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KINETIC PROPERTIES AND RELATED CHANGES OF MOLECULAR WEIGHT IN A FRUCTOKINASE FROM *STREPTOMYCES VIOLACEORUBER*

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Summary

1. A study of the initial reaction rates at variable substrate concentrations and of the molecular weight of the enzyme in the presence of different effectors, has been carried out using fructokinase (ATP: fructose 6-phosphotransferase, EC 2.7.1.4) from *Streptomyces violaceoruber*.

2. Saturation curves for MgATP or CoATP are sigmoidal and they change to hyperbolic in the presence of 10 mM Mg^{2+} or Co^{2+} in excess over the nucleoside triphosphate.

3. Saturation curves for fructose show intermediary plateaux at high (but not at low) concentrations of ATP or Mg^{2+}.

4. The molecular weight of the enzyme in the presence of high concentrations of MgATP is 80 000. In the presence of fructose, and/or Mg^{2+}, the molecular weight is 20 000.

5. The effects of MgADP, uncomplexed ADP or ATP, and low concentrations of detergent on the kinetics have been studied. The results are interpreted as showing the existence of cooperative effects.

Introduction

In a preceding paper [1] the characterization and partial purification of a fructokinase (ATP: fructose 6-phosphotransferase, EC 2.7.1.4) from *Streptomyces violaceoruber* were described. This inducible enzyme is highly specific for the sugar substrate while showing a broad specificity for the nucleotide substrate. Preliminary studies have shown it exhibits kinetic behaviour markedly different from that usually found among hexose kinases. While the kinetic pattern usually found in kinases for sugars corresponds to mechanisms of "quasi-equilibrium" involving the formation of a ternary complex of the enzyme with both substrates [2,3], the saturation curves of this fructokinase are non-hyperbolic.

In the present paper a study of the initial-rate kinetics and other related properties of this kinase is reported. The effect of ATP, ADP and MgADP on the saturation curves for MgATP has been investigated in an attempt to elucidate whether these curves could be indicative of cooperative phenomena or be due to any other effect that would mimic cooperativity behaviour as reported in some cases [4,5]. Positive cooperativity effects induced by the nucleotide substrates and, apparently, negative cooperativity effects induced by fructose, are interpreted as being related to changes in the state of aggregation of the enzyme. A preliminary account of some of the results discussed herein has been presented earlier [6].

Materials and Methods

Enzymes and other biochemicals

Fructokinase from *Str. violaceoruber* was partially purified as described previously [1]. The specific activity of the enzyme at the final stage of purification was 40 μmoles/min. Protein content in the enzyme solution was 0.5 mg/ml. The preparation was essentially free of interferring activities [1]. The enzyme was dissolved in 50 mM imidazole buffer, pH 7.5, containing 2 mM dithioerythritol. All the auxiliary and marker enzymes were from Boehringer. Fructose (Pfanstiehl) was recrystallized from 90% ethanol and its contamination with glucose was less than 0.1%. NADP, NADH, ADP, and all the nucleoside triphosphates were of the highest quality available from Sigma. Hemoglobin and cytochrome *c* were from Calbiochem. All other chemicals were of analytical reagent grade.

Initial reaction rates measurement

The routine measurements were carried out in spectrophotometer cuvettes of 10 mm light-path containing 1.0 ml of reaction mixture. Changes in absorbance at 340 nm were recorded in a Gilford 2400 spectrophotometer equipped with a thermostatic circulator at 30°C. The auxiliary system consisted of about 0.2 units of glucosephosphate isomerase (EC 5.3.1.9), 0.5 units of glucose 6-phosphate dehydrogenase (EC 1.1.1.49), and 0.5 mM NADP in 50 mM imidazole buffer, pH 7.5. Substrates and Mg^{2+} (as $MgCl_2$) or other metal ions, as indicated in Figs 2 and 3. The reaction was started by addition of the enzyme. Preliminary tests indicated that the order of addition of the reagents does not change the reaction rates. One unit is defined as the activity capable of phosphorylating 1 μmol/min.

