Direct Evidence for the Involvement of Two Glucose 6-Phosphate-binding Sites in the Glucose-6-phosphatase Activity of Intact Liver Microsomes

CHARACTERIZATION OF T1, THE MICROSMAL GLUCOSE 6-PHOSPHATE TRANSPORT PROTEIN BY A DIRECT BINDING ASSAY*

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S 5627 is a synthetic analogue of chlorogenic acid. S 5627 is a potent linear competitive inhibitor of glucose 6-phosphate (Glc-6-P) hydrolysis by intact microsomes (K_i = 41 nM) but is without effect on the enzyme in detergent- or NH_4OH-disrupted microsomes. S 5627 was synthesized and used as a ligand in binding studies directed at characterizing T1, the Glc-6-P transporter. Binding was evaluated using Ca^{2+}-aggregated microsomes, which can be sedimented at low g forces. Aside from a modest reduction in K values for both substrate and S 5627, Ca^{2+} aggregation had no effect on glucose-6-phosphatase (Glc-6-Pase). Scatchard plots of binding data are readily fit to a simple “two-site” model, with K_i = 21 nM for the high affinity site and K_i = 2 μM for the low affinity site. Binding to the high affinity site was competitively blocked by Glc-6-P (K_i = 9 mM), whereas binding was unaffected by mannos-6-phosphate, Pi, and PP_i, and only modestly depressed by 2-deoxy-D-glucose 6-phosphate, a poor substrate for Glc-6-Pase in intact microsomes. Thus the high affinity K_i for S 5627 binding site fits the criteria for T1. Permeabilization of the membrane with 0.3% (3-[chloramidopropyl]-dimethylammonio]-1-propanesulfonate) activated Glc-6-Pase and broadened its substrate specificity, but it did not significantly alter the binding of H-S 5627 to the high affinity sites or the ability of Glc-6-P to block binding. These data demonstrate unequivocally that two independent Glc-6-P binding sites are involved in the hydrolysis of Glc-6-P by intact microsomes. The present findings are the strongest and most direct evidence to date against the notion that the substrate specificity and the intrinsic activity of Glc-6-Pase in native membranes are determined by specific conformational constraints imposed on the enzyme protein. These data constitute compelling evidence for the role of T1 in Glc-6-Pase activity.

Glc-6-Pase* (EC 3.1.3.9) catalyzes the terminal reaction in the glucogenic processes of glycogenolysis and gluconeogenesis. In 1951 Hers et al. (3) showed that the hydrolysis of Glc-6-P is catalyzed by an enzyme within the hepatic endoplasmic reticulum. Since then a major research effort has been made to understand the physiological significance of this membrane association and the role of the membrane in the function of Glc-6-Pase. Two sharply contrasting models have been proposed to explain the role of the membrane in the function of Glc-6-Pase. The first, developed by Arion and co-workers (4, 5), is called substrate transport model or alternatively the translocase-catalytic unit model (6). In the substrate transport model Glc-6-Pase is viewed as a multicomponent system in the hepatic and renal endoplasmic reticulum. Hydrolysis of Glc-6-P by intact membranes is postulated to involve the coupled functions of at least three integral proteins: 1) a Glc-6-P-specific translocase, denoted T1, that mediates penetration of the hexose phosphate through the membrane; 2) a relatively nonspecific phosphohydrolase situated with its active site facing the luminal cavity; and 3) a second translocase, denoted T2, that mediates efflux of P_i, T2 also mediates the transport of PP_i, and carbamoyl phosphate across the membrane (5). The mechanism of glucose efflux remains undefined (5, 7).

The second model has its origin in Mitchell’s oriented group translocation model (8), which posits substrate binding on one side of a membrane with release of a product (i.e. “group”) on the other side. Variations on this theme have been proposed by Stetten and Burnett (9), Nordlie (10), Zakim and Dannenberg (11), Schulze et al. (12), and van de Werve and co-workers (13–15). The most recent formulation of this model of Glc-6-Pase is called the “combined conformational flexibility-substrate-transport model” (13, 15). The conformation-based models hypothesize a single, membrane-spanning protein whose active site is directly accessible to Glc-6-P but whose catalytic activity is conformationally “constrained” within the environment of the native (i.e. intact) membrane. More recently it was conceded (15) that to account for all the characteristics of Glc-6-Pase of intact microsomes, other polypeptides must participate in the hydrolytic process.

