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GLYCOGEN PHOSPHORYLASE OF POTATOES PURIFICATION AND THERMODYNAMIC PROPERTIES OF THE ADSORPTION ON GLYCOGEN

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SUMMARY

1 Glycogen phosphorylase from potato tubers was purified by a rapid procedure including chromatography on starch grains followed by DEAE-Sephadex chromatography

2 By gel filtration on Sephadex G-200 the purified enzyme showed a molecular weight of 180 000 Electrophoresis in gels with laurylsulphate revealed a molecular weight of 90 000 for the protein

3 The enzyme activity with glycogen as a primer substrate is 30% higher than with starch

4 Adsorption of the enzyme on glycogen and starch occurs over a pH region of pH 4–10

5 Thermodynamic data were obtained for the adsorption of the glycogen phosphorylases 1 and 3 on glycogen by polyacrylamide-gel electrophoresis at different temperatures and with varying amounts of glycogen in the gels The enthalpy change of the absorption is −58 6 kJ/mole for phosphorylase 3 and −66 5 kJ/mole for phosphorylase 1 The difference between the free energy change of the adsorption of isoenzymes 1 and 3 is 8 4 kJ/mole, whereas the entropy change is almost the same for both enzymes

INTRODUCTION

The enzyme phosphorylase (EC 2 4 1 1) plays a major role in the metabolism of the reserve polysaccharides In plants this enzyme has a function both in breakdown and synthesis of reserve polysaccharides [1–4] It is likely that these two functions are not exerted by one and the same form of the enzyme For that reason it is of interest to obtain a more detailed information about the multiple forms of phosphorylase that have been described already [1, 5, 6]

This paper deals mainly with the two forms in potato tubers called phosphorylase 1 and phosphorylase 3 that show affinity to glycogen and starch grains These two have several physicochemical properties in common which are different from those of the other phosphorylase forms in potatoes [6]

METHODS

Preparation of crude extract

Potato tubers (*Solanum tuberosum*, c v Bintje) stored in the dark at 8 °C, were cut into small pieces and homogenized for 1 min, with an equal volume of the buffer used for the elution of the different columns To the extract 4 mM Na_2SO_4 and 3 mM $Na_2S_2O_5$ was added to prevent browning The homogenate was pressed through perlon gauze and centrifuged at 10 000 × *g* for 15 min The supernatant was used as crude extract

Partial purification of starch phosphorylase

Starch phosphorylase (isoenzyme 6) [6] was purified from crude potato extract by gel filtration on a Sephadex G-200 column, followed by chromatography of the enzyme containing fractions on a DEAE-Sephadex A25 column Disc electrophoresis showed that the partly purified enzyme was slightly contaminated with other proteins Staining the gels with I_2–KI revealed that the starch phosphorylase (isoenzyme 6) was not contaminated by other forms of phosphorylase

Partial purification of the glycogen phosphorylases

Crude potato extract was subjected to chromatography on a DEAE-Sephadex A25 column The first peak with phosphorylase activity eluted by a NaCl gradient contains the glycogen phosphorylases (isoenzymes 1 and 3) [6] not contaminated by other phosphorylases [6]

Separation methods

Sodium dodecylsulphate–gel electrophoresis was performed by the method of Weber and Osborn [7] in gels containing 10% acrylamide and 0 1% dodecylsulphate

Disc electrophoresis was carried out according to Ornstein [8] and Davies [9] as previously described [6] The protein zones in the gels were stained using Coomassie Brilliant Blue R 250 (Serva) Phosphorylase activity in the gels was stained with I_2–KI, after incubation of the gels during 4 h in 0 25 M glucose 1-phosphate (Boehringer) and 0 1 M sodium citrate buffer (pH 5 0) at 25 °C [6, 10] When the gels did not contain glycogen this was added to the incubation mixture (1% w/v)

Gel filtration was performed according to Andrews [11], on columns of Sephadex G-200 (2 5 cm × 40 cm) equilibrated with 0 01 M Tris–HCl buffer (pH 7 5), containing 0 1 M KCl

The procedure of chromatography on DEAE-Sephadex A25 was the same as previously described [6]

