Three Thiol Groups Are Important for the Activity of the Liver Microsomal Glucose-6-phosphatase System

UNUSUAL BEHAVIOR OF ONE THIOL LOCATED IN THE GLUCOSE-6-PHOSPHATE TRANSLOCASE*

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Liver microsomal glucose-6-phosphatase (Glc-6-Pase) is a multicomponent system involving both substrate and product carriers and a catalytic subunit. We have investigated the inhibitory effect of N-ethylmaleimide (NEM), a rather specific sulfhydryl reagent, on rat liver Glc-6-Pase activity. Three thiol groups are important for Glc-6-Pase system activity. Two of them are located in the glucose-6-phosphate (Glc-6-P) translocase, and one is located in the catalytic subunit. The other transporters (phosphate and glucose) are not affected by NEM treatment. The NEM alkylation of the catalytic subunit sulfhydryl residue is prevented by preincubating the disrupted microsomes with saturating concentrations of substrate or product. This suggests either that the modified cysteine is located in the protein active site or that substrate binding hides the thiol group via a conformational change in the enzyme structure. Two other thiols important for the Glc-6-Pase system activity are located in the Glc-6-P translocase and are more reactive than the one located in the catalytic subunit. The study of the NEM inhibition of the translocase has provided evidence of the existence of two distinct areas in the protein that can behave independently, with conformational changes occurring during Glc-6-P binding to the transporter. The recent cloning of a human putative Glc-6-P carrier exhibiting homologies with bacterial phosphoethanolamine transporters, such as Escherichia coli UhpT (a Glc-6-P translocase), is compatible with the fact that two cysteine residues are important for the bacterial Glc-6-P transport.

The strategic position of Glc-6-Pase \(^1\) (EC 3.1.3.9) in carbohydrate metabolism, at the connection between gluconeogenesis and glycogenolysis, makes it a key enzyme in blood glucose homeostasis. \(^2\) The enzyme is a nonspecific phosphohydrolase tightly associated with the endoplasmic reticulum and nuclear membranes of liver and kidney cells. \(^3\) It has been identified as a 38-kDa protein \(^2,\) and has been cloned in several animal species \(4–7\). \(^2\) The active site of the enzyme is located in the endoplasmic reticulum lumen. \(8\) In vitro, with the use of microsomes (small endoplasmic reticulum vesicles), the protein activity exhibits a phenomenon termed latency, with part of its activity expressed only when the membrane is disrupted, increasing both activity and affinity \(9\). To explain the role of the membrane in the function of Glc-6-Pase, two models have been proposed, the conformational model \(10, 11\) and the transport model \(12, 13\).

The conformational model \(10, 11, 14–18\) explains all of the kinetic observations by assuming that the protein can be found in several active conformations, each one possessing particular properties. The transport model involves specific permeases termed T1 (Glc-6-P), T2 (phosphate), and T3 (glucose). These carriers allow the translocation of the substrate molecules (Glc-6-P) through the membrane into the endoplasmic reticulum lumen, followed by its hydrolysis by the Glc-6-Pase catalytic subunit and then the elimination of the reaction products (phosphate and Glc) from the endoplasmic reticulum \(cisternae\) (Fig. 1; for review see Ref. 19). However, direct evidence, such as the unequivocal identification of one of the transporters, has to be produced. Recently, an elegant study using a very specific Glc-6-Pase inhibitor has provided compelling evidence for the existence of T1 \(20\). The first indications for the existence of such a Glc-6-P translocase were provided using chemical modification of thiol groups of the Glc-6-Pase system. Although the presence of an accessible sulfhydryl residue(s) necessary for the transport activity of the Glc-6-P translocase T1 is clearly established \(21–24\), the number of such residues is still to be elucidated. The difficulty with determining this is that the catalytic subunit can also be inhibited by thiol reagents \(25, 11\).