Molecular weight determinations

Equilibrium sedimentation measurements were carried out in a Spinco L 50 ultracentrifuge equipped with a SW 39 rotor, in tubes containing 4.4 ml of a gradient of glycerol ranging from 5 to 25%. Hemoglobin (M_r 64 000), adenylate kinase (EC 2.7.4.3) (M_r 21 000), and glucose 6-phosphate dehydrogenase (M_r 128 000) were included as markers. Different effectors were also included as indicated in Results. On the top of each tube 1.5 units of fructokinase were layered and the centrifuge was operated at 38 000 rev./min for 16 h at 2°C. At the end of the run a hole was punctured in the bottom of each tube and

fractions of 8 drops were collected and assayed for the different enzyme activities, hemoglobin (absorbance at 414 nm), and protein. Adenylate kinase activity was assayed with 5 mM ATP and AMP, 15 mM $MgCl_2$, 100 mM KCl, 2.5 mM phosphoenolpyruvate, 0.15 mM NADH and 2 units each pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27), following the decrease in absorbance at 340 nm. Glucose 6-phosphate dehydrogenase activity was assayed with 2 mM glucose 6-phosphate and 0.5 mM NADP by following the increase in absorbance at 340 nm.

Column gel filtration was carried out in columns of 42 × 3 cm with Sephadex G-100 equilibrated and eluted with 50 mM imidazol pH 7.5, 2 mM dithioerythritol and effectors as indicated in Results. Adenylate kinase, hemoglobin (which in gel filtration behaves as a dimer of M_r 32 000), aspartate aminotransferase (EC 2.6.1.1) (M_r 90 000), and glucose 6-phosphate dehydrogenase (M_r 128 000) were used as markers. Aspartate aminotransferase activity was assayed in 50 mM imidazol pH 7.5 with 1 mM α-ketoglutarate and 3 mM L-aspartate, in the presence of 0.2 mM NADH and 0.1 unit of malate dehydrogenase in 1.0 ml of total volume.

Thin layer gel filtration was carried out in the apparatus developed by Pharmacia for this purpose, using Sephadex G-100 superfine. The layers, 0.6 mm thick, were equilibrated in the cold room with 50 mM imidazol buffer containing 1 mM dithioerythritol and the effectors to be tested with regard to their influence on the molecular weight. Hemoglobin and cytochrome *c* were employed as references. After elution for 8—10 h with an inclination of 20 degrees, the slides were placed horizontally and the distance run by the colored markers carefully measured. The path of proteins was cut into 14 slices 1.8 cm wide and each slice was eluted with buffer, centrifuged to remove Sephadex, and tested for activity.

Disc electrophoresis

Gels were produced by polymerization of 7% polyacrylamide and 0.2% bis-acrylamide in a buffer containing 100 mM Tris, 25 mM boric acid, and 3 mM EDTA, catalyzed by ammonium persulfate. Conventional tubes rinsed with Photo-Flow (Kodak) were used in a Polyanalyst Chamber from Buchler. The samples (1—2 units of fructokinase) in 20% sucrose were layered onto the surface of the gels. The electrode chamber contained the above Tris—boric acid buffer. After electrophoresis for 3 h in the cold room at 2 mA/column the gels were removed and either stained with Amido black 10B in 7% acetic acid by conventional procedures, or cut into slices of about 1 mm. Elution was carried out by repeated freezing and thawing and homogenisation in the Tris—boric acid buffer with 5 mM dithioerythritol. The extracts were centrifuged and assayed for fructokinase activity in the presence of 10 mM fructose, 5 mM MgATP, and the standard auxiliary system. The stained gels were recorded at 675 nm in a Gilford 2400 spectrophotometer equipped with the scanning gel attachment.

Results

Evidence for a single fructokinase activity

In order to obtain a good interpretation of the kinetics of fructokinase

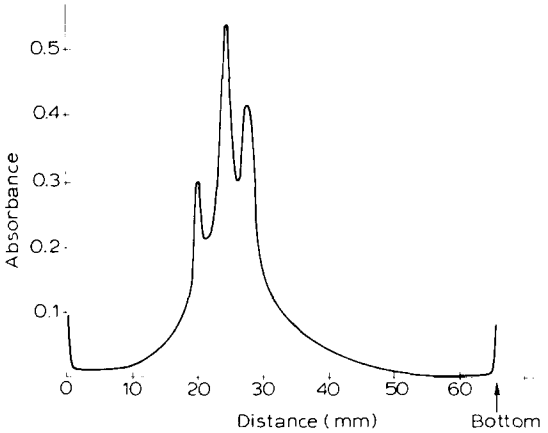

Fig. 1. Profile of the stained bands found by disc electrophoresis of partially purified fructokinase.

described in the following paragraph, it seemed important to test the possibility that the enzyme preparation used in these studies contained a mixture of two activities with similar specificity but marked differences in their affinity for both sugar and nucleotide substrates. Therefore, the activity recovered from the last step of purification [1] emerging as an apparently single peak from the DEAE-Sephadex column was tested by disc polyacrylamide electrophoresis as described under Methods. Fig. 1 shows the trace given by a densitometer after fixation and staining. The preparation was resolved into three bands, clearly separated. A non-stained gel was cut into slices of about 1 mm and eluted with a recovery of about 70% of the total activity and about 80% of protein. The activity was exclusively in the band of greatest mobility.

