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COLD-LABILITY OF PHOSPHOFRUCTOKINASE FROM POTATO TUBERS

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; tuber; low temperature sweetening; phosphofructokinase; differential scanning calorimetry; protein dissociation; cold-lability.

Abstract—The aim of this work was to study the cold-lability of phosphofructokinase from tubers of *Solanum tuberosum* cv Record, a variety that exhibits low temperature sweetening. The enzyme was purified by affinity chromatography and samples were examined by differential scanning calorimetry. Power–time curves were recorded for cooling and warming between 293 and 265 K. This revealed an exothermic dissociation, centred on 286 K, as the temperature was lowered. The latter temperature is close to that at which the tubers start to sweeten. It is suggested that hydrophobic interactions that contribute to the stability of the active configuration of the oligomeric enzyme are weakened at low temperatures, and that this causes spontaneous dissociation and consequent loss of activity of the enzyme. The results are discussed in relation to low temperature sweetening of potatoes.

INTRODUCTION

There is now appreciable evidence that cold-lability of phosphofructokinase (ATP: D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) is an important cause of low temperature sweetening of potato tubers. Measurements of temperature coefficients show that lowering the temperature to that which leads to sweetening reduces the activity of phosphofructokinase *in vitro* more than that of sucrose phosphate synthetase and some other enzymes of carbohydrate metabolism [1]. Phosphofructokinase dominates control of hexose phosphate oxidation in the tubers [2]. Thus, differential sensitivity of phosphofructokinase to low temperature could reduce carbohydrate oxidation to a greater extent than other reactions that metabolize hexose phosphates, and thereby lead to a diversion of these compounds to sucrose. The manner in which tubers metabolize [^{14}C]glucose provides considerable evidence that lowering the temperature to 2° causes just such a diversion [3,4].

The aim of the work reported in this paper was to use differential scanning calorimetry to investigate the effect of temperature on the behaviour of phosphofructokinase from potato tubers. Phosphofructokinase is an oligomeric enzyme [5]. Lowering the temperature could weaken hydrophobic bonds in the oligomeric complex: this could lead to dissociation and a consequent loss of catalytic activity. Cold-labile phosphofructokinase has been reported from rabbit muscle [6], chicken liver [7] and *Bacillus licheniformis* [8]. In these instances light scattering or sucrose density gradient centrifugation were used to study the effects of temperature on the enzyme and the results were consistent with the view that lowering the temperature caused dissociation into inactive subunits. The above two methods provide an estimate of the equilibrium constant, and hence the free energy, governing the dissociation, on the assumption that the process is fully reversible.

In order to obtain a clearer picture of the mechanism, as well as the thermodynamic and kinetic details, of temperature-induced dissociation and association, various calorimetric techniques can be used. In particular, differential scanning calorimetry monitors the heat capacity (Cp) as a function of temperature. As

$$\Delta H = \int \Delta Cp \, dT$$

the enthalpy of any thermal transition can also be obtained, provided that the standard states are properly defined [9]. In the present study we have estimated ΔH that accompanies the low temperature dissociation of phosphofructokinase from potatoes. This direct calorimetric method is greatly to be preferred to estimates based on light scattering or sucrose density gradient centrifugation. This is because the errors in ΔH introduced by the van't Hoff treatment of equilibrium constants are large, and because, in the absence of any knowledge about the degree of cooperativity of dissociation, the van't Hoff value for ΔH must be suspect and is unlikely to coincide with the true ΔH that can be determined directly by calorimetry. In addition, the high concentrations of sucrose employed in density gradients may affect the dissociation and thus lead to spurious results.

RESULTS AND DISCUSSION

The first requirement was pure phosphofructokinase from potato tubers. We obtained this by affinity chromatography. We had to pay considerable attention to minimizing deleterious effects of phenolics during the homogenization of the tissue; success depended upon the inclusion of bovine serum albumin, dithiothreitol and

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Dowex resin in the homogenizing medium. The protein that was precipitated by 60% $(NH_4)_2SO_4$ was dialysed, desalted, and applied to a column of Blue Sepharose which was then eluted with ATP and $MgCl_2$. Nearly all of the phosphofructokinase added to the Blue Sepharose was recovered in fractions 2–5 (Fig. 1a). These fractions were subjected to SDS/polyacrylamide-gel electrophoresis and each showed only two major bands of protein (Fig. 2a). The three fractions were combined, concentrated with $(NH_4)_2SO_4$, applied to a second column of Blue Sepharose, and phosphofructokinase was then eluted with a linear gradient of ATP and $MgCl_2$ (Fig. 1b). The fractions with the highest phosphofructokinase activities were examined by SDS/polyacrylamide-gel electrophoresis and fraction 3 was found to show only a single band (Fig. 2b). This single fraction was used for calorimetry.

