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Modulation of the activity of hepatic glucose-6-phosphatase by methylthioadenosine sulfoxide

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Methylthioadenosine sulfoxide (MTAS), an oxidized derivative of the cell toxic metabolite methylthioadenosine has been used in elucidating the relevance of an interrelationship between the catalytic behavior and the conformational state of hepatic glucose-6-phosphatase and in characterizing the transmembrane orientation of the integral unit in the microsomal membrane. The following results were obtained: (1) Glucose 6-phosphate hydrolysis at 37 °C is progressively inhibited when native microsomes are treated with MTAS at 37 °C. In contrast, glucose 6-phosphate hydrolysis of the same MTAS-treated microsomes assayed at 0 °C is not inhibited. (2) Subsequent modification of the MTAS-treated microsomes with Triton X-114 reveals that glucose-6-phosphatase assayed at 37 °C as well as at 0 °C is inhibited. (3) Although excess reagent is separated by centrifugation and the MTAS-treated microsomes diluted with buffer before being modified with Triton the temperature-dependent effect of MTAS on microsomal glucose-6-phosphatase is not reversed at all. (4) In native microsomes MTAS is shown to inhibit glucose-6-phosphatase noncompetitively. The subsequent Triton-modification of the MTAS-treated microsomes, however, generates an uncompetitive type of inhibition. (5) Preincubation of native microsomes with MTAS completely prevents the inhibitory effect of 4,4′-diisothiocyanostilbene 2,2′-disulfonate (DIDS) as well as 4,4′-diazidostilbene 2,2′-disulfonate (DASS) on glucose-6-phosphatase. (6) Low molecular weight thiols and tocopherol protect the microsomal glucose-6-phosphatase against MTAS-induced inhibition. (7) Glucose-6-phosphatase solubilized and partially purified from rat liver microsomes is also affected by MTAS in demonstrating the same temperature-dependent behavior as the enzyme of MTAS-treated and Triton-modified microsomes. From these results we conclude that MTAS modulates the enzyme catalytic properties of hepatic glucose-6-phosphatase by covalent modification of reactive groups of the integral protein accessible from the cytoplasmic surface of the microsomal membrane. The temperature-dependent kinetic behavior of MTAS-modulated glucose-6-phosphatase is interpreted by the existence of distinct catalytically active enzyme conformation forms. Detergent-induced modification of the adjacent hydrophobic microenvironment additionally generates alterations of the conformational state leading to changes of the kinetic characteristics of the integral enzyme.

Introduction

Hepatic glucose-6-phosphatase has been proposed to represent a multicomponent system which, according to the 'translocase-catalytic unit concept' from Arion et al. [1,2], is constituted of a nonspecific hydrolase and three separate transport proteins. It is believed that the catalytic component is located inaccessibly on the luminal membrane side of the microsomal vesicles and glucose 6-phosphate translocated by a specific transporter into the luminal space. Substrate hydrolysis at the luminal membrane surface liberates glucose and P_i which are expected to cross the membrane by distinct transport proteins. In postulating a variability in the number of the distinct translocase protein molecules relative to the phosphohydrolase protein this

Abbreviations: DIDS, 4,4′-diisothiocyanostilbene 2,2′-disulfonate; DASS; 4,4′-diazidostilbene 2,2′-disulfonate; MTAS, methylthioadenosine sulfoxide (5′-deoxy-5′-*S*-adenosylmethyl sulfoxide).

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concept may explain virtually all changes of the enzyme activity as induced by genetic disorders, nutritional or hormonal states (Ref. 3 and for review Ref. 4). Unfortunately, however, neither the three putative transport proteins nor the phosphohydrolase itself have been definitely identified and purified as active, homogeneous entities. Several experimental findings from recent studies, moreover, have been brought into conflict with the proposals of this working hypothesis: as concluded from pre-steady state and steady state kinetic data microsomal glucose 6-phosphate hydrolysis does not require the action of a separate glucose 6-phosphate specific transporter [5]. Immunochemical investigations [6,7] and studies with peptidases [6] and trypsin [8–10] have shown that the enzyme must be deeply embedded within the lipid membrane in such a way that neither the hydrolase nor a carrier protein is accessible to antibody or proteolytic attack from the cytoplasmic membrane surface. Enzyme protein modifiers with low molecular weight, however, are expected to interact directly from the cytoplasmic membrane surface with the integral phosphohydrolase [11–13]. Analyzing the effect of the nonionic detergent Triton X-114 on microsomal glucose 6-phosphate hydrolysis has given rise to suggestions that changes of the enzyme kinetic characteristics, induced by detergent treatment of the membrane are generated by alterations of the native conformational state of the integral phosphohydrolase itself [14]. Recently published studies on the thermal stability of microsomal glucose-6-phosphatase have indicated that changes of the kinetic properties induced by heat treatment indeed result from different enzyme states and therefore do not reflect participation of putative separate transport proteins [15].

In addition, using *p*-mercuribenzoate [11] and pyridoxal-5′-phosphate [16] in chemically modifying the integral phosphohydrolase it has been implicated for the first time that the microsomal enzyme may exist in temperature-dependent, flexible conformational forms which allow profound changes of the catalytical behavior. The 'conformation-substate transport concept' developed from Schulze et al. (Ref. 11 and for review Ref. 4) combining aspects of both, the earlier 'conformation hypothesis' [17,18] and the 'translocase-catalytic unit concept' [1,2], includes these important experimental findings additionally to explain the transverse topology of the functional glucose-6-phosphatase within the microsomal membrane. In terms of this concept, glucose-6-phosphatase "represents at the time of reaction a topographical unit which traverses the microsomal membrane in a precise spatial arrangement" [6].

It is suggested that the enzyme may exist as an integral channel-protein which, "however, constructed and possibly created by the appropriate association of several proteins (subunits), would resolve some transport phenomena" [11]. The catalytic part of glucose-6-phosphatase expected to be located within the aqueous pore is maintained by the native conformation form of the enzyme elicited by the intact membrane structure. Therefore, specificity for glucose 6-phosphate as well as activation of glucose-6-phosphatase by detergent treatment of the membrane, which is designated as latency, are considered to be properties of the enzyme per se. The hypothesis that microsomal glucose-6-phosphatase might be created by the appropriate association of protein subunits originated from Schulze et al. [11] has been supported by data obtained from purification attempts. It has been concluded that the partially purified enzyme representing a protein complex is constructed by several specific components [19]. Further substantial support has also been given by a recent review on glycogen storage diseases [20]. In clear contrast to earlier reports [21,22] postulating the existence of single and separately operating entities distributed within the microsomal membrane, now it finally has been concluded that the microsomal enzyme indeed may represent a protein complex. The weakness of the 'conformation-substrate transport concept', however, is that it is difficult to explain the mechanism of regulation and variation of the catalytic activity under different in vivo conditions. Therefore, we have evaluated the relationship between the enzyme kinetic properties and the conformational state of the glucose-6-phosphatase proposed as well as we have characterized the topographical orientation of functionally important sides of the enzyme within the microsomal membrane. In this respect, methylthioadenosine sulfoxide (MTAS) has been used which is an oxidized derivative of the cell toxic metabolite methylthioadenosine [23]. MTAS is highly watersoluble and more polar than the nonoxidized precursor and therefore is not expected to penetrate the entire lipid barrier, but to act on the cytoplasmic membrane side under mild treatment of the microsomes.