In this paper, we demonstrate that two thiol groups of the Glc-6-P translocase and one in the Glc-6-Pase catalytic subunit are important for the activity of the system. We also show that conformational changes in the translocase protein are triggered by Glc-6-P binding.

EXPERIMENTAL PROCEDURES

Chemicals—Glc-6-P (sodium salt), mannose 6-phosphate (disodium salt), glucose, alamethicin, histone IIA, NEM, bovine serum albumin (free fatty acid), and orthovanadate were from Sigma. Mannose, potassium phosphate, PIPES, sucrose, EDTA, β-mercaptoethanol, ascorbic acid, ammonium molybdate, HEPES, and sodium dodecylsulfate were from Merck.

Preparation of Rat Liver Microsomes—Fed Wistar rats \(220–250\) g were used. Liver microsomes were made as described elsewhere \(26\). The microsomes were resuspended in a 0.25M sucrose, 5 mM HEPES, pH 7.4; quickly frozen; and kept at \(−70°C\) until used. The protein concentrations were determined using the Lowry method \(27\) as modified by Peterson \(28\) using bovine serum albumin as standard.

Activity Measurements—A 96-well microplate assay derived from the
calorimetric technique previously described was used (30). The substrates were dissolved at various concentrations in a 24 mM HEPES, 3 mM EDTA buffer, pH 6.5. In some substrate sets, histone II A at a 1 mg/ml final concentration was added in order to measure the Glc-6-Pase activity in disrupted vesicles (31). In the assay, 5 μl of microsomal suspension were incubated with 25 μl of substrate stock solutions for time periods between 10 and 30 min (depending on the substrate concentration) at 30 °C. Then, 250 μl of a stop solution (0.28% ammonium molybdate, 2.2% SDS, 1.1% ascorbic acid in 0.33 mM sulfuric acid) were added in order to measure colorimetrically the amount of phosphate formed. Under our conditions, at least three identical measurements were made, and blanks, in which stop solution was added before the substrates, were used to correct the assay values. A standard curve with known amounts of phosphate was made under identical conditions.

The microplates were then incubated at 46 °C for 20 min and read with an ELISA plate reader at 820 nm. The microsomal intactness was determined. The samples were kept on ice until assayed for activity or for transport.

**Calculations of Inhibition Kinetics**—With nondisrupted microsomes (chemical modification performed in absence of histones), the Glc-6-Pase activity inhibition curves observed were fitted to the following equation (32, 33),

\[
[S_{\text{A}}]_i = \left( \frac{M_{\text{Glc-6-P}}}{M_{\text{Glc-6-P}}} \right) \times S_{\text{A}}; \quad \text{1} - \left( \frac{M_{6P}}{M_{6P}} \right)_i

\]

where \(S_{\text{A}}\) is the Glc-6-Pase specific activity measured without histones, \(S_{\text{A}}\), is the Glc-6-Pase specific activity measured with histones, and \(M_{\text{Glc-6-P}}\) and \(M_{6P}\) are the activity measured with mannose 6-phosphate as a substrate in absence or presence of histones, respectively.

**NEM Chemical Modification**—A 0.25 mM stock solution of NEM in sucrose/HEPES buffer, pH 7.4, was prepared by warming up the mixture at 46 °C for 5 min. This solution or dilutions of this stock solution were used to modify rat liver microsomes. In a typical experiment, a pool of microsomal vesicles (1 mg/ml) was incubated in sucrose/HEPES buffer, pH 7.4, at room temperature, in the presence of various concentrations of NEM. Then, at different times, aliquots were withdrawn, and a 2-fold molar excess (with respect to NEM concentration) of β-mercaptoethanol was added in order to prevent further modification by the alkylating reagent. The samples were kept on ice until assayed for activity or for transport.

