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ATP: D-gluconate 6-phosphotransferase of *Mycobacterium phlei*

6-фосфотрансфераза АТФ: Д-глюконат из *Mycobaterium phlei*

6-Fosfotransferaza ATP: D-glukonian z *Mycobacterium phlei*

As demonstrated in a previous paper from this laboratory (10), *Mycobacterium phlei* possesses considerable phosphorylating activity toward D-gluconic acid. In enzyme preparations obtained from Lowenstein- or gluconate-grown cells the reaction could also proceed, though very slowly, at the expense of inorganic polyphosphates (Kurrol's salt). The product of the reaction was identified in either case as 6-phosphogluconate, but properties of the enzyme were not studied.

The present report deals with the mycobacterial gluconokinase (D-gluconate 6-phosphotransferase, EC 2.7.1.12) which utilizes adenosinetriphosphate (ATP) as P donor, and is a part of work on the carbohydrate metabolism in mycobacteria.

MATERIAL AND METHODS

M. phlei was cultured statically on liquid glutamate-citrate-salt medium with glucose as the main source of carbon (2). After growth for 7 days at 37°C, cells were collected and washed with dist. water by centrifugation.

Active extracts were prepared either by grinding acetone-dried cells in the cold as described previously (10), or more often by exposing fresh cell suspension (20% in 0.05 M-tris-HCl buffer, pH 7.4) to 24 kc/s ultrasonic vibration from a MSE 100 W generator for 2 successive 5 min. periods, the temperature being maintained within 0—20°. Homogenates were centrifuged for 20 min. at 9,000 rev./min. (MSE refrigerated centrifuge) and the resulting supernatants preserved for further experiments. The protein concentration was approx. 4—6 mg/ml and the specific activity, as measured under the conditions described below, amounted to 9 μmoles/hr/mg protein.

A 20-fold increase in specific activity was achieved by a procedure which involved: 1) incubation of extract with deoxyribonuclease, 2) fractionation with ammonium sulfate, and 3) filtration on a Sephadex G-100 column (9, 10). Some attempts to purify the enzyme further by means of starch block electrophoresis resulted in considerable loss of activity (Fig. 1). Therefore, in the majority of experiments Sephadex G-100 eluates were employed.

The rate of reactions was measured by the acid-labile P disappearance method. If not otherwise indicated in the text, the reaction mixture contained in a final volume of 0.40 ml: 2 μmoles of ATP (Sigma), 2 μmoles of potassium gluconate (Pfizer), 2 μmoles of $MgSO_4$ or $MgCl_2$, 40 μmoles of tris-HCl buffer (pH 9) and enzyme preparation. At zero time and at appropriate time intervals of incubation (37°), 0.1 ml samples were withdrawn for acid-labile phosphate determinations (10 min. in N H_2SO_4 at 100°). For this purpose the Fiske-Subbarow method was used with elon (p-methylaminophenyl sulfate) as the reducing agent. The unit was defined as that amount of enzyme which catalysed phosphorylation of 1 μmole of D-gluconate per minute under the above conditions.

Protein was determined by the Lowry et al. procedure (5).

The extinctions were measured in a photoelectric colorimeter of Chinese production (model 581), filter No. 65.

RESULTS

Fig. 2 shows the requirement of the mycobacterial gluconokinase for Mg ions. In the presence of excess of gluconate, the activity increased with increasing concentration of Mg^{2+}, maximum activity being

Table 1. Effect of various metal ions on the mycobacterial gluconokinase activity. A concentration of 5 mM-ATP was used with a molar ratio Metal/ATP = 1. Other conditions as in Methods except for sample No. 3 which was incubated at pH 7.5 to avoid precipitation

Sample No.	Divalent metal ion added	Rate, per cent of control with $MgSO_4$
1	None	25
2	$MgSO_4$	100
3	$MgSO_4$ + 1 mM $ZnSO_4$	35 §
4	$CaCl_2$	80
5	$MnSO_4$	75
6	$CoCl_2$	70

§ Referred to a control incubated at pH 7.5.

attained at a molar ratio Mg^{2+}/ATP = 0.5. At higher relative concentrations, Mg was inhibitory. Activation of the enzyme also occurred when Mg was replaced by such divalent metal ions as Ca, Mn and Co. Zn ions (1 mM) exhibited a negative influence (Table 1). Addition of 0.2 M-NaCl caused a small (about 10%) but reproducible decrease in activity.