Materials and Methods

Materials

Glucose 6-phosphate, inosine 5′-diphosphate, adenosine 5′-monophosphate, NADH, NADPH and cytochrome *c* were obtained from Boehringer (Mannheim).

Methylthioadenosine, mannose 6-phosphate and L-cysteine were from Sigma (Deisenhofen) and 4,4′-diisothiocyanostilbene 2,2′-disulfonic acid disodium salt and 4,4′-diazidostilbene 2,2′-disulfonic acid disodium salt from Fluka (Neu-Ulm). Triton X-114 (average molecular weight 536) and dithiotreithol were purchased from Serva (Heidelberg), glutathione was from Merck (Darmstadt) and α-tocopherol from Hofmann-La Roche (Basel).

Preparation of microsomes

Microsomes were prepared by differential centrifugation from livers of male rats (200–300 g body weight) fasted for 16 h [24]. 'Intactness' was determined on the basis of low K_m mannose-6-phosphatase activity [25,26] which was routinely ≥ 96%. Microsomes which were not 96% intact were omitted. Glucose 6-phosphate hydrolysis by nonspecific phosphatases determined as described in Refs. 27, 28 was lower than 1% of the total glucase-6-phosphatase activity.

Partial purification of glucose-6-phosphatase

Glucose-6-phosphatase was partially purified according to the procedure previously described in detail [29]. After Triton-solubilization of the microsomes the suspension was centrifuged at 105 000 × g_{max} and the proteins obtained in the pellet resolubilized with 0.8 M KCl and then chromatographed on phenyl-Sepharose. Glucose-6-phosphatase obtained after hydrophobic chromatography was approx. 20–30-fold purified over native microsomes and was taken as partial purified glucose-6-phosphatase in our present experiments. Glucose 6-phosphate hydrolysis by nonspecific phosphatases [27,28] was zero.

Sonication of microsomes

10 ml native microsomes (20 mg protein/ml 0.15 M sodium acetate/acetic acid (pH 7.4)) were sonicated with the micro tip of a Branson sonifier (model B-15) at position 3 for 30 5-s intervals at 0 °C. Periods of 60 s

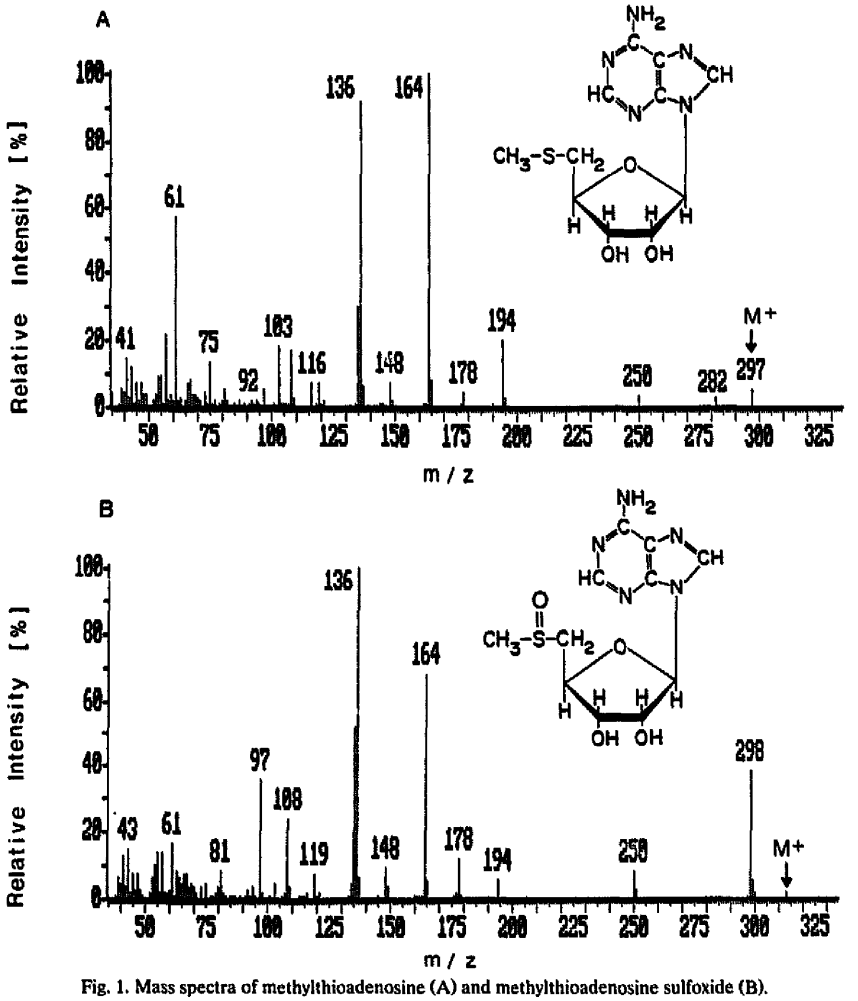

Fig. 1. Mass spectra of methylthioadenosine (A) and methylthioadenosine sulfoxide (B).

were used to cool the sample on ice since after each sonication interval the temperature increased to 5–7 °C.

Oxidation of methylthioadenosine

2.5 ml of a 20 mM methylthioadenosine solution (about 45 μmol) in 4% acetic acid were oxidized by adding 10 μl of 30% H_2O_2 (about 90 μmol) and incubation for 60 min at 37 °C in a water bath. Unreacted H_2O_2 was immediately removed by evaporating in vacuo with 40 ml methanol at 37 °C. For incubation with microsomal or partial purified glucose-6-phosphatase preparations the sample was redissolved in 2.5 ml 0.15 M sodium acetate/acetic acid (pH 7.4) and kept on ice.

Identification of the oxidized product by mass spectrometry

Nonoxidized and oxidized methylthioadenosine were analyzed by mass spectrometry to improve complete conversion of the sulfide to the corresponding sulfoxide and to exclude overoxidation to the sulfone. The mass spectra were obtained with a Varian MAT 311-A mass spectrometer using a source temperature of 180 °C and an ionization voltage of 70 eV by direct probe inlet. Typically, methylthioadenosine showed a molecular ion (M^+) peak at m/z 297 (Fig. 1A). The main fragmentation process yielded the loss of the methyl residue and subsequently the sulfur giving the expected peaks at m/z 282 and m/z 250. The nitrogen base gave a doublet at m/z 135 (base + 1H) and m/z 136 (base + 2H); the sugar fragment showed a peak at m/z 164 (sugar + 1H). Minor peaks were detectable at m/z 178 and m/z 194 representing the base including distinct fragments of the sugar part. Peaks occurring at m/z 148 and around m/z 100 were due mainly to distinct fragments of the sugar part [30].

