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 MICROSOMAL 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 (Kᵢ = 41 nM) but is without effect on the enzyme in detergent- or NHL2OH-disrupted microsomes. 3H-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 Ca²⁺-aggregated microsomes, which can be sedimented at low g forces. Aside from a modest reduction in K values for both substrate and S 5627, Ca²⁺ 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 Kᵢ = 21 nM for the high affinity site and Kᵢ = 2 µM for the low affinity site. Binding to the high affinity site was competitively blocked by Glc-6-P (Kᵢ = 9 mM), whereas binding was unaffected by mannose-6-phosphate, Pi, and PP, 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 3H-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 3H-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 consistent with the proposal that there may be two distinct transport mechanisms for 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 P₃, T2 also mediates the transport of PP, 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-translocate 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 existence continues.

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1 The abbreviations used are: Glc-6-Pase, glucose-6-phosphatase; Glc-6-P, glucose 6-phosphate; 2-deoxy-n-Glc-6-P, 2-deoxy-n-glucose 6-phosphate; Chaps, (3-[chloramidopropyl]-dimethylammonio-1-propanesulfonate).
istence 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 ($K_i = 260 \mu M$) 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 ($K_i = 100 \mu M$) 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 $-70^\circ C$ 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 activity in intact liver and kidney microsomes (16–18). The development of a class of chlorogenic acid analogues (17, 18) S 5627 is a linear competitive inhibitor of Glc-6-Pase from detergent-disrupted microsomes, microsomes and intact liver microsomes when assayed at 1 mM Glc-6-P. Fig. 1 also shows the sensitivity of Glc-6-P hydrolysis to S 5627. Disruption with other detergents (e.g. 0.1% Triton X-100) or ammonium hydroxide (26, 29) was as effective as Chaps (data not shown). Thus S 5627 inhibits only in intact microsomes with about equal potency in normal and calcium aggregated microsomes.

**RESULTS**

Inhibition by S 5627 in Normal Microsomes and Microsomes Aggregated by Ca$^{2+}$—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$^{2+}$, which causes aggregation of the vesicles and enables them to be sedimented at low speeds (typically 300 $\times$ 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$^{2+}$ microsomes in binding studies, it was necessary to evaluate whether aggregation with Ca$^{2+}$ 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 normal and intact calcium microsomes when assayed at 1 mM Glc-6-P. Fig. 1 also shows that disruption of microsomes with 0.3% Chaps abolished the sensitivity of Glc-6-P hydrolysis to S 5627. Disruption with other detergents (e.g. 0.1% Triton X-100) or ammonium hydroxide (26, 29) was as effective as Chaps (data not shown). Thus S 5627 inhibits only in intact microsomes with about equal potency in normal and calcium aggregated microsomes.

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\(_s\) = 2.7 mm, K\(_{\text{H} (5627)}\) = 52 ± 4 nm, and V\(_{\text{max}}\) = 325 milliunits/mg protein for Ca\(^{2+}\)-aggregated microsomes, K\(_s\) = 1.9 mm, K\(_{\text{H} (5627)}\) = 42 ± 3 nm, and V\(_{\text{max}}\) = 303 milliunits/mg protein. Kinetic constants for the enzyme in fully disrupted microsomes were for control microsomes K\(_s\) = 0.93 mm and V\(_{\text{max}}\) = 725 milliunits/mg protein, and for calcium microsomes K\(_s\) = 0.83 mm and V\(_{\text{max}}\) = 705 milliunits/mg protein.

Fig. 2. The kinetics of inhibition of Glc-6-Pase activity by S 5627 in control (A) and Ca\(^{2+}\)-aggregated (B) microsomes. Assay media, pH 7.0, contained 50 mM Hepes, Glc-6-P varied from 1 to 10 mM as indicated, S 5627 as indicated, and 7.5 (A) or 5.5 (B) \(\mu\)g of microsomal protein. Slopes and intercepts of the primary plots are plotted in the insets against the concentration of S 5627. Kinetic constants for Glc-6-P hydrolysis were for control microsomes K\(_{\text{H}}\) = 0.93 mM and V\(_{\text{max}}\) = 303 milliunits/mg protein. Kinetic constants for the enzyme in fully disrupted microsomes were for control microsomes K\(_{\text{H}}\) = 0.93 mm and V\(_{\text{max}}\) = 725 milliunits/mg protein, and for calcium microsomes K\(_{\text{H}}\) = 0.83 mm and V\(_{\text{max}}\) = 705 milliunits/mg protein.

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 conformation possessing the high

FIG. 3. Binding of \(^3\)H-S 5627 to control microsomes in the absence and the presence of 30 mM Glc-6-P or 30 mM 2-deoxy-d-Glc-6-P. Details of the binding assay and fitting of binding data are given under "Experimental Procedures." The common fitting parameters used in all curves were: K\(_{\text{H} (5627)}\) = 1900 nm, [S1] = 44 pmol/mg protein, and [S2] = 240 pmol/mg protein. The best fit values for inhibition of binding to S1 were K\(_{\text{H} (5627)}\) = 9.0 nm for 30 mM Glc-6-P and K\(_{\text{H} (5627)}\) = 160 nm for 30 mM 2-deoxy-d-Glc-6-P.

\(\text{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 effect 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-P (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 identity and function of the lower affinity site, S2, is unknown.
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 \(^3\)H-S 5627 or the ability of Glc-6-P to competitively block \(^3\)H-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 \(^3\)H-S 5627 and the properties of the site that imposes the substrate and inhibitor specificity on Glc-6-Pase (4, 5, 7, 16, 18, 33), when the 0.3N 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 \(^3\)H-S 5627 binding following other modes of membrane permeabilization. Exposure to 0.1 N NH\(_4\)OH, which also 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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