CARBAMYL PHOSPHATE: GLUCOSE PHOSPHOTRANSFERASE ACTIVITY OF HEPATIC MICROSOMAL GLUCOSE 6-PHOSPHATASE AT PHYSIOLOGICAL pH*

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SUMMARY

Evidence is presented indicating that classical hepatic microsomal D-glucose 6-phosphate phosphohydrolase (EC 3.1.3.9) possesses potent carbamyl phosphate:glucose phosphotransferase activity. Unlike phosphotransferase activity of this enzyme observed with nucleotides, phosphoenolpyruvate, or to a less pronounced extent inorganic pyrophosphate as phosphoryl donors, activity with carbamyl phosphate remains high even at pH 7.0 and 7.5. For example, at the latter pH in the presence of deoxycholate glucose 6-phosphate production with 10 mM carbamyl phosphate and 180 mM D-glucose is approximately double the rate of hydrolysis of 10 mM glucose 6-phosphate. A physiologically significant synthetic role for this phosphotransferase is suggested.

During the past six years a large body of experimental evidence has accumulated establishing the catalysis of a variety of synthetic phosphotransferase reactions by classical microsomal D-glucose-6-P phosphohydrolase (EC 3.1.3.9) (1-4). Unlike relatively minor phosphotransferase activities displayed by non-specific acid and alkaline phosphatases (5), the synthetic activities of glucose 6-phosphatase can, under certain conditions, equal or actually exceed the rate of hydrolysis of glucose-6-P catalyzed by this enzyme (6,7).

While this enzyme catalyzes glucose-6-P hydrolysis over a wide range of pH values on both sides of neutrality (8), phosphotransferase activity of the enzyme with various phosphoanhydride compounds as phosphoryl donors drops off rapidly as assay pH approaches 7 from the acid side (1,2). These effects are particularly pronounced with nucleotide substrates, which demonstrate little or no activity at or above pH 7.0 (3). In

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contrast, mannose-6-P:glucose phosphotransferase activity and the hydrolysis of this hexose phosphate are manifest above, as well as below, pH 7, as is the hydrolysis of glucose-6-P (9). The former reaction, however, involves simply the interconversion of hexose-6-P compounds.

On the basis of the above observations of the effects of assay pH on the various activities, and in view of the additional fact that phosphoanhydrides can competitively inhibit the hydrolysis of glucose-6-P between pH 7 and 8.5, as well as at lower pH values (10), Nordlie, Hanson and Johns (9) have concluded that a) various phosphate substrates all may bind to the active enzymic site over a wide range of acid and alkaline pH values, but b) binary enzyme-substrate complexes formed from PP_i^{-3}, ADP^{-3}, or ATP^{-4} (in contrast with PP_i^{-2}, ADP^{-2}, ATP^{-3}, glucose-6-P^{-2} or mannose-6-P^{-2} which are active substrates) are catalytically inert and do not undergo dissociation leading to the production of the phosphoryl-enzyme complex postulated (6) as a requisite intermediate in the catalytic mechanisms of both synthetic and hydrolytic activities of this multifunctional catalyst. The inability of the electron-rich oxygen atom of water molecules to approach phosphorus atoms of highly electronegative binary complexes formed from enzyme and the more extensively ionized species of phosphoanhydrides found in the alkaline pH range is believed to explain this lack of reactivity of these latter compounds at higher pH.

These observations and theory were employed as a rationale in our search for additional compounds which might possibly serve as active, physiologically significant phosphoryl donor substrates for the synthetic activity of this enzyme system. Ideally, such a substrate should exhibit the following characteristics:

i) By analogy with glucose-6-P and mannose-6-P (and in contrast with PP_i and the various nucleoside diphosphates and triphosphates thus far studied (1-4)), it should carry no more than two negative charges when fully ionized at pH values ≥ 7.

ii) It should be an energy-rich "high-energy phosphate" compound.

iii) It must be formed in physiologically significant amounts in mammalian livers.

iv) It must be relatively highly active when tested as a phosphoryl donor with this system.