The pH profile of the mycobacterial enzyme was characterized by a broad optimum of about 8 to 10.5 (Fig. 3). The enzyme was most stable in the same range of pH and underwent partial denaturation at pH below 7 (Fig. 4).

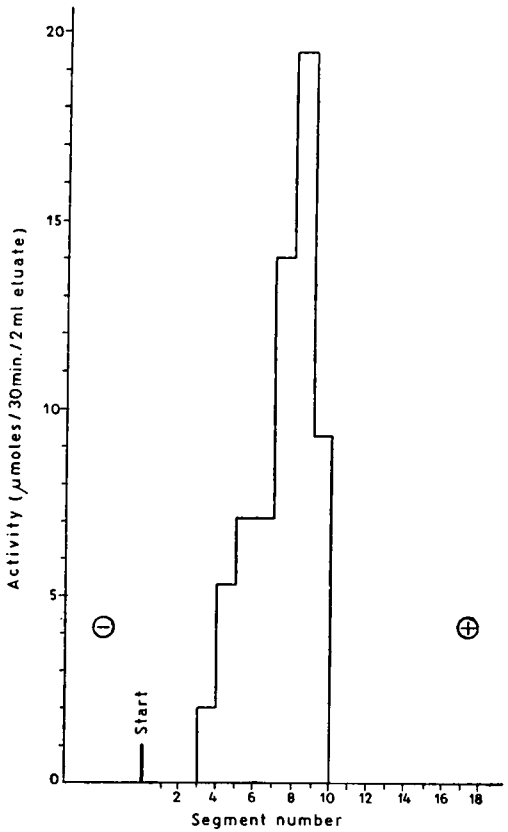

Fig. 1. Starch block electrophoresis of a gluconokinase preparation from *M. phlei*. 2.1 ml of Sephadex G-100 eluate (about 5.5 units) were concentrated with Carbowax to 0.5 ml volume and applied to a block of starch prepared as described previously (9). Electrophoresis was conducted in 0.05 M-veronal buffer, pH 8.6, at 200 V and 7.5 mA in the cold. After 14 hr of migration, the block was cut into 0.5 cm width segments which were extracted with 2 ml of 0.10 M-tris-HCl, pH 9, and gluconokinase activity determined. Reaction mixtures contained 2.8 μmoles of ATP, 2.8 μmoles of D-gluconate, 1.4 μmole of $MgSO_4$ and 0.2—0.4 ml of supernatant in a final volume of 0.47 ml. Incubation 30 min.

Fig. 5 shows the influence of temperature on the enzymatic activity. It is evident that reaction rate was higher at 40° than at 50°. When, however, the heating was carried out at pH 7.4 in absence of the reactants, the enzyme appeared less sensitive to temperature (Fig. 6), and this was true both for Sephadex G-100 eluate and dialysed 35—75% sat. ammonium sulfate fraction.

Addition of p-chloromercuribenzoate (PCMB) to the reaction mixture in a final concentration of 0.1 mM abolished enzymatic activity in 100%.