All separation procedures were performed at 4 °C, unless otherwise stated

Phosphorylase assay

Phosphorylase activity was determined by measuring the liberation of P_i from glucose 1-phosphate according to Lowry and Lopez [12] The incubation mixture contained 11 mM glucose 1-phosphate, 11 mM glycerophosphate and 0 03% (w/v) starch (nach Zulkowsky, Merck) or glycogen (Merck) and was adjusted to pH 6 5 The reaction was started by adding 0 1 ml enzyme solution to the incubation mixture

After incubation for 10 min at 30 °C, the reaction was stopped by 5 ml sodium acetic acid buffer (pH 4 0) after which the P_i content was determined

Determination of protein content

Protein content was measured following the method of Lowry et al [13]

Isoelectric focusing

Isoelectric focusing on polyacrylamide gels was carried out in Pyrex glass tubes (10 cm × 0 6 cm) Gels were made from a mixture of 4% (w/v) acrylamide, 0 1% (w/v) *N,N'*-methylene-bis-(acrylamide), 0 05% (w/v) tetramethylenediamine, 4% (v/v) Ampholine (LKB), 0 7% (w/v) $(NH_4)_2S_2O_8$, 0 1% (w/v) glycogen (Merck) and the enzyme solution After polymerization the gels were inserted into an electrophoresis apparatus The anode (lower compartment) was filled with 250 ml 0 25 M H_2SO_4 and the cathode compartment with 700 ml 2% (v/v) ethylenediamine The voltage was gradually increased to 100 V and then maintained constant for 5 h After finishing the focusing some gels were cut into pieces of 1 cm These were minced in 1 ml destilled water and the pH was measured in each suspension In other gels the phosphorylase activity was determined by the I_2–KI staining procedure, after which densitograms were recorded at 700 nm using a Unicam SP 591 densitometer acessoire on a Unicam SP-500 spectrophotometer

RESULTS

Purification of phosphorylase 1

Step 1 Preparation of the extract Tuber tissue of potatoes (200 g) was homogenized in a Braun Multipress MP 50 The juice was caught in a beaker glass containing 4 ml of a sulphite mixture (5% Na_2SO_4 and 3 7% $Na_2S_2O_5$ in water) to prevent browning

The Multipress was rinsed with 250 ml 0 1 M glycine–NaOH buffer (pH 10 0), to wash the enzyme from the remaining starch and cell debris The juice and rinsing buffer were combined giving crude extract (approx 500 ml) The crude extract was adjusted to pH 7 5 with HCl These operations were conducted at 4 °C

Step 2 Adsorption on starch grains The crude extract was layered upon a starch column in a Buchner funnel (5 cm × 15 cm) and sucked through by a light vacuum After the surface of the column did run dry, it was washed 3 times with 250 ml washing buffer (10 mM Tris, 10 mM maleic acid, adjusted to pH 7 0 with NaOH) By this treatment the phosphorylase isoenzymes which do not adsorb on starch are washed from the column The adsorbed enzyme was eluted with 0 2 M glycine–NaOH (pH 9 0) in six fractions of 50 ml The highest activity was found in the second and third fraction (see Table I) These operations were conducted at room temperature

Step 3 DEAE-Sephadex column chromatography The second and third elution fractions were combined and dialysed overnight against 1 l buffer (50 mM Tris, 20 mM NaCl, 20 mM glycine adjusted to pH 7 0 with HCl) After dialysis the enzyme was applied to a column of DEAE-Sephadex A25, equilibrated with the same buffer The column was washed and was then eluted by a linear gradient of NaCl (in 500 ml) ranging from 0 02 to 0 5 M By this procedure the enzyme was eluted at 0 15 M NaCl,

TABLE I

PURIFICATION OF GLYCOGEN PHOSPHORYLASE BY ADSORPTION ON STARCH GRAINS AND DEAE-SEPHADEX CHROMATOGRAPHY

The glycogen phosphorylase from potato extract was adsorbed on a column (15 cm × 5 cm) of starch grains at pH 7 5 and was eluted with 0 2 M glycine–NaOH buffer (pH 9 0) and after that chromatographed on a DEAE-Sephadex A25 column (2 5 cm × 35 cm) equilibrated with 50 mM Tris–HCl buffer, 20 mM NaCl and 20 mM glycine (pH 7 2) The enzyme was eluted by a linear NaCl gradient (500 ml) ranging from 0 02 to 0 5 M Enzyme activity is expressed as P_i liberated from glucose 1-phosphate in 15 min The purification factor was calculated on the assumption that glycogen phosphorylase accounts for 10% of the total phosphorylase activity in crude extract