Recent studies in this laboratory, some of which are described here, have revealed that carbamyl phosphate (carbamyl-P), which meets the first three of the above criteria, is a highly active substrate over a broad pH range in a phosphotransferase reaction (Reaction 1) catalyzed by liver microsomal glucose 6-phosphatase.

$$\text{Carbamyl-P} + \underline{D}\text{-glucose} \rightarrow \text{glucose-6-P} + \text{carbamate} \quad (1)$$

MATERIALS AND METHODS

Carbamyl-P (dilithium salt) was purchased from Sigma Chemical Co., St. Louis, Mo., as were other substrates. N-2-Hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES) and cacodylic acid were obtained, respectively, from Calbiochem, Los Angeles, Calif., and Fisher Scientific Co., Fair Lawn, N.J. Experimental methods and enzymic assays were as described by Nordlie and Arion (11). Carbamyl-P solutions were prepared immediately prior to use. Freshly prepared microsomal fractions (12), from male rat (Sprague-Dawley strain) livers, either unsupplemented or supplemented with cetrimide (to 0.1%, w/v) or sodium deoxycholate (to 0.2%, w/v) as previously described (13,14), were employed as enzyme source in the studies depicted in Figure 1. Partially purified enzyme preparations, obtained as described previously (1,6) and supplemented with cetrimide to 0.05% (w/v), were employed in kinetic studies. Additional details are given in the legends to Figures 1 and 2. Enzymic activities were in all instances measured under conditions of linearity with respect to protein concentration and incubation time.

RESULTS

Various enzymic activities, measured at pH 5.5, 6.5, 7.0 and 7.5

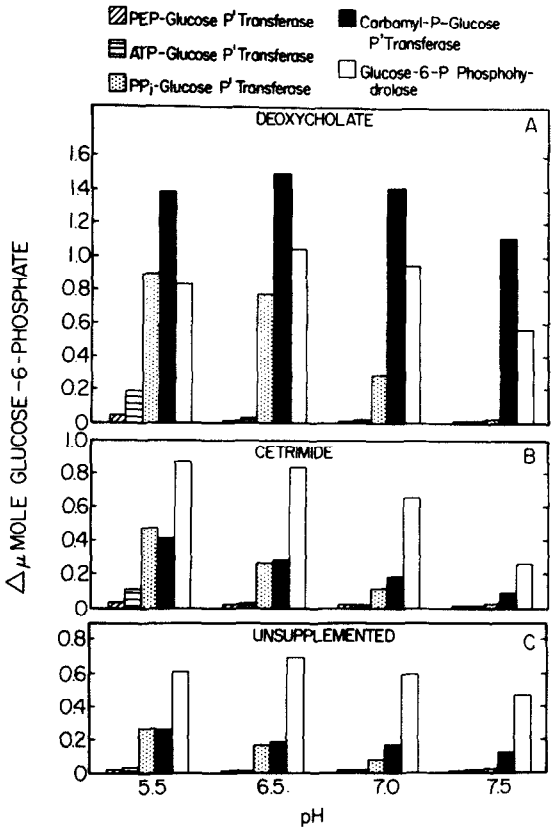

Fig. 1. Effects of assay mixture pH on the phosphotransferase and phosphohydrolase activities of glucose-6-phosphatase of rat liver microsomes. All reaction mixtures contained, in 1.5 ml, 60 μmoles sodium cacodylate (pH 5.5 and pH 6.5) or 60 μmoles HEPES (pH 7.0 and pH 7.5) buffer, 15 μmoles of either glucose-6-P or indicated phosphoryl donor, and sufficient NaCl such that $\mu = 0.15$. Phosphotransferase reaction mixtures contained 270 μmoles D-glucose. Freshly prepared microsomes untreated (C) or pretreated with 0.1% cetrimide (B) or 0.2% deoxycholate (A) as described in "Methods" were used; 0.40 mg protein was added to each reaction. Activity is expressed as μmoles glucose-6-P formed (phosphotransferase) or utilized (phosphohydrolase)/1.5 ml/10 min at 30°C.

with 10 mM phosphoryl substrates and 180 mM D-glucose (phosphotransferase) are compared in Figure 1, A-C. Activities were measured with freshly prepared hepatic microsomes (C), and with such a preparation to which either the detergent sodium deoxycholate (A) or cetyltrimethylammonium bromide (cetrimide) (B) had been added prior to assay, as described above. Significant glucose-6-P phosphohydrolase activity was noted under all conditions. Activities of all phosphotransferases studied (phosphoenol-

pyruvate(PEP):glucose, ATP:glucose, inorganic pyrophosphate(PP_i):glucose, and carbamyl-P:glucose phosphotransferases) were apparent in all instances at pH 5.5. However, only traces of activity with ATP and PEP as phosphoryl donors were noted at pH 7.0 or 7.5, and PP_i:glucose phosphotransferase, although still reasonably active at pH 7.0, also was almost totally absent at pH 7.5. In marked contrast, carbamyl-P:glucose phosphotransferase remained quite active even at the highest pH studied, as did glucose-6-P phosphohydrolase activity. This phenomenon was especially apparent in the presence of deoxycholate (Figure 1A) were carbamyl-P:glucose phosphotransferase activity (1.2 units) is approximately twice that of glucose-6-P phosphohydrolase.

