Detection and Characterization of the Phosphorylated Form of Microsomal Glucose-6-Phosphatase

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SUMMARY

Incubation of rat liver microsomes with ^{32}P-glucose-6-phosphate results in rapid incorporation of ^{32}P into protein as N-3-phosphoryl histidine.

A mechanism involving a phosphoryl enzyme intermediate for microsomal glucose-6-phosphatase was originally proposed on the basis of exchange of ^{14}C-glucose into glucose-6-phosphate (G6P) (1). The enzyme has been shown to catalyze several phosphohydrolase and phosphotransferase activities (2,3); kinetic data (4) are consistent with involvement of a common phosphoryl enzyme as intermediate in all these reactions, with transfer of the phosphoryl group to water or other acceptor. Lack of exchange of water oxygens into the phosphoryl group of G6P is consistent with a covalent phosphoryl enzyme, but does not rule out stereospecific participation of the phosphoryl oxygens of a non-covalent complex of the enzyme with P_i (5). Using ^{32}P labelled substrates we have detected incorporation of ^{32}P into the enzyme, and we report here the chemical nature of the phosphoryl enzyme. After completion of this work, Parvin and Smith (6), in an investigation of the broad substrate specificity of this enzyme, reported incorporation of ^{32}P from ^{32}P-G6P into microsomal protein, with discharge of the ^{32}P upon mild acid treatment.

MATERIALS AND METHODS

Microsomes were prepared from homogenates of the liver of 200 g Wistar rats which had been starved for 18 hours. The fraction (in 0.25 M

sucrose, 0.1 mM EDTA) sedimenting between 8,000g (10 min) and 45,000g (60 min) was resuspended, recentrifuged and stored frozen in small portions. Prior to use, the enzyme was activated by treatment at pH 10 (7).

^{32}P-G6P was obtained via the hexokinase reaction from ATP-γ-^{32}P (8), and was purified on Dowex 1-X-8 formate, using a linear gradient from 0.3 to 1.0 M formic acid. N-3-phosphoryl histidine was synthesized from phosphoramidate (9), and purified as described by Hultquist, et al. (10). Hydrolysis of ^{32}P-G6P and phosphoryl peptides was followed by rapid extraction of $^{32}P_i$ as the phosphomolybdic acid complex into organic solvents from cold aqueous solution (11). After hydrolysis for 30 min in 1 N HCl at 100°, total phosphate (P_i plus phosphoryl histidine) was determined by the Fiske-SubbaRow procedure (12), and histidine by a modification of the Pauly method (13). Protein was estimated by the method of Lowry, et al. (14).

Incubation of microsomes with ^{32}P-G6P was done at 2-3° in 0.5 M acetate (Na^+), pH 6. Incubation was terminated by addition of 2-4 volumes of 88% aqueous phenol into which the labelled protein is quantitatively and irreversibly extracted (15). Radioactive G-6-P and P_i were removed by repeated (6X) extraction of the phenol layer with aqueous (pH 9) buffer containing 10 mM EDTA, 1 mM G6P, 10 mM P_i, and 16% phenol. For determination of the level of isotope incorporated into protein, the phenol extracts were quantitatively transferred to planchets, dried and counted. For characterization of labelled protein, phenol was omitted from the last 3 extractions so that the protein precipitated due to the removal of phenol.

RESULTS AND DISCUSSION

As can be seen in Table I, the 78 mμmoles of ^{32}P-G6P utilized contained about 0.4 mμmole (0.5%) $^{32}P_i$ and about 13 μμmoles (0.017%)

Table I

Incubation Time (Sec)	Protein	mμmoles $^{32}P_i$ Present	μμ moles ^{32}P in Phenol	μμ moles ^{32}P Incorporated
15	microsomes	7.8, 10.2	22, 23	9, 9
0	microsomes	0.35, 0.48	14, 13	-
15	serum albumin	0.48, 0.44	13, 12	-
15	none	0.38, 0.41	12, 12	-