Initial velocity patterns and effect of Mg^{2+} on them

Figs 2 and 3 show saturation curves for either substrate at several fixed

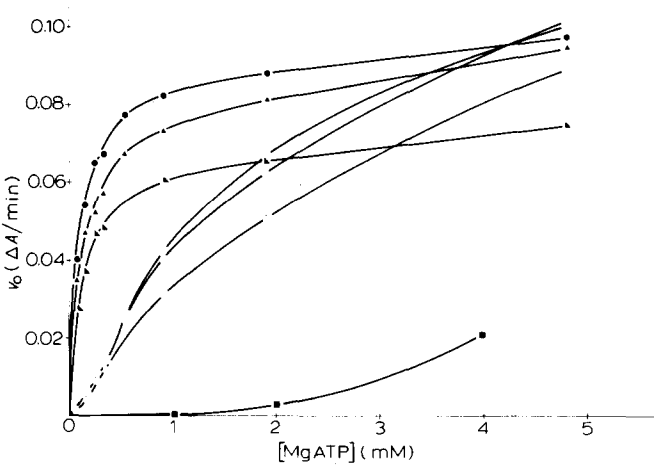

Fig. 2. Saturation curves for MgATP at three fixed concentrations of fructose in the absence and in the presence of 10 mM $MgCl_2$ in excess over the ATP present in each case. Open points correspond to absence of excess Mg^{2+}. Concentrations of fructose: ○, 2.5 mM; △, 10 mM; ▷, 30 mM. Full points correspond to the same concentrations of fructose in the presence of excess Mg^{2+}. ■, corresponds to reaction rates in the presence of 16 mM ATP in excess over MgATP at 5 mM fructose.

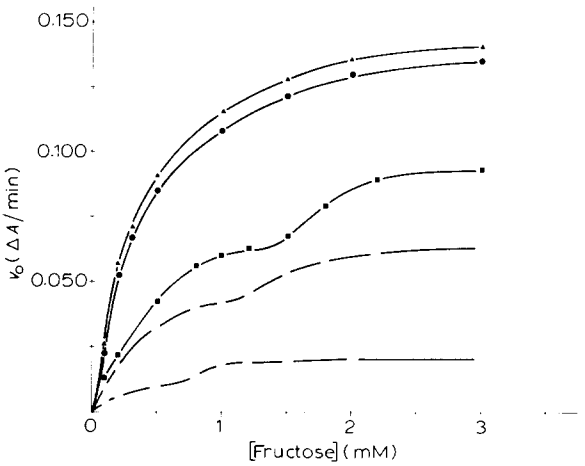

Fig. 3. Saturation curves for fructose at several fixed concentrations of MgATP. Concentrations of MgATP: ○, 0.2 mM; △, 0.4 mM; ■, 0.5 mM; ●, 4 mM. In the latter case one half of the actual rates have been plotted. ▲, 0.4 mM MgATP in the presence of 10 mM $MgCl_2$.

concentrations of the other substrate. The concentrations of the nucleotide substrate have been calculated assuming that $MgATP^{2-}$ is the donor substrate at pH 7.5 and deducing the concentrations of this species [5] for each mixture of $MgCl_2$ and ATP. In all cases the curves became hyperbolic in the presence of an excess of $MgCl_2$ over the nucleoside triphosphate. Fig. 2 shows a selection of these saturation curves for MgATP at three fixed concentrations of fructose, either in the presence of 10 mM $MgCl_2$ in excess over ATP, or at $MgCl_2$ equimolar with ATP. For the three hyperbolic curves, those with excess Mg^{2+}, K_m values deduced from double reciprocal plots were found to be very similar, about 0.2 mM. For the three sigmoid curves, those without excess of Mg^{2+}, $S_{0.5}$ were graphically estimated. No marked differences were found either in $S_{0.5}$, all of which were in the range 2—4 mM, or in the Hill coefficients deduced from plots of $\log(S)$ vs $\log v/(V-v)$. The differences in the three sigmoidal curves were not considered significant, since the estimation of the parameters involves some uncertainty in the evaluation of V which was found by extrapolating the initial part of a double reciprocal plot.