	Volume (ml)	Total enzyme activity (μmole)	Yield (%)	Total protein (mg)	Spec act (μmole/mg)	Purification factor
Crude extract	450	1224	100	6750	0 18	1
Starch column						
not adsorbed	1800	1087	88 9	6780		
1 elution fraction	50	1		5 5	0 18	
2 elution fraction	50	38		12 0	3 17	
3 elution fraction	50	39		15 0	2 30	
4 elution fraction	50	10		9 5	1 05	152
5 elution fraction	50	2 5		5 5	0 45	
6 elution fraction	50	1 5		4 0	0 38	
subtotal		92	7 5	51 5		
DEAE-Sephadex						
Fraction 18	11 4	8 4		0 912	9 25	
Fraction 19	11 4	9 6		0 912	10 50	583
Fraction 20	11 4	5 8		0 789	7 29	
Fraction 17, 21–23	45 6	9 0				
subtotal		32 8	2 7			

whereas the other forms of phosphorylase are eluted at NaCl concentration above 0 25 M [6]

Electrophoresis of purified enzyme

After DEAE-Sephadex chromatography the purity of the enzyme was checked by disc electrophoresis Gels containing glycogen (0 1 %), stained with I_2–KI revealed that only phosphorylase 1 was present Electrophoresis was also conducted in gels without glycogen, staining for protein yielded three coloured zones and staining for phosphorylase activity by I_2–KI yielded one zone (see Fig 1) The R_F of the band with enzyme activity did not correspond with that of the most pronounced protein band that moved faster

After gel filtration on a Sephadex G-200 column the enzyme activity was eluted together with all detectable protein Therefore it is supposed that the enzyme is partly inactivated during electrophoresis probably by dissociation into subunits

Molecular weight

The molecular weight of the purified enzyme was determined by gel filtration on a Sephadex G-200 column [11], with bovine serum albumin, apoferritin, R-phycoery-

Fig 1 Polyacrylamide-gel electrophoresis of purified potato glycogen phosphorylase (A) Stained by I_2–KI for enzyme activity, (B) stained by Coomassie Brilliant Blue R 250, (C) separated in gels with 0 1% dodecylsulphate and stained by Coomassie Brilliant Blue R 250

thrin and catalase as reference proteins The molecular weight of the phosphorylase proved to be 180 000 The same value was found previously for the enzyme in crude potato extract [6]

Polyacrylamide-gel electrophoresis with sodium dodecylsulphate

The subunit structure of the enzyme was studied by polyacrylamide-gel electrophoresis with sodium dodecylsulphate After electrophoresis there appeared two protein bands in the gels on staining with Coomassie Brilliant Blue (see Fig 1) The protein of the main zone had a molecular weight of 90 000, the protein of the other more faint zone had a molecular weight of 40 000, determined with ovalbumin and bovine serum albumin as reference proteins

Enzyme activity with glycogen or starch as primer

The ability of the purified enzyme to use starch or glycogen as primer polysaccharide was tested and compared with partly purified starch phosphorylase (phosphorylase 6) [6] The results (see Table II) indicate that the purified enzyme shows greater activity with glycogen than with starch as a primer, whereas phosphorylase 6 shows the highest activity with starch, like the potato phosphorylase described in literature [14]

The influence of the pH on the adsorption of phosphorylase on starch and glycogen

Columns of starch grains on which the purified glycogen phosphorylase was

TABLE II

ENZYME ACTIVITY WITH STARCH OR GLYCOGEN AS PRIMER

The phosphorylase activity (P_i liberated from glucose 1-phosphate per 15 min) is expressed as a percentage of the activity with starch as primer

	Phosphorylase activity		
	With starch	With glycogen	Without primer
Glycogen phosphorylase (phosphorylase 1)	100	130	8
Starch phosphorylase (phosphorylase 6)	100	41	11

adsorbed were eluted by buffers of different composition and pH The enzyme could not be eluted by Tris–maleic acid (50 mM) of pH 7 5 By a Tris–HCl buffer (50 mM) of pH 8 5 some enzyme was eluted The enzyme could easily be desorbed from starch by Tris (5 mM)–glycine (30 mM) buffer (pH 8 5), or by 30 mM glycine NaOH buffer (pH 8 5)