Incubation mix, 0.5 ml, contained in addition to buffer, 78 mμmoles ^{32}P-G6P (5.02×10^4 cpm/mμmole) and 6 mg of either microsomal protein, or bovine serum albumin, or neither. Assays are described in Methods.

phenol-extractable impurity (16). These values are indifferent to the presence or type of protein. In this and all subsequent data, therefore, ^{32}P incorporation has been corrected by subtraction of values obtained on a control sample of identical final composition, to which isotope was added after termination of the incubation with phenol (0 time). Even at this low temperature and short incubation time, incorporation of ^{32}P

Table II

Incubation Time (Sec)	Preincubation	Added at 30 Sec	mμmoles ^{32}P-G6P Hydrolyzed	μμmoles ^{32}P Incorporated
60	-	-	29	17.5
60	P_i	-	30	21.5
60	G6P	-	6.2	2.5
60	PP_i	-	7.6	0
60	-	G6P	16.5	3.5
60	-	PP_i	16.5	4.5

Incubation mix, 0.5 ml, contained in addition to buffer, 6 mg microsomal protein and 125 mμmoles of ^{32}P-G6P (3.21×10^4 cpm/mμmole). Preincubations were for 30 seconds with G6P or PP_i (2.5 μmoles) or with P_i (60 mμmoles). G6P or PP_i (5 μmoles) was added midway through the incubation (30 sec) where shown. The average value of duplicate analyses is reported.

into protein was significant and reproducible.

Increased incorporation of isotope is observed after 60 sec incubation (Table II). Prior addition of a large excess of nonradioactive G6P or PP_i almost completely prevented incorporation, but P_i had no effect (when measured at lower concentration). Rapid turnover of the phosphoryl group is shown by the large loss of ^{32}P when unlabelled G6P or PP_i is added at 30 sec. This decrease provides additional evidence that the same enzyme acts upon both of these substrates. In contrast, no effect was observed, in a similar set of experiments, upon addition of β-glycerolphosphate. No incorporation from $^{32}P_i$ was detected, demonstrating that labelling from ^{32}P-G6P is not due to hydrolysis of ^{32}P-G6P followed by incorporation of $^{32}P_i$ into the same or a different protein.

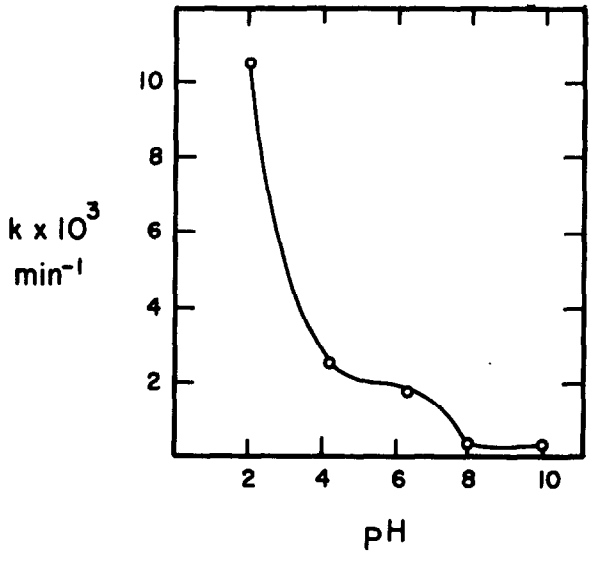

Figure 1. Rate of hydrolysis of phosphorylated peptides as a function of pH. Denatured microsomal protein (36 mg), labelled with ^{32}P and isolated as described in Methods, was suspended in 1 ml of 5 mM P_i, pH 9, by sonication (Branson Sonifier) for 10 min. Pronase (2 mg)(Calbiochem) was added and digestion allowed to proceed for 10 hours at room temperature. After removal of insoluble material by centrifugation, 0.1 ml aliquots of the soluble peptide fraction were warmed to 51°, and 2.5 ml of .05 M citrate-borate buffer at 51° was added. Release of $^{32}P_i$ at time intervals up to 160 min was determined on small portions of the mix as described in Methods. The first order rate constant for the hydrolytic reaction is plotted vs pH.

