] except that change in absorbance was measured

 North-Holland Publishing Company – Amsterdam

directly with a Gilford Model 2000 recording spectrophotometer. Each cuvette contained 50 μmoles of tris-Cl, pH 7.4, 4 μmoles of α-ketoglutarate and 0.5 or 1 μmole of 3-NADH in 3 ml. After equilibration at 30°, 2 mg of glutamate dehydrogenase in 0.2 ml were added. After 1 min (to allow for reaction of endogenous ammonia) NH_4Cl, cyanate, carbamyl phosphate or urea were added. When using the last two substrates, after one minute equilibration, a large excess of muscle acyl phosphatase or urease was added (5 units or 0.2 mg respectively).

3. Results and discussion

It seemed possible to take advantage of the rapid interaction of carbamyl phosphate or cyanate with NH_2OH as well as the relatively long half-life of the cyanate in comparison to that of carbamyl phosphate to ascertain whether cyanate is an intermediate in the enzyme catalyzed hydrolysis of carbamyl phosphate. In the presence of a large excess of acyl phosphatase, one should be able to measure an appreciable quantity of cyanate if indeed cyanate is an intermediate formed during enzymic hydrolysis of carbamyl phosphate. As shown in table 1, with three different enzyme preparations which should have cleaved rapidly the carbamyl phosphate in the incubation period, there were but traces of cyanate and/or carbamyl phosphate left. Interestingly, and as shown, essentially the same results were found in the pH range of 5 to 8.

Additional evidence against cyanate as an intermediate is shown in fig. 1. These experiments were based upon the recommended utilization of a large excess of glutamate dehydrogenase for determination of ammonia [10], a procedure which allows such measurements at or near neutral pH and which theoretically should allow the detection of unstable intermediates yielding ammonia, i.e. differential rates. The experiments reported in the figure demonstrate a rapid reaction with ammonia as well as the fact that there is no reactivity with cyanate. Indeed the half-life of cyanate is longer than previously reported. Therefore cyanate is not an intermediate in the enzymatic hydrolysis of carbamyl phosphate or urea which was used as a control. As shown in fig. 1, in all cases when the reaction has reached completion, the change in absorbance corresponds to the theoretically expected

Table 1
Hydrolysis of carbamyl phosphate at different pH's and with several enzyme preparations.

Enzyme added	Hydroxyurea present (μmoles) after hydrolysis at pH:		
	5.0	7.4	8.1
None	5.9	7.6	8
Beef brain	0.6	0.7	0.8
Beef liver	0.6	1.0	1.2
Horse muscle	0.1	0.2	0.2

The incubation mixture contained 8 μmoles carbamyl phosphate, 100 μmoles of buffer (sodium acetate, pH 5.0, imidazole, pH 7.4, or tris-chloride, pH 8.1) and acyl phosphatase (100 I.U. of muscle enzyme, 30 I.U. of liver or brain enzyme) in a final volume of 0.5 ml. Incubation was for 20 min at 30°. At the end of the incubation, 0.2 ml of 1 M neutralized NH_2OH was added to each tube. The tubes were then heated in boiling water for 15 min, cooled and 3 ml of H_2O were added to each tube. After centrifugation aliquots of the supernatants were assayed [8].

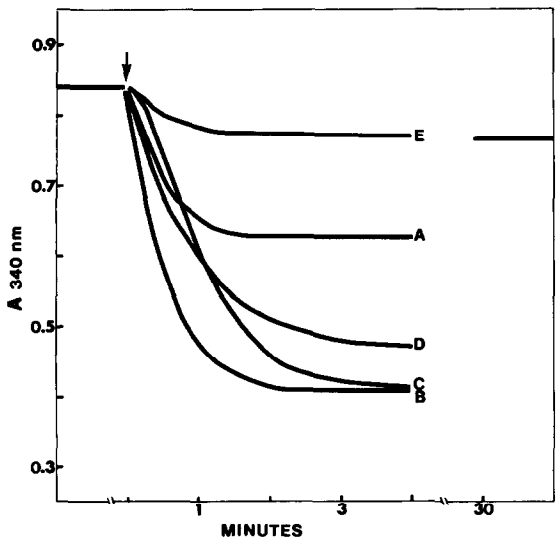

Fig. 1. Comparison of reactivity of glutamate dehydrogenase to added ammonia and to ammonia formed from enzymatic hydrolysis of carbamyl phosphate and urea. The changes in absorbance with the different substrates were measured as indicated in the text. Curve A, 0.1 μmole of ammonia; curve B, 0.2 μmole of ammonia; curve C, 0.1 μmole of urea; curve D, 0.18 μmole of carbamyl phosphate; curve E, 5 μmoles of potassium cyanate. At the point indicated by the arrow, the reactions were initiated by the addition of ammonium chloride (A and B), urease (C), acyl phosphatase (D), or cyanate (E).