**Determination of the NEM Concentration Suitable for Glc-6-Pase Activity Inhibition in "Untreated" (Absence of Histones) Microsomes**—Liver microsomes were incubated in a sucrose/HEPES buffer with different NEM concentrations at room temperature for 1 h and then blocked with β-mercaptoethanol. Glc-6-Pase activity assayed in nondisrupting conditions started to be inhibited at a NEM concentration higher than 100 μM; the IC₅₀ was 500 μM (not shown). When the activity was measured in NEM-modified vesicles in the presence of histones (permeabilized membranes), the activity loss was very low; the residual activity slightly decreased after 1–2 mM NEM. In the presence or absence of 0.1 mM vanadate, a competitive inhibitor of the Glc-6-Pase catalytic subunit (39), the inhibition in intact vesicles was identical to the control (without vanadate), and when the activity was assayed in the presence of histones, no significant loss of activity was observed (with 1–2 mM NEM concentrations).

**RESULTS**

**NEM Kinetic Inhibition in Untreated Microsomes**—Nondisrupted microsomes in sucrose/HEPES buffer, pH 7.4 (Fig. 2), were incubated at room temperature with various concentrations of NEM (between 0.1 and 1 mM). At different times, aliquots were withdrawn, and a 2-fold molar excess of β-mercaptoethanol (with respect to NEM concentration) was added. The microsomal intactness remained unchanged and was between 90 and 95% whatever the NEM concentration and incubation time used (not shown). A logarithmic representation of Glc-6-Pase activity in intact microsomes, modified with 0, 0.25, 0.5, or 1 mM NEM and assayed with 15 mM Glc-6-P as substrate (Fig. 2A), shows two inhibition phases (chemical modification of two different sites), one faster than the other, both of which are the second-order rate constants of the reactions for the quickly and slowly modified sites, respectively.

For a given NEM concentration, the dependence on Glc-6-P concentrations of \(k_{\text{app}}\) values allowed us to estimate the affinity of this compound for the first site modified by NEM using the following relationship,

\[
k_{\text{extrapolated}} = k_1/k_{\text{extrapolated}} - 1 = f([\text{Glc-6-P}]) \quad (\text{Eq. 3})
\]

where \(k_1\) is the \(k_{\text{app}}\) for a Glc-6-P concentration \(x\), and \(k_1\) equals the \(k_2\) value found for the NEM concentration considered. The saturation curve obtained can be linearized via a double reciprocal plot. The intercept with the abscissa axis gave us \(-1/k_{p}\), being the affinity constant of Glc-6-P for the first (quickly) NEM-modified site.

With disrupted microsomes (modification performed in presence of histones), the Glc-6-Pase activity inhibition curves observed were fitted to the following equation (35),

\[
A_{\text{obs}} = A_0 e^{-kt} \quad (\text{Eq. 4})
\]

where \(k\) is the first order rate constant of the mechanism, \(A_0\) is the starting specific activity, and \(t\) is the time in minutes. As described previously, \(k\) was dependent on the NEM concentration used to inhibit the enzyme activity, indicating that the NEM inhibition of Glc-6-Pase activity in "disrupted" microsomes was also a second order mechanism.

**NEM Kinetic Inhibition in Untreated Microsomes**—Nondisrupted microsomes in sucrose/HEPES buffer, pH 7.4 (Fig. 2), were incubated at room temperature with various concentrations of NEM (between 0.1 and 1 mM). At different times, aliquots were withdrawn, and a 2-fold molar excess of β-mercaptoethanol (with respect to NEM concentration) was added. The microsomal intactness remained unchanged and was between 90 and 95% whatever the NEM concentration and incubation time used (not shown). A logarithmic representation of Glc-6-Pase activity in intact microsomes, modified with 0, 0.25, 0.5, or 1 mM NEM and assayed with 15 mM Glc-6-P as substrate (Fig. 2A), shows two inhibition phases (chemical modification of two different sites), one faster than the other, both of which are the second-order rate constants of the reactions for the quickly and slowly modified sites, respectively.
Fig. 2. Inhibition of Glc-6-Pase activity by NEM in intact microsomes. Microsomes were incubated at room temperature in a sucrose/HEPES buffer, pH 7.4 (identical results were obtained with 4 mM PIPES buffer, pH 7.1). NEM was then added, and at the time indicated in the figure, an aliquot was withdrawn and mixed with a 2-fold excess of β-mercaptoethanol. The activity was measured using different concentrations of substrate in the absence or presence of histones. A, Glc-6-Pase residual activity was assayed in nondisrupted conditions with 15 mM Glc-6-P as substrate for three sets of NEM-modified rat liver microsomes (○; 0.25; □, 0.5; ▲, 1 mM). The logarithm of the residual specific activity corrected for intactness is plotted against time. B, the residual specific activity in nondisrupted microsomes modified by 0.5 mM NEM was assayed for activity using the same concentrations of Glc-6-P as reported in B but in the presence of histones (○; 0.6; □, 2; △, 5; ▲, 20 mM). The logarithm of the residual specific activity is plotted against time.