Partial loss of carbamyl-P:glucose phosphotransferase activity with heating of microsomal preparations at various temperatures between 37^o and 50^o was, at each temperature tested, equal to the concurrent loss of glucose-6-P phosphohydrolase and PP_i:glucose phosphotransferase activities (Figure 2A), suggesting catalysis of these activities by a single microsomal enzyme (1,3). Further supporting the involvement of one enzyme is the competitive nature of inhibition by carbamyl-P of glucose-6-P hydrolysis in the experiment described in Figure 2B. The K_i value for carbamyl-P (1.6 mM) calculated from these data was in excellent agreement with the K_m value for this same compound (1.5 mM) which was determined for carbamyl-P:glucose phosphotransferase activity under identical conditions in supplementary experiments. The common identity of the enzyme catalyzing carbamyl-P:glucose phosphotransferase and glucose-6-P phosphohydrolase reactions is further supported by results of preliminary experiments which indicate: a) The presence of both activities in livers of man, rabbit, and fetal and adult rat; b) common patterns of fractionation by ammonium sulfate precipitation from deoxycholate-dispersed microsomal preparations; c) parallel patterns of pre- and post-natal development of both activities; d) identical responses to

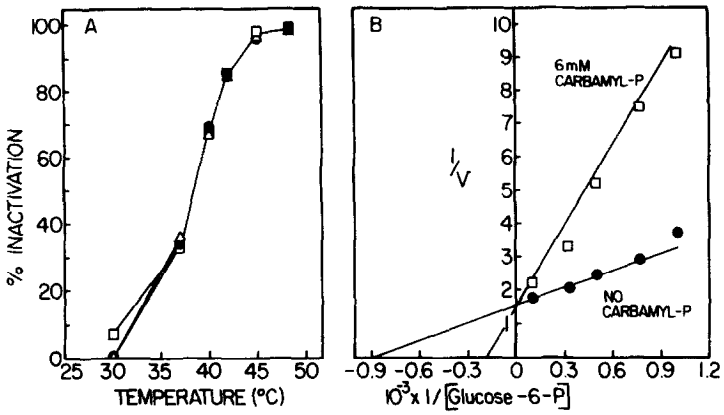

Fig. 2. A. Thermal inactivation of glucose-6-P phosphohydrolase (△), PP_i:glucose phosphotransferase (●), and carbamyl-P:glucose phosphotransferase (□) activities. Reaction mixtures, pH 6.0, contained 60 μmoles cacodylate buffer and 15 μmoles of glucose-6-P, carbamyl-P, or PP_i. Phosphotransferase reaction mixtures contained 270 μmoles D-glucose. The partially purified preparation was preincubated at the indicated temperature for 5 min before assay which was carried out at 30°C for 10 min (see ref. 1 and 3); 0.29 mg protein was added to each reaction tube. Inactivation is expressed as activity relative to activity obtained with unheated preparation. B. Effect of substrate concentration on the hydrolysis of glucose-6-P in the absence (●) and presence (□) of 6.0 mM carbamyl-P. Reaction mixtures, pH 7.0, contained 60 μmoles HEPES buffer, glucose-6-P, sufficient NaCl such that $\mu = 0.10$, and 9 μmoles carbamyl-P where pertinent (□) in a total volume of 1.5 ml including enzyme (0.16 mg protein). Activity (v) is expressed as μmoles P_i formed/1.5 ml/10 min.

administrations, in vivo, of dibutyryl 3',5'-cyclic-AMP (stimulation); and e) identity of K_m values for glucose in both carbamyl-P: and PP_i: glucose phosphotransferase reactions, and agreement of this parameter with the K_i value for glucose determined with this hexose functioning as an apparent non-competitive inhibitor of glucose-6-P phosphohydrolase activity (see refs. 1 and 3).

DISCUSSION

Carbamyl-P serves as the carbamyl group donor for citrulline and carbamyl aspartate biosynthesis, the initial steps in two important reaction sequences, the formation of urea and pyrimidines, respectively. It recently has been demonstrated that a glutamine-dependent carbamyl-P synthetase, quite distinct from the mitochondrial enzyme, exists in the

cytosol of livers of adult rats (15). In view of the relatively high carbamyl-P:glucose phosphotransferase activity observed at pH values near that of intracellular pH of rat livers (16), we would suggest that carbamyl-P produced in the soluble phase of the cell may be utilized in the synthesis of glucose-6-P as well as for pyrimidine biosynthesis.

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