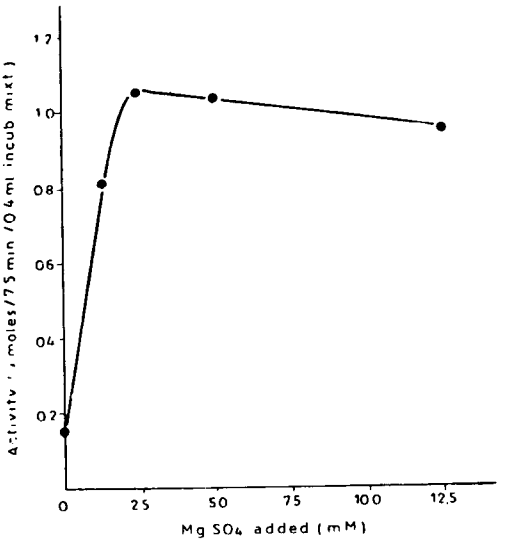

Fig. 2. Influence of $MgSO_4$ on the mycobacterial gluconokinase activity at a constant level of ATP. The reaction mixture contained: 5 mM-ATP, 5 mM-potassium gluconate, 100 mM-tris-HCl buffer, pH 9, 0.05 ml of enzyme (Sephadex eluate) and indicated amounts of $MgSO_4$ in a final volumne of 0.40 ml. Incubation 7.5 min.

10 mM-iodoacetate was, however, completely inefficient. On the other hand, 25 mM-NaF caused a fall of activity by 60% (Table 2).

In a number of specificity tests, the enzyme proved to be specific for ATP and D-gluconate. The saturating level of the latter substrate must have been 1.5 mM or less since higher concentrations up to 15 mM did not increase the activity. There was no reaction towards D-galactonate,

Table 2. Inhibition of the mycobacterial gluconokinase activity. Standard assay conditions. Inhibitors were added at the beginning of incubation

Sample No.	Inhibitor	Concn (mM)	Rate, per cent of control without addition
1	None		100
2	NaF	25	40
3	Iodoacetate	10	100
4	p-Chloromercuribenzoate (PCMB)	0.1	0

D-xylonate, D-ribonate or 2-oxo-D-gluconate (Northern Regional Res. Lab., USA). When either ITP (Bohringer, Germany) or CTP (K. a. K. Lab., USA) were substituted for ATP, the initial rate was about 5—10% of that with ATP, and might be due to the contamination of these

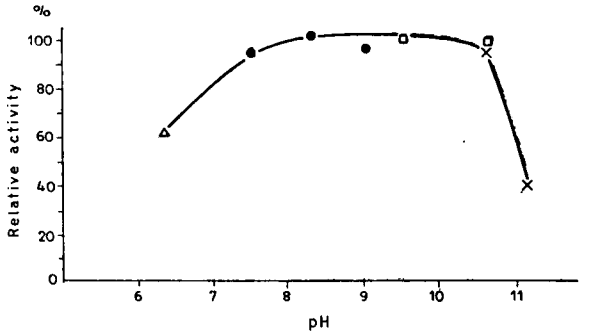

Fig. 3. Influence of pH on the mycobacterial gluconokinase activity. Standard assay conditions except for buffer as indicated: △, maleate; ●, tris-HCl; □, glycine-NaOH; ×, carbonate. Incubation 5 min. pH values of the buffers were adjusted with use of glass electrode (Radiometer, Copenhagen).

nucleotides by traces of ADP. Inorganic polyphosphate was not utilized by the enzyme preparations obtained from glucose-grown *M. phlei*.

Peak eluates from the Sephadex G-100 column were devoid of gluco-, manno-, fructo- or phosphofructokinases. They did not reveal phosphatase activity either toward ATP or 6-phosphogluconate.

