*Biochimica et Biophysica Acta*, 616 (1980) 389—394


**BBA Report**

BBA 61384

# EFFECT OF PHOSPHO*ENOL*PYRUVATE ANALOGS ON THE ACTIVITY OF ENOYLPYRUVATE TRANSFERASE AND THE EFFECT OF UDP-*N*-ACETYLGLUCOSAMINE ON THE REACTIVITY OF THE ACTIVE SITE SH GROUP

RASHID A. ANWAR and MARIANNA VLAOVIC

*Department of Biochemistry, University of Toronto, Toronto M5S 1A8 (Canada)*

(Received August 19th, 1980)

*Key words: Enoylpyruvate transferase; Phosphoenolpyruvate analog; Thiol group; Active site; Enzyme mechanism*

## Summary

Effect of several phospho*enol*pyruvate analogs on the activity of enoylpyruvate (phospho*enol*pyruvate:UDP-2-acetamido-2-deoxy-D-glucose 2-enoyl-1-carboxyethyltransferase, EC 2.5.1.7) transferase was examined. The results suggest that the phospho*enol*pyruvate binding site of the transferase is very similar to that of pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40). Evidence is presented to show that the binding of UDP-GlcNAc to the transferase enhances the reactivity of the active site SH group.

The enzyme, enoylpyruvate transferase (phospho*enol*pyruvate:UDP-2-acetamido-2-deoxy-D-glucose 2-enoyl-1-carboxyethyltransferase, EC 2.5.1.7) catalyzes the transfer of *enol*pyruvate moiety of phospho*enol*pyruvate to 3′-position of *N*-acetylglucosamine moiety of UDP-GlcNAc to form UDP-GlcNAc-*enol*pyruvate with the liberation of inorganic phosphate and is the first committed reaction of bacterial cell wall peptidoglycan biosynthesis [1]. Previously, the enzyme was purified to homogeneity [2] and evidence presented to show the formation of an enzyme *enol*pyruvate intermediate in the reaction and that the formation of E-*enol*pyruvate intermediate requires the prior binding of the second substrate, UDP-GlcNAc [3].

The work with phospho*enol*pyruvate analogs, described in this communication, suggests that the phospho*enol*pyruvate binding site of the transferase is very similar to that of pyruvate kinase. This work also led to the finding that

UDP-GlcNAc binding to the transferase enhances the reactivity of the active site SH group.

Phospho*enol*pyruvate analogs, phospho*enol*-α-ketovalerate, phospho*enol*-3-bromopyruvate, phospho*enol*-3-phenylpyruvate, were synthesized by reacting corresponding bromoketo acids with trimethyl phosphite essentially as described in the literature [4,5]. Pyruvate, bromopyruvate and fluoropyruvate were purchased from Sigma. α-Ketovaleric acid was from Aldrich and trimethyl phosphite from Eastman. Other reagents were the same as described previously [2].

The enzyme was purified as described previously and assayed by measuring $P_i$ released [2]. The assay was carried out in 50 mM Tris-HCl buffer, pH 7.4, containing 0.5 mM dithiothreitol, 1.0 mM UDP-GlcNAc and appropriate concentrations of phospho*enol*pyruvate or its analogs. After incubation, the reaction was stopped by making the mixture 6% (w/v) in trichloroacetic acid and the entire supernatant was used for $P_i$ determination by a modified method of Polley [6] using $SnCl_2$ as the reducing agent.

Effects of phospho*enol*pyruvate analogs, (Z)-phospho*enol*-α-ketobutyrate, phospho*enol*-α-ketovalerate, phospho*enol*-3-bromopyruvate, phospho*enol*-3-phenylpyruvate, pyruvate, fluoropyruvate and bromopyruvate, on the activity of enoylpyruvate transferase were examined. As reported previously [3], (Z)-phospho*enol*-α-ketobutyrate was found to act as a substrate for the enzyme and UDP-GlcNAc-*enol*-α-ketobutyrate was characterized as the product. Phospho*enol*-α-ketovalerate was found to be an effective inhibitor of the enzyme with a $K_i$ value of 1.7 mM as shown in Fig. 1. No detectable substrate activity could be observed with phospho*enol*-3-bromopyruvate or phospho*enol*-3-phenylpyruvate. It was not possible to determine the competitive inhibition of the reaction by phospho*enol*-3-bromopyruvate or phospho*enol*-3-phenylpyruvate, due to non-enzymatic hydrolysis of these analogs under the assay conditions. The situation was further complicated with phospho*enol*-3-bromopyruvate: non-enzymatic hydrolysis generated bromopyruvate which inhibited the enzyme irreversibly (see below). Our inability to detect substrate activity

