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FUNCTIONAL MULTIPLICITY OF PHOSPHOGLUCOSE ISOMERASE FROM *LACTOBACILLUS CASEI*

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Summary

Phosphoglucose isomerase (D-glucose-6-phosphate ketolisomerase, EC 5.3.1.9), purified from *Lactobacillus casei*, showed multiplicity with respect to electrophoretic mobility, molecular weight, kinetic properties and responses to erythrose 4-phosphate. Among the three forms isolated, one having a dimeric conformation, was specific for glucose 6-phosphate. Erythrose 4-phosphate inhibited this preparation in a sigmoid fashion, while this compound activated the enzyme for isomerization of ribose 5-phosphate. In tetrameric conformation of the similar subunits, the enzyme was more specific for ribose 5-phosphate and the inhibition exerted by erythrose 4-phosphate was hyperbolic. The possible implications of these observations have been discussed.

Introduction

Earlier studies from this laboratory have shown that *Lactobacillus casei* could be adapted either to glucose or to ribose with induced changes in the fermentative and gluconeogenic enzymes [1—3]. Among the systems not amenable to adaptive variations was phosphoglucose isomerase [1]. The existence of separate isomerases, specific for hexose phosphates and pentose phosphates, has been presumed in most of the reported investigations [4,5]. However, the possibility could not be ruled out that structural variations in the same protein could influence the substrate specificity. The present studies were therefore initiated to define the characteristics of phosphoglucose isomerase from *L.*

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casei and to identify differences with ribose-5-phosphate isomerase (D-ribose-5-phosphate ketolisomerase, EC 5.3.1.6). It was observed that the purified phosphoglucose isomerase had multiple forms showing subtle differences in the substrate specificity and other properties.

Materials and Methods

Materials. The substrates, coenzymes and DEAE-cellulose used in these studies were obtained from Sigma Chemical Co. (U.S.A.) and the components of the growth media from Difco. All other chemicals of Analar grade were procured from British Drug Houses (India).

Culture conditions. The conditions for growth of *L. casei* ATCC 7469 in a medium containing glucose as carbon source, have been described earlier [1—3]. The organism was grown in batches of 6—7 l of the culture medium composed of glucose, bactotryptone, yeast extract and salts [6,7]. After 22 h of growth, the cells were harvested in a refrigerated Sorvall RC-2B centrifuge at 7000 rev./min for 20 min using a GS-3 rotor and were washed three times with precooled isotonic saline.

Preparation of cell extract. The cells (30—40 g wet weight) were suspended in 150 ml of cold 0.05 M sodium phosphate buffer, pH 6.0 and were subjected to mechanical disruption in an Aminco-French pressure cell at 4000—6000 lb/inch^2. The cell debris was discarded after centrifugation at 17 500 rev./min using a SS-34 rotor for 25 min and the supernatant obtained was used for purification of phosphoglucose isomerase.

Protein was estimated by the method of Lowry et al. [8] using bovine serum albumin as standard.

Assay of phosphoglucose isomerase. The enzyme activity of phosphoglucose isomerase was estimated by the method of Bodansky and Schwartz [9]. The reaction mixture contained in a total volume of 1.0 ml, 100 μmol Tris-HCl buffer (pH 7.5) containing 220 μmol sodium chloride, 20 μmol glucose 6-phosphate and a 0.1 ml of the appropriately diluted (5—10 μg protein) enzyme extract. The reaction was stopped by adding 1.0 ml 10% trichloroacetic acid within 2 min of incubation at 37°C. For each assay the corresponding blank was kept, in which glucose 6-phosphate was added only after stopping the reaction with trichloroacetic acid. The tubes were centrifuged to remove the precipitated protein and the supernatant was taken for estimation of fructose 6-phosphate formed by the colorimetric procedure of Roe [10]. The activity has been expressed as μmol of the product formed/min per mg protein.