On prolonged incubation, the uptake of ATP always exceeded 50%

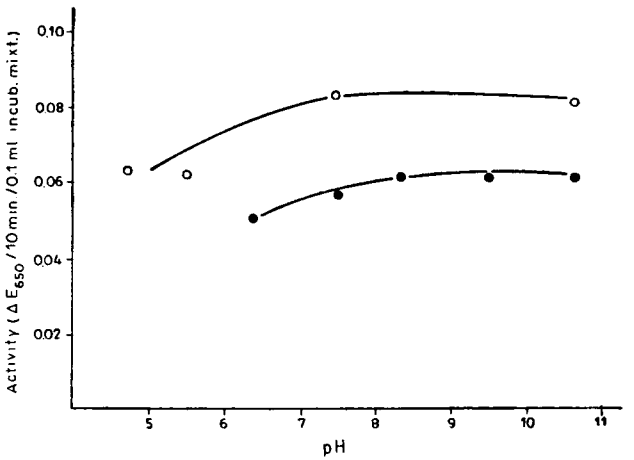

Fig. 4. Influence of pH on the mycobacterial gluconokinase stability. ○, Sephadex G-100 eluate; ●, 35—75% ammonium sulfate sat. fraction (dialysed). To 0.1 ml--aliquets of either enzyme preparation were added 0.05 ml of 0.2 M-buffer as specified in Fig. 2, and kept in stoppered tubes for 24 hr at 4°. Thereafter, 0.05 ml-samples were removed and incubated with 0.30 ml-volumes of standard mixture (pH 9.5) for 10 min. Further procedure as in Methods. After incubation, pH was checked with phenolphtalein and found to be above 8 in all tubes.

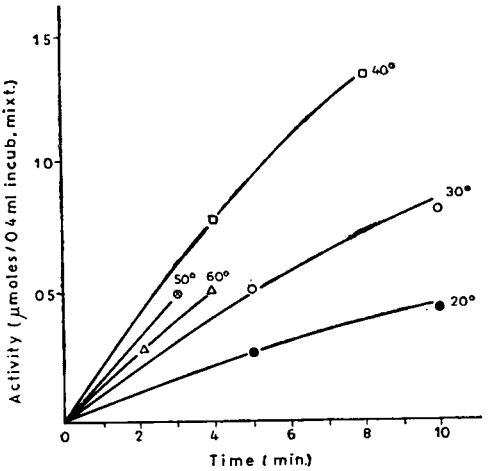

Fig. 5. Influence of temperature on the mycobacterial gluconokinase activity. Standard reaction mixtures.

of easily hydrolysable phosphorus (Fig. 7) thus indicating the presence of active adenylate kinase, which had been reported to occur in *M. phlei* by Oliver and Peel (7). This possibility was tested with a mixture consisting of: ATP (2 μmoles), AMP (2 μmoles), $MgSO_4$ (2 μmoles),

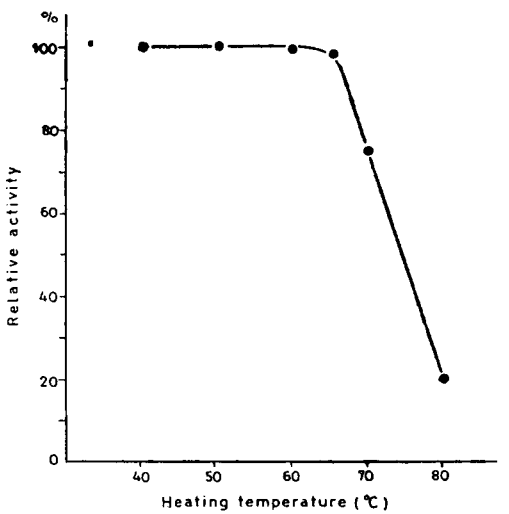

Fig. 6. Influence of temperature on the mycobacterial gluconokinase stability. 0.1 ml-portions of Sephadex G-100 eluate (0.05 M-tris-HCl buffer, pH 7.4) were transferred into small tubes and heated in water bath at the indicated temperatures for 60 s. After cooling in ice-bath, to each tube 0.25 ml of standard reaction mixture was added and the enzyme activity assayed as in Methods.

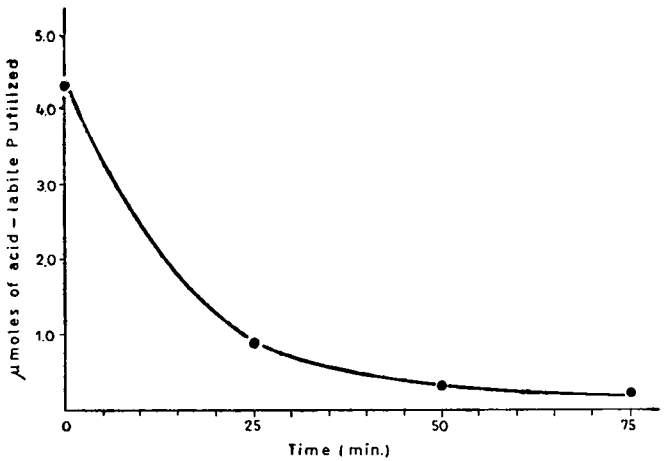

Fig. 7. Time course of ATP utilization for the phosphorylation of D-gluconate. Reaction mixture contained: 2.16 µmoles of ATP, 4 µmoles of $MgSO_4$, 10 µmoles of potassium gluconate, 80 µmoles of glycine buffer (pH 10.6) and Sephadex G-100 eluate (0.1 ml) in a total volume of 0.78 ml.