At fixed concentrations of MgATP and variable concentrations of fructose, saturation curves for the latter were obtained showing an intermediary plateau with two well defined inflection points as shown in Fig. 3. As in the case of the saturation curves for MgATP, those for fructose became hyperbolic in the presence of an excess of $MgCl_2$ over the nucleoside triphosphate. At increasing concentrations of MgATP the saturation curves of fructose progressively lose the inflection points so that at about 4 mM MgATP the curve is sensibly hyperbolic as shown in Fig. 3.

In Fig. 4 the double reciprocal plots of one curve from Fig. 2 (that corresponding to 10 mM fructose) and one from Fig. 3 (that corresponding to 0.4 mM MgATP) are shown. These curves have been chosen because of their evident deviation from the hyperbolic shape. In Fig. 5 Hill plots for the same two curves are shown. As pointed out by Engel & Dalziel [7] and by Koshland [8] each of these plots may be more sensitive than the conventional ones in reveal-

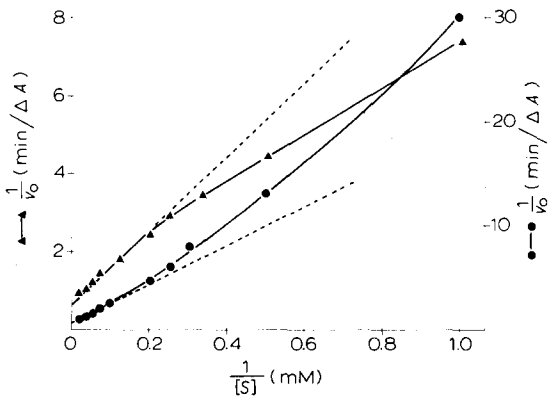

Fig. 4. Double reciprocal replot of one curve of Fig. 2 (that at 10 mM fructose) (●) and another of Fig. 3 (that at 0.4 mM MgATP) (▲).

ing kinetic features of enzyme reactions possibly involving cooperative phenomena.

Besides the above experiments, carried out at pH 7.5, a few measurements were made at pH 9.0 (50 mM glycine). No significant differences were found either in the saturation curves for substrates or in the presence of excess Mg^{2+}. 0.1 mM K^+ did not modify the kinetic properties of the enzyme in experiments similar to those described above.

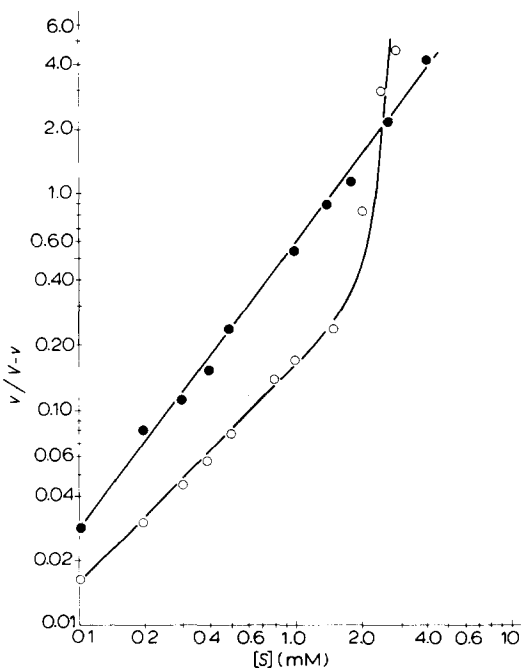

Fig. 5. Double log plot of the same two curves whose double reciprocal plot is shown in Fig. 4: ○, 0.4 mM MgATP and variable concentrations of fructose; ●, 10 mM fructose and variable concentrations of MgATP.

Effect of uncomplexed ATP and of MgADP on the reaction rate

It is well documented that a common feature of most ATP-dependent kinases is to be inhibited by ATP in excess over Mg^{2+} as well as by ADP either uncomplexed or as Mg complex. This inhibition is currently interpreted as being due to a competitive binding on the subsite of the nucleotide substrate. In the case of the fructokinase under study, since a cooperative effect seems to be induced by the nucleotide substrates, the question was raised as to whether or not the patterns of inhibition by these inhibitors could be interpreted as involving two antagonistic effects, one of competitive inhibition and other of apparent activation due to induction of the same cooperative effect that MgATP induces. The effect of an excess of 16 mM ATP over a set of concentrations of MgATP on the fructokinase reaction carried out in the presence of 5 mM fructose is shown in the lower part of Fig. 2 (full squares). The $S_{0.5}$ appears markedly shifted towards higher concentrations of MgATP without suppression of the sigmoidicity. If the uncomplexed ATP were capable of inducing a cooperative effect in a similar way to MgATP, at a concentration as high as 16 mM the shape of the curve ought to be hyperbolic with an apparent K_m more or less increased depending on the affinity of ATP for the site of MgATP.

