Anomeric Specificity of the Alkaline Form of Fructose 1,6-Diphosphatase from Rabbit Liver

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SUMMARY

The preferred configuration of the active substrate for rabbit liver fructose 1,6-diphosphatase has been determined by techniques based on rapid quench kinetics to be the α anomer of fructose-1,6-P₂. Utilization of the β anomer, however, is also catalyzed by the enzyme with a rate coefficient 5- to 10-fold less than that for the α anomer.

The anomeric distribution of fructose-1,6-P₂ in neutral aqueous solution previously has been shown by ¹³C NMR to be in a ratio of 4:1 (β:α) (1) with essentially no acyclic (keto) form present (< 1%) (2, 3). In order to determine the anomeric substrate specificity of fructose 1,6-diphosphatase, there was added an amount of enzyme capable of reacting with all of the substrate of a particular anomeric configuration within a time span that is short with respect to the rate of anomerization. Thus, if one anomer were reactive, it should be able to detect the rapid conversion to fructose-6-P followed by subsequent product formation that would be determined by the anomerization rate. This latter phase, ideally, should be independent of enzyme concentration if the rate-determining process is simply spontaneous or buffer-catalyzed anomerization. On the other hand, if both anomers can be utilized by the enzyme, i.e. the enzyme either catalyzes anomerization to the reactive configuration or lacks absolute anomeric specificity, the duration of both the initial and latter phases of product formation should depend on enzyme concentration. This phenomenon has been observed previously for phosphoglucose isomerase (4).

The results of the rapid quench experiments are exhibited in plots of mole fraction fructose-6-P formed (f₆) at various times. Under the preselected conditions where the initial ratios of [ET]:K_M and [β-fructose-1,6-P₂]:K_M are 18 to 36 and 200, respectively, the true velocity is effectively that of V_max (5). The kineties remain zero order in substrate to greater than 50% hydrolysis for the β anomer at all enzyme levels. Some justification for the assumption that K_M for both α and β anomers is identical is derived from earlier studies utilizing analogs including α- and β-methyl-1-fructofuranoside-1,6-P₂ (6) where K_I is estimated as about 10⁻⁴ M.

In Fig. 1 are displayed plots of f₆ versus time for two enzyme concentrations. The graph is biphasic for the two experimental sets. An initial rapid formation of about 35% fructose-6-P is followed by a slower rate of product formation. It is apparent that the slopes of the initial and second phases are dependent on enzyme concentration, both slopes doubling (within experimental error) for a 2-fold increase in fructose 1,6-diphosphatase. Extrapolation of the second phase to zero yields in the two cases an f₆ value of 0.2, signifying that 20% of fructose-1,6-P₂ is more rapidly converted to product. The agreement between extrapolated values at the two levels of enzyme supports the premise that the enzyme concentration is sufficiently high to reduce rapidly to a limiting negligible value the equilibrium concentration of the form of fructose-1,6-P₂ utilized by the enzyme. The observed percentage corresponds to preferred utilization of the α anomer of fructose-1,6-P₂.

Values for the slopes of the initial and second phases have been plotted as a function of enzyme concentration (Fig. 2). The lower line which describes only the conversion of β anomer to product permits direct calculation of kₐ, the first order rate constant for decomposition of a presumed enzyme-β-fructose-1,6-P₂ complex. That the upper line contains contributions for the conversion of both α and β anomers to fructose-6-P is indicated by the point of intersection of the two phases being >20% (Fig. 1). Insular as the analog data are applicable, the initial phase may be solved for kₐ, the first order constant for decomposition of the enzyme-α-fructose-1,6-P₂ complex as follows. Assuming K_Mα ≈ K_Mβ, and since under our initial conditions [fructose-1,6-P₂] > ET, the initial distribution of the two complexes is 4:1 favoring the enzyme-β-fructose-1,6-P₂ complex, provided binding is not rate-determining. Consequently, 0.2 kₐ = k_Mα - 0.8 k_Mβ where k_Mα is the observed first order rate constant for the initial phase. The values for k_Mα and k_Mβ determined according to this procedure are 0.07 ± 0.02 and 0.009 ± 0.002 (μmol/min/unit) respectively. Under the usual assay conditions, i.e. S₀ > E_I, the defined value for 1 unit of fructose 1,6 diphosphatase is 0.017 μmol/s⁻¹. The value calculated from the above parameters, 0.021 ± 0.005 μmol/s⁻¹, is in good agreement. Therefore the enzyme appears to effectively utilize the α anomer about 5 to 10 times more rapidly than the β anomer, although, as a consequence of the greater concentration of the β anomer in a analogs to possess within an order of magnitude an affinity for fructose 1,6-diphosphatase similar to that exhibited by fructose 1,6-P₂ as measured by K_M or K_Mβ. Moreover, α and β analogs behave identically at concentrations <0.1 M.