Kinetic Constants of NEM Modification in Untreated Microsomes—We calculated the $k_{1\text{app}}$ and $k_2$ values for various NEM concentrations. Because $k_{1\text{app}}$ values are dependent on the Glc-6-P concentration used to measure the activity decrease, the rate constants remain unchanged. From measurements using microsomes modified with a given NEM concentration (0.5 mM in the case of Fig. 2B) and assayed with different concentrations of Glc-6-P, we can observe that, as expected, the second (slow) inhibition phase is not affected by the Glc-6-P concentration used to monitor the residual activity. However, the first (fast) phase is greatly accentuated for low concentration of substrate and tends to disappear for high substrate concentrations ($\geq 20$ mM). The $k_1$ rate constants are apparent rate constants ($k_{1\text{app}}$), dependent on substrate concentrations, whereas $k_2$ (rate constant of the second inhibition phase) are not. The activity measured with the same NEM-modified microsomes, which have subsequently been permeabilized by histones, remains constant with time whatever the Glc-6-P concentration used for assaying the residual activity (Fig. 2C).

Dissociation Constant Value of Glc-6-P for the First NEM-modified Site—From the data shown in Fig. 3A, we calculated the dissociation constant between Glc-6-P and the quickly modified site using the relation described under “Experimental Procedures.” A saturation curve was obtained (Fig. 4, inset). The double reciprocal representation of the data gives a straight line that allows the calculation of the $K_D$ value of Glc-6-P for the fast NEM-modified site. The affinity constant is $2.02 \pm 0.26$ mM.

Protection by Glc-6-P and Vanadate—NEM inhibition experiments were performed in the presence of 50 mM Glc-6-P or 100 μM vanadate as described in Table I (legend). The rate constant of the second (slow) NEM-modified site is not dependent on the Glc-6-P concentration used to assay the Glc-6-Pase residual activity. However, when the microsomes were incubated in the presence of both 0.5 mM NEM and 50 mM Glc-6-P, then there was a 2-fold decrease of $k_2$ ($< 0.005$ compared with control), showing a protection from NEM (Table I). The $k_2$ was unchanged (not significantly different compared with control) when 100 μM vanadate was added to the microsomes. The first (fast) NEM-modified site rate constant ($k_{1\text{extrapolated}}$) was doubled when the microsomes were incubated with 50 mM Glc-6-P but unchanged (compared with control) when 100 μM vanadate was used (Table I).

Effect of NEM on Microsomal Transport of Glc-6-P, Phosphate, and Glucose—We investigated the transport of both substrate (Glc-6-P) and products (phosphate and glucose) from Glc-6-Pase reaction, in untreated microsomes incubated for various time periods with 1 mM NEM (Fig. 5). Typical traces are presented for sucrose (Fig. 5a), a nonpermeating compound, Glc-6-P (Fig. 5b), phosphate (Fig. 5c), and glucose (Fig. 5d) with microsomes incubated with 2 mM β-mercaptoethanol. The same type of traces are shown for microsomes treated for 1 h with 1 mM NEM and in which 2 mM β-mercaptoethanol was added in order to stop the NEM alkylation reaction (Fig. 5, e–h). Traces for sucrose (Fig. 5e), phosphate (Fig. 5g), and glucose (Fig. 5h) were identical to those obtained in normal (no NEM) microsomes (Fig. 5, a, c, and d). The Glc-6-P transport...
we calculated the apparent values of the first (fast) phase modified site of 2.02 estimate of the dissociation constant of Glc-6-P for the first NEM–

centrations). The calculation (Equation 3) described under “Experimental Procedures.”

