BBA 66154

KINETICS OF GLUCOSEPHOSPHATE ISOMERASE ACTIVITY ON FRUCTOSE IN THE PRESENCE OF ARSENATE

REACTIVITY OF ALCOHOLIC GROUPS WITH ARSENATE

ROSARIO LAGUNAS

Instituto de Enzimología, Centro de Investigaciones Biológicas, C.S.I.C., Madrid (Spain)

(Received March 20th, 1970)
(Revised manuscript received June 1st, 1970)

SUMMARY

1. The kinetics of glucosephosphate isomerase activity (D-glucose-6-*P* ketol-isomerase, EC 5.3.1.9) on fructose in the presence of arsenate, and the effect of phosphate, fructose-6-*P*, and erythrose-4-*P* on this activity, have been studied.

2. The results suggest that arsenate interacts with the hydroxyl group of C-6 of the sugar resulting in, perhaps, an ester bond, and that the resulting compound, fructose-6...arsenate, may be isomerized by the enzyme with about the same *V* as that of the physiological substrate fructose-6-*P*.

3. Studies on the mechanism by which the interaction proceeds seem to indicate that, subsequent to the arsenate binding, the enzyme binds fructose, and then the interaction occurs between arsenate and the hydroxyl group of fructose.

INTRODUCTION

Enzymes whose main substrates are phosphorylated sugars show little or no activity on the corresponding dephosphorylated sugars. Nevertheless, an increase in or the appearance of this activity has been described with a variety of enzymes when arsenate, but not phosphate, is present[1]. GRISOLIA AND CASCALES[2] observed that phosphoglyceromutases, dependent on 2,3-phosphoglycerate as cofactor, were specifically activated by arsenate, and not by phosphate, when the cofactor was absent. It seems likely that 3-phosphoglycerate, present in the assay mixture as substrate, and arsenate, were together able to substitute for 2,3-phosphoglycerate.

The diversity of enzymic reactions that can be activated by arsenate, the different nature of some reactant molecules whose common characteristic is the hydroxyl group, and the kinetic behaviour of glucosephosphate isomerase with respect to the arsenate-induced activity on fructose, suggest an interaction between certain hydroxyl groups and arsenate forming ester-like compounds capable of substituting for the homologous phosphate esters[1,3]. In this paper the kinetics of the glucose-

phosphate isomerase activity on fructose induced by arsenate have been studied in an attempt to understand the mechanism involved.

MATERIALS AND METHODS

Reagents

Yeast glucosephosphate isomerase, glucose-6-*P* dehydrogenase (D-glucose-6-*P*: NADP oxidoreductase, EC 1.1.1.49), glucose oxidase (*β*-D-glucose:oxygen oxidoreductase EC 1.1.3.4), and fructose-6-*P* (disodium salt) were obtained from Boehringer and Soehne. Peroxidase (donor:hydrogen peroxide oxidoreductase, EC 1.11.1.7) was obtained from Worthington Biochem. Corp.; *o*-dianisidine and imidazole from Sigma Chem. Co.; Triton X-100 from Rohm and Haas; EDTA, sodium arsenate, and sodium phosphate from Merck AG., Darmstadt. Fructose was obtained from Pfanstiehl Laboratories Inc. and recrystallized from 98% ethanol before use.

Assay of enzymic activities

The activity of glucosephosphate isomerase on the free sugar was measured by following the formation of glucose from fructose, employing a colorimetric method based on the oxidation of glucose by glucose oxidase and later oxidation of *o*-dianisidine[4]. Usually, unless otherwise indicated, the reaction mixture contained: 0.3 M arsenate–HCl (pH 7.0), 0.4 M fructose, 5 mM EDTA, 0.2% Triton X-100, 10 μg peroxidase, 200 μg glucose oxidase and 100 μg *o*-dianisidine. All reagents were placed in a microtube of a Klett–Summerson photoelectric colorimeter to a total volume of 2 ml. The reaction was started by adding 0.75–5 μg of the glucosephosphate isomerase. Colour development was followed in the colorimeter with the 42 filter.