The oxidized probe, however, showed a molecular ion (M^+) peak of low intensity at m/z 313. The most important fragments were detectable at m/z 298 and m/z 250, representing the loss of the methyl residue and the monooxygenated sulfur (Fig. 1B). Mass fragments giving peaks at m/z 148, 164, 178 and 194 were identical with those produced from the nonoxidized probe, methylthioadenosine.

Identification of the oxidized product by NMR-spectrometry

^{13}C-NMR spectra were recorded on a Bruker AM-400 spectrometer operating at 100 MHz, 25000 Hz spectral width, 74° pulse angle and 0.76 s relaxation time. The oxidized probe (about 36 μmol) was redissolved in water containing tetramethylsilan. The assignment of resonances in the ^{13}C-NMR spectra was based on published ^{13}C chemical shift values catalogized in Ref. 31 which are expressed as ppm relative to the internal standard tetramethylsilan. The ^{13}C-NMR spectra of oxidized methylthioadenosine showed prominent peaks at 156.6, 153.6, 145.0, 142.7, 118.7, 89.2, 79.3, 73.8, 57.2 and 54.6 ppm which can be assigned to the carbons of adenosine. The important signal observed at 38.5 ppm represents the $CH_3-\overset{\overset{O}{\|}}{S}-$ resonance. Instead, signals of the $CH_3-\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}-$ or the CH_3–S– carbon which would be expected around 40.4 or 16.3 ppm, respectively, were not detectable.

Enzyme assays

Glucose-6-phosphatase (EC 3.1.3.9) activities were assayed in a final volume of 800 μl 0.2 M imidazole-HCl (pH 6.5) containing 20 mM glucose 6-phosphate [32]. The assay was started by the addition of 100 μl sample and after 5 min at 37 °C or 60 min at 0 °C the reaction was stopped by the addition of 200 μl 3 M trichloroacetic acid to the test medium. Specific enzyme activity (nmol/min per mg protein) is expressed as inorganic phosphorus released. Specific glucose-6-phosphatase activity of native microsomes ranged around 7–8 nmol/min per mg protein at 0 °C and 350–400 nmol/min per mg protein at 37 °C, respectively. Microsomal nucleoside diphosphatase (EC 3.6.1.6) was measured according to Kuriyama [33] using inosine 5′-diphosphate as substrate. Activity of 5′-nucleotidase (EC 3.1.3.5) was assayed with adenosine 5′-monophosphate as described by Mitchell et al. [34]. NADPH : ferricytochrome *c* oxidoreductase (EC 1.6.2.4) and NADH : ferricyanide oxidoreductase (EC 1.6.99.3) were assayed according to the procedure described in Refs. 35 and 36, respectively.

Protein determination

Protein concentrations were determined by the Biuret-method [37] using bovine serum albumin as standard.

Reproducibility and data presentation

All experiments were done at least five times ($n \geq 5$) with different microsomal preparations and partial purified enzyme preparations. Each determination was carried out in duplicate. The data presented in the figures and tables are from one representative experiment out of $n \geq 5$.

Results and Discussion

Time-course of the effect of MTAS on microsomal glucose phosphatase and nucleoside diphosphatase

Microsomal nucleoside diphosphatase undoubtably has been characterized as a typical enzyme located at the luminal side of the microsomal membrane [6,7,33].

Latent activity is developed, when the native physical state of the membrane is changed. In terms of the 'translocase-catalytic unit concept' [1,2] glucose 6-phosphate : phosphohydrolase is also positioned at the inner surface of the microsomal membrane. It is expected, therefore, that within the native membrane neither the nucleoside diphosphatase nor the phosphohydrolase are directly accessible to hydrophilic, polar chemical probes as MTAS from the cytoplasmic membrane surface and, in addition, that both enzymes should release latent activity when the native membrane barrier is affected by the agent.

In the following experiment we have studied the effect of MTAS on both enzymes as a function of time. Native rat liver microsomes were preincubated with 2 mM MTAS at 37 °C and subsequently one half of each sample modified with Triton X-114. As illustrated in Fig. 2A preincubation of native microsomes with MTAS does not induce latent activity but inhibits glucose 6-phosphate hydrolysis progressively with increasing time of exposure of the microsomes to the reagent. When these MTAS-treated microsomes are subsequently modified with Triton neither detergent-induced latency as observed with untreated microsomes is released, nor is the inhibition of glucose 6-phosphate hydrolysis reversed. This is valid for any time of reaction. Moreover, parallel with the inhibition of glucose-6-phosphatase, the hydrolysis of mannose 6-phosphate catalyzed by the phosphohydrolase of MTAS-treated and Triton-modified microsomes is inhibited.

In contrast, as the data in Fig. 2B reveal, MTAS does not inhibit the nucleoside diphosphate. Furthermore, nucleoside diphosphatase even is not inhibited, when these MTAS-treated microsomes are subsequently modified by Triton and the same latent activity released as observed with the untreated control preparation. It is, however, found that prolonging the time of exposure of the microsomes to MTAS more than 60 min slightly increases nucleoside diphosphatase activity. This observation would indicate that MTAS might act as membrane modifier generating gradually a mild perturbation and rearrangement of the membrane structure upon prolonging incubation of the microsomes with the reagent more than 60 min. Since nucleoside diphosphatase is not as tightly associated with the microsomal membrane as glucose-6-phosphatase [6,7,33] the enzyme is solubilized from the microsomes by sonication. It can be seen from Fig. 2B that liberation of the enzyme by sonication of the microsomes results in complete release of latency. As shown with Triton-modified microsomes nucleoside diphosphatase even of sonicated microsomes is not inhibited by MTAS. Therefore, it is concluded, that resistance of nucleoside diphosphatase in native, Triton-modified and sonicated microsomes against inhibition of MTAS is provided by the absence of important reactive groups