recovered baseline; see Fig. 5 and light scattering variations (from the top of the trace to the 30, and 60 min are shown in Fig. 5 shown in Fig. 5 systems, we chose to use the light scattering variation (as the To monitor the NEM modification of the different transport

ner. The value of the inhibition rate constant (0.025 min

However, Glc-6-P transport was reduced in a first order manner transport were not affected by NEM-microsome alkylation. [50x547]19394

ONED inhibition phase rate constant, \( k_1 \). The NEM concentrations used to modify the microsomes were identical to those used in A, C, the values of \( k_{1 app} \) (A) were extrapolated to 0 mM Glc-6-P. Then, the \( k_{1 extraplated} \) values were plotted against the concentration of NEM (second order rate constant \( k_1 = 2300 \text{ min}^{-1} \text{ mM}^{-1} \)). D, the second order constant of the second inhibition phase is independent of the Glc-6-P concentration (B). The calculated value of \( k_1 \) is 33 min \(^{-1} \text{ mM}^{-1} \).

traces obtained with 1 mM NEM-treated microsomes for 2, 20, 30, and 60 min are shown in Fig. 5f. In that case, both half-lives and light scattering variations (from the top of the trace to the recovered baseline; see Fig. 5h, double arrow line) are modified. To monitor the NEM modification of the different transport systems, we chose to use the light scattering variation (as shown in Fig. 5h) as a parameter reflecting the percentage of microsomal vesicles still exhibiting transport. Then we plotted the logarithm of these values against time for the three different transport systems (Fig. 6). The phosphate and glucose transport were not affected by NEM-microsome alkylation. However, Glc-6-P transport was reduced in a first order manner. The value of the inhibition rate constant (0.025 min \(^{-1} \)) is not significantly different from the value of the rate constant obtained from Glc-6-Pase activity measurements with the same NEM-treated microsomes: 0.028 min \(^{-1} \) (corresponds to the second inhibition phase rate constant, \( k_2 \)).

Determination of the NEM Concentration Suitable for Glc-6-Pase Inhibition in Disrupted Microsomes—Liber microsomes (1 mg/ml) previously permeabilized for 30 min with 1 mg/ml of histones were incubated at room temperature with various amounts of NEM. After 1 h, a 2-fold excess (over NEM) of \( \beta \)-mercaptoethanol was added to prevent further modification. The residual Glc-6-Pase specific activity was assayed using 20 mM Glc-6-P and plotted against NEM concentration (not shown). Inhibition of Glc-6-Pase activity was observed for concentrations over 20–25 mM. The IC \(_{50} \) measured in these conditions was 7–8 mM.

\( k_2 \) was in all cases independent of the Glc-6-P concentrations used to assay the residual enzyme activity, whereas \( k_1 \) is dependent on the Glc-6-P concentration. \( k_{1 extraplated} \) values were then determined as described in Fig. 3A. The values of \( k_2 \) are mean ± S.D. \((n = 5)\).

\begin{table}
\begin{tabular}{|c|c|c|c|}
\hline
& Control & + 100 \( \mu \)M vanadate & + 50 mM Glc-6-P \\
\hline
\( k_2 \) & 0.025 ± 0.003 & 0.025 ± 0.004 & 0.012 ± 0.001* \\
\hline
\( k_{1 extraplated} \) & 1.41 & 1.41 & 2.95 \\
\hline
\end{tabular}
\end{table}

* Very significant \((p < 0.005)\) compared to both control and 100 \( \mu \)M vanadate values.