Assay of ribose-5-phosphate isomerase. Ribose-5-phosphate isomerase activity was assayed by using the method of Axelrod [11]. The reaction mixture contained in a total volume of 1.0 ml, 100 μmol Tris-HCl buffer, pH 7.0, 1.2 μmol ribose 5-phosphate and 0.1 ml of the enzyme preparation containing 5—10 μg protein. The mixture was incubated for 10 min at 37°C. Appropriate blanks were kept and the product ribulose 5-phosphate formed was measured by the cysteine-carbazole method [12]. Since the form A of the enzyme showed pH optimum at 3.0, the enzyme assay was also carried out at pH 3.0. The enzyme activity has been expressed as units/mg protein as defined by Urivetzky and Tsuboi [13].

Polyacrylamide disc gel electrophoresis. The polyacrylamide disc gel electrophoresis was carried out according to the procedure described by Davis [14]. After electrophoresis, the gel columns were removed and stained for protein with Amido black (0.1%).

Results

Purification of phosphoglucose isomerase from L. casei

The protocol employed for purification was similar to that described by Nakagawa and Noltmann [15]. Table I summarises the data on the recoveries of the enzyme activity at various stages of purification. The cell-free extract was brought to 50% saturation by adding precooled acetone at −10°C with constant stirring. The precipitate formed was allowed to stand for 30 min and was centrifuged at 12 000 × *g* for 30 min in a GSA rotor of Sorvall RC-2B centrifuge. The precipitate containing the enzyme activity was taken for further purification. The precipitate was dissolved in 0.05 M magnesium acetate (pH 6.0) and was kept at −10°C for 2 h. This was centrifuged again and the supernatant was subjected to precipitation with precooled (−10°C) 95% ethanol to 65% saturation. Most of phosphoglucose isomerase activity was recovered in the 65% ethanol precipitate. The precipitate obtained was dissolved in a minimal volume of 0.05 M magnesium acetate (pH 6.0) and was then dialysed overnight in a cold room against 2 l water. The dialysed preparation was subjected to calcium phosphate gel adsorption on which the enzyme was negatively adsorbed.

Multiplicity of phosphoglucose isomerase

The partially purified preparation thus obtained was subjected to DEAE-cellulose chromatography which gave an elution profile as shown in Fig. 1. Though there were several protein peaks only three of these had measurable phosphoglucose isomerase activity. The enzymatically active components were

TABLE I

PURIFICATION OF PHOSPHOGLUCOSE ISOMERASE FROM *L. CASEI*

The unit of enzyme activity is expressed as μmol fructose 6-phosphate formed/min.

Preparation	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Fold	Recovery (%)
Crude	160	704	10 560	15	—	100
50% acetone ppt.	215	430	10 020	23	1.5	95
65% alcohol ppt.	93	260	9 720	45	3	92
Ca-phosphate gel	83	110	7 755	70.5	4.7	74
DEAE-cellulose						
A	25	0.6	124	211	14	
B	166	6.1	3 206	502	34	67
C	166	7.6	3 811	501	33	
Sephadex gel filtration						
A	5	0.56	106	191	13	
B	5	2.0	1 480	740	49	15
C	5	2.9	2 146	740	49	

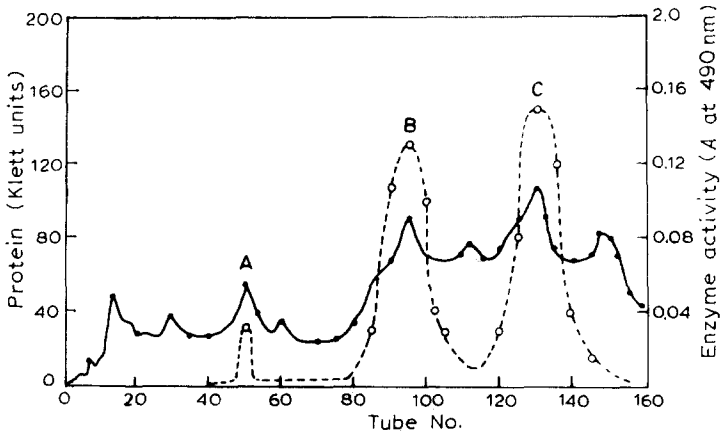

Fig. 1. Chromatography on DEAE-cellulose of partially purified preparation of phosphoglucose isomerase from *L. casei*. The protein (100 mg) was loaded on the column (2.2 × 50 cm) previously equilibrated with 0.005 M sodium-phosphate buffer, pH 6.0. Elution was carried out using a gradient (0.01—0.07 M) of sodium-phosphate buffer, pH 6.0. Fractions of 5.0 ml size were collected and tested for enzyme activity and total protein. The enzyme activity has been shown by the dotted line.

