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UDPGlucose 4-Epimerase from *Saccharomyces fragilis*

ALLOSTERIC KINETICS WITH UDP-GLUCOSE AS SUBSTRATE

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SUMMARY

UDPglucose 4-epimerase from *Saccharomyces fragilis* catalyzes a freely reversible reaction between UDP-galactose and UDP-glucose. With UDP-galactose as the substrate the enzyme shows a classical hyperbolic kinetics but when UDP-glucose is used as the substrate a distinct allostericity is observed. As a consequence, at low concentrations of UDP-glucose, the enzyme fails to establish the equilibrium at a significant rate. Glucose 6-phosphate acts as a strong activator for the enzyme with low concentrations of UDP-glucose as the substrate. In view of these rather unusual kinetic data for an enzyme catalyzing a freely reversible reaction, UDPglucose 4-epimerase may play a regulatory role in controlling the flux of galactose metabolism.

The enzyme UDPglucose 4-epimerase (EC 5.1.3.2) has been extensively purified from various sources including calf liver (1), *Escherichia coli* (2), and *Saccharomyces fragilis* (3). In all cases the enzyme catalyzes a freely reversible reaction between UDP-glucose and UDP-galactose. Most of the kinetic studies for the enzyme were conducted with UDP-galactose as the substrate employing a coupled assay system. UDP-glucose, produced during the course of the reaction was oxidized to UDP-glucuronic acid by reduction of NAD in the presence of UDPglucose dehydrogenase and the rate of formation of NADH was measured (4). Recently, we have reported the presence of an effector site for the epimerase from *S. fragilis* (5). The site is specific for some sugar phosphates and the catalytic activity as measured by the above assay method was significantly enhanced in presence of these sugar phosphates. In an effort to investigate the possible effect of these sugar phosphates on the rate with UDP-glucose as the substrate, we have observed that the enzyme shows distinct allostericity when epimerization is catalyzed from this side of the reaction. Further, the allostericity progressively decreased in presence of increasing concentrations of glucose 6-phosphate.

All biochemicals including UDPglucose dehydrogenase were purchased from Sigma Chemical Co. For some of the experiments, UDPglucose 4-epimerase from *S. fragilis* was purchased from the same source. The lyophilized enzyme was taken into solution with 0.1 M sodium citrate, pH 7.0. Generally however, the enzyme was purified up to Stage 3 of the method described by Darrow and Rodstrom (6). The specific activity of the enzyme at this stage of purification was about 3 to 5 units/mg. One unit of the enzyme was defined as the amount of enzyme needed to convert 1 μmol of UDP-galactose to UDP-glucose per min under standard conditions of the coupled assay system. For this assay, 1 ml of the final assay medium contained 100 μmol of glycine-sodium hydroxide buffer, pH 8.8, 0.5 μmol of NAD, and 0.03 unit of UDPglucose dehydrogenase and the requisite amount of the enzyme. The reaction was started with UDP-galactose as the substrate and the rate was measured by following the increase in absorbance at 340 nm between the 2nd and the 5th min.

The rate with UDP-glucose as the substrate was measured by following a two-step assay procedure. In the first step, in a final volume of 1 ml of the incubation medium containing 100 μmol of glycine-sodium hydroxide buffer, pH 8.8 and requisite amount of the enzyme, varying concentrations of UDP-glucose were added. The reaction was allowed to proceed for 5 min at the end of which time it was terminated by the addition of 1 ml of chloroform with rapid shaking. After complete separation of the layers, about 0.7 ml of the upper aqueous layer was carefully transferred with a pasteur pipette. Faint smell of chloroform that often persisted was removed by putting the tubes on a 63° bath for 2 min. Depending on the initial amount of UDP-glucose present, an aliquot containing about 20 nmol of UDP-glucose was now transferred to an assay medium of final volume of 1 ml containing 100 μmol of glycine-sodium hydroxide buffer, pH 8.8 and 0.01 unit of UDPglucose dehydrogenase. The initial absorbance of this sample at 340 nm was noted. The reaction was started with 10 μl of 35 mM NAD and followed every 3 min until a constant value at 340 nm was obtained. The difference in absorbance gave a direct measure of the amount of UDPglucose that remained unconverted during the reaction. When this value was subtracted from the original amount of UDPglucose present, which was also measured by the same procedure, the amount of UDP-glucose converted to UDP-galactose during the incubation period was obtained. Five microliters of UDPglucose 4-epimerase containing 0.005 unit was now added to the same incubation mixture. The reaction was followed every 2 min until a constant value was obtained. The difference between this final reading and the previous constant reading gave a direct measure of UDP-galactose formed during the course of the reaction. The formation of UDP-galactose as measured by the difference in UDP-glucose content and also by direct determination of UDP-galactose corresponded extremely well. With UDP-galactose as the substrate, the rate was also determined by the same two-step procedure. In this case however, only the formation of UDP-glucose was estimated.