The inhibition caused by MgADP in the presence of excess of Mg^{2+}, and hence with hyperbolic kinetics, was found to be purely competitive with respect to MgATP with a K_i of 0.2 mM. This observation suggests that MgADP can bind at the nucleotide subsite with an apparent affinity very similar to that of MgATP. When the excess of Mg^{2+} is omitted, the situation becomes complicated by the fact that binding of the nucleotide substrates could induce cooperative effects. As in the case of ATP, it seemed interesting to investigate the possibility that MgADP would induce cooperative effects by mimicking the interaction of MgATP with the protein. In an attempt to analyze this possibility, at least in a qualitative way, a curve was drawn representing the reaction rates to be expected if the following assumptions are made: 1) MgADP can bind at the site of MgATP with the same intrinsic affinity of the latter. 2) The total sites of the enzyme bound to nucleotide is given by [MgATP] + [MgADP] and the fraction of them bound to MgATP should be [MgATP] / {[MgATP] + [MgADP]}. 3) The reaction rate is given by the Hill equation where $K = 1.2$ and $n = 1.3$ as deduced from Fig. 5. 4) When a mixture of MgATP and MgADP is present, the actual concentration of substrate in the Hill equation should be taken as that of MgATP by a coefficient indicative of the ratio of MgATP to total nucleotide. For instance, if [MgATP] is 0.4 mM and [MgADP] is 1.0 mM, the ratio of ATP to total nucleotide is 0.4/1.4 = 0.3 and therefore the actual [S] in the Hill equation should be $0.4 \times 0.3 = 0.12$ mM. In Fig. 6 the theoretical reaction rates to be expected under the above assumptions and the real ones found for different values of [MgADP] at a fixed concentration of MgATP are represented. From this figure it becomes apparent that the inhibition by MgADP is less than expected in the range of total nucleotide concentration in which the cooperative effects are more marked.

Kinetic behavior at low temperature

In order to test the possibility that low temperatures could modify the

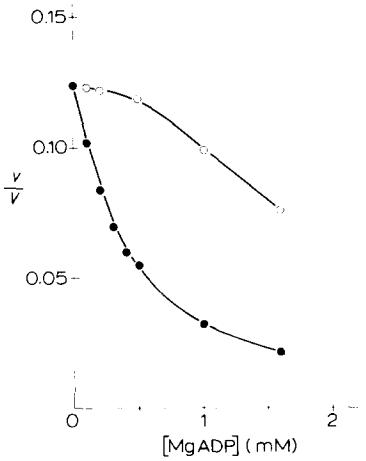

Fig. 6. Effect of MgADP on the initial reaction rates at 0.4 mM MgATP and 5 mM fructose. •, theoretical curve to be expected under the assumptions discussed in Results. ○, actual curve found.

cooperative effects shown by the nucleotide substrate, by modifying the interactions between subunits, a set of determinations of initial rate was carried out at 7° C. In Table I the reaction rates found at two concentrations of MgATP within the range of maximal cooperative effect at 30° C are shown (see Fig. 2), namely 0.2 and 0.4 mM MgATP. The ratio of the corresponding velocities rises from 3 at 30° C to 4 at 7° C. On the other hand, the activating effect of excess Mg^{2+} is also more pronounced at 7° C. These results are interpreted as indicative of the fact that at 7° C the sigmoidicity of the saturation curves for MgATP is more marked than at 30° C.

Effects of detergents on the saturation curves for MgATP

The non-ionic detergent Triton X-100 was assayed at 0.1% and was found to be without effect on the reaction rate. Sodium dodecylsulphate at 0.06 mM was effective in modifying the saturation curves for MgATP. The enzyme was incubated with the detergent during 20 min at 28° C before the reaction mixture was completed. No difference was detected due to the presence of either substrate or Mg^{2+} during the incubation time. A set of measurements were carried out including the same manipulations but in the presence of detergent.

TABLE I

REACTION RATES AT 7°C AT TWO CONCENTRATIONS OF MgATP

Experimental conditions as in Fig. 3. Fructose concentration 5 mM.