tris-HCl buffer, pH 9 (25 µmoles) and Sephadex G-100 eluate (0.05 ml) in a total volume of 0.25 ml. After incubation for 30 min. at 37°, suitable aliquots of the mixture were chromatographed on Whatman No. 1 filter paper with isobutyrate-ammonia-water as the solvent and molibdate--perchloric acid as the reagent (3). Under these conditions a separate spot of ADP appeared on the chromatogram with concomitant diminution of the two spots of AMP and ATP originally present in the reaction mixture. A control sample incubated in parallel without enzyme revealed no ADP formation.

DISCUSSION

Phosphorylation of gluconic acid was frequently encountered in biological material but only in a few cases the corresponding enzymes were subjected to further studies. These are the gluconokinases of *Escherichia coli* (1), *Pseudomonas fluorescens* (6), *Saccharomyces cerevisiae* (8), rat and hog kidney (4). The results obtained in the present paper allow to compare gluconokinase of *Mycobacterium phlei* with similar enzymes of other origin.

All the gluconokinases so far described in sufficient detail were activated by Mg or other divalent cations. Where tested, optimum pH was sharp: 6.2 for the hog kidney enzyme and 7.2 for yeast. The *E. coli* enzyme preparation proved to be absolutely specific for D-gluconate, while that of *Pseudomonas fluorescens* contained a separate kinase

phosphorylating 2-oxo-D-gluconate as well. PCMB and heavy metals caused inactivation (though Zn^{2+} stimulated the hog kidney enzyme), which indicates that SH groups were essential for gluconokinase activity.

The mycobacterial enzyme differs from animal gluconokinases in that it was inhibited by NaF and $ZnSO_4$. Another difference lies in the broad optimum pH which covers a range from about 8 to 10.5. Its substrate specificity was restricted to D-gluconate and ATP, and the phosphorylation of D-gluconate with inorganic polyphosphates described earlier (10) might have been due to another type of enzyme analogous to that phosphorylating D-glucose (9). The ATP gluconokinase of *M.phlei* withstood dehydration of cells with cold acetone and was more stable in alkaline pH's than below pH 7. Sodium chloride in concentrations exceeding 0.1 M was inhibitory. This behaviour resembles that of yeast hexokinase (11) but contrasts with the *M. phlei* polyphosphate glucokinase which was more stable at slightly acidic pH's and required, besides Mg^{2+}, 0.25 M-NaCl or other neutral salt for maximum activity (9).

It may be of interest to add, that the gluconokinase activity of *M. phlei* as measured in cell-free extracts was 5—150 times higher than that of *Pseudomonas fluorescens* or yeast and hog kidney, respectively. The 20-fold purified enzyme revealed an activity of about 3 units/mg protein and utilized ATP almost completely due to the combined action of both gluconokinase and adenylate kinase. Attempts to separate the latter enzyme by means of starch block electrophoresis were unsuccessful.

It seems that the enzyme preparation from *M. phlei*, as obtained in the present study, could be used for the quantitative determination of gluconate or ATP.

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STRESZCZENIE

Komórki *M. phlei* dezintegrowano w aparacie ultradźwiękowym MSE i zawartą w ekstrakcie 6-fosfotransferazę ATP:D-glukonian (glukonokinazę) oczyszczano stosując kolejno: 1) inkubację z dezoksyrybonukleazą, 2) frakcjonowanie siarczanem amonu, i 3) sączenie na kolumnie sefadeksu G-100. W kilku przypadkach zastosowano również elektroforezę w bloku skrobiowym. Ok. 20-krotnie oczyszczony enzym użyto do badania jego własności. Otrzymane wyniki pozwalają na wyciągnięcie następujących wniosków.