arbitrarily designated as A, B and C forms, respectively. These fractions were pooled, dialysed against water and lyophilised. The dried preparations were dissolved in 2.0 ml of 0.05 M phosphate buffer, pH 6.0, and were further subjected to Sephadex G-100 gel filtration. Form A had to be passed through Sephadex G-150 as it otherwise emerged in the void volume. Each of these gave a single band on protein staining by polyacrylamide gel electrophoresis. These results point to the apparent homogeneity of the preparations. The purified preparations were found to be stable for several weeks when stored in magnesium acetate solution in a freezer (−10°C). These forms showed subtle differences in their characteristics as shown in Table II. Form A seemed to have more affinity for ribose 5-phosphate while forms B and C, which appeared to be similar, were more specific for glucose 6-phosphate. The separation of forms B and C could presumably be due to variations in their net conformations. However, detailed studies were undertaken only with form B because of the regulatory responses shown by this component as described later. Comparisons were sought essentially between forms A and B.

The pH optima for forms A and B of phosphoglucose isomerase using glucose 6-phosphate and ribose 5-phosphate as substrate have been presented in

TABLE II

DIFFERENTIAL CHARACTERISTICS OF MULTIPLE FORMS OF PHOSPHOGLUCOSE ISOMERASE FROM *L. CASEI*.

	A	B	C
Molecular weight	135 000	67 000	67 000
Subunits	4	2	2
K_m for glucose 6-phosphate	$1.1 \cdot 10^{-3}$ M	$1.1 \cdot 10^{-5}$ M	$1.6 \cdot 10^{-5}$ M
Ribose 5-phosphate	$1.8 \cdot 10^{-5}$ M	$2.0 \cdot 10^{-4}$ M	$2.0 \cdot 10^{-4}$ M

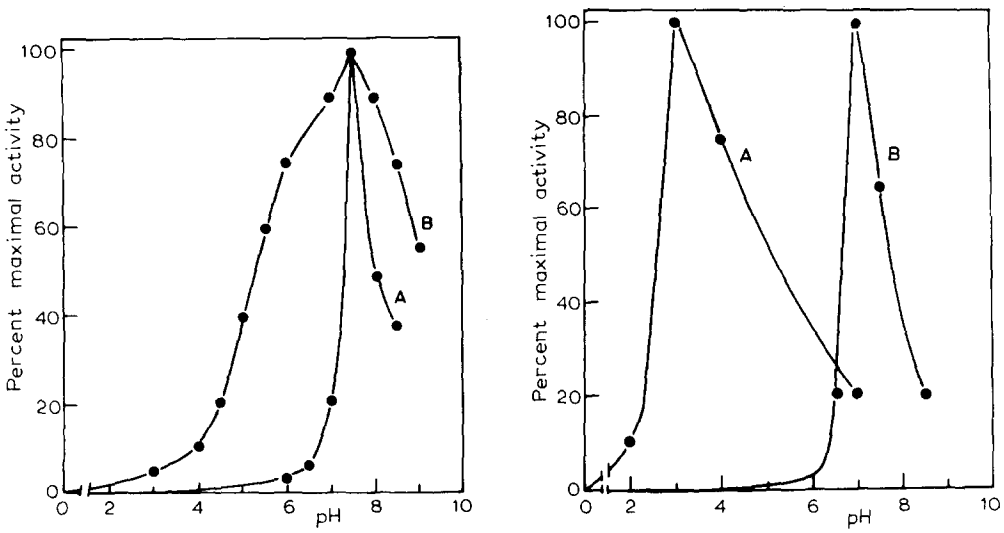

Fig. 2. The effect of pH on activities of forms A and B of phosphoglucose isomerase of *L. casei* with glucose 6-phosphate as substrate. The maximal activities of forms A and B were 236 and 614 μmol fructose 6-phosphate formed/min per mg of protein, respectively, as saturating concentration of the substrate.