MgATP (mM)	$MgCl_2$ (mM)	Velocity (ΔA/min X 10^3)
0.2	-	8
0.2	10	93
0.4	-	32
0.4	10	160

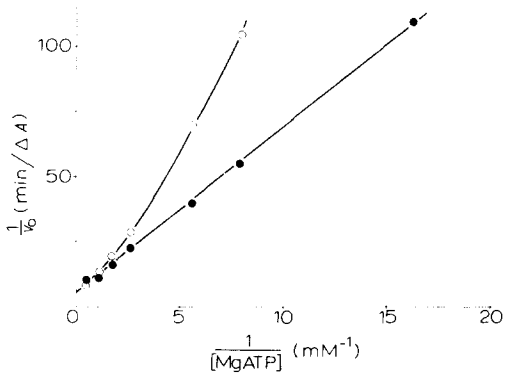

Fig. 7. Double-reciprocal plot of a saturation curve for MgATP in the presence (●) and in the absence (○) of 0.06 mM sodium dodecylsulfate. Fructose concentration was 5 mM.

The saturation curves are represented in Fig. 7 in double reciprocal plots. While the curves obtained in the presence of $MgCl_2$ in excess (5 mM) over the nucleotide did not show appreciable difference due to the presence of the detergent (except a slight lowering in the K_m value) the ones at equimolar Mg^{2+} and ATP showed a clearly hyperbolic pattern in the presence of detergent with a K_m about an order of magnitude higher than that found in excess of Mg^{2+}.

Specificity for nucleotide substrate and metal ion

Preliminary studies [1] showed that fructokinase can use a variety of nucleoside triphosphates as phosphoryl donors. The question arises as to whether a unique kinetic mechanism is operating in every case or whether the ability to induce cooperative effects should be ascribed only to determined structural features in the donor substrate. With dATP and ITP complete saturation curves were obtained and found to be quite similar to those of ATP. In the case of UTP, GTP, and CTP, reaction rates were measured at 1 mM nucleoside triphosphate in the presence and in the absence of 5 mM $MgCl_2$ in excess over the nucleotide. A marked activation by uncomplexed Mg^{2+} found in all the above mentioned cases was taken as evidence that the corresponding nucleotide behaves in a similar way to ATP, at least from a qualitative point of view.

Several divalent metal ions were assayed with regard to their ability to substitute for Mg^{2+} in the fructokinase reaction. In the case of Co^{2+}, curves of saturation were found with ATP or ITP closely resembling those found with Mg^{2+}. Moreover, an activating effect was observed when Co^{2+} was added in excess over the nucleotide. The concentration of Co^{2+} which produces an activating effect a half of the maximal one is 5 mM whereas that of Mg^{2+} is 1.8 mM. The ions Zn^{2+}, Cd^{2+}, and Ca^{2+} did not show detectable activity when complexing with ATP. In the case of Be^{2+} and Mn^{2+} it was found that the corresponding complexes with ATP are active as phosphoryl donors at 0.2 mM concentration, but no increased activity was observed when these ions were added in an excess of 1 mM over the nucleotide. A detailed kinetic study was not possible because of the low solubility of the complexes formed by these ions with ATP or ITP.

Determinations of molecular weight of fructokinase

Molecular weight of fructokinase in a variety of experimental conditions was measured by ultracentrifugation in a glycerol gradient, by column gel filtration on Sephadex G-100 and by thin-layer gel filtration on superfine Sephadex G-100 as detailed under Methods, with the adequate markers as reference.

In a glycerol gradient ranging from 5 to 25%, the equilibrium–sedimentation behavior of fructokinase was studied in the presence of fructose, Mg^{2+}, ATP, or binary mixtures of these. When only 10 mM fructose or 10 mM $MgCl_2$ was present the activity was lost during the centrifugation. When both of them were present the sedimentation pattern (open triangle in Fig. 8) indicated a molecular weight of about 20 000. In the presence of 10 mM MgATP the sedimentation pattern (open circle in Fig. 8) indicated a molecular weight of about 80 000 for fructokinase. Fig. 8 combines the results found in both tubes. In a tube without any effector the results were coincident with those in the tube containing fructose plus $MgCl_2$.

The elution profile from a column of Sephadex G-100 was consistent with the above results since elution with buffer alone, or buffer containing 10 mM fructose, or 10 mM $MgCl_2$, or both of them, gave in all cases a molecular weight of 20 000 for fructokinase. In thin-layer gel filtration with Sephadex G-100 superfine developed with the same imidazol buffer containing 1 mM ATP plus 10 mM $MgCl_2$ fructokinase ran as corresponding to a molecular weight of 20 000. When developed with buffer containing 10 mM MgATP the situation of fructokinase activity corresponded to a molecular weight of 80 000.