The isomerizing activity on the phosphorylated sugar was measured by following the formation of glucose-6-*P* with glucosephosphate dehydrogenase. The assay mixture contained, in a final volume of 1 ml, 50 mM imidazole (pH 7.0), 5 mM fructose-6-*P*, 0.4 mM $NADP^+$ and 0.4 unit of glucosephosphate dehydrogenase. The reaction was started by the addition of 0.063 μg of the glucosephosphate isomerase. The increase in absorbance, at 340 nm, accompanying the reduction of $NADP^+$, was followed in

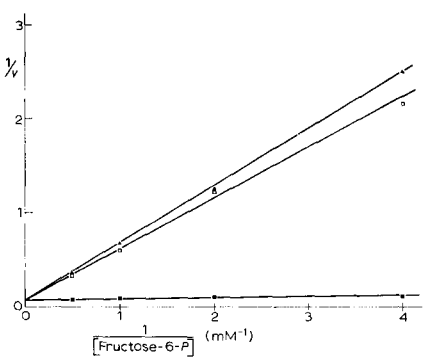


Fig. 1. Competitive inhibition by arsenate and phosphate of the isomerization of fructose-6-*P* by glucosephosphate isomerase. ■—■, no added anion; ▲—▲, 0.3 M phosphate (pH 7.0); □—□, 0.3 M arsenate (pH 7.0). The reaction was carried out by 0.063 μg of the enzyme. Velocities are expressed as nmoles of fructose-6-*P* transformed per min.

a Beckman DB spectrophotometer in a cuvette of 1-cm light path. Enzymes were diluted in 0.1% bovine albumin solution.

RESULTS

Effect of arsenate and phosphate on the activity of glucosephosphate isomerase with fructose-6-P and fructose

Arsenate as well as phosphate were competitive inhibitors of fructose-6-*P* binding (Fig. 1) with about the same K_i for both anions, 10 and 8 mM, respectively.

The activity of the enzyme on fructose was dependent upon the presence of arsenate and all attempts to find some activity in the presence of phosphate, sulphate or in the absence of added anion were unsuccessful. The arsenate-induced activity with fructose increased linearly with the concentration of arsenate up to a concentration of about 0.050 M, reached a maximum at about 0.2 M and decreased at higher concentrations (Fig. 2). By extrapolation of the Lineweaver–Burk plot an apparent K_m for arsenate of 0.25 M is obtained.

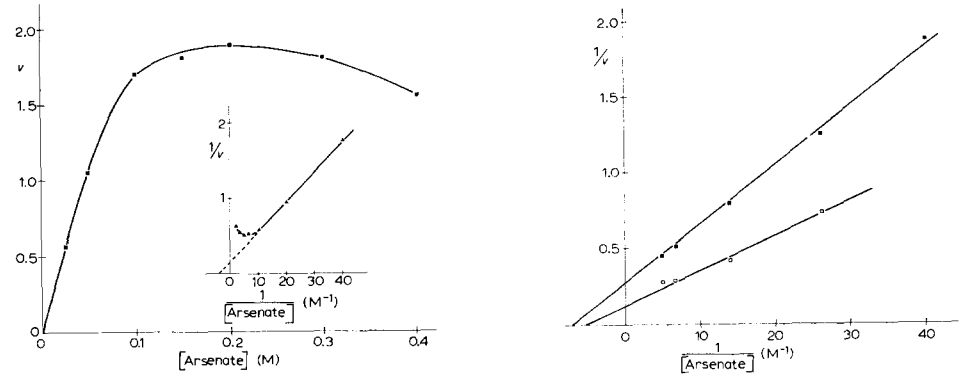


Fig. 2. Kinetics of fructose isomerization by glucosephosphate isomerase with respect to arsenate concentration. 0.4 M fructose and 0.75 μg of the enzyme. Velocities are expressed as nmoles of fructose transformed per min.

Fig. 3. Inhibitory effect of phosphate on arsenate-induced activity of glucosephosphate isomerase on fructose. 0.4 M fructose. □—□, no phosphate; ■—■, 0.1 M phosphate and 1 μg of enzyme was used. Velocities are expressed as nmoles of fructose transformed per min.

Phosphate can inhibit the arsenate-induced activity. In Fig. 3 the results obtained at various concentrations of arsenate in the presence and absence of 0.1 M phosphate are shown.

Effect of concentration of fructose on the arsenate-induced activity of glucosephosphate isomerase

The effect of the concentration of fructose was studied at two different concentrations of arsenate. At both concentrations, except for the departure from linearity at concentrations of fructose over 0.5 M in the presence of low concentrations of arsenate, close to first-order kinetics were obtained, as shown in Figs. 4A and 4B.