value for the complete hydrolysis and conversion to glutamate of all of the ammonia from carbamyl phosphate or urea. For example, with 0.1 and 0.2 μmole of ammonia, the total change in absorbance was 0.21 and 0.43 (curves A and B respectively). The hydrolysis of 0.18 μmole of carbamyl phosphate resulted in the change in absorbance corresponding to that given by 0.18 μmole of ammonia (curve D). Again with 0.36 μmole of carbamyl phosphate, we measured an absorbance change of 0.77, essentially the theoretical value. As shown in the figure, these changes were essentially as rapid as those measured with ammonia. Interestingly, as shown in the figure, the (very rapid) change in absorbance due to hydrolysis of 0.1 μmole of urea was 0.43, exactly that observed with 0.2 μmoles of ammonia. Moreover, although not shown in the figure, with 0.2 μmole of urea, we observed again a very rapid change in absorbance of 0.88, corresponding to that given by 0.4 μmole of ammonia. Other experiments under similar conditions confirmed these results.

Therefore, the present work definitely excludes the formation of cyanate as an intermediate in the enzyme-catalyzed hydrolysis of carbamyl phosphate and incidentally also during urea hydrolysis by urease (a reaction in which carbamyl phosphate has been postulated to be an intermediate [11]). Whether or not there is intermediate formation of carbamate or direct splitting of carbamyl phosphate or urea to ammonia and CO_2 remains to be demonstrated. As shown by the kinetic data, if carbamate is formed, it decomposes very rapidly. This is in contradiction to an earlier report [10], wherein, under essentially the same conditions, the rapid release of 1 mole of ammonia per mole of urea by urease as well as presumed stability of carbamate for a lengthy period (at least 18 min) was noted. Our observations are in agreement with the chemistry of carbamate which is unknown in the free state [12]. Thus, if carbamate is an intermediate in carbamyl phosphate (as well as urea) hydrolysis, demonstration of its presence will require rapid and/or special techniques.

In view of our finding, a review of the literature has failed to reveal reliable data which directly demonstrate carbamate as an intermediate in the enzymatic hydrolysis of urea.

Acknowledgements

Supported by Public Health Service Grant AM01855. We wish to thank Carol Bettis for excellent technical assistance.

References

[1] C.M. Allen, Jr. and M.E. Jones, Biochemistry 3 (1964) 1238.
[2] S. Grisolia and R. Marshall, in: A Symposium on Amino Acid Metabolism, eds. W.D. McElroy and H.B. Class (Johns Hopkins Press, Baltimore, 1955) p. 258.
[3] D. Diederich and S. Grisolia, Biochim. Biophys. Acta (1970) in press.
[4] G. Ramponi, A. Guerritore, C. Treves, P. Nassi and V. Baccari, Arch. Biochem. Biophys. 130 (1969) 362.
[5] D. Diederich and S. Grisolia, J. Biol. Chem. 244 (1969) 2412.
[6] G. Ramponi, P. Nassi, C. Treves, G. Cappugi and M. Orienti, FEBS Abstracts (6th Meeting of the Federation, Madrid, 1969) p. 323.
[7] R.O. Marshall, Doctoral Dissertation, University of Wisconsin (1955).
[8] D. Hunninghake and S. Grisolia, Anal. Biochem. 16 (1966) 200.
[9] R.L. Levine and N. Kretchmer, private communication, December (1969).
[10] M. Rubin, Prog. Clinico-Chemical Methods 3 (1966) 6.
[11] J.E. Varner, in: The Enzymes, Vol. 4, eds. P.D. Boyer et al. (Academic Press, New York, 1960) p. 247.
[12] V. Von Richter's Organic Chemistry, eds. R. Anschütz (Blakiston, Philadelphia, 1899) p. 393.