During calorimetry, samples were cooled from 293 K to 265 K at various rates within the range 5–10 K/min. After holding the temperature at 265 K for 5 min, the samples were warmed to 293 K at the same rates. Power–time curves were recorded for cooling and warming and are exemplified by Fig. 3, which strongly suggests the existence of a low temperature enzyme transition and also provides information about the probable mechanism of this transition. The dissociation is exothermic and is centred on 286 K independently of the scanning rate. The

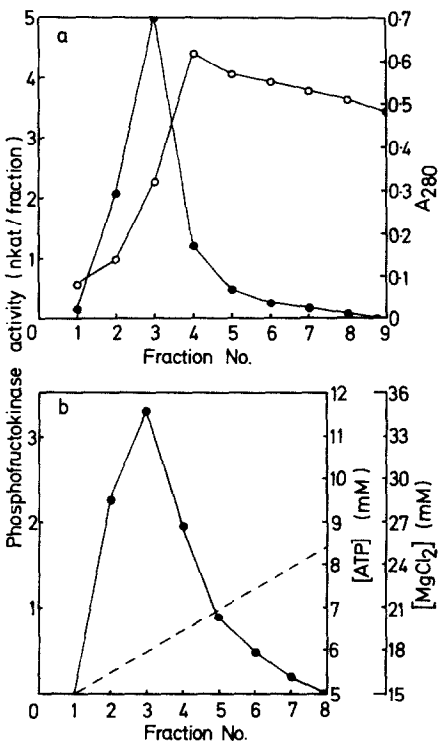

Fig. 1. (a) Elution of phosphofructokinase (●——●) and protein (○——○) from Blue Sepharose. Protein was precipitated with 60% $(NH_4)_2SO_4$, desalted and applied to a column of Blue Sepharose which was then eluted with 10 mM ATP–30 mM $MgCl_2$; 5-ml fractions were collected. (b) Fractions 2–4 were combined, concentrated with 60% $(NH_4)_2SO_4$, desalted and applied to a second column of Blue Sepharose which was then eluted with a linear gradient of 5–12 mM ATP and 15–36 mM $MgCl_2$; 3-ml fractions were collected.

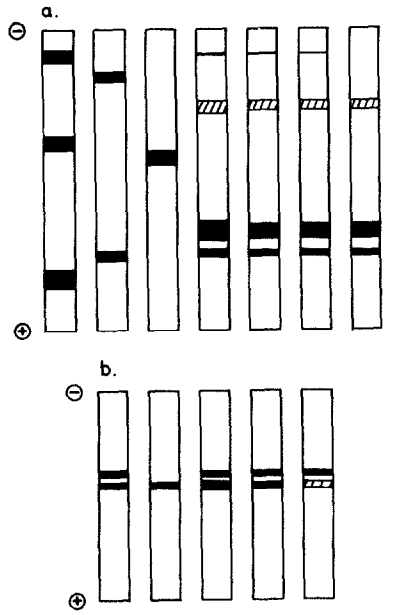

Fig. 2. SDS/polyacrylamide-gel electrophoresis of fractions eluted from Blue Sepharose shown in Fig. 1. The samples were: 2a, from the first column; lane 1, bovine serum albumin, carbonic anhydrase, cytochrome *c*; lane 2, myoglobin, ovalbumin; lane 3, α-chymotrypsinogen; lane 4, 50 μl fraction 2; lane 5, 25 μl fraction 3; lane 6, 25 μl fraction 4; lane 7, 25 μl fraction 5: 2b; lane 1, 15 μl fraction 2 from first column; lane 2, 15 μl fraction 3 from second Blue Sepharose column; lane 3, 15 μl fraction 4 from second column; lane 4, 15 μl fraction 5 from second column; lane 5, 15 μl fraction 6 from second column.