The contrasting kinetic data with UDP-glucose and UDP-galactose as substrates are presented in Fig. 1. With UDP-glucose concentration below 0.2 mM, a distinct and reproducible sigmoidicity was observed. When UDP-galactose was used as the substrate, even at low concentrations of UDP-galactose the typical Michaelis-Menten kinetics was followed. The rates, which were expressed in terms of NADH formation, were measured by the two-step assay procedure. Assuming the equilibrium for the reaction to be approximately 3:1 in favor of UDP-glucose, the formation of UDP-galactose at higher concentrations of UDP-glucose as substrate, was only about 15% of the maximum attainable value. With UDP-galactose as the substrate, about 12% of the equilibrium value of UDP-glucose was obtained. The K_m for

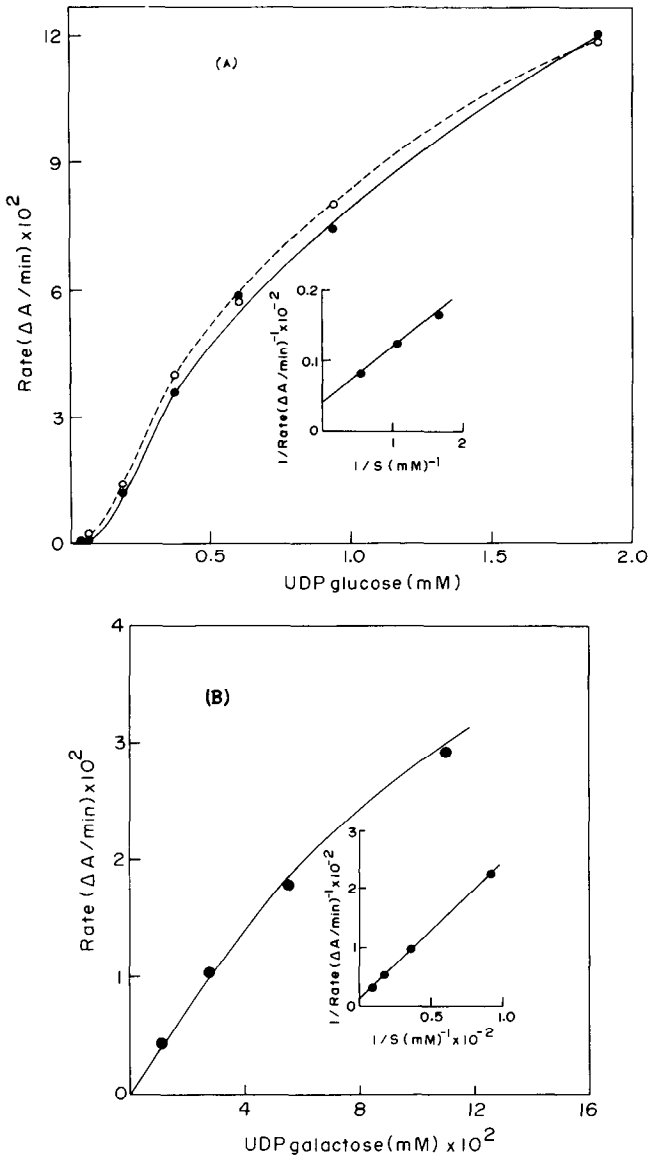

FIG. 1. Kinetics of UDPglucose 4-epimerase with UDP-glucose and UDP-galactose as substrates. In *A*, the incubation mixture contained in a total volume of 1 ml, 100 μmol of glycine-sodium hydroxide buffer, pH 8.8, 0.01 unit of the epimerase, and varying concentrations of UDP-glucose. Incubation time was 5 min. UDP-galactose formed was estimated by the two-step assay procedure. O- - -O indicates the value obtained by difference in UDP-glucose content. ●——● gives the values obtained by direct estimation of UDP-galactose formed. In *B*, the incubation mixture contained in a total volume of 1 ml, 100 μmol of glycine-sodium hydroxide buffer, pH 8.8, 0.004 unit of epimerase, and varying concentrations of UDP-galactose. Incubation time was 5 min. The UDP-glucose formed during the course of the reaction was assayed by a two-step procedure. The *insets* show the inverse plots of the same data. In *A*, the average values of the three higher concentrations of UDP-glucose were utilized only for the inverse plot.