Whereas both models of Glc-6-Pase have their origin in the results of kinetic measurements, in each instance the respective interpretation of these data was seen as evidence against the alternative model. However, methodological problems may be at the center of the existing controversy (see “Discussion”). Considering that 25 years have passed since its postulation and T1 remains to be identified, the skepticism concerning its ex-

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1 The abbreviations used are: Glc-6-Pase, glucose-6-phosphatase; Glc-6-P, glucose 6-phosphate; 2-deoxy-D-Glc-6-P, 2-deoxy-D-glucose 6-phosphate; Chaps, (3-[chloramidopropyl]-dimethylammonio]-1-propanesulfonate).

2 W. J. Arion and E. S. Callaway, manuscript in preparation.
sitence is understandable.

In the present investigation we have applied methodology previously unavailable to investigations on Glc-6-Pase. Specifically, we have made direct measurements of binding to evaluate whether one or two binding sites for Glc-6-P are involved in Glc-6-P hydrolysis by intact liver microsomes. The outcome of this evaluation bears directly on whether substrate transport (two Glc-6-P binding sites) or group translocation (a single binding site) is the underlying mechanism. Although the affinities of T1 and enzyme for Glc-6-P are too low to enable direct binding measurements, we were able to exploit the recent development of a class of chlorogenic acid analogues, which function as highly specific competitive inhibitors of Glc-6-Pase activity in intact liver and kidney microsomes (16–18). The potency of the high affinity analogue S 3483 (K<sub>i</sub> = 260 μM) was markedly depressed when the concentration of microsomal protein in the assay system was elevated, whereas protein concentration had no effect on the potency of chlorogenic acid (K<sub>i</sub> = 260 μM) (18). This suggested that T1 may reside in microsomes in the concentration range of 50–100 pmol/mg protein (18) and raised the possibility that use of a high affinity (viz. K<sub>i</sub> < 100 nM) radiolabeled chlorogenic acid analogue would permit quantitative characterization of T1 by, for example, Scatchard analysis (19). We report here on studies with the chlorogenic acid analogue designated S 5627. 3H-S 5627 was synthesized and used in binding studies to characterize T1.

**EXPERIMENTAL PROCEDURES**

S 5627 is an analogue of compound II of patent EP 95–106769 950504 (20). Rat liver microsomes were prepared as described previously (21) and stored frozen at −70 °C at a concentration of 15–20 mg protein/ml. Unless otherwise indicated microsomes from 24-h fasted rats were used. Microsomal intactness, quantified from the latency of mannose-6-phosphatase (22, 23), was always greater than 96%. Data from assays of untreated microsomes were corrected for the contribution of enzyme residing in disrupted membranes (24, 25). Fully disrupted microsomes were prepared by exposing microsomes (1–2 mg protein/ml) for 20 min at 0 °C to 0.1% Triton X-100 (26) or 0.3% Chaps (22). Enzyme assays were carried out at 30 °C in a volume of 100 μl (22). Phosphohydrolase activity was assayed by quantifying P<sub>i</sub> formation as described previously (22).

S 5627 binding was evaluated using Ca<sup>2+</sup>-aggregated microsomes (or simply calcium microsomes), which can be sedimented at low g forces. (27, 28). To prepare calcium microsomes from control microsomes, one volume of microsomes (15–20 mg protein/ml) was thawed on ice and added to one volume of an ice-cold solution containing 8.9 mM calcium chloride, 5.0 mM Hepes, and 14 mM sucrose, final pH 7.0. The suspension was mixed well and centrifuged at 250 × g for 15 min, and the pellet was resuspended to the original volume of microsomes in 0.25 M sucrose in 5 mM Hepes, pH 7.4. Protein recovery was usually about 75%. To prepare calcium microsomes from detergent-disrupted microsomes, calcium microsomes were disrupted with detergent as described above and then sedimented by centrifugation at 100,000 × g for 45 min. The membranes were resuspended to their original volume in 0.25 M sucrose in 5 mM Hepes, pH 7.4, and transformed to calcium microsomes as described for control microsomes.

3H-S 5627 binding was quantified at pH 7.0 as follows. Calcium microsomes (1 mg protein/ml), S 5627 varied from 6 to 500 nM, 3H-S 5627 (2 nCi/tube), and 50 mM Hepes in 1 ml of final volume were incubated at 22 °C. After 10 min, 0.4 ml was removed for determination of total 3H-S 5627, and the remaining 0.6 ml was centrifuged at 3000 × g for 5 min. The supernatant fluid was counted to determine free ligand, and bound ligand was determined by difference.