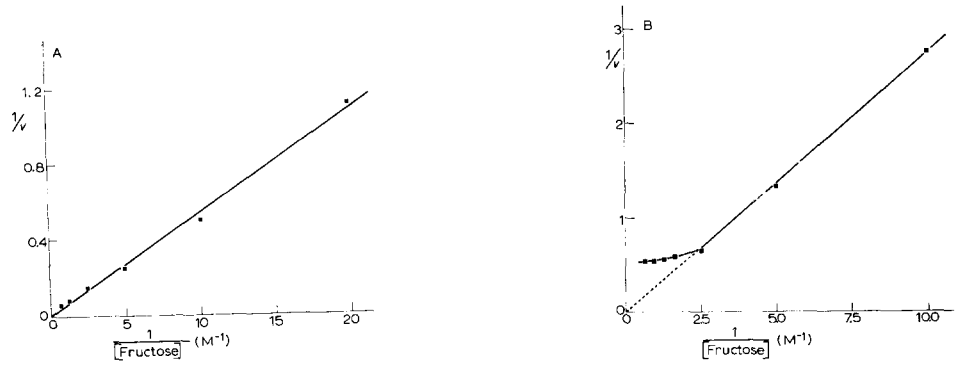


Fig. 4. Effect of fructose concentration on its rate of isomerization by glucosephosphate isomerase in the presence of arsenate. A. 0.3 M arsenate. When the fructose concentration was between 0.050 and 0.4 M, the amount of enzyme was 5 μg. Between 0.4 and 1.6 M fructose, 0.25 μg of the enzyme was used. The results of this series are referred to 5 μg of the enzyme, and are expressed as nmoles of fructose transformed per min. The activities between 0.4 and 1.6 M fructose are multiplied by 3.3, the correction factor for the departure from linearity at the widely different enzyme concentration used. B. Arsenate 0.025 M. The reaction was carried out by 4 μg of the enzyme.

Therefore, it is not possible to establish either the K_m of the enzyme for fructose, which is > 2 M, or the V value. Nevertheless, an approximate calculation of the latter parameter can be made: the maximal activity wiht fructose-6-*P* as substrate was 194 units/mg protein, and that with 1.6 M fructose and 0.3 M arsenate, 3 units/mg protein, so the ratio of these two velocities is 65. Taking into account the non-saturation conditions for both fructose and arsenate and the fact that the activity was not strictly proportional in this range of enzyme concentration (from 5 to 0.063 μg), in the sense of increasing as the enzyme becomes more diluted, it is quite possible that, in both cases, the V is nearly the same, and, indeed, it seems likely that they are not markedly different.

The low affinity of the enzyme for fructose is in agreement with the observation

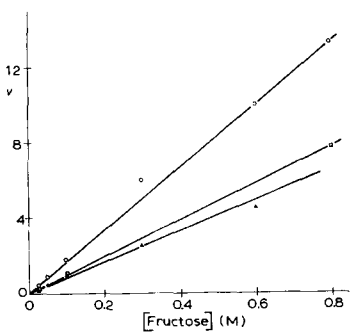


Fig. 5. Inhibitory effect of fructose-6-*P* and erythrose-4-*P* on arsenate-induced activity on fructose. 0.3 M arsenate. ○—○, no added inhibitor. ▲—▲, 5 mM fructose-6-*P*. □—□, 0.03 mM erythrose-4-*P*. 0.5 μg of the enzyme was used when the fructose concentration was between 0.025 M and 0.1 M. Between 0.1 and 0.8 M the amount of enzyme was 2.0 μg. The results are referred to 2 μg of the enzyme. Activities are expressed as nmoles of fructose transformed per min.

of no inhibition of the activity on 0.2 mM fructose-6-*P* with 0.8 M fructose. NATAKE[3] has reported for the enzyme from *Escherichia intermedia* a K_m for glucose of 1.6 M.

Effect of fructose-6-P and erythrose-4-P on the activity with fructose

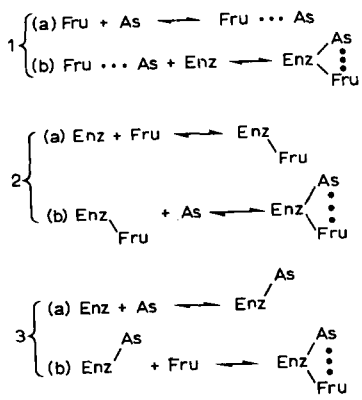
Erythrose-4-*P* is a competitive inhibitor of glucosephosphate isomerase with a K_i value of 0.002 mM (ref. 5). This compound, when assayed at 0.03 mM concentration, produced a 45% inhibition in the rate of isomerization of fructose by glucosephosphate isomerase with 0.3 M arsenate (Fig. 5). Fructose-6-*P*, the physiological substrate of the enzyme, at 5 mM concentration produced 50% inhibition (Fig. 5). These inhibitions may seem too low considering that the concentrations of the inhibitors were 15 and 25 times their respective apparent affinity constants and that the substrate was far below saturation concentration. This paradoxical result will be discussed below.