observation that $\Delta H < 0$ is consistent with a dissociation promoted by weakening of hydrophobic interactions at low temperature [10, 11]. It is not consistent with suggestions that aggregation of the subunits is stabilized by salt bridges or any other interactions of an electrostatic nature [7]. The heating trace in Fig. 3 does not reflect a quantitative reversal of the dissociation, although a broad endotherm does suggest reassembly of the enzyme. From our data, which at this stage are still preliminary, it appears that reassembly is slow compared to dissociation. Alternatively, the form of the heating curve may be due to

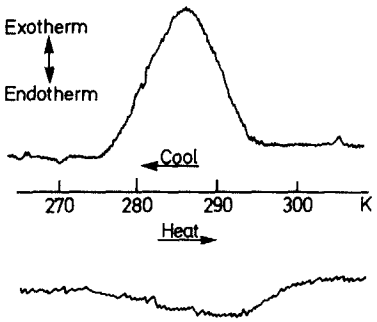

Fig. 3. Power–time differential scanning calorimeter traces for the low temperature dissociation and reassembly of phosphofructokinase from potato tubers. The enzyme was cooled/heated at 10 K/min. With the instrumental sensitivity of 1 mcal/sec full-scale deflection and a sample weight of *ca* 5 mg, the peak height corresponds to a heat evolution of 0.15 mcal/sec.

partial irreversibility caused by the high concentration of protein used, or to a low rate of renaturation in comparison to the scanning rate employed.

We suggest that phosphofructokinase from potatoes owes its cold-lability to denaturation of the oligomeric enzyme complex. The facts that the dissociation is exothermic, and that the corresponding entropy change must therefore be negative, strongly suggest that hydrophobic interactions which contribute to the stability of the enzyme at ordinary temperatures are so weakened at lower temperatures that the enzyme spontaneously dissociates into subunits. We suggest that, *in vivo*, this dissociation lowers the activity of phosphofructokinase relative to those of other enzymes that metabolize hexose phosphates, and that this leads to an accumulation of hexose phosphates which are subsequently converted to sucrose. We also suggest that this whole process is a significant cause of cold induced sweetening. The maximum sweetening of potatoes occurs at a lower temperature than the transition temperature which we observed on cooling phosphofructokinase. This may reflect additional causes of sweetening but is more probably due to differences in the environment of the protein. Our experiments were carried out with the purified protein. *In vivo* the behaviour of the enzyme is likely to be affected by the cytoplasmic solutes which could afford some protection from the effects of cold so that dissociation occurs at a lower temperature.

Finally, we suggest that our experimental approach is worth further development and application. At present, our calorimetric results are limited and semi-quantitative. This is due to the fact that the molar concentration of enzyme in the sample was not known. Thus, while the total energy output was obtained with a reasonable degree of precision, we cannot express this in terms of energy per mole of enzyme. A better supply of pure enzyme will allow this deficiency to be remedied. This, in turn, will allow more information to be obtained from the power–time curves. Heat capacity measurements provide an estimate of the amplitudes of the energy fluctuations of the two protein structures, and, in the isothermal mode, scanning calorimetry can yield rate constants and activation energies of reactions with half-lives $t_{\frac{1}{2}} > 2$ min [12].

EXPERIMENTAL

Materials. Sephadex G-25 and Blue Sepharose CL-6B were from Pharmacia, Uppsala, Sweden. Tubers of *Solanum tuberosum* L. cv Record were from the National Institute of Agricultural Botany, Cambridge and were kept in the dark at 10° until used. Experiments were carried out using mature tubers within 6 months of harvest.