To characterize the radioactive material, larger amounts of labelled, insoluble protein, after removal of phenol, were subjected to enzymatic digestion with Pronase, releasing 90% of the ^{32}P in soluble form, largely as acid-labile ^{32}P-peptides. The rate of hydrolysis of the phosphorylated peptides to give free peptide and P_i^{32} was studied as a function of pH. The first order rate constant for hydrolysis of the phosphoryl group as a function of pH (Fig. 1) parallels that of N-3-phosphoryl histidine (10).

Phosphorylated peptides were separated from the bulk of unlabelled protein by gel filtration, as shown in Fig. 2. The first large peak of radioactivity was shown to be acid labile phosphorylated peptides and the second, smaller peak contained predominantly $^{32}P_i$. In other experiments complete separation of phosphorylated peptides and $^{32}P_i$ has been achieved. Microsomes inactivated by phenol prior to addition of

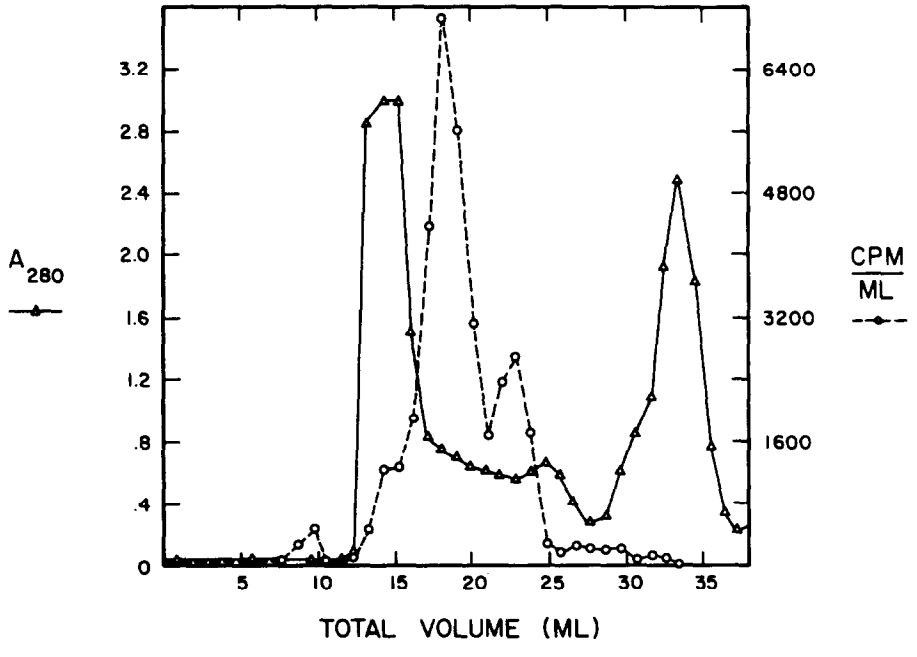

Figure 2. Purification of phosphorylated peptides by gel filtration. Peptides prepared as described in Fig. 1 were placed on a Biogel P-2 column (0.8 x 100 cm) which had been equilibrated with 0.1 M ammonium formate, 1 mM P_i, pH 9. The column was eluted with the same buffer at a flow rate of 11.5 ml/hr; fraction size was 0.96 ml.

^{32}P-G6P gave no detectable phosphorylated peptides.

The labelled peptides were subjected to alkaline hydrolysis and chromatographed on Dowex-1 (Fig. 3). The first peak of radioactivity was shown to be $^{32}P_i$, and the second peak released radioactivity as $^{32}P_i$ only after mild acid hydrolysis. The location of synthetic phosphohistidine is shown by the peak with approximately 1:1 correspondence of histidine and phosphate. As can be seen, the migration of the biologically formed ^{32}P compound was coincident with that of authentic N-3-phosphoryl histidine. The second radioactive peak was pooled, concentrated, and shown to migrate with authentic N-3-phosphorylhistidine on paper electrophoresis (10) and paper chromatography (17). N-1-phosphoryl histidine is separable from the N-3 isomer under these electrophoretic conditions, and is more labile (10).