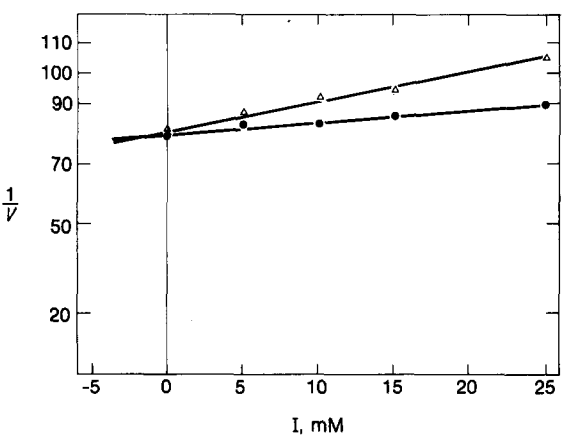



**Fig. 1. Dixon plot of phospho*enol*-α-ketovalerate inhibition of enoylpyruvate transferase. Higher phospho*enol*pyruvate concentration, 1.2 mM (●———●); lower phospho*enol*pyruvate concentration, 0.6 mM (△———△); UDP-GlcNAc, 1 mM; transferase, 0.05 unit of activity; $V = A_{660}$ per min ($P_i$ released).**

TABLE I

EFFECTS OF PHOSPHO*ENOL*PYRUVATE ANALOGS ON THE ACTIVITY OF ENOYLPYRUVATE TRANSFERASE

| Phospho*enol*pyruvate analog | Effect |
|---|---|
| (Z)-Phospho*enol*-α-ketoburyrate | Active as a substrate |
| Phospho*enol*-α-ketovalerate | Effective inhibitor; $K_i$ = 1.7 mM |
| Phospho*enol*-3-bromopyruvate<br>Phospho*enol*-3-phenylpyruvate | No detectable activity as substrate |
| Pyruvate<br>Fluoropyruvate | No inhibition |
| 3-Bromopyruvate | Irreversible inhibition |

with phospho*enol*-3-bromopyruvate at saturation concentrations may be attributable to this effect. No inhibition of the reaction was detected with pyruvate or fluoropyruvate at concentrations as high as 100 mM (250-fold excess over phospho*enol*pyruvate). These results are summarized in Table I.

The effects of phospho*enol*-α-ketobutyrate and phospho*enol*-α-ketovalerate on the activity of the transferase are very similar to those reported with pyruvate kinase [4,5] (with pyruvate kinase, phospho*enol*-α-ketobutyrate acts as substrate and phospho*enol*-α-ketovalerate is an effective inhibitor with a $K_i$ value of 1.07 mM). This suggests that the phospho*enol*pyruvate binding site in transferase and pyruvate kinase are very similar. Even the $K_m$ values for phospho*enol*pyruvate with these enzymes are very similar (with pyruvate kinase [5], $K_m = 2.6 \cdot 10^{-5}$ M and with transferase [2], $K_m = 3 \cdot 10^{-5}$ M). This is interesting because the reactions catalyzed and the mechanisms of catalysis of these two enzymes are apparently quite different (transferase [2] has no metal requirement and forms E-enolpyruvate intermediate). The result with pyruvate (i.e., no inhibition of the reaction) is not really inconsistent with the phospho*enol*pyruvate binding site of transferase being similar to that of pyruvate kinase. The pyruvate kinase reaction is essentially irreversible and pyruvate is known to bind pyruvate kinase very poorly [7].