Fig. 3. The effect of pH on activities of forms A and B of phosphoglucose isomerase of *L. casei* with ribose 5-phosphate as substrate. The maximal activities of forms A and B were 376 and 79 units/mg of protein, respectively.

Figs. 2 and 3. It can be seen that with glucose 6-phosphate as substrate, form B exhibited a broad range of pH for activity with an optimum at 7.5, while A also showed a sharp pH optimum at 7.5. On the other hand, with ribose 5-phosphate as substrate, form A showed a pH optimum of 3.0 while form B was still active in a narrow range with a sharp optimum at pH 7.0. Also, form B, preincubated after 10 min at 30°C, at pH 3.0, was partially inactivated when the assay was carried out at pH 7.5 with glucose 6-phosphate as substrate.

Activity responses in presence of erythrose 4-phosphate

The activities of the multiple forms of phosphoglucose isomerase were estimated in the presence of varying concentrations of erythrose 4-phosphate, which has been reported to be an inhibitor of this enzyme [16]. Both glucose 6-phosphate (20 mM) and ribose 5-phosphate (10 mM) were used as the substrates. Erythrose 4-phosphate inhibited both the forms of phosphoglucose isomerase as shown in Fig. 4. It was observed that form B showed sigmoidal inhibition responses, while form A was inhibited in an hyperbolic fashion. The Hill plot of inhibition of form B by erythrose 4-phosphate gave a coefficient of 0.5 indicating negative cooperativity. The K_i values obtained from the respective Dixon plots [17] were $1.6 \cdot 10^{-4}$ M for A and $2.2 \cdot 10^{-4}$ M for B.

With ribose 5-phosphate as the substrate it was observed that erythrose 4-phosphate activated the form B in a sigmoidal fashion in the same range of effector concentration as shown in Fig. 5, though with glucose 6-phosphate as substrate this compound was inhibitory, as observed above. The responses with the two substrates were, thus found to be converse. On the other hand, eryth-

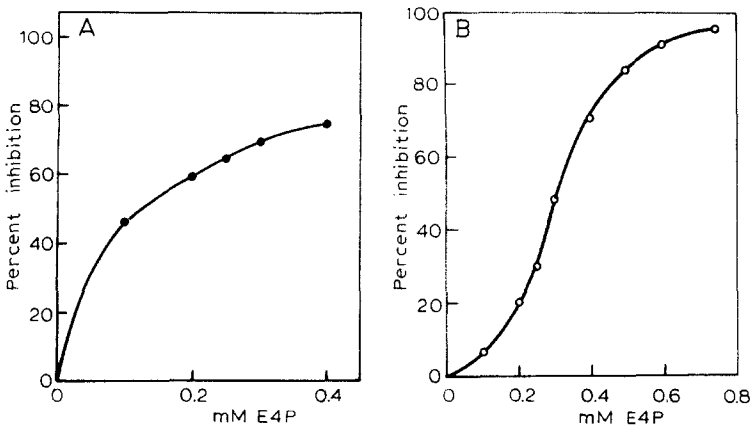

Fig. 4. Inhibitory responses of *L. casei* phosphoglucose isomerase with erythrose 4-phosphate (E4P). The assay system with glucose 6-phosphate as substrate used in these experiments has been outlined in the text. The hyperbolic response given by form A is shown on the left and the sigmoid curve obtained with form B is shown on the right.

rose 4-phosphate still exerted inhibitory action on form A in a hyperbolic manner, with ribose 5-phosphate as substrate with a K_i value of $1.5 \cdot 10^{-5}$ M.