Stability of fructokinase in the presence of fructose

Earlier observations concerning the stability of fructokinase on storage at

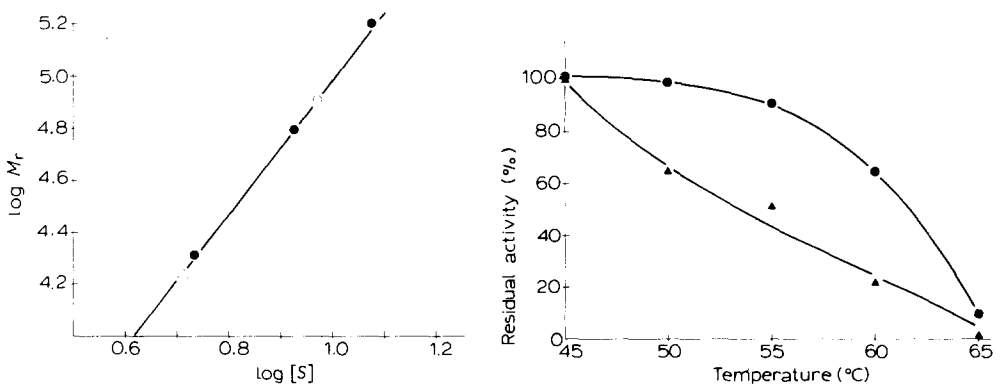

Fig. 8. Molecular weight of fructokinase measured by sedimentation equilibrium. △, in the presence of either 10 mM fructose, 10 mM $MgCl_2$, or both of them; ○, in the presence of 10 mM MgATP. The position of the markers was closely coincident in both cases so that results have been combined. 1, adenylate kinase; 2, hemoglobin; 3, glucose 6-phosphate dehydrogenase. S means the total number of fractions (i.e. 22) less the number corresponding to the fraction containing the maximal concentration of the marker.

Fig. 9. Heat inactivation of fructokinase as influenced by fructose. ●, a buffered solution of the enzyme; ▲, the same containing 10 mM fructose.

4°C [1] and the above mentioned results of ultracentrifugation revealed that fructokinase becomes less stable in the presence of fructose. This fact was confirmed by heat inactivation experiments described in Fig. 9, that showed residual activity after incubation for 5 min at temperatures ranging from 45—65°C in imidazol buffer pH 7.5, both in the presence and in the absence of 10 mM fructose. The increased lability in the presence of fructose suggests an interaction of fructose with the protein, probably inducing a conformational change in the latter.

Discussion

The kinetic behaviour of fructokinase from *Str. violaceoruber* is rather unusual among hexose kinases from bacterial, yeast or animal origin. The existence of non-hyperbolic saturation curves for either substrate and the possibility of changing them into hyperbolic ones with the concomitant occurrence of changes in molecular weight suggests that this enzyme may be compared with the well-known increasing group of enzymes endowed with allosteric properties associated with changes in kinetics and molecular properties. In the case of hexokinase from animal tissues, allosteric regulation exists indeed but it does not seem to involve cooperativity effects [9].

The sigmoidal saturation curves for MgATP, as discussed by Purich and Fromm [5], could be explained assuming that uncomplexed ATP would have high affinity (K_i in the range μM) for the substrate site of MgATP. In this case, the small amount of ATP formed by dissociation of MgATP would competitively inhibit the reaction. Obviously, both high concentrations of MgATP and excess Mg^{2+} would tend to remove the inhibition, giving rise to saturation curves apparently sigmoidal at low concentrations of MgATP in the absence of excess Mg^{2+}. In order to investigate such a possibility, the reaction rate at 0.5 mM ATP and 0.5 mM $MgCl_2$ was compared with the reaction rate at 0.6 mM ATP and 0.5 mM $MgCl_2$. No difference was found. This result allows us to exclude the possibility that free ATP is a competitive inhibitor with an affinity an order of magnitude, at least, higher than that of MgATP for the nucleotide—substrate site.

The saturation curves of fructose clearly show well defined intermediary inflections similar to those described by Koshland and coworkers [10—12], as being due to negative cooperativity phenomena. Of the proposals by these authors to explain the negative cooperativity, only one seems to meet the characteristics of the present case. Repulsive forces among the ligand molecules bound stepwise to the enzyme may be excluded in the case of fructose. The possibility of different kinds of subunits may also be excluded on the basis of the effect of Mg^{2+} in excess over ATP. A plausible explanation of the effect of Mg^{2+} or high concentrations of MgATP on the saturation curves for fructose requires us to admit the possibility of conformational changes in subunits being induced by a variety of ligands. While MgATP seems to induce a change leading to positive cooperativity, fructose seems to induce a change giving rise to negative cooperativity. Both kinds of cooperativity appear to be suppressed by Mg^{2+} in excess over the nucleotide. The hyperbolic curves shown in Fig. 3 for high MgATP or excess of Mg^{2+} tend to exclude the possibility that the non-hyperbolic curves are due to an experimental artifact.

