Liver Microsomal Glucose-6-phosphatase: Protection by Inhibitors and Substrates Against Thermal Inactivation[1] (36439)

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Multifunctional (1, 2) microsomal glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) remains the only principal enzyme of gluconeogenesis which has not been extensively purified (3, 4). Difficulty in purification appears to be due principally to the enzyme instability (1, 4, 5) as well as its particulate nature (3, 4, 6). With the objective of overcoming, at least in part, the former problem, we have carried out a series of systematic studies on the protection of the enzyme against thermal inactivation by a variety of substrates and inhibitors. The results of some of these studies are described below.

Materials and Methods. Young, adult rats (150–200 g) obtained from Sprague-Dawley, Inc., Madison, WI, and fed Purina Laboratory Chow *ad libitum* served as experimental animals. Substrates, inhibitors, and other chemicals were from Sigma Chemical Co., St. Louis, MO. The pH of all substrates, inhibitors, and buffer was adjusted to 6.5 with dilute HCl or NaOH. Methods for preparation of liver microsomal suspensions by differential centrifugation (7) and for assay of glucose-6-P phosphohydrolase activity were as described previously by Nordlie and Arion (3). Microsomal preparations, after washing, were suspended in 5 ml of 0.25 *M* sucrose solution/original g of wet liver.

Thermal inactivation studies were carried out in the following manner: Preincubation mixtures were prepared containing, in 1.0 ml, 20 m*M* sodium cacodylate buffer, pH 6.5, 0.8 ml of microsomal preparation, and either the desired concentration of inhibitor or substrate compound or an equal volume of distilled water. Half of each such preparation was maintained at 0° in ice, while the other half of each was heated in a water bath at 40 ± 0.1° for 20 min. Such heated preparations were rapidly cooled in ice. Glucose-6-P phosphohydrolase activity was then measured with all such preparations. Assay mixtures, pH 6.5, contained in 1.5 ml, 40 m*M* sodium cacodylate buffer, 10 m*M* sodium glucose-6-P, and 0.15 ml of the above enzyme preparations (approx 0.5 mg microsomal protein). Assay incubations were carried out for 10 min at 30 ± 0.1°, with shaking. Unincubated, "zero-time" control mixtures also were prepared, maintained in ice, supplemented with enzyme preparations after addition of trichloroacetic acid, and assayed as with the incubated samples. Enzymic activity is expressed (see Table I) as micromoles of glucose-6-P hydrolyzed during the 10-min assay incubation period.

Results and Discussion. The protective effects of various concentrations of a number of inhibitors and substrates for this multifunctional enzyme, determined under such conditions, are described in Table I. Significant protection against loss of activity was provided by the inhibitors citrate (8), bicarbonate (9), ammonium molybdate (1, 6), and to a lesser extent P_i (10–12); and by the phosphoryl substrates glucose-6-P, PP_i (1, 2), ADP (2, 13), and ATP (2, 13, 14). The binding of all of these compounds, in a competitive fashion, to a common enzymic active site probably involving protein-bound divalent cation (15), previously has been

[1] This work was supported in part by a grant from the Hill Family Foundation and by research grants from the National Science Foundation (GF-14752), the National Institutes of Health, U.S. Public Health Service (AM07141), and the American Diabetes Association.

TABLE I. Protection by Substrates and Inhibitors Against Thermal Inactivation of Microsomal Glucose-6-phosphate Phosphohydrolase.[a]