DISCUSSION

The activity of glucosephosphate isomerase on fructose has been shown to be dependent on the presence of arsenate. The apparent K_m of this enzyme for fructose in the presence of arsenate is > 2 M. This low affinity very likely depends not only on the lack of a phosphoryl group on C-6, but also on the fact that this enzyme binds the open form of the sugars much more efficiently[5]. Therefore the high K_m for fructose should be, to a considerable extent, determined by the low proportion of the open form present in equilibrated solutions of the free sugar, much lower than for phosphorylated sugars[5–7].

In the isomerization of fructose-6-*P*, a natural substrate of the enzyme, arsenate and phosphate are competitive inhibitors. In addition, phosphate has been shown to be an inhibitor of the isomerization of fructose induced by arsenate. These observations indicate that arsenate and phosphate bind at the active site of the enzyme. The kinetics of the arsenate induction of activity of glucosephosphate isomerase on fructose, described in this paper and by NATAKE[3], and the generality of the phenomenon which occurs with molecules whose common characteristic is the hydroxyl groups, suggest an interaction between hydroxyl groups and arsenate forming, perhaps, an ester bond[1]. The resulting "ester-like" compounds are able to substitute for the corresponding phosphorylated esters in enzymic reactions. The inability of the phosphate to "induce" activity could indicate that in the conditions used, phosphate either does not interact with the hydroxyl groups of the sugars, or it interacts to a much less extent than does arsenate. In phosphorolytic reactions, arsenate can replace phosphate, supposedly through the formation of transient arsenic–organic compounds, anhydrides or esters. Although these postulated compounds have not been isolated, experiments with ^{18}O-labelled arsenate support this theory[8,9]. Moreover, CHAN *et al.*[10] report that, during respiration of rat liver mitochondria in the presence of radioactive arsenate, a radioactive arsenic substance is formed, which has been purified and whose properties are consistent with those expected for a stable arsenate ester.

If we assume the formation of "ester-like" compounds of sugars with arsenate (represented as As in the scheme below), there are at least three possible mechanisms that could in principle explain the arsenate-dependent fructose isomerization.



After the formation of the complex, $Enz\begin{matrix}As\\ \vdots\\ Fru\end{matrix}$ it could be assumed that the isomerization proceeds as in the case of the natural substrate fructose-6-*P*. The product of the reaction, glucose-6...As, would be promptly hydrolysed to glucose and arsenate.

Mechanism 1 postulates that the "ester-like" compound is formed between arsenate and fructose in solution depending on an association constant. Taking into account the fact that arsenate is a competitive inhibitor with respect to fructose-6-*P* (Fig. 1), the non-combined arsenate, free in solution, would compete for the active site of the enzyme with the formed fructose-6...As. On the basis of arsenate as competitive inhibitor of fructose-6...As, by applying the equation of velocity in the presence of a competitive inhibitor

$$\frac{1}{v_i} = \left(1 + \frac{[\text{arsenate}]}{K_i(\text{arsenate})}\right)\frac{K_m}{V}\cdot\frac{1}{[\text{fructose-6...As}]} + \frac{1}{V}$$

and since [fructose-6...As] $= C \cdot$ [arsenate], "v_i" would be nearly constant when the arsenate concentration is changed within a range well over the K_i value (10 mM). At concentrations of arsenate well over its K_i value, the enzyme would be almost saturated. Although an increase of the arsenate concentration would increase the concentration of fructose-6...As, the degree of saturation of the enzyme would not allow a noticeable increase of the velocity. This prediction is in contrast with our results (Fig. 2) and those of NATAKE[3] because almost linear increases of activity were found on increasing the arsenate concentration up to 0.1 M.

The contrast between the 10 mM K_i of arsenate for glucosephosphate isomerase in the isomerization of fructose-6-*P* (Fig. 1), and the 0.25 M apparent K_m of arsenate in the isomerization of fructose (Fig. 2) cannot be understood by Mechanisms 2 or 3. This contrast could be explained on the assumption that glucosephosphate isomerase in the presence of its substrate fructose-6-*P* experiences some conformational change lasting long enough to produce a significant increase in the apparent affinity for phosphate and arsenate. As a consequence the K_i of 10 mM found for these anions would be lower than the dissociation constants of these anions in the absence of the natural substrate. With this assumption, any of the three proposed mechanisms could be consistent, in principle, with the kinetics found for fructose and arsenate.