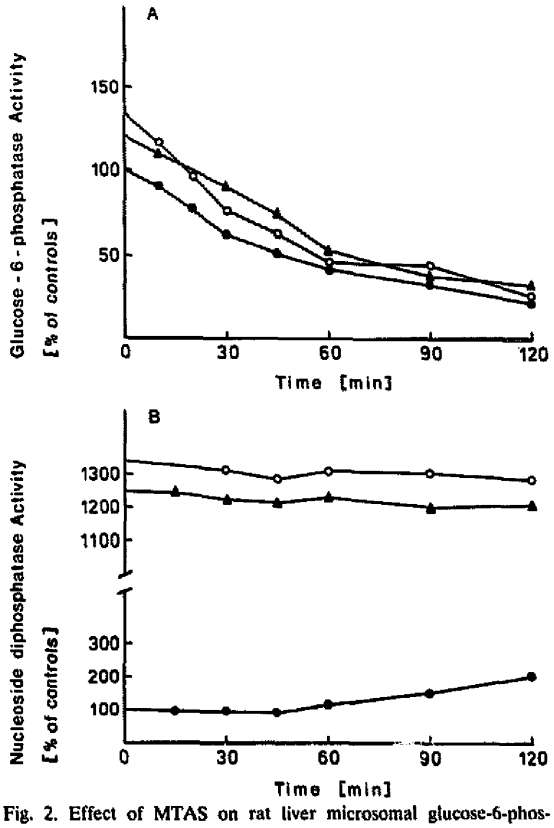

Fig. 2. Effect of MTAS on rat liver microsomal glucose-6-phosphatase and nucleoside diphosphatase. Rat liver microsomes (2 mg protein/ml 0.15 M sodium acetate/acetic acid (pH 7.4)) were incubated without or with 2 mM MTAS at 37 °C as the time indicated and the sample chilled in ice devided into two equal parts. One part was supplemented with Triton X-114 to give a final Triton concentration of 0.08% (w/v) and a Triton/protein ratio of 0.4 (w/w) and incubated for 30 min at 0 °C. Glucose-6-phosphatase activity (A) was assayed for 5 min at 37 °C with 100 μl of native (●) or Triton-modified (○) microsomes using glucose 6-phosphate; and with 100 μl of Triton-modified (▲) microsomes using mannose 6-phosphate as substrate. 100% control activity corresponds to the activity of native microsomes without any additives which was in a range of 400 nmol glucose 6-phosphate/min per mg protein. Nucleoside diphosphatase activity (B) was assayed for 5 min at 37 °C with 100 μl of native (●); Triton-modified (○) or sonicated (▲) microsomes with inosine 5'-diphosphate as substrate. 100% control activity corresponds to80 nmol inosine 5'-diphosphate/min per mg protein.

specifically interacting with the agent. More strikingly, however, is that glucose-6-phosphatase is even progressively inhibited by MTAS before latency of the luminal nucleoside diphosphatase is developed and the native membrane structure might be affected severely by the reagent. Therefore, the effect of MTAS on microsomal glucose 6-phosphate hydrolysis most obviously results from interaction between the glucose-6-phosphatase and the reagent at the cytoplasmic membrane side.

TABLE I

Effect of MTAS on the catalytic activity of various microsomal enzymes

Rat liver microsomes (2 mg protein/mg 0.15 M sodium acetate/acetic acid (pH 7.4)) were incubated for 60 min at 37 °C without or with 2 mM MTAS. Specific enzyme activities were determined as indicated in Materials and Methods.

Enzyme	Specific enzyme activity (nmol/min per mg protein)	
	without MTAS	with MTAS
Glucose-6-phosphatase	402	75
5'-Nucleotidase	95	98
NADPH: ferricytochrome *c* oxidoreductase	552	519
NADH: ferricyanide oxidoreductase	8500	7750

To further evaluate the effect of MTAS on glucose-6-phosphatase and the microsomal membrane we have measured the activity of microsomal enzymes located at the cytoplasmic membrane surface after treatment of the microsomes with 2 mM MTAS for 60 min at 37 °C. As shown by Table I, however, MTAS neither inhibits 5'-nucleotidase, NADPH: ferricytochrome *c* oxidoreductase nor NADH: ferricyanide oxidoreductase and does not induce a latent activity of these enzymes which would indicate the beginning of disruption and breakdown of the membrane barrier. Glucose-6-phosphatase, on the other hand, which is inhibited within native microsomes, therefore, must have been affected directly by specific interaction with MTAS or indirectly by modification of the intimate microenvironment of the membrane generating the inactive enzyme. The fact, that glucose-6-phosphatase of MTAS-treated microsomes cannot be reactivated by detergent-modification of the microsomes as well as the observation that mannose-6-phosphatase is concomitantly inhibited by MTAS would indicate that not a putative separate transport protein but the phosphohydrolase has been the target of the inhibitory agent. This, however, is in conflict with the view that the catalytic component is localized inaccessibly at the inner surface of the native membrane and therefore

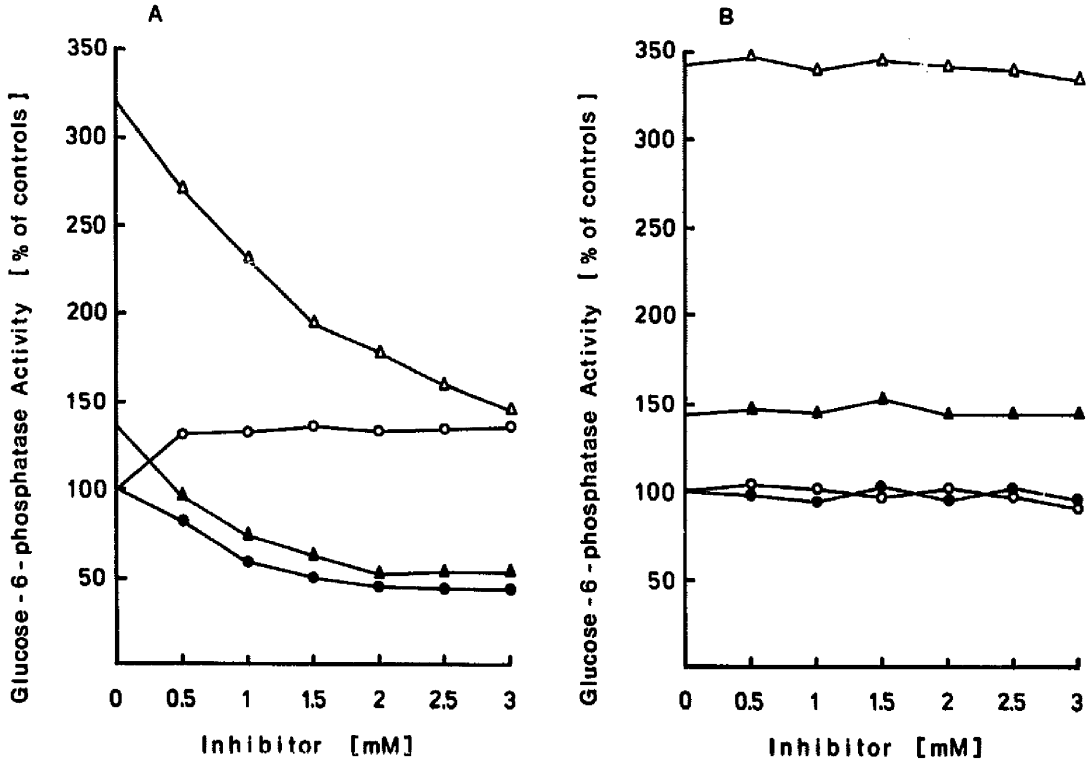

Fig. 3. Concentration- and temperature-dependent effect of MTAS on microsomal glucose-6-phosphatase. Microsomes (2 mg protein/ml 0.15 M sodium acetate/acetic acid (pH 7.4)) were incubated for 60 min at 37 °C with increasing concentrations of MTAS. The samples were diveded in two equal aliquots which either were incubated without or with Triton X-114 (final concentration of 0.08%, w/v, and Triton/protein ratio of 0.4, w/w) for 30 min at 0 °C. Glucose-6-phosphatase activity was assayed for 5 min at 37 °C with 100 μl of native (●) or Triton-modified (▲) as well as for 60 min at 0 °C with 100 μl of native (○) or Triton-modified (△) microsomes. 100% control activity corresponds to the activity of native microsomes without any additives which was in a range of 420 nmol/min per mg protein at 37 °C and 8 nmol/min per mg protein at 0 °C.

should be largely protected against inhibitor attack from the opposite cytoplasmic membrane side. Instead, the apparent controversy is easily resolved by the 'conformation-substrate transport concept' suggesting a direct accessibility of the integral phosphohydrolase (protein-channel) to low molecular weight inhibitors from the cytoplasmic membrane side. In addition, this concept has postulated that alterations within the adjacent microenvironment of glucose-6-phosphatase indeed may have consequences on the native, catalytically active enzyme conformation form [11,14].