The effect of UDP-GlcNAc on the reactivity of the active site SH group was investigated. As mentioned above, there was non-enzymatic hydrolysis of phospho*enol*-3-bromopyruvate under the assay conditions and there was evidence of irreversible inhibition of the transferase at high concentrations of phospho*enol*-3-bromopyruvate. To test the possibility that 3-bromopyruvate, generated as a result of the non-enzymatic hydrolysis of phospho*enol*-3-bromopyruvate, was alkylating the enzyme, the effect of 3-bromopyruvate on the activity of the transferase was examined. The results (Table II) show that the enzyme was preferentially inhibited by 3-bromopyruvate in the presence of UDP-GlcNAc. Preincubation of the enzyme for 10 min with 0.2 mM 3-bromopyruvate in the presence of UDP-GlcNAc (Table II, No. 4) resulted in 70% loss of activity. On the other hand, in the absence of UDP-GlcNAc, the loss of activity was 45% (Table II, No. 3) and was similar to the inhibition observed when 3-bromopyruvate was omitted during preincubation but was present in the assay incubation mixture (No. 2). The transferase was not inhibited by phospho*enol*-3-

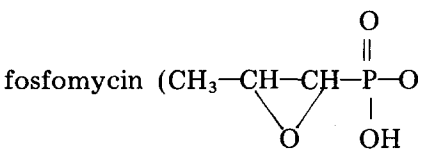
TABLE II

INHIBITION OF ENOYLPYRUVATE TRANSFERASE BY BROMOPYRUVATE

The enzyme (0.03 unit) was preincubated for 10 min in 50 mM Tris-HCl buffer, pH 7.4, containing 0.5 mM dithiothreitol, with or without bromopyruvate as indicated. Activity ($P_i$ liberated; $A_{660}$) was measured after incubation for 30 min as indicated. Pep, phospho*enol*pyruvate.

| Sample | Preincubation mixture (10 min) | Incubation mixture | Activity ($A_{660}$) |
|---|---|---|---|
| 1 | Enzyme | Enzyme + 1 mM Pep + 1 mM UDP-GlcNAc | 0.24 |
| 2 | Enzyme | Enzyme + 1 mM Pep + 0.2 mM bromopyruvate + 1 mM UDP-GlcNAc | 0.15 |
| 3 | Enzyme + 0.2 mM bromopyruvate | Enzyme + 1 mM Pep + 0.2 mM bromopyruvate + 1 mM UDP-GlcNAc | 0.14 |
| 4 | Enzyme + 0.2 mM bromopyruvate + 1 mM UDP-GlcNAc | Enzyme + 1 mM Pep + 0.2 mM bromopyruvate + 1 mM UDP-GlcNAc | 0.075 |

bromopyruvate at concentrations (0.1—0.2 mM) comparable to those used for bromopyruvate. This preferential inhibition of the transferase by 3-bromopyruvate in the presence of UDP-GlcNAc could either be due to the enhanced binding of 3-bromopyruvate to the active site or the enhanced reactivity of the active site SH group in the presence of UDP-GlcNAc, or both.

Previously, enoylpyruvate transferase has been shown to form a covalent intermediate with *enol*pyruvate and the intermediate forms only in the presence of UDP-GlcNAc [3]. Similarly, the enzyme is irreversibly inhibited with fosfomycin (  ), active site SH is involved in this reaction and the inhibition occurs only in the presence of UDP-GlcNAc [8,9]. On the basis of these observations, it was suggested that for the proper binding of phospho*enol*pyruvate the prior binding of UDP-GlcNAc to the active site is required. Bromopyruvate is known to act as an active site-directed inhibitor for a number of enzymes (e.g., pyruvate carboxylase [10]; 2-keto-3-deoxy-6-phosphogluconic aldolase [11], *N*-acetylneuraminic acid aldolase [12], pyruvate kinase [13] and DHAP synthase (EC 4.1.2.14) [14]). In all of these cases, one of the substrates or products is pyruvate. However, in the case of enoylpyruvate transferase, pyruvate is neither a substrate nor a product. Further, pyruvate and 3-fluoropyruvate show no competitive inhibition of this enzyme even at very high concentrations (Table I; 100 mM 250-fold excess over phospho*enol*pyruvate) and thus do not bind to the active site of the transferase. These observations suggested that the enhanced reactivity of the active site SH group in the presence of UDP-GlcNAc may be responsible for the preferential inhibition of this enzyme by 0.2 mM bromopyruvate in the presence of UDP-GlcNAc.