Molecular weight and subunit structure

The molecular weights of the purified preparations of the multiple forms of *L. casei* phosphoglucose isomerase, as determined by Sephadex gel filtration [18] were observed to be 135 000 for A and 67 700 for B as indicated in Table II. The standard proteins used were bovine serum albumin (69 000), hexokinase (96 000) and cytochrome *c* (12 800). These preparations also gave differential movements on polyacrylamide gel electrophoresis. The enzyme preparations when subjected to treatment with 1.0% SDS and gel filtration on Sephadex G-100, previously equilibrated with SDS (1.0%), eluted as a single peak having an apparent molecular weight of 34 000. Therefore, form A appeared to be a

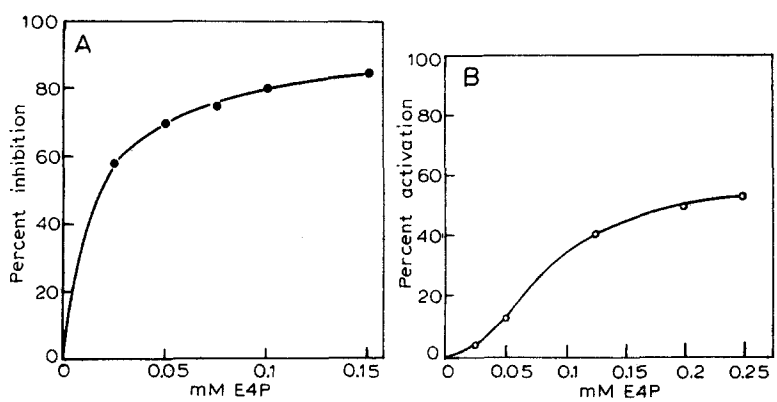

Fig. 5. The effector responses with erythrose 4-phosphate (E4P) on *L. casei* phosphoglucose isomerase with ribose 5-phosphate as substrate. The hyperbolic inhibitory responses shown by form A are presented on the left and the sigmoidal activation exerted on form B has been indicated on the right.

TABLE III

EFFECT OF MALEIC ANHYDRIDE ON ACTIVITY OF PHOSPHOGLUCOSE ISOMERASE FROM *L. CASEI*

The enzyme preparation (0.1 mg/ml) in 0.05 M sodium phosphate buffer, pH 6.0, in a total volume of 1.0 ml was incubated with 2 M maleic anhydride (in acetone) for 20 min at 30°C. Suitable aliquots (0.1 ml) were drawn from this mixture and tested for enzyme activity using the assay system outlined in the text. The additions were made in the sequence indicated. DTT, dithiothreitol.

Treatment	Spec. act. μmol fructose 6-phosphate formed/min per mg of protein	Percentage of initial activity
Enzyme	502	100
Enzyme + acetone	502	100
Enzyme + maleic anhydride	0	0
Enzyme + DTT + maleic anhydride	241	48
Enzyme + glucose 6-phosphate maleic anhydride	502	100

tetramer, while the other forms were dimeric though composed of similar subunits. The preparations when subjected to polyacrylamide gel electrophoresis in presence of 0.1% SDS, yielded a single band with similar migration. However, the identical nature of the subunits could not be established in terms of amino acid composition.

Functional residues in the enzyme

Phosphoglucose isomerase (form B) from *L. casei* was treated with maleic anhydride according to the procedure of Sia and Horecker [19]. Table III presents the inactivation responses of the preparation when treated with this reagent. The addition of glucose 6-phosphate before the treatment with maleic anhydride prevented inactivation suggesting the involvement of lysine residue for substrate binding. The activity was partially retained when dithiothreitol was added along with maleic anhydride.

The preparation did not show any change in the activity after preincubation with tetranitromethane at different pH values [20], precluding the functional involvement of tyrosine residues and thiol groups. Photooxidation of the enzyme in presence of Rose bengal or methylene blue [21] was also without any effect, thus suggesting that histidine residues had no role in catalysis. This was also confirmed by treatment of the enzyme preparation with diethyl pyrocarbonate [22].