Concentration dependence of the effect of MTAS on microsomal glucose-6-phosphatase at 37 °C and 0 °C

The effect of MTAS on microsomal glucose-6-phosphatase at 37 °C and 0 °C as a function of concentration is illustrated in Fig. 3A and B. Native rat liver microsomes were treated for 60 min at 37 °C with increasing concentrations of MTAS and subsequently one half of each sample was modified with Triton X-114. As the data in Fig. 3A show, glucose-6-phosphatase activity assayed at 37 °C is drastically inhibited by MTAS depending on the concentration of the reagent. In contrast, enzyme activity of the same MTAS-treated microsomes assayed at 0 °C is not inhibited but slightly increased as compared to the activity of untreated microsomes. Subsequent modification of these MTAS-treated microsomes with Triton, however, reveals that glucose-6-phosphatase activity at 0 °C as well as 37 °C is inhibited by MTAS.

The experimental findings described here give rise to important conclusions to explain the mechanism of inhibition of microsomal glucose 6-phosphate hydrolysis by MTAS. First, the observation that inhibition of glucose 6-phosphate hydrolysis by MTAS in native microsomes at 37 °C cannot be reversed by modification of the microsomes with detergent indicates again that the glucose 6-phosphate : phosphohydrolase itself and not a putative separate transporter within the membrane has been inhibited by MTAS. Second, the phenomenon that the effect of MTAS on microsomal glucose 6-phosphate hydrolysis is dependent on the temperature during glucose-6-phosphatase assay of the same MTAS-treated microsomes, makes it appear that the integral enzyme exists in at least two temperature-dependent conformational states with distinct kinetic properties. Third, the data obtained with MTAS-treated and subsequently Triton-modified microsomes at 0 °C not only confirm the inhibitory effect of MTAS on the phosphohydrolase, but they also suggest that subsequent Triton-induced modification of the microsomal membrane additionally has modulated the kinetic properties of the integral enzyme.

In the second experiment, native microsomes were treated for 60 min at 0 °C instead at 37 °C with increasing concentrations of MTAS. One half of each sample was modified with Triton X-114 and glucose-6-phosphatase activity was determined at 37 °C and 0 °C. The data shown in Fig. 2B, however, demonstrate that glucose-6-phosphatase of native Triton-modified microsomes has not been affected neither upon treatment of the microsomes with MTAS at 0 °C nor during the short-term testincubation of the microsomes at 37 °C or 0 °C. It is obvious that exposure of the native microsomes to MTAS for 60 min at 37 °C prior to modification with Triton X-114 and enzyme assay is necessary to obtain the inhibitory effect of the reagent on microsomal glucose 6-phosphate hydrolysis.

Finally, it should be important to note that other sulfoxides as dimethylsulfoxide, methionine sulfoxide or methylphenyl sulfoxide tested under the same conditions as MTAS, do not affect microsomal glucose-6-phosphatase at all (data not shown).

Obviously, the presence of a sulfoxide moiety within a chemical probe alone is not efficient to enable inhibition of microsomal glucose-6-phosphatase but specific molecular design of the agent (e.g., molecular size, stereochemical properties, hydrophilic and hydrophobic sites) is necessary to induce the inhibitory effect.

Effect of centrifugation and dilution on glucose 6-phosphate activity of MTAS-treated microsomes

To establish whether the effects of MTAS on glucose 6-phosphate hydrolysis observed are indeed generated during exposure of the native microsomes to MTAS exclusively, the MTAS-treated samples were separated from excess reagent by centrifugation and diluted with 10 volumes of buffer before being modified with Triton X-114 and assayed for glucose-6-phosphatase activity. Native microsomes without MTAS were used as controls which have been subjected to the same incubation, centrifugation and dilution procedure. As shown in Table II centrifugation and dilution of microsomes incubated with 2 mM MTAS for 60 min at 37 °C does not prevent or reverse inhibition of glucose-6-phosphatase assayed at 37 °C, neither in native nor in Triton-modified microsomes (experiment 1). Enzyme activity of the same MTAS-treated microsomes assayed at 0 °C, however, is slightly increased in the absence of Triton but demonstratably decreased, when the microsomes have been modified by the detergent. Additionally, native microsomes have been treated with MTAS for 60 min at 0 °C, immediately centrifuged and diluted with buffer (experiment 2). One half of the sample has been subsequently incubated for 60 min at 37 °C additionally, whereas the other half has been kept on ice (experiment 3). Glucose-6-phosphatase activity has been measured at 37 °C and 0 °C in both sample aliquots, both without and with application of Triton-modification. As can be seen, MTAS-treatment of the microsomes at 0 °C alone does not significantly affect glucose 6-phosphate hydrolysis.

TABLE II

Glucose-6-phosphate hydrolysis of MTAS-treated microsomes after separation of unbound reagent and dilution

Microsomes (20 mg protein in 10 ml 0.15 M sodium acetate/acetic acid (pH 7.4)) treated for 60 min at 37 °C (experiment 1) or 60 min at 0 °C (experiment 2) without or with 2 mM MTAS were centrifuged at 105000× g_{max} for 60 min at 0 °C. The four pellets obtained were each resuspended in 10 volumes of 0.15 M sodium acetate/acetic acid (pH 7.4) and kept on ice. The samples treated at 0 °C were divided in two parts of which one was subjected to heating for 60 min at 37 °C (experiment 3). All samples (experiment 1–3) additionally were divided in two equal aliquots which were incubated either without or with Triton X-114 (final Triton concentration of 0.08%, w/v and Triton/protein ratio of 0.4, w/v) for 30 min at 0 °C. Glucose-6-phosphatase was assayed with 100 μl each sample for 5 min at 37 °C or 60 min at 0 °C. 100% control activity of the native microsomes, measured at 37 °C or 0 °C, was 400 or 6.5 nmol/min per mg protein.