Mykobakteryjna glukonokinaza jest enzymem o kwaśnym charakterze (w pH 8,6 wędruje do anody) i mniejszym od heksokinaz ciężarze cząsteczkowym (z kolumny sefadeksu G-100 wypływa w ostatnich frakcjach). Enzym ten posiada czynne grupy SH (inhibicja pod wpływem PCMB) i wykazuje maksimum aktywności w szerokim zakresie pH od 8 do 10,5. Ta cecha zdecydowanie różni glukonokinazę *M. phlei* od glukonokinaz innego pochodzenia. Badany enzym wymaga jonów metali dwuwartościowych (Mg, Ca, Mn, Co). Metale ciężkie (Zn) oraz stosunkowo duża siła jonowa (0,2 M NaCl) wywierają działanie hamujące. Enzym jest mniej trwały w pH poniżej 7 i w warunkach oznaczenia wykazuje niskie optimum temperatury (ok. 40°), jakkolwiek w nieobecności reaktantów (pH 7,6) znosi 1-minutowe ogrzewanie w 65° bez straty aktywności. Specyficzność mykobakteryjnej glukonokinazy wydaje się być ograniczona do ATP i kwasu D-glukonowego. Częściowo oczyszczony preparat nie zawiera heksokinaz ani enzymów hydrolizujących ATP wzgl. 6-fosfoglukonian, zawiera natomiast kinazę adenylanową, która umożliwia zużytkowanie ATP praktycznie w 100%.

OBJAŚNIENIA DO TABEL

Tab. 1. Wpływ różnych jonów metali na aktywność mykobakteryjnej glukonokinazy. Stosowano 5-milimolarne stężenia ATP przy molarnym stosunku metal/ATP = 1. Pozostałe warunki jak w Metodach z wyjątkiem próby nr 3, którą inkubowano przy pH 7,5, aby uniknąć precypitacji cynku. Próba nr, dodany jon metalu dwuwartościowego, szybkość, % kontroli bez dodatku, §— odniesiona do kontroli inkubowanej przy pH 7,5 z $MgSO_4$ lecz bez $ZnSO_4$.

Tab. 2. Hamowanie aktywności mykobakteryjnej glukonokinazy. Standardowe warunki oznaczania. Inhibitory dodano na początku inkubacji. Próba nr, inhibitor, stężenie, szybkość, % kontroli inkubowanej bez dodatku.

OBJAŚNIENIA DO RYCIN

Ryc. 1. Elektroforeza w bloku skrobiowym preparatu glukonokinazy z *Mycobacterium phlei*. 2.1 ml eluatu z sefadeksu G-100 (ok. 5,5 jednostek) zagęszczono karbowaksem do 0,5 ml objętości i naniesiono na blok skrobiowy, przygotowany

jak w poprzedniej pracy (9). Elektroforezę przeprowadzano w 0,05 M buforze weronalowym, pH 8,6, przy 200 V i 7,5 mA w pokoju chłodni. Po 14-godzinnej migracji blok pocięto na 0,5 cm-segmenty, które ekstrahowano 2 ml 0,1 M buforu tris-HCl, pH 9, a następnie oznaczano w ekstraktach aktywność enzymatyczną. Mieszanina inkubacyjna zawierała: 2,8 μmole ATP, 2,8 μmole D-glukonianu, 1,4 μmola $MgSO_4$ i 0,2—0,4 ml ekstraktu w końcowej objętości 0,47 ml. Inkubacja 30 min.

Ryc. 2. Wpływ $MgSO_4$ na aktywność mykobakteryjnej glukonokinazy przy stałym stężeniu ATP. Mieszanina reakcyjna zawierała: ATP (5 mM), glukonian potasu (5 mM), tris-HCl, pH 9 (0,1 M), 0,05 ml enzymu (eluat z sefadeksu oraz zmienne ilości $MgSO_4$ w końcowej objętości 0,40 ml. Inkubacja 7,5 min.