As pointed out above, erythrose-4-*P* and fructose-6-*P*, when assayed as inhibitors of the isomerization of fructose in the presence of 0.3 M arsenate, at concen-

trations 15 and 25 times their respective K_i and K_m, caused inhibitions of only 45 and 50%, respectively (Fig. 5). We will now consider these results on the basis of the three possible mechanisms described above.

If Mechanism 1 were involved to the greater extent, the substrate fructose-6...As would be at a concentration far below saturation of the enzyme, since its actual concentration would be dependent on the fructose concentration, and first-order kinetics have been found for this sugar. In these experimental conditions, *i.e.* low concentration of substrate and concentration of inhibitors 15 and 25 times their respective apparent affinities, the predictable inhibitions would be about 95%. Since only 50% inhibition was found, it seems unlikely that Mechanism 1 has a significant function.

In mechanism 2, fructose would compete with fructose-6-*P* or erythrose-4-*P* for the active site. Because of the very low affinity of glucosephosphate isomerase for fructose and for reasons similar to those discussed for Mechanism 1, it seems that Mechanism 2 can also be eliminated as the main one.

In Mechanism 3, arsenate would compete with fructose-6-*P* or erythrose-4-*P* for the active site. The dissociation constant of the complex enzyme–arsenate in the presence of fructose-6-*P* has been found to be 10 mM, and presumably the same constant would apply in the presence of erythrose-4-*P*. In the experiments described, arsenate was present at 0.3 M, that is 30 times the above dissociation constant, while fructose-6-*P* or erythrose-4-*P* were at 25 and 15 times their respective K_m and K_i. In these conditions about 50% inhibition is to be expected. Therefore, on the assumption that the dissociation constant of the enzyme–arsenate complex is higher in the absence than in the presence of the phosphorylated substrate, only Mechanism 3 is consistent with the results and seems likely to be quantitatively important in the "induction" of the activity of yeast glucosephosphate isomerase on fructose.

The involvement of Mechanism 1, while possible in qualitative terms, is likely limited by the low association constant of the fructose-6...As compound. The low affinity of the enzyme for fructose would be an important limitation in Mechanism 2 as it would be also in Mechanism 3. However, in the latter this limitation could be sidestepped on the assumption that the binding of arsenate to the enzyme increases the affinity of the enzyme for fructose, perhaps involving some conformational change in the protein.

Probably most hydroxyl groups have potential reactivity with arsenate, as is suggested by the generality of the activation or induction of activity by arsenate of certain enzymes[1,2] with their corresponding dephosphorylated substrates or co-enzymes. The precise mechanism of activation by arsenate would depend in each case on the peculiar characteristics of the different enzymes. ANDERSON *et al.*[12], according to their experimental observations with glucosephosphate dehydrogenase, suggest a Koshland's "induced fit" produced by sulphate, phosphate and bicarbonate, to be responsible for the increase of activity on free glucose. We have also found a 3-fold stimulation by phosphate of the glucosephosphate dehydrogenase on free sugar, but as much as 16-fold stimulation by arsenate[1]. Consequently, it may be suggested in the case of glucosephosphate dehydrogenase that arsenate, as well as phosphate, produce a conformational change in the enzyme that facilitates its catalytic activity on free sugar. On the other hand, there probably occurs an interaction of arsenate with the hydroxyl group of C-6 of glucose, giving a glucose-6...As that would closely resemble

glucose-6-*P* and which could explain the greater increase of activity in the presence of arsenate as compared with phosphate.

In general, it is suggested that, for enzymes whose substrate or coenzyme are phosphorylated compounds and which bind arsenate in the active site, the enzymes themselves are likely to play an important role in the interaction of arsenate and hydroxyl groups. The specific binding to the enzyme would facilitate the proximity and the precise spatial orientation of the groups able to interact giving an "ester-like" compound structurally related to the physiological substrate or coenzyme. In addition, if the active site were located in a hydrophobic part of the protein probably the interaction could be favoured, and the resultant bond stabilized.

ACKNOWLEDGEMENTS

The author is indebted to Drs. A. Sols, C. Gancedo, G. DelaFuente and E. J. Bell for stimulating discussion and advice, and greatly appreciates the technical assistance of Miss Clotilde Estévez and Mr. Lorenzo Seguido.

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