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² Competitive kinetic inhibition constants (K_I) as well as fluorescence binding measurements (K_M) have shown the furanose...
solution of fructose-1,6-P₂, the rate of turnover of substrate arises from reaction of both anomers.

Finally one may note the significance of these results as they bear on the mechanism of fructose 1,6-diphosphatase action. The fact that both initial and second phases are dependent on enzyme concentration argues against a scheme in which the enzyme interacts solely with one configuration of the sugar-P₃. If this were the case, the rates associated with the second phase would be independent of enzyme concentration. Two main possibilities remain: (a) the enzyme catalyzes the hydrolysis of α-fructose-1,6-P₂ and conversion of β to the reactive α anomer or (b) the enzyme is relatively nonspecific and can act on both anomers. A variation of (a) is to catalyze the ring opening of both α and β to the common keto intermediate prior to dephorylation.³

Regardless of the actual scheme, fructose 1,6-diphosphatase evidently is able to employ the greater thermodynamic instability of the α anomer for a rate advantage.

Finally, it should be noted that these studies were performed with the alkaline form of fructose 1,6-diphosphatase. Although the inhibition patterns for a series of substrate analogs with both the alkaline and neutral forms of the enzyme are identical (7), one cannot entirely discount the possibility that the anomic specificity may be dependent to some degree on the nature of the enzyme preparation employed.

MATERIALS AND METHODS

Rabbit liver fructose 1,6-diphosphatase was purified according to published procedures (8, 9). Mean specific activity unless otherwise specified was 1.5 units per mg. α-Fructose-1,6-P₂ was obtained from Sigma. Rapid quench kinetic experiments utilized a Durrum Multi Mixer as modified by the authors.

The technique employed in determining the anomic specificity for the substrate of fructose 1,6-diphosphatase may be outlined as follows. Experiments were initiated at room temperature by mixing equal volumes of enzyme and reactant solutions followed by rapid quenching with two volumes of 10% HClO₄. Approximately 0.4 to 0.5 ml of quenched solution was collected.

As a precaution against a systematic unrecognized error, such as enzyme denaturation, the reaction times were varied randomly during the course of an experiment with a given E₇. Stock enzyme solutions varied from 35 to 85 units/ml of fructose 1,6-diphosphatase in a dimal containing 0.1% bovine serum albumin, 0.5 mm MnCl₂ and 0.04 M glycine (pH 9.2). Enzyme in 5 x 10⁻⁴ M malonate (pH 6.8) and 5 x 10⁻⁴ M fructose-1,6-P₂ (as eluted from a column) was concentrated via an Amicon ultrafiltration cell, repeatedly flushed with 0.04 M glycine (pH 9.2), treated with MnCl₂ at 25°, rechilled, and refushed extensively with 0.04 M glycine. Enzymatic analysis showed the fructose 1,6-diphosphatase solution to be free of fructose phosphates.

The composition of the stock reactant solution was 0.04 M glycine (pH 9.2), 0.5 mm MnCl₂ and 0.40 mm fructose-1,6-P₂. The quenched reaction mixture, maintained at 0°, was then centrifuged and the supernatant was removed and adjusted to pH 9.2 with 50% KOH. Sample aliquots initially were analyzed for fructose-6-P by the standard coupled enzyme assay (10) and the unreacted fructose-1,6-P₂ then was measured by addition of fructose-1,6-diphosphatase. The total recovered sugar phosphates were >90% that of the stock reactant regardless of enzyme concentration.

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