\( NEM \) Kinetic Inhibition in “Disrupted” Microsomes—Disrupted microsomes were subjected to different concentrations of NEM and then assayed for residual Glc-6-Pase activity with different concentrations of Glc-6-P. The activities were normalized to the value obtained for time 0 and plotted against time (Fig. 7A). The figure shows that there was no effect (within the experimental error) of Glc-6-P concentration on the inhibition rate constant for a given NEM concentration. Moreover, the NEM inhibition is a pseudo-first order mechanism with one modified site. The second-order rate constant was calculated plotting the first order rate constant against NEM concentra-
Influx of various compounds into liver microsomal vesicles modified or not with 1 mM NEM evaluated by a light scattering technique. Liver microsomes (1 mg/ml) were modified with 1 mM NEM in 4 mM PIPES, pH 7.1. At various times, aliquots were withdrawn and NEM modification was stopped by adding 2 mM β-mercaptoethanol. During this procedure, microsomes were kept on ice and, before being used for light scattering experiments, were diluted 5-fold (in 4 mM PIPES, pH 7.1). Osmotically induced changes in microvesicle size and shape were induced by addition of 25 μl (black arrowhead) in a microsome volume of 500 μl of 4 mM PIPES (a, bottom trace) or of concentrated solutions of sucrose (a and e), Glc-6-P (b and f), phosphate (c and g), and glucose (d and h), leading to final concentrations of 50, 15, 50, and 50 mM, respectively. Alamethicin was added to fully permeabilize the microvesicle (open arrowhead). Traces are representations of two separate experiments: traces a, b, c, and d were observed with untreated microsomes, and traces e, f, g, and h were observed with 1 mM NEM-modified microsomes (e, g, and h, 60-min incubation; f, 2, 20, 30, and 60 min incubation). In h, the double arrow line shows a light scattering variation that is proportional to the number of microvesicular vesicles transporting the substrate.

FIG. 6. Evidence that translocase for Glc-6-P transport but not for phosphate or glucose is affected by NEM modification. The traces (some are shown in Fig. 5) obtained both with substrate (15 mM Glc-6-P) and products (50 mM phosphate and 50 mM glucose) of Glc-6-Pase reaction were analyzed as follows. The light scattering intensity variations, comprising the difference between the top of the trace (initiation of the transport) and the recovered baseline (when the compound concentrations outside and inside the vesicles are presumed to be identical) were measured (see double arrow in Fig. 5h) and normalized to the value obtained for time 0. We assumed that these values are proportional to the number of microvesicles that are still able to exhibit transport (i.e., to the number of still functional transporters). We plotted the logarithm of these values against time. Each point is the average of two separate experiments (○, Glc-6-P; △, phosphate; ■, glucose).

DISCUSSION

NEM is a relatively specific sulphydryl reagent commonly used to assess the structure of proteins (35, 41, 42). We have to note that reactions with amino groups can occur (43, 44) but need high pH conditions (∼8.5–9.0). NEM can cross phospholipid membranes. Thus, studying the topology of a membrane protein using only this reagent is difficult. However in our case, the high reactivity of this compound toward cysteine residues makes it a good tool to examine the structure of the liver microsomal Glc-6-Pase system.