Experiment		Glucose-6-phosphatase activity (% of native microsomes)			
		37 °C assay		0 °C assay	
		without Triton	with Triton	without Triton	with Triton
(1)	native microsomes	100	135	100	422
	MTAS-treated microsomes	36	46	144	110
(2)	native microsomes	100	146	100	451
	MTAS-treated microsomes	102	152	99	439
(3)	native microsomes	100	132	100	419
	MTAS-treated microsomes	46	51	140	105

On the contrary, when these microsomes have been subjected to heating for 60 min at 37 °C (experiment 3) the data obtained are virtually identical with those obtained from experiment 1, in which the microsomes have been incubated for 60 min at 37 °C with MTAS before being centrifuged and diluted with buffer. From these results it has been concluded that the effect of MTAS on glucose 6-phosphate hydrolysis in native and Triton-modified microsomes measured at 0 °C as well as 37 °C is initiated and completed by the exposure of the microsomes to the chemical probe for 60 min at 37 °C. Inhibition of glucose-6-phosphatase observed in Triton-modified samples therefore cannot be attributed to excess reagent possibly interacting with the phosphohydrolase exposed within the detergent-modified membrane. Furthermore, our results indicate that MTAS also interacts with the glucose-6-phosphatase by a treatment of the microsomes for 60 min at 0 °C. The inhibitory effect of MTAS on glucose 6-phosphate hydrolysis, however, is only elicited when the MTAS-treated and centrifuged microsomes additionally are subjected to heat treatment for 60 min at 37 °C. With regard to the close correlation of these important observations to our earlier experimental findings with *p*-mercuribenzoate [11], we propose that at 0 °C and 37 °C glucose-6-phosphatase of MTAS-treated microsomes indeed exists in distinct conformational forms which exhibit different enzyme kinetic properties.

Kinetic characterization of the inhibition of glucose-6-phosphatase by MTAS

The inhibition of glucose-6-phosphatase induced by treatment of native microsomes with MTAS for 60 min at 37 °C has been analyzed kinetically by the Lineweaver-Burk approach [38]. Results of representative experiments in which the microsomes have been assayed without and with Triton X-114 are shown in Fig. 4A and B. Substrate concentrations were used in a range of 2–30 mM of glucose 6-phosphate. As illustrated in Fig. 4A, the kinetic type of inhibition of glucose-6-phosphatase of native microsomes by MTAS assayed without Triton X-114 is apparently noncompetitive with the substrate, as indicated by the convergence of the Lineweaver-Burk plots on the *x*-axis. The observation that MTAS decreases the maximal velocity without affecting the K_m of microsomal glucose 6-phosphate hydrolysis would imply that the site of modification is not essential for substrate binding. It might be responsible, however, for maintaining the enzyme conformation required to enable maximal velocity of glucose 6-phosphate hydrolysis.

The analysis of the double-reciprocal plots obtained from MTAS-treated microsomes assayed with Trition X-114 reveals a second and curious change in the kinetic characteristics of microsomal glucose-6-phosphatase. As shown by Fig. 4B, in the presence of Triton X-114 the type of inhibition of glucose-6-phosphatase by MTAS changes to a kinetic pattern of an uncompetitive inhibition. In the classical sense this would indicate that either the inhibitor interacts with the enzyme substrate complex preventing the formation of the phosphoryl enzyme or, on the other hand, that MTAS prevents the subsequent phosphoryl transfer to water, when the phosphoryl enzyme has been formed. Since, however, the effect of MTAS on glucose-6-phosphatase of Triton-modified microsomes is shown to be initiated and terminated by the preincubation of the native microsomes with the probe for 60 min at 37 °C (Table II) this kinetic behavior most obviously is not generated by the inhibitor itself. Therefore, uncompetitive inhibition of glucose-6-phosphatase in MTAS-treated and Triton-modified microsomes may rather represent an artifact. In the light of this evaluation, we would suggest an immediate kinetic response of the MTAS-inhibited enzyme to alterations of the intimate environment of the microsomal membrane induced by the nonionic detergent.

Effect of DIDS and DASS on glucose-6-phosphatase of MTAS-treated microsomes

From several lines of evidence it has been suggested recently that 4,4′-diisothiocyanostilbene 2,2′-disulfonic

acid (DIDS) [12] and a photoreactive derivative of 4,4'-diazidostilbene 2,2'-disulfonic acid (DASS) [13], both inhibit microsomal glucose 6-phosphate hydrolysis at 0 °C as well as 37 °C, obviously by interacting with essential sulfhydryl groups of the integral phosphohydrolase, accessible from the cytoplasmic membrane surface.

In contrast, MTAS inhibits glucose 6-phosphate hydrolysis of native microsomes only, when the MTAS-treated microsomes are assayed at 37 °C. Enzyme activity of the same MTAS-treated microsomes assayed at 0 °C, however, is not inhibited by the chemical probe (Fig. 2A, Table II). This quite differing effect of the stilbene derivatives and MTAS on glucose-6-phosphatase of native microsomes observed at 0 °C, has led us to the following experiment. Native rat liver microsomes were treated for 60 min at 37 °C without or with 2 mM MTAS and immediately centrifuged to remove excess reagent. The pellets were redissolved in buffer and each sample was divided into multiple aliquots of equal volume. One half of these aliquots were incubated for 30 min at 0 °C with increasing concentrations of DIDS. The other half were preequilibrated with increasing concentrations of DASS at 0 °C and each sample mixture was irradiated with a long wave ultraviolet-light source for 10 min at 0 °C [13]. This has been necessary, since DASS (but not DIDS!) is converted to a photoreactive derivative which is the actual reactant. Glucose 6-phosphate hydrolysis was subsequently assayed in each of the samples at 0 °C.

The experimental data illustrated by Fig. 5 demonstrate that DIDS and DASS progressively inhibit glucose-6-phosphatase, when the microsomes have not been treated with MTAS before being incubated with the stilbene derivatives. When, however, the microsomes have been subjected to MTAS-treatment, the inhibitory effect of both, DIDS as well as DASS on glucose-6-phosphatase is completely prevented. From these important results it is concluded, therefore, that either MTAS acts directly on or very near the DIDS and DASS binding site preventing interaction of the stilbene derivatives with reactive groups of the phosphohydrolase, accessible from the outer membrane surface. Alternately, insensitivity of glucose-6-phosphatase against inhibition by DIDS and DASS could be created by MTAS-induced changes of the native