The influence of pH on the adsorption of phosphorylase on glycogen was studied by isoelectric focusing of the purified enzyme in polyacrylamide gels containing 0 1 % glycogen with Ampholine ranging from pH 3 to 10 and from pH 7 to 10 During focusing the originally homogeneous distributed enzyme tends to move towards the zone with the pH of its isoelectric point (pH 5 1) [6], but is kept by the immobile glycogen at pH values at which adsorption occurs between pH 4 and 9 (see Fig 2)

Thermodynamics of the adsorption

The thermodynamics of the adsorption process were studied by investigating

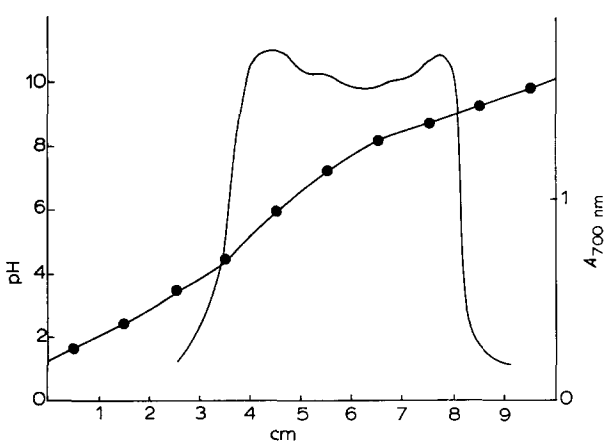

Fig 2 The pH dependence of the adsorption of phosphorylase on glycogen Purified glycogen phosphorylase was focused in 7% acrylamide gels (0 6 cm × 10 cm) containing 0 1% glycogen and 4% Ampholine (pH 3–10) After focusing for 5 h at 100 V some gels were stained for phosphorylase activity by the I_2–KI method and scanned at 700 nm (———) Other gels were cut into pieces of 1 cm and minced in 1 ml water to measure the pH (●—●)

the influence of different temperatures on the dissociation constant (K) of the enzyme glycogen complex K was electrophoretically determined according to the equation [6]

$$(R_{F_m} - R_F)^{-1} = K(R_{F_m} \cdot S)^{-1} + R_{F_m}{}^{-1} \quad (1)$$

The electrophoretic mobility (R_F) of the isoenzymes was determined with bromophenol blue as a front marker R_{F_m} is the R_F in glycogen free gels, S is the free glycogen concentration in the gels which is supposed to be equal to the concentration of the glycogen polymerized in the gel By plotting $(R_{F_m} - R_F)^{-1}$ against S^{-1}, a linear relation is obtained from which K can be derived, for the intercept on the abcissa corresponds with $-K^{-1}$

After electrophoresis some gels were crushed in 2 ml water and the pH of this mixture was measured at the temperature of the electrophoresis The pH was 9 62 at 5 °C and 9 36 at 18 °C A possible influence of these small pH differences on the adsorption of phosphorylase on glycogen was neglected

To obtain accurate dissociation constants at different temperatures a series of fully identical gels was prepared out of one stock solution with a certain glycogen concentration Electrophoresis was performed with phosphorylase 1 and 3 which were partly purified by DEAE-Sephadex chromatography The values of log K were plotted against the reciprocal of the temperature (Arrhenius plot) (see Fig 3) From these

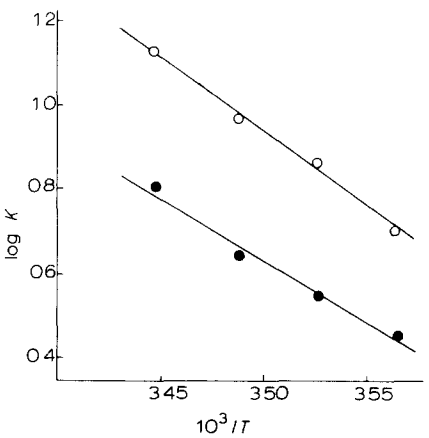

Fig 3 Arrhenius plots of the adsorption of the glycogen phosphorylases of potatoes on glycogen The dissociation constants were calculated by means of Eqn 1 [6] from the R_F values of the enzymes after electrophoresis in 7% acrylamide gels (0 6 cm × 10 cm) containing varying amounts of glycogen, with bromophenol blue as a front marker ○—○, phosphorylase 1, ●—●, phosphorylase 3

graphs the enthalpy change of formation (ΔH_f) of the enzyme–glycogen complex was obtained by multiplying the slope of the lines by 4 56 (=e R) [15] From five experiments a ΔH_f value of −58 6 (± 5 4) kJ/mole for phosphorylase 1 and −66 5 (±5 4) kJ/mole for phosphorylase 3 was calculated