The results shown in Figs 2—5 could conceivably be ascribed to a mixture of two isozymes with different K_m values for their substrates both showing hyperbolic saturation curves. The fact that a single band with fructokinase activity appears in disc electrophoresis does not allow us to rule out the possibility of an unresolved mixture. The fact that excess of Mg^{2+} renders the saturation curves hyperbolic in every case is much more conclusive. The observation that, among the several divalent metal ions that form complexes with ATP which are capable of phosphorylating fructose, only Mg^{2+} and Co^{2+} are suitable as activators at low ATP concentrations, suggests the existence of a specific binding site for those metal ions on the enzyme. The activating effect of these two ions shows hyperbolic curves of saturation from which K_a values that reasonably represent dissociation constants are deduced.

Since relatively high concentrations of MgATP or CoATP suppress the intermediary "plateau" of the saturation curves for fructose, the possibility exists that these complexes could bind at the activator site by their metal moiety and induce the same effect as free metal ions do. In this connection it may be pointed out that the activating effect of Mg^{2+} takes place also at pH 9, at which Mg^{2+} are complexed with OH^-. Other observations that could be related to this question are those concerning the inhibition by uncomplexed ATP and by MgADP as competitive analogs for MgATP. While ATP does not suppress the sigmoidal shape of the saturation curves of MgATP, the inhibition pattern of MgADP is consistent with an activation concomitant with the competitive inhibition to be expected at the nucleotide site, as indicated by experiments shown respectively in Figs 2 and 6.

The changes in molecular weight of fructokinase in different experimental conditions suggests that the sigmoidal curves found at increasing concentrations of MgATP (see Fig. 2) are due to association of four subunits with concomitant increasing affinity for the nucleotide substrate, so that the tetrameric enzyme should only exist at high concentrations of MgATP. The possibility that dissociation into subunits in sedimentation equilibrium experiments could be an effect of glycerol may be excluded since the gel filtration experiments are consistent. On the other hand, the effect of increasing concentrations of fructose seems to correspond to a sequence of events primarily related to conformational changes in the protein and accompanied by a peculiar pattern of kinetic behavior described as negative cooperativity [10]. A marked effect of these changes induced by fructose on the binding of MgATP is not apparent, since the saturation curves of the latter are only slightly modified by the concentration of fructose as indicated in Fig. 2.

The effect of an excess of Mg^{2+} could be interpreted in two alternative ways. The first assumes that Mg^{2+} (or Co^{2+}) stabilizes a conformation of the monomeric protein which renders it active with low K_m for MgATP and hyperbolic kinetics for both substrates. The second assumes that Mg^{2+} stabilizes a tetrameric form of the enzyme with low K_m and hyperbolic kinetics requiring the presence of both substrates. In the absence of free Mg^{2+}, only high concentrations of MgATP are capable of stabilizing the tetrameric form as suggested by the results shown in Fig. 8. Since in the assay mixture for activity both substrates are present, the possibility exists that an enzyme eluted from Sephadex or taken from the centrifuge tube as a monomeric protein, becomes a

tetrameric one in the assay cuvette if the association of subunits occurs without a measurable lag. Therefore, the question whether or not an active monomer can exist, remains unsolved. In any case it seems evident that MgATP promotes association of the subunits, accompanied or unaccompanied by cooperativity phenomena, depending on the presence of free Mg^{2+} or Co^{2+}. The interpretations are supported by observations at low temperature and in the presence of sodium dodecylsulphate. Both conditions are known to modify interactions among subunits in multimeric proteins.

With regard to the effect of MgADP (see Fig. 6), it is interesting to point out that other allosteric enzymes have been described in which a substrate analog capable of binding to the enzyme at the same site as the substrate with a concomitant effect as competitive inhibitor, behaves as an activator at low concentrations of substrate by mimicking the cooperative effect of the latter in terms of increasing the affinity of the remaining groups for it. Such phenomena, exemplified by aspartate transcarbamylase [13], are discussed by Monod et al. [14] as a prediction that may be made in any K system of their model.

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