Relatively low concentrations of NEM (<1–2 mM) inhibited the activity of the Glc-6-Pase system when microsomes were incubated in nondisrupting conditions. However, when the membrane was subsequently treated with histones, the activity measured was identical to those of the NEM-unmodified controls (Fig. 2C), showing that the catalytic subunit activity is not affected by an alkylation by 1–2 mM NEM of one or more of its cysteine residues. When an inhibition time course is made in nondisrupted microsomes (Fig. 2A, A and B), a two-site (two different thiol groups), first order kinetic inhibition is demonstrated; the first site was modified after only 2 min, and the second site needed more time to be alkylated by NEM. The modification rate of the two sites depends on NEM concentration (Fig. 2A). The rate constant for the second modified site was unchanged, whatever the Glc-6-P concentration used to assay the residual activity (Figs. 2B and 3B), but surprisingly, the rate constant of the first modified site depends on the substrate concentration used to determine residual activity (Figs. 2B and 3A). Indeed, the inhibition was released when the concentration of Glc-6-P used was higher than 20 mM (Figs. 2B and 3A). Here in the first phase, the rate constants (k1) are apparent constants. An extrapolation of the k1app to 0 mM Glc-6-P has been done to calculate the second order constant of the reaction. The value of the second order rate constant k2 for the first (quickly) modified thiol is 2360 min−1 M−1 (Fig. 3C), whereas the calculated value for the second site is 33 min−1 M−1.

A light scattering technique (36) has demonstrated that the Glc-6-P translocase was the only transporter inhibited by NEM alkylation (Figs. 5 and 6) and that the Glc-6-P transport inhibition was closely correlated to the activity loss. Therefore, the two thiol groups, modified with different velocities, are in the T1 protein.
first reactive site depends on the Glc-6-P concentration used to assay the residual activity (Fig. 2B). This dependence is a saturation process (Fig. 4). We explain the effect of Glc-6-P on the first inhibition phase by the binding of this compound to T1, which causes a conformational change releasing the effects of NEM modification. Whether this Glc-6-P molecule is regulatory and/or transported is still unclear. In 1991, Nordlie and co-workers reported that T1 could be regulated by intramicrosomal levels of Glc-6-P (45). The conformational change observed in our conditions could be the reflection of such a regulation.

Protection experiments, performed with nondisrupted vesicles in the presence of Glc-6-P or vanadate, have shown that vanadate has no effect on the inhibition process (Table I). However, a high concentration of Glc-6-P, the transported compound, partially prevents the second site from NEM modification and increases the reactivity of the first site, by improving its accessibility to NEM. From these observations, it seems that two areas or domains, each containing a reactive cysteine residue, can be found in T1. A description of their particular features is attempted in the working model proposed in Fig. 8.

Recently, a human membrane protein that exhibits sequence similarities with bacterial phosphoester transporters, such as UhpT (the sugar phosphate carrier of Escherichia coli), and that is mutated in patients suffering from glycogen storage disease 1b (no Glc-6-P transport), has been cloned (46). In bacteria, two UhpT cysteines can be modified by sulfhydryl reagents leading to an inactive protein (47, 48). The behavior of the mammalian T1 reported in this paper is rather close to that observed with UhpT. Hence, it seems possible that the putative Glc-6-P translocase recently cloned is T1.

Experiments performed with histone-disrupted microsomes allow us to look directly at the effects of NEM on the Glc-6-P-catalytic subunit activity without the rate limitations imposed by the translocases. The catalytic subunit loses its activity at NEM concentration higher than those used to inhibit the whole system in nondisrupting conditions (IC50 = 7–8 mM instead of 0.5 mM). The incubation of the histones with dithionitrobenzoate (Ellman’s reagent) did not result in a yellow coloration of titrable cysteines. Thus, the higher IC50 cannot be attributed to the presence of cysteines in the histones. We therefore have to assume that a thiol group of the Glc-6-P-catalytic subunit can be modified but needs rather high NEM concentrations.

The NEM inhibition of the Glc-6-P-catalytic subunit (Fig. 7A) is a second order mechanism (k = 1.1 min-1 M-1). The presence of substrates in the NEM incubation assay fully protects the enzyme from the inhibition, which suggests that the modified cysteine is located in the protein active site or that substrate binding hides the thiol group via a conformational change in the Glc-6-Pase structure. The identification of the residue alkylated by NEM would be helpful for completing the recently proposed Glc-6-Pase topology (49, 50) and improving our knowledge of the Glc-6-Pase active site.

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