When enthalpy data and dissociation constants are available the other thermo-

dynamic parameters can be calculated [15] The free energy change (ΔG) by $\Delta G = RT\ln K$ and the entropy change (ΔS) by the formule $\Delta S = (\Delta H - \Delta G)T^{-1}$ Because the glycogen concentrations in the gel could not be expressed in molarities, only the difference between ΔG and ΔS for both isoenzymes could be calculated At 20 °C the difference between ΔG for the two phosphorylases was 8 4 kJ/mole, whereas both enzymes showed almost no differences in ΔS (1 7 J/mole/°K)

The influence of some metabolites on adsorption

The effect of some metabolites on the affinity of phosphorylase to glycogen was tested by electrophoresis of a partly purified mixture of phosphorylase 1 and 3 (1st eluted phosphorylase peak from a DEAE-Sephadex column) in gels containing varying amounts of glycogen To the cathode buffer 5-AMP (10 mM), ATP (10 mM) or glucose 1-phosphate (10 mM) were added These metabolites had a retarding effect on the electrophoretic mobility of the enzymes, they did, however, not influence the K values

Heterogenity of the phosphorylase 1 and 3

The partly purified phosphorylase 1 was separated into three bands that showed little differences in R_F, at glycogen concentrations yielding a R_F value greater than 0 05 The band with the lowest R_F showed the highest enzyme activity, all data described concern this band The middle one showed little activity whereas the one with the greatest R_F was hardly visible Because these bands could not be distinguished well at low glycogen concentrations their R_{F_m} values could not be obtained Therefore it is not yet clear whether these two bands differ from the main component with respect to their affinity to glycogen or that the difference in electrophoretic behaviour is caused by a more negative electric charge

Phosphorylase 3 separated into 2 bands the slow moving band showed the highest enzyme activity and only for this one dissociation constants could be determined

DISCUSSION

Phosphorylase 1 prepared from potato tuber tissue is proved to be a glycogen phosphorylase that uses glycogen more efficiently as a substrate then starch This enzyme shows not only a great affinity to glycogen but also to starch grains, probably because the surface of both substrates possess many end groups of polysaccharide chains Therefore it is likely that the data obtained with glycogen are also valid for starch grains The enzyme shows its high affinity to starch grains and glycogen over a broad pH region Consequently it is unlikely that the degree of adsorption and thereby the enzyme activity is regulated in vivo by changes of pH

The reaction catalysed by phosphorylase uses two substrates and follows a random bi-bi-mechanism [16] This implicates that the binding of one substrate does not influence the affinity of the enzyme to the other substrate and further that K is almost equal to K_m That glucose 1-phosphate does not influence K of phosphorylase 1 and 3 is in agreement with this view

The potato glycogen phosphorylases exhibit a great affinity to glycogen compared with other glycogen phosphorylases At 20 °C, K of the enzyme glycogen com-

plex is 17 4 10^{-5}% for phosphorylase 1 and 9 5 10^{-3}% for phosphorylase 3, whereas Takeo and Nakamura [17] reported K values for the enzyme isolated from muscle, liver or brain tissue of 0 11, 0 40 and 0 25%, respectively A relative great affinity to the substrate was also described for amylopectine phosphorylase of potatoes, the K_m for amylopectin end groups being 4–6 6 10^{-5} M [18–20] and for pentaose 7 10^{-5} M [20] whereas the K_m of muscle phosphorylase for pentaose is 1 5 10^{-2} M [22] and for glycogen 1 2 10^{-4} M [21]

The high values of $1H_f$ (−58 6 kJ/mole and −66 5 kJ/mole) mean that the enzymes show a tendency to adsorb on glycogen to a greater extent at low temperatures The K for the enzyme glycogen complex is 17 4 10^{-5}% for phosphorylase 1 at 20 °C and 2 3 10^{-5}% at 0 °C (calculated by extrapolation from Fig 3) Because glycogen and starch grains are interchangeable as substrates for the phosphorylase, the temperature dependency of the association probably plays a role in the sweetening of potato tubers at low temperatures Lower temperatures retard metabolic processes, but phosphorylase association with starch is enhanced and consequently sugar formation from starch may be retarded to a lesser extent

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