Ryc. 3. Wpływ pH na aktywność mykobakteryjnej glukonokinazy. Standardowe warunki oznaczeń z wyjątkiem buforu jak niżej: △ — maleinian; ● — tris-HCl; □ — glicyna-NaOH; × — węglan. Inkubacja 5 min. pH buforów nastawiano przy pomocy elektrody szklanej (Radiometer, Copenhagen).

Ryc. 4. Wpływ pH na stabilność mykobakteryjnej glukonokinazy. ○ — Eluat z sefadeksu G-100; ● — dializowana frakcja po strąceniu siarczanem amonu (35—75%). Do 0,1-mililitrowych próbek wymienionych preparatów dodano 0,05 ml 0,2 M buforu jak w legendzie do ryc. 2, i pozostawiono w zamkniętych probówkach na 24 godziny w 4°. Po upływie tego czasu pobrano 0,05-mililitrowe objętości i inkubowano z dodatkiem 0,30 ml mieszaniny standardowej (pH 9,5) przez 10 min. Dalej postępowano jak w Metodach.

Ryc. 5. Wpływ temperatury na aktywność mykobakteryjnej glukonokinazy. Stosowano standardowe mieszaniny reakcyjne.

Ryc. 6. Wpływ temperatury na stabilność mykobakteryjnej glukonokinazy. 0,1-mililitrowe porcje eluatu (0,05 M bufor tris-HCl, pH 7,4) przeniesiono do małych probówek i ogrzewano na łaźni wodnej w odpowiednich temperaturach przez 60 sek. Po oziębieniu do każdej probówki dodano 0,25 ml standardowej mieszaniny i wykonano oznaczenia jak w Metodach.

Ryc. 7. Przebieg czasowy zużytkowywania ATP do fosforylacji D-glukonianu. Mieszanina reakcyjna zawierała: 2,16 μmole ATP i 4 μmole $MgSO_4$, 10 μmoli glukonianu potasu, 80 μmoli buforu glicynowego (pH 10,6) i eluat z sefadeksu G-100 (0,10 ml) w całkowitej objętości 0,78 ml.

РЕЗЮМЕ

Клетки *M. phlei* дезинтегрировали в ультразвуковом аппарате MSE и содержащуюся в экстракте 6-фосфотрансферазу АТФ-Д-глюконат (глюконокиназу) очищали, применяя последовательно: 1) инкубацию с дезоксирибонуклеазой; 2) фракционирование сульфатом аммония и 3) фильтрацию на колонке Sephadex G-100. В нескольких случаях применяли также электрофорез в крахмальном блоке. Очищенный приблизительно 20 раз энзим использовали для исследований его свойств. Полученные результаты дают следующие выводы.

Микобактериальная глюконокиназа — это энзим кислого характера (в pH 8,6 мигрирует к аноду) с меньшим молекулярным весом, чем гексокиназы (из колонки Sephadex G-100 выплывает в последних

фракциях). Этот энзим имеет активные группы SH (замедление происходит под действием PCMB) и проявляет максимальную активность в широком диапазоне pH от 8 до 10,5. Эта черта отличает глюконокиназу *M. phlei* от глюконокиназ иного происхождения. Исследованный энзим требует двухвалентных ионов (Mg, Ca, Mn, Co). Тяжелые металлы (Zn) и относительно большая ионная сила (0,2 M NaCl) оказывают тормозящее действие. Энзим является менее прочным в pH ниже 7 и в условиях обозначения показывает низкий оптимум температуры (около 40°), хотя при отсутствии субстратов (pH 7,6) выдерживает минутное нагревание при 65° без утраты активности. Специфичность микобактериальной глюконокиназы, кажется, будет ограничена до АТФ и Д-глюконовой кислоты. Частично очищенный препарат не содержит гексокиназ и энзимов, гидролизирующих АТФ и 6-фосфоглюконат, зато содержит адениловую киназу, которая способствует практическому использованию АТФ на 100%.

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