Expt. no.	Addition and conc (*M*)	Observed activity[b]		100 × (activity of heated preparation / activity of unheated preparation) (%)
		Heated preparation	Unheated preparation	
I	None	0.31	0.67	45
	Citrate, 1×10^{-4}	0.32	0.64	50
	1×10^{-3}	0.40	0.68	59
	1×10^{-2}	0.54	0.64	84
II	None	0.27	0.73	37
	Bicarbonate, 1.1×10^{-4}	0.33	0.80	41
	1.1×10^{-3}	0.36	0.78	46
	1.1×10^{-2}	0.64	0.77	83
III	None	0.22	0.63	35
	P_i, 1×10^{-4}	0.18	0.58	31
	1×10^{-3}	0.23	0.64	36
	1×10^{-2}	0.31	0.60	52
IV	None	0.31	0.77	40
	Ammonium molybdate, 1×10^{-7}	0.31	0.82	38
	1×10^{-6}	0.37	0.81	46
	1×10^{-5}	0.52	0.81	64
V	None	0.23	0.74	31
	1,10-Phenanthrene, 4.1×10^{-4}	0.02	0.66	3
	4.1×10^{-3}	0.03	0.74	4
VI	None	0.27	0.71	38
	D-Glucose, 1×10^{-2}	0.29	0.76	38
	1×10^{-1}	0.33	0.76	43
	2×10^{-1}	0.33	0.74	45
VII	None	0.24	0.63	38
	Glucose-6-P, 1×10^{-4}	0.20	0.63	32
	1×10^{-3}	0.36	0.68	53
	1×10^{-2}	0.42	0.60	70
VIII	None	0.35	0.85	41
	AMP, 3×10^{-2}	0.32	0.68	38
	4×10^{-2}	0.34	0.71	41
IX	None	0.35	0.79	44
	ADP, 3×10^{-2}	0.34	0.79	43
	4×10^{-2}	0.55	0.76	72
	5×10^{-2}	0.55	0.81	68
X	None	0.30	0.76	38
	ATP, 2×10^{-3}	0.37	0.77	48
	6×10^{-3}	0.50	0.75	67
	1×10^{-2}	0.61	0.80	80
	3×10^{-2}	0.67	0.76	88
XI	None	0.21	0.63	33
	PP_i, 1×10^{-4}	0.18	0.59	31
	1×10^{-3}	0.38	0.64	59
	1×10^{-2}	0.66	0.83	80

[a] Enzymic activity values are expressed in terms of micromoles of glucose-6-P hydrolyzed per 10 min per assay mixture. Other details are given in the text.

[b] Enzymic activity is defined in text.

suggested (2, 4, 15). Initially effective concentrations of these compounds correlate with previously demonstrated K_m or K_i values. Such attachment of inhibitors or substrates appears, on the basis of the present study, to stabilize the enzyme.

5′-AMP, which is inactive as a substrate and ineffective as an inhibitor (4), afforded little or no protection.

The metal-binding agent 1,10-phenanthroline, which earlier studies (15) suggest also binds to enzyme-bound divalent metal ion, behaved quite differently than the above compounds, since mild heating of the enzyme in the presence of this compound led to a further loss, rather than protection, of activity. This observation is, however, consistent with the fact that, unlike the other compounds studied, 1,10-phenanthroline is an *irreversible* inhibitor (15, 16). Preincubation of the enzyme with this inhibitor consequently only served to allow for reaction of the enzyme with inhibitor to proceed essentially to completion. And subsequent assay incubation with substrate, of the enzyme-inhibitor complex thus produced, failed to effect reversal.

The observation that glucose provided very little protection of the enzyme to heating also is consistent with, and provides additional evidence for, the previously proposed mechanism of action of this enzyme (2, 4, 11, 17, 18). According to such a mechanism, reaction occurs through a compulsory sequence of enzyme-substrate interactions in which phosphoryl substrate binds first to the enzyme, and then dissociates to produce a phosphoryl-enzyme intermediate which finally interacts alternatively with glucose (phosphotransferase) or water (phosphohydrolase) (17). Thus, by itself glucose would not be predicted to thermally stabilize the enzyme since, in the absence of phosphoryl substrate, that form of the enzyme to which glucose may attach [see Refs. (17 and 18)]—the phosphoryl-enzyme complex—would be essentially absent.

The results described thus provide additional supportive evidence for earlier mechanistic concepts regarding this multifunctional catalyst (2, 4, 16–18), and also indicate the advisability of including certain phosphoryl substrates or reversible inhibitors with the enzyme for stabilizing purposes during future attempts at purification.

Summary. The ability of a variety of inhibitors and substrates to stabilize liver microsomal glucose-6-phosphatase (D-glucose-6-P phosphohydrolase, EC 3.1.3.9) against thermal inactivation has been investigated. Studies were carried out by incubating microsomal suspensions in sodium cacodylate buffer at pH 6.5 for 20 min at 40°, either in the absence or presence of various concentrations of certain inhibitors or substrates, cooling; and then assaying for enzymic activity. Significant protection was afforded by the inhibitors citrate, bicarbonate, P_i, and ammonium molybdate, and by the phosphoryl substrates glucose-6-P, PP_i, ADP, and ATP. D-Glucose had little effect, and inhibition by 1,10-phenanthroline was irreversible. These observations support the reaction mechanism previously suggested for this multifunctional catalyst, and indicate the advisability of including phosphoryl substrates or certain competitive inhibitors as stabilizers of the enzyme preparations during future attempts at purification.

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Received Jan. 7, 1972. P.S.E.B.M., 1972, Vol. 140.