Regression analyses of plotted data were carried out using MicroCal Origin 5.0 (MicroCal Software Inc., Northampton, MA). Linear regressions were weighted. Binding parameters were obtained from Scatchard plots of binding data (19) using a two-site, mathematical model in which the binding affinity at the higher affinity site (S1) was assumed to be 2 orders of magnitude greater than binding affinity at the second site (S2). The variables were the concentration of binding sites, [S1] and [S2], the dissociation constants for complexes of S 3483-S 5627 with S1 and S2, and the K<sub>i</sub> values for competing ligands, Glc-6-P and 2-deoxy-o-Glc-6-P. For each concentration of 3H-S 5627 and competing ligand the binding was fit to a linear graph superimposed on the scatter plot of experimental binding data. The results of the variables were adjusted until the line defined by the calculated values yielded the lowest value for the sum of the squares. In most cases a single set of parameters gave a “best fit” to all data sets in an experiment. These are noted in the figures.

**RESULTS**

**Inhibition by S 5627 in Normal Microsomes and Microsomes Aggregated by Ca<sup>2+</sup>**—At the outset we were concerned with methodology for evaluating binding of S 5627 to liver microsomes. We considered the advantages and disadvantages of equilibrium dialysis, vacuum filtration, filtration under centrifugal force, and centrifugation. Our primary concern with filtration was the likelihood of a high background due to “non-specific” binding to the filter media and trapping of label between membrane vesicles. Equilibrium dialysis and ultra-centrifugation of normal microsomes is relatively slow and would markedly limit the number of measurements per experiment. Such a limitation would preclude rigorous competitive binding studies. To measure transport in microsomes, Ballas and Arion (27) used microsomes exposed to 8 mM Ca<sup>2+</sup>, which causes aggregation of the vesicles and enables them to be sedimented at low speeds (typically 300 × g) (28). The ease of separating free and bound ligand made calcium microsomes especially well suited to the present task, but before employing the Ca<sup>2+</sup>-microsomes in binding studies, it was necessary to evaluate whether aggregation with Ca<sup>2+</sup> altered the interaction of the Glc-6-Pase system with S 5627.

Fig. 1 shows the concentration dependence of inhibition of Glc-6-Pase by S 5627 in intact and disrupted microsomes. Assay media, pH 7.0, contained 50 mM Hepes, pH 7.0, 1 mM Glc-6-P, the indicated concentrations of S 5627, and about 7 or 2 μg of microsomal protein for assays of intact and disrupted microsomes, respectively. Hyperbolic lines were fit to data by unweighted, nonlinear regression.

Fig. 2 shows that like chlorogenic acid (16) and other chlorogenic acid analogues (17, 18) S 5627 is a linear competitive
inhibitor of Glc-6-P hydrolysis in intact microsomes and Ca\(^{2+}\) exposure did not alter the pattern of inhibition or the potency of S 5627. Other than a modest decrease in the K values for both substrate and competitive inhibitor, the Glc-6-Pase system is unaffected by Ca\(^{2+}\) aggregation.

**Evaluation of \(^{3}\)H-S 5627 Binding**—Fig. 3 is a Scatchard plot (19) of typical binding data with rat liver microsomes. The data are readily fit (see solid lines in Fig. 3) to a two-site model in which the binding affinities of the two sites differ by about 2 orders of magnitude. Binding to S1, the high affinity site (K\(_s\) = 21 nM), was competitively reduced by the presence of Glc-6-P. The estimate of K for Glc-6-P (9 mM) agrees well with the expected value for K\(_{\text{m(T1)}}\), because we expect the value for K\(_{\text{m(T1)}}\) to be two to three times greater than the K\(_s\) for Glc-6-P hydrolysis by intact microsomes (see Fig. 2 and Ref. 30). In contrast, S 5627 binding was unaffected by 60 mM mannose-6-phosphate, 50 mM P\(_i\), or 50 mM PP\(_i\) (data not shown). 2-Deoxy-d-Glc-6-P, which is a weak substrate for Glc-6-Pase in intact microsomes (31), also was relatively ineffective in blocking \(^{3}\)H-S 5627 binding (Fig. 3).

Fig. 1 documents that treatment with Chaps abolished the inhibitory potency of S 5627. Thus S 5627 has no affect on Glc-6-P binding to the catalytic site on the enzyme. The experiment shown in Fig. 4 directly addressed the question of whether membrane permeabilization with 0.3% Chaps also abolished S 5627 binding or the ability of Glc-6-P to block S 5627 binding. The results are definitive; Binding to Chaps-disrupted microsomes was essentially unchanged from that observed for control microsomes, and Glc-6-P was still effective in preventing S 5627 binding. After disruption, 2-deoxy-d-Glc-6-P was hydrolyzed at a rate equivalent to Glc-6-Pase (results not shown, but see Ref. 31), yet, in contrast to Glc-6-P, 2-deoxy-d-Glc-6-P remained ineffective in preventing binding of \(^{3}\)H-S 5627.