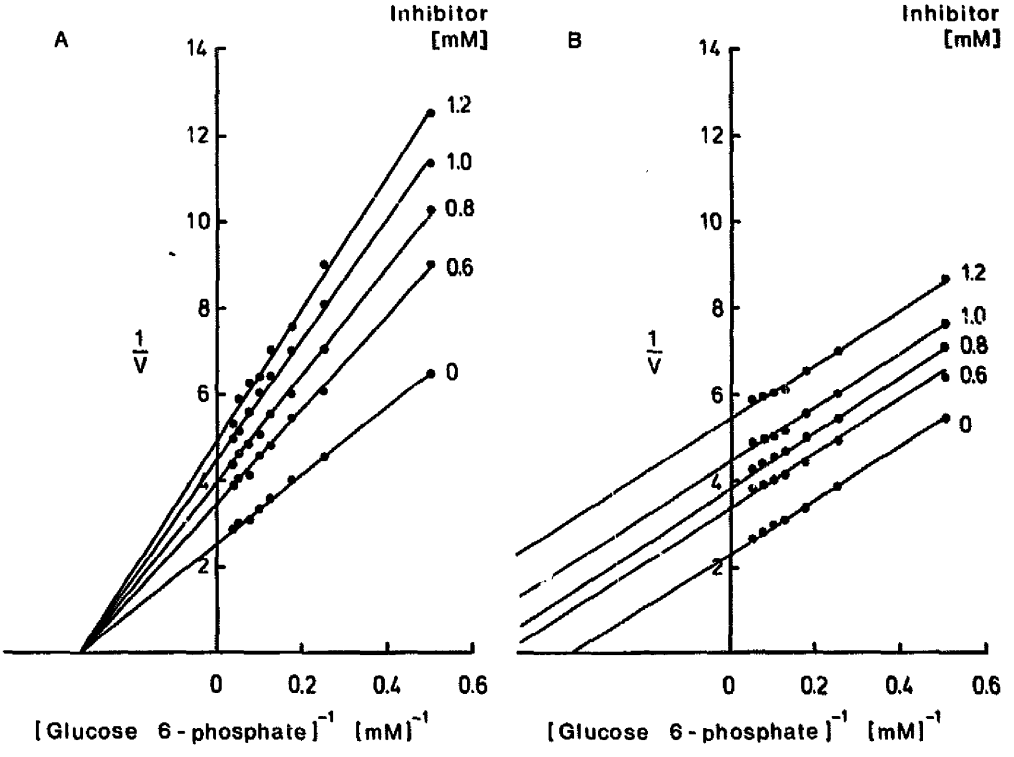

Fig. 4. Kinetic analysis of the inhibition of microsomal glucose-6-phosphatase by MTAS. Rat liver microsomes treated with different concentrations of MTAS for 60 min at 37 °C and chilled in ice were divided in two equal aliquots and subsequently incubated for 30 min at 0 °C without (A) or with (B) Triton X-114 (final Triton concentration of 0.08%, w/v and Triton/protein ratio of 0.4, w/w). Glucose-6-phosphatase activity was assayed with 100 μl for 5 min at 37 °C.

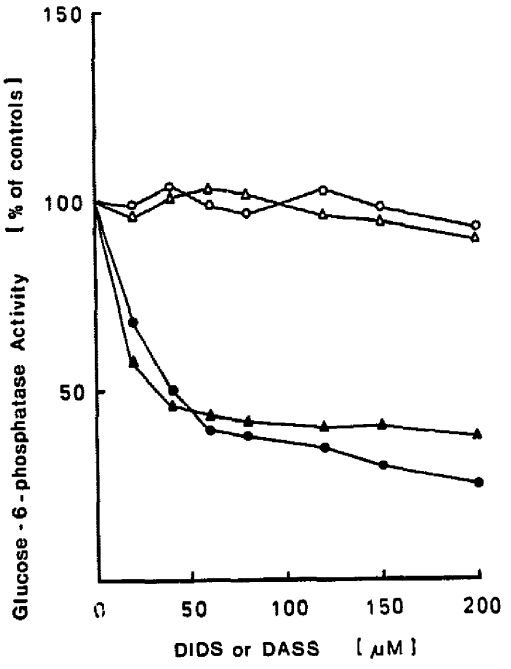

Fig. 5. Protective effect of MTAS on glucose-6-phosphatase against inhibition by DIDS and DASS. Previously, microsomes (2 mg protein/ml 0.15 M sodium acetate/acetic acid (pH 7.4)) were treated for 60 min at 37 °C without (dark symbols) or with 2 mM MTAS (light symbols) and then centrifuged at 105000× g_{max} for 60 min at 0 °C. The pellets were resuspended in 0.15 M sodium acetate/acetic acid (pH 7.4) and the protein concentration determined. Both samples were diveded into multiple aliquots of equal volume which either were incubated for 30 min at 0 °C with increasing concentrations of DIDS (●; ○) or irradiated at 366 nm for 10 min at 0 °C with increasing concentrations of DASS (▲; △). The final protein concentration of each sample was adjusted to 2 mg/ml. Glucose-6-phosphatase activity was determined with 100 μl each sample for 60 min at 0 °C.

enzyme conformation resulting in inaccessibility of the reactive groups interacting with DIDS and DASS or simply in an enzyme conformer which binds DIDS and DASS but is no longer inhibited.

Protective effect of sulphydryl reagents and tocopherol on glucose-6-phosphatase against inhibition by MTAS

Dithiotreithol, glutathione and tocopherol have been characterized as membrane protecting agents preventing oxidative attack of oxygen radicals and other oxygen species on membrane proteins and lipids [39–42]. The capacity of oxidants to promote severe changes in the native conformational state of membrane proteins including concomitant consequences on their biological function has also been well established [43–45]. In order to prove, whether MTAS mediates inhibition of microsomal glucose-6-phosphatase by oxidative modification reaction we have preequilibrated the native microsomes with dithiotreithol, glutathione, cysteine or tocopherol before being treated with the inhibitor for 60 min at 37 °C. As illustrated by Fig. 6 inhibition of glucose-6-phosphatase by MTAS observed at 37 °C is almost completely prevented by any of the three antioxidants as well as by cysteine. When, however, these agents have been added after the treatment of microsomes with MTAS has been completed, glucose-6-phosphatase remains inhibited to the same degree as observed with MTAS-treated microsomes without antioxidant additives. Thus, from these experimental results it is suggested that the sulfhydryl reagents as tocopherol prevent MTAS induced inhibition of microsomal glucose-6-phosphatase by scavenging either the sulfoxide itself or the reaction product(s) derived from modification of membrane lipids initiated by MTAS. Since oxidation of protein sulfhydryl groups is shown to be one of the first events upon oxidative attack on the cellular membrane systems [39–43,45] and in regard of the special requirement of cytoplasmic accessible and highly reactive sulfhydryl groups of microsomal glucose-6-phosphatase, participation of these reactive groups on MTAS-induced inhibition unavoidably has to be implicated. In Fig. 7 two reaction pathways are proposed by which MTAS may affect microsomal glucose 6-phosphate hydrolysis. As illustrated, immediate oxidation of enzyme sulfhydryl groups by the sulfoxide could promote the formation of a disulfide prerequisiting the presence of a vincinal dithiol or a close stereochemical neighborhood of sulfhydryl groups from two distinct protein species. Alternately, MTAS could induce lipid peroxidation generating products highly reactive towards protein sulfhydryl groups. Indeed, lipid peroxides are shown to induce disulfide formation and crosslinking of membrane proteins [39,43], whereas α,β-unsaturated aldehydes (e.g., 4-hydroxynonenal [46]) form stable adducts with protein sulfhydryl groups by hydro-alkylthioaddition. In light of the alternative mechanisms proposed it is suggested that MTAS-induced inhibition of glucose-6-phosphatase either results from stable covalent linkage insensitive to the reducing agents applied or from an inactive enzyme conformation which cannot be regenerated to the native catalytically active state although the scissile bond is cleavable upon reduction.