To explore this point further, the effect of iodoacetate and iodoacetamide

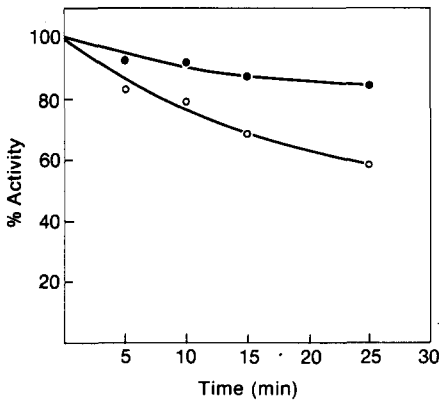



Fig. 2. Effect of UDP-GlcNAc on the reactivity of the active site SH group of enoylpyruvate transferase with iodoacetate: The enzyme (0.06 units/ml) was preincubated with 0.2 mM iodoacetate in 50 mM Tris-HCl buffer, pH 7.4, in the presence (1.0 mM; ○———○) and in the absence (●———●) of UDP-GlcNAc. Aliquots (1.0-ml) were removed at the indicated time intervals, cysteine was added to a final concentration of 10 mM to stop the alkylation of the enzyme and the residual enzyme activity was then determined by measuring the $P_i$ released (final incubation vol. was 1.02 ml; 0.97 mM UDP-GlcNAc and 0.97 mM phospho*enol*pyruvate). The enzyme preincubated in the absence of iodoacetate was used as a control.

on the activity of the transferase, in the presence and absence of UDP-GlcNAc, was examined. The results of the iodoacetate experiment are shown in Fig. 2. The enzyme was preincubated with 0.2 mM iodoacetate in the presence and absence of UDP-GlcNAc, aliquots were removed at indicated time intervals, cystein added to a final concentration of 10 mM to stop the alkylating reaction and the remaining enzyme activity was then measured (for details see Fig. 2). The rate of inhibition of the enzyme was higher in the presence of UDP-GlcNAc than in its absence. After preincubation with iodoacetate, for 25 min, in the presence of UDP-GlcNAc, 41% of the enzyme activity was lost and in the absence of UDP-GlcNAc the loss was only 15%. Similar results were obtained with iodoacetamide; the loss of enzyme activity after 15 min preincubation with 0.2 mM iodoacetamide in the presence of UDP-GlcNAc was 38% and in the absence of UDP-GlcNAc the loss was only 20%. These results clearly show the enhanced reactivity of the active site SH group after the binding of UDP-GlcNAc to the active site of the transferase. These results also explain our observations that although fosfomycin irreversibly inhibits the transferase, it shows no competitive inhibition of pyruvate kinase even at very high concentrations (300 mM; 750-fold excess over phospho*enol*pyruvate), which one would expect, on the basis of similarities in phospho*enol*pyruvate binding sites of the transferase and pyruvate kinase, if fosfomycin was acting as an analog of phospho*enol*pyruvate. Thus, it appears that the fosfomycin inhibition of the transferase in the presence of UDP-GlcNAc is also due to the enhanced reactivity of the active site SH group and may have very little to do with its being an analog of phospho*enol*pyruvate.

## Acknowledgements

This work was supported by the Medical Reseârch Council of Canada. Fosfomycin was a gift from the Merck Institute for Therapeutic Research, U.S.A.

## References


1 Gunetileke, K.G. and Anwar, R.A. (1968) J. Biol. Chem. 243, 5770—5778
2 Zemell, R.I. and Anwar, R.A. (1975) J. Biol. Chem. 250, 3185—3192
3 Zemell, R.I. and Anwar, R.A. (1975) J. Biol. Chem. 250, 4959—4964
4 Wood, A.E., O'Bryan, J.M., Mui, P.F.K. and Crowder, R.D. (1970) Biochemistry 9, 2334—2338
5 Strubbe, J.A. and Kenyon, G.L. (1971) Biochemistry 10, 2669—2677
6 Polley, R.J. (1949) Can. J. Res. Sect. E. Med. Sci. 27, 265—274
7 MacFarlane, N. and Ainsworth, S. (1972) Biochem. J. 129, 1035—1037
8 Cassidy, P.J. and Kahan, F.M. (1973) Biochemistry 12, 1364—1374
9 Anwar, R.A., Sodek, J. and Zemell, R.I. (1971) Fed. Proc. 30, 1284
10 Gale, G.R. (1961) Arch. Biochem. Biophys. 94, 236—240
11 Meloche, H.P. (1965) Biochem. Biophys. Res. Commun. 18, 277—282
12 DeVries, G.H. and Binkley, S.B. (1972) Arch. Biochem. Biophys. 151, 234—242
13 Yun, S.-L. and Suelter, C.H. (1979) J. Biol. Chem. 254, 1811—1815
14 Staub, M. and Denes, G. (1969) Biochim. Biophys. Acta 178, 599—608