Purification of phosphofructokinase. Our procedure follows closely that designed by J. E. Isaac and M. J. C. Rhodes (personal communication). Tubers were taken straight from storage and 100 g fr. wt were immediately homogenized in an equal vol. of 50 mM Tris–HCl, pH 8, containing 2 mM EDTA, 5 mM dithiothreitol, 0.5% (w/v) bovine serum albumin (BSA) and 5% (w/v) Dowex-1 resin (Cl^- form). The homogenate was filtered through 4 layers of muslin and then centrifuged at 35 000 g for 25 min. Solid $(NH_4)_2SO_4$ was added to the resulting supernatant to 60% satn and the ppt. was resuspended in 30 ml resuspension medium (5 mM Tris–HCl, pH 8, 2 mM EDTA, 1 mM dithiothreitol) and then dialysed for 6 hr against 100 vol. resuspension medium. The dialysis residue was desalted by passage through a column (1 × 15 cm) of Sephadex G-25 (coarse) with resuspension buffer being used for elution. The protein was then applied to a column (1.6 × 15 cm) of Blue Sepharose CL-6B which had been equilibrated with resuspension buffer. The Blue Sepharose was then washed with 50 ml resuspension buffer, and phosphofructokinase was eluted with 50 ml 10 mM ATP–30 mM $MgCl_2$ dissolved in resuspension buffer. Fractions of 5 ml were collected and fractions 2–5 (Fig. 1a) were combined. Solid $(NH_4)_2SO_4$ was added to 60% satn and the ppt. redissolved in resuspension buffer, dialysed for 3 hr against 100 vol. resuspension buffer and then applied to a second column (1.6 × 15 cm) of Blue Sepharose CL-6B. This column was then washed with 50 ml resuspension buffer and eluted with a linear gradient of 5–12 mM ATP and 15–36 mM $MgCl_2$. Fractions (3 ml) were collected and fraction 3 (Fig. 2b) was concd by precipitation with $(NH_4)_2SO_4$ and by ultrafiltration to yield a soln of *ca* 200 mg/ml which was made up to 50 mM Tris–HCl, pH 8, 2 mM EDTA and 1 mM dithiothreitol. This concentrate was used for calorimetry. All stages in the purification of phosphofructokinase were carried out at 2–4°. The enzyme was assayed spectrophotometrically at 25° by following NAD^+ production at 340 nm in a reaction mixture, 3 ml, containing 0.5 mM fructose 6-phosphate, 0.33 mM ATP, 0.1 mM NADH, 1 mM $MgCl_2$, 4 mM cysteine, 0.14 unit aldolase, and 12 μg of a mixture of α-glycerolphosphate dehydrogenase, and triosephosphate isomerase in 40 mM Tris–HCl, pH 8. Protein was measured as in ref. [13].

Gel electrophoresis. SDS–PAGE was performed as described in ref. [14]. The stacking gel contained 5% (w/v) acrylamide, 0.13% (w/v) *N,N'*-methylene-bisacrylamide, 0.1% (w/v) SDS, 0.25% (v/v) *N,N,N',N'*-tetramethyl-1,2-diaminoethane (TEMED) and 0.05% (w/v) ammonium persulphate buffered with 0.056 M Tris–HCl, pH 6.7. The separating gel contained 15% (w/v) acrylamide, 0.4% (w/v) *N,N'*-methylenebisacrylamide, 0.1% (w/v) SDS, 0.25% (v/v) TEMED and 0.05% (w/v) ammonium persulphate buffered with 0.38 M Tris–HCl, pH 8.9. The electrode buffer was 0.005 M Tris–HCl, pH 8.3, 0.039 M glycine, 0.1% (w/v) SDS. The samples, 4–40 μg protein, were taken directly from the fractions eluted from Blue Sepharose and made up to 62.5 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) mercaptoethanol and 0.001% (w/v) bromophenol blue. Marker proteins (5 μl at 1 mg/ml) were put in the flanking wells. Electrophoresis was at 18° for 16 hr at 90 V. Gels were fixed and stained in 9.2% (v/v) HOAc, 45% (v/v) MeOH, 0.25% (w/v) Coomassie Brilliant Blue R250. Destaining was in 7.5% (v/v) HOAc, 5% (v/v) EtOH.

Calorimetry. A Perkin Elmer differential scanning calorimeter, DSC-2, fitted with a sub-ambient temperature accessory, was used. Samples (4–5 mg) of fraction 3 from the second Blue Sepharose column were sealed in standard Al pans, and reference pans were prepared which contained the identical wt (±0.02 mg) of H_2O. The samples were loaded with the temp. of the calorimeter block at 293 K. After thermal equilibration the samples were cooled and warmed as described under Results. In order to check the reversibility of the thermal transition, several cooling/warming cycles were performed with the same sample and the areas under the power curves were compared.

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