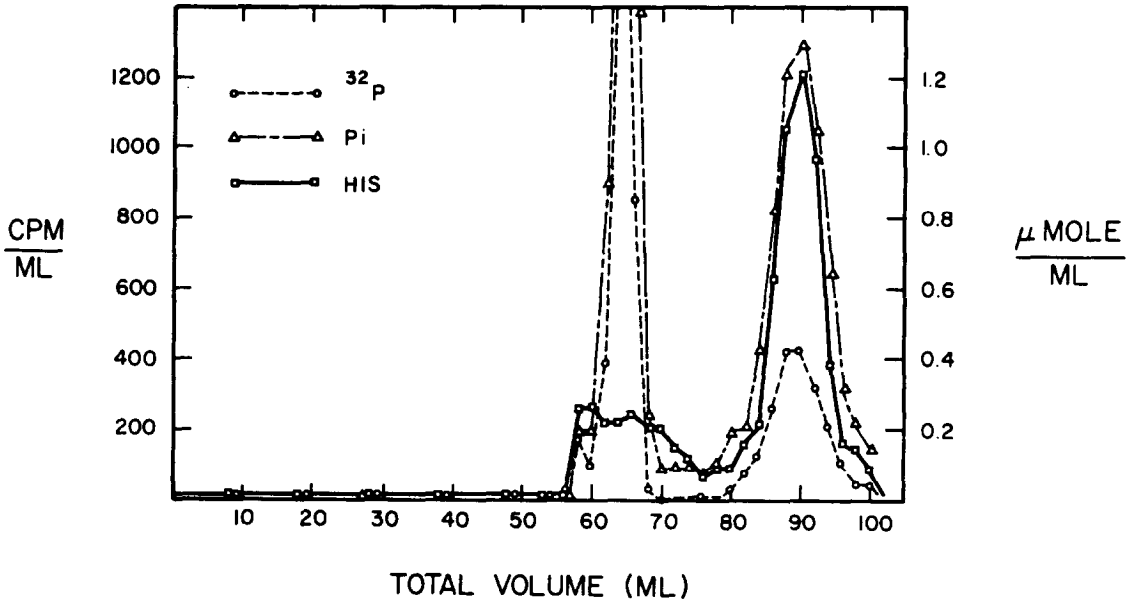

Figure 3. Apparent chromatographic identity of alkali-digested radioactive component with synthetic phosphohistidine. Fractions from 16.3 to 25 ml of the Biogel column were combined, 20 μmoles N-3-phosphoryl histidine were added and the material was hydrolyzed with 3M KOH for 4 hours at 100° in a sealed tube. After neutralization to pH 9 with $HClO_4$ and removal of the precipitated $KClO_4$ by centrifugation, the hydrolysate was chromatographed at room temperature on a Dowex 1-X-8 OH column (2.5 x 10 cm) with a linear gradient of 0.2M - 1.5M NH_4HCO_3 pH 8.5.

Parvin and Smith (6) suggested the possibility that labelling of microsomal protein resulted from phosphorylation in the active site of the enzyme. Binding of ^{32}P by microsomal protein may not be a sufficient indication for formation of covalent phosphoryl protein, for we have observed in other experiments with ^{32}P-PP_i that the relatively large amount of ^{32}P bound to protein after phenol extraction was largely substrate ^{32}P-PP_i. The experiments reported here demonstrate that the incorporated ^{32}P cannot be accounted for as bound substrate or product. Isolation of N-3-phosphoryl histidine, as postulated previously (18,6), confirms the covalent nature of the linkage. An investigation of whether all substrates phosphorylate the same histidine residue, as well as a study of the residues around the presumed active site is underway in our laboratory.

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