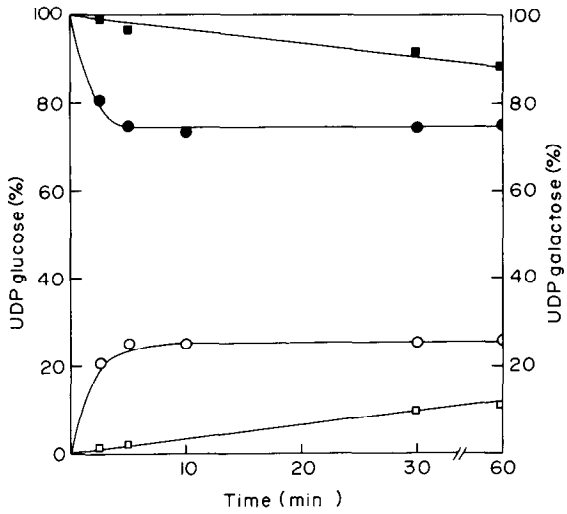

FIG. 2. Approach to equilibrium with UDP-glucose as the substrate. ●——● and ■——■ indicate the amount of UDP-glucose left unreacted after specified reaction times using 0.25 mM and 0.08 mM UDP-glucose, respectively. The other components of the incubation mixture in each tube were 100 μmol of glycine-sodium hydroxide buffer, pH 8.8 and 0.03 unit of the enzyme. O——O and □——□ indicate the amount of UDP-galactose formed in the same tube as determined by the two-step assay procedure.

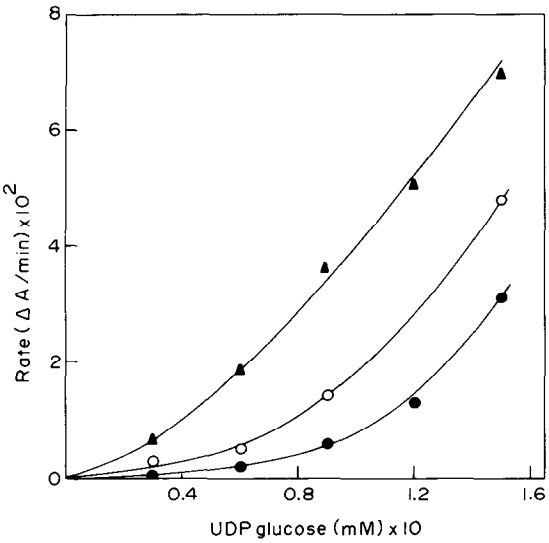

FIG. 3. Activation of epimerase in presence of glucose 6-phosphate with UDP-glucose as substrate. The *lowermost curve* (●——●) indicates the rate in absence of glucose 6-phosphate. The *upper two curves*, (O——O) and (▲——▲) represent the rates with 1.6 and 3.2 mM glucose 6-phosphate, respectively.

UDP-glucose and UDP-galactose were calculated to be 1.93 mM and 0.13 mM, respectively. These, obviously, are approximate values since the backward reactions due to the formation of the products are not negligible in these cases. The K_m for UDP-galactose however, agreed fairly well with the reported value of 0.11 mM which was determined by the coupled assay procedure (1).

An immediate consequence of the allostericity of the kinetics with UDP-glucose as the substrate, is that at low concentrations of UDP-glucose the equilibrium is established very slowly. This is evident in Fig. 2, when 0.25 mM substrate was used the equilibrium was established in less than 5 min. However, with the same amount of enzyme but with 0.08 mM UDP-glucose, even after 60 min of incubation, equilibrium could not be established. At still lower concentrations of the substrate virtually all UDP-glucose remained unreacted. With identical concentration of UDP-galactose (0.08 mM) however, rapid restoration of equilibrium was observed (not shown).

At low concentrations of UDP-galactose as the substrate, glucose 6-phosphate acts as an activator of the enzyme. Furthermore, in this case glucose 6-phosphate interacts with the enzyme at a site away from the active site (5). With UDP-glucose as substrate, at low concentrations of UDP-glucose, the enzyme is strongly activated in presence of glucose 6-phosphate (Fig. 3). With increasing glucose 6-phosphate concentration (1.6 and 3.2 mM) the sigmoidicity was progressively decreased.

UDPglucose 4-epimerase from *Saccharomyces fragilis* appears to be a rather unusual enzyme in that this enzyme catalyzes a

freely reversible reaction but whereas the kinetics from one side is strictly conventional, the kinetics from the other side shows a distinct sigmoidicity. The allosteric nature of the protein with UDP-glucose as the substrate and the possible effector role of glucose 6-phosphate strongly suggests a regulatory role for this enzyme in controlling the flux of galactose metabolism. A more detailed and intensive study of the epimerase as a regulatory protein may therefore considerably enhance our understanding of the control of galactose metabolic pathway.

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