**DISCUSSION**

The combination of binding and inhibition data presented here constitute compelling proof that two independent Glc-6-P binding sites are involved in the hydrolysis of Glc-6-P by intact microsomes. In intact microsomes Glc-6-P hydrolysis is effectively inhibited when S 5627 binds to the high affinity binding site, S1. Significantly, the relative potency of the phosphate compounds in interfering with the binding of S 5627 mirrors the substrate and inhibitor specificity of the Glc-6-Pase activity in intact microsomes (4, 5, 23, 31). In Chaps-disrupted microsomes, Glc-6-P is hydrolyzed at a site that is unaffected by S 5627 or other chlorogenic acid derivatives (16–18). In terms of the substrate transport model, Glc-6-P binding to S1 is equivalent to Glc-6-P binding to T1, and Glc-6-P binding to the S 5627-insensitive site is equivalent to Glc-6-P binding to the enzyme. The identity and function of the lower affinity site, S2, is unknown.

Proponents of the combined conformational flexibility-substrate-transport model for Glc-6-Pase have argued (13, 32) that detergent treatment prevents a hysteretic transformation that is purported to occur in intact microsomes within the early seconds of the assay and that presumably involves a transformation of the enzyme from a conformer possessing the high...
catalytic turnover, seen in detergent-disrupted microsomes, to the lower activity conformer seen during steady-state hydrolysis in intact microsomes. The conformational model posits that Glc-6-P hydrolysis involves a single Glc-6-P binding site that may exist in a high specificity conformation in untreated microsomes under steady-state conditions (13, 32) and in a low specificity conformation in detergent-treated microsomes (13) or in the early seconds of assay preceding the hysteretic transformation (32). Thus the finding that Chaps and other membrane-permeabilizing agents (see below) do not abolish either the high affinity binding site for 3H-S 5627 or the ability of Glc-6-P to competitively block 3H-S 5627 binding at this site allows a definitive choice between the substrate and group translocation models of Glc-6-Pase.

The present data demonstrate unequivocally that the broadening of substrate specificity and increased activity that follows exposure to detergents and other membrane disruptive agents (31) does not involve a change, conformational or otherwise, in the site to which Glc-6-P initially binds to facilitate its eventual hydrolysis. Indeed, the properties of the binding site for Glc-6-P and 3H-S 5627 and the properties of the site that imposes substrate and inhibitor specificity on Glc-6-Pase (4, 5, 7, 16, 18, 31) are in fact retained following Chaps exposure. Accordingly, the present results do not support the notion of a conformational transition from a catalytically constrained but highly specific enzyme to a more active but less specific conformation. Moreover, the present results gain critical support from the earlier finding that the catalytic turnover number of the enzyme rather than the burst is predicted.

We have examined in detail the kinetics of Glc-6-Pase during the early seconds of the reaction, and we have consistently failed to see either a burst of activity or any indication of a slowing in rate to a new steady-state, i.e. a hysteretic transformation. Moreover, the substantial difference in initial rate observed at steady-state between intact and disrupted microsomes is observed at the earliest time, i.e. by 2 s. However, in contrast to these results, which involved the use of harsh protein denaturants to rapidly stop the hydrolysis reaction (22, 33), when the 0.3 mM zinc sulfate solution used by others (13, 14, 32) was employed as the stop reagent, the progress curves gave the appearance of the burst in assays of intact but not disrupted microsomes. This and other observations lead us to conclude that the burst is a highly reproducible artifact caused by the relatively long quench time (viz. about 5 s) for zinc sulfate in intact microsomes.

We have examined S 5627 binding following other modes of membrane permeabilization. Exposure to 0.1 N NH₄OH, which also fully activates Glc-6-Pase (26, 29), had essentially no effect on high affinity binding. In contrast, disruption with 0.1% Triton X-100 significantly depressed the concentration of S1 binding sites and decreased binding affinity. The latter observation provides an explanation for the inhibition of Glc-6-Pase that is observed at low concentrations of Triton X-100 (9), sodium taurocholate (35), and perhaps other agents such as fatty acids and long chain fatty acyl-CoA esters (36–38).

The existence of a site-specific probe of Glc-6-P binding to T1 provides a valuable new tool to investigate Glc-6-Pase. Among the many potential applications of this reagent is that it provides a direct assay to follow the isolation and purification of the T1 protein, and thus it offers the hope that this evasive protein may soon be identified.

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