Discussion

The observations presented in this report point to the multiplicity of phosphoglucose isomerase in *L. casei*. This may perhaps be one of the first instances of the observed presence of multiple molecular forms of this enzyme in a bacterial species, though the multiplicity has been reported in higher forms of life [4,5,23]. The present preparations also showed distinct identities with respect to pH optima, preferences for the substrates and responses with erythrose 4-phosphate. One of these (form B) showed sigmoid inhibitory responses with erythrose 4-phosphate which has not been reported so far for the enzyme from

other sources. The inhibitory action of this compound on rabbit muscle phosphoglucose isomerase was shown to be essentially competitive [23]. Another interesting observation was that, with ribose 5-phosphate as substrate, form B was activated in the presence of erythrose 4-phosphate. The competitive inhibition exerted by the compound on form A with ribose 5-phosphate as substrate, may be an instance of negative feedback inhibition, in as much as this protein was also more specific for ribose 5-phosphate. It has been shown that the intermediates of the pentose phosphate pathway could exert inhibitory action on the isomerase reaction [24]. This has been suggested as a mechanism for channeling glucose 6-phosphate utilization through pentose phosphate pathway.

The peculiar responses of the multiple forms of *L. casei* phosphoglucose isomerase with respect to pH optima with the two substrates could also suggest a regulatory role in metabolism. Form A exhibited maximal activity at pH 3.0 while no activity was observed with glucse 6-phosphate. This fraction alone therefore showed pH-dependent discrimination for the substrate. Pentose phosphate isomerases from other species are also reported to be active at acidic pH [4]. In *E. coli*, phosphoglucose isomerase has been shown to be inactivated at acidic pH [25]. On the other hand, form B did not reveal any differential responses, with respect to pH optima, with either of the substrates.

The data presented here indicate that the form A of phosphoglucose isomerase from *L. casei* was a tetramer while forms B and C were dimers. It is apparent that the dimeric forms were more specific towards glucose 6-phosphate as the substrate, while in the tetrameric conformation, the enzyme had preference for the pentose phosphate. It is difficult to presume that different states of aggregation of the same subunits could alter the substrate specificity, particularly in the absence of data on amino acid composition. Also, form B seemed to be partially inactivated at pH 3.0 while form A was not. Alterations in substrate specificities of some bifunctional enzymes have been correlated with shifts in the equilibrium of subunits towards association of dissociation in polymeric proteins. It has been shown that the binding of NADH to glutamate dehydrogenase could cause dissociation of the polymer into monomers and that the resulting proteins could catalyze dehydrogenation of alanine causing loss of its original function of glutamate oxidation [26,27]. Similarly, fructose-1,6-diphosphatase in chloroplasts existed as a dimer at pH 5.5 and that with a change in pH from 5.5 to 8.5 the monomers formed by dissociation of the dimer exhibited catalytic responses with sedoheptulose 1,7-diphosphate but no activity with fructose diphosphate [28]. These reported alterations have been attributed either to the binding of specific ligand to the protein or a change in pH, while metabolic implications are still not clear. It may seem reasonable to assume that the different states of an enzyme may arise as a physiological need of an organism. Such situations could be envisaged with respect to in vivo isomerization of either glucose 6-phosphate or ribose 5-phosphate in the two adaptive states of *L. casei* during growth on glucose or ribose [1—3].

The subunit composition of form B showed similarities with phosphoglucose isomerase from brewer's yeast [29], rabbit muscle [30] and human erythrocyte [31]. Though multiplicity of the enzyme was shown in yeast [32], the differences in molecular weight were not reported as observed here. The enzyme

from *L. casei* also resembled the mammalian enzyme with respect to pH optimum and kinetic properties. Earlier studies have indicated that fructose-1,6-diphosphate aldolase from *L. casei* had typical characteristics of the mammalian alsolase in contrast to class II enzyme normally encountered in the bacterial systems [7,33,34].

The functional involvement of lysine and histidine was suggested in phosphoglucose isomerase from rabbit muscle [35,36]. However, in the present preparation histidine residues did not seem to be involved in phosphoglucose isomerase activity. The inactivation caused by treatment with maleic anhydride and the protection afforded by glucose 6-phosphate, suggested the role of lysine in catalysis. The partial retention of the activity, when dithiothreitol was added along with maleic anhydride could perhaps indicate the essentiality of -SH groups in maintaining the conformation rather than the actual participation in catalysis.

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