Response of the partial purified glucose-6-phosphatase to MTAS treatment

Glucose-6-phosphatase has been partially purified from rat liver microsomes according to the procedure developed in our laboratory, involving solubilization of the microsomes with Triton X-114 and separation of the enzyme from nonspecific proteins and the membrane lipids by hydrophobic chromatography on phenyl-Sepharose [29]. The specific activity of the partial purified glucose-6-phosphatase measured at 0 °C

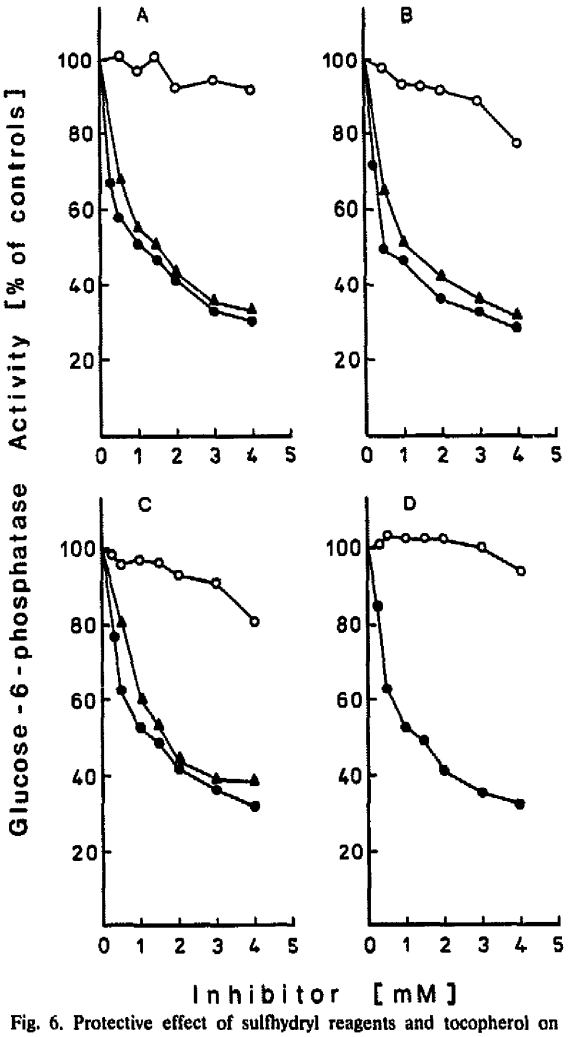

Fig. 6. Protective effect of sulfhydryl reagents and tocopherol on microsomal glucose-6-phosphatase against inhibition by MTAS. Microsomes (2 mg protein/ml 0.15 M sodium acetate/acetic acid, pH 7.4) were preequilibrated for 10 min at 0 °C without (●) or with (○): (A) 10 mM dithiotreithol; (B) 10 mM glutathione; (C) 20 mM cysteine or (D) mixed for 60 min at 22 °C by gentle stirring under nitrogen atmosphere without (●) or with (○) 0.5 mg α-tocopherol/mg microsomal protein. MTAS was added as the concentrations indicated and the final volume each sample adjusted to 1 ml. Subsequently, all samples were incubated for 60 min at 37 °C. The black triangles (▲) represent samples which were first incubated with MTAS for 60 min at 37 °C and then treated with dithiotreithol, glutathione or cysteine. Glucose-6-phosphatase was assayed with 100 μl each sample for 5 min at 37 °C. 100% control activity represents the activity of native microsomes without any additives which was in a range of 380 nmol/min per mg protein. Specific enzyme activity of native microsomes incubated with either dithiotreithol, glutathione, cysteine or α-tocopherol alone was in the same range.

and 37 °C ranging around 160 and 8000 mU/mg protein, respectively, is approx. 20-fold over the activity of native microsomes. Simultaneously, during hydrophobic chromatography the nonionic Triton X-114 is separated and thus the enzyme preparation obtained is largely protected against detergent-induced inactivation upon short-term heat treatment at 37 °C [29]. When the partial purified glucose-6-phosphatase is subjected to prolonged heat treatment for 60 min at 37 °C, 60–70% of the initial activity remains which is approx. 4608 and 133 mU/mg protein, measured at 37 °C and 0 °C, respectively.

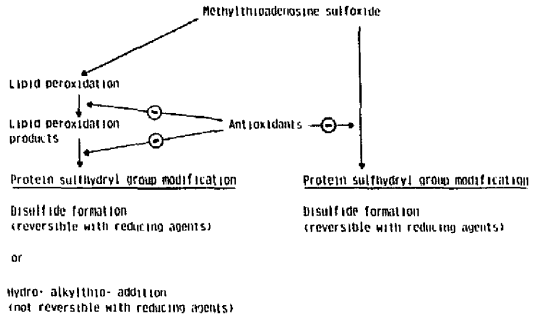

Fig. 7. Schematic illustration of two possible reaction pathways by which MTAS may affect hepatic microsomal glucose-6-phosphatase.

With the fortune of a relative thermostable enzyme preparation we have performed inhibition studies with MTAS and the partial purified glucose-6-phosphatase. Native rat liver microsomes and the partial purified glucose-6-phosphatase were incubated for 60 min at 37 °C with 2 mM MTAS and enzyme activity assayed at 37 °C and 0 °C was compared. The data presented in Table III reveal that at 37 °C MTAS both inhibits

TABLE III

Treatment of the partial purified glucose-6-phosphatase with MTAS

Glucose-6-phosphatase was partially purified from rat liver microsomes as indicated. Native microsomes (2 mg protein/ml 0.15 M sodium acetate/acetic acid (pH 7.4)) and the partial purified enzyme (0.1 mg/ml 0.15 M sodium acetate/acetic acid (pH 7.4)) were incubated for 60 min without or with 2 mM MTAS and the samples chilled on ice. Specific enzyme activity was assayed with 100 μl each sample for 5 min at 37 °C or 60 min at 0 °C. Additionally, native microsomes and partial purified glucose-6-phosphatase were preequilibrated each with 10 mM glutathione for 10 min at 0 °C before being incubated without or with MTAS and enzyme activity assayed.

Preparation	Specific enzyme activity (nmol/min per mg protein)			
	37 °C assay		0 °C assay	
	without MTAS	with MTAS	without MTAS	with MTAS
Native microsomes	310	125	8.8	9.4
Partial purified enzyme	4608	1024	133	62
Native microsomes with glutathione	304	306	9.7	9.2
Partial purified enzyme with glutathione	4672	4736	130	135

the partial purified glucose-6-phosphatase as well as the enzyme of native microsomes. At 0 °C, however, partial purified glucose-6-phoshatase is also significantly inhibited by MTAS, whereas glucose-6-phosphatase of native microsomes is not affected by the reagent.

Thus, the data obtained clearly correlate with the experimental findings described in the preliminary inhibition studies on glucose-6-phosphatase of detergent-modified and native microsomes (Figs. 2A, 3A, Table II). Since the majority of microsomal membrane lipids has been separated from the enzyme by solubilization and hydrophobic chromatography it seems very unlikely that MTAS-induced inhibition of the partial purified glucose-6-phosphatase is a result of lipid peroxidation. Moreover, it is found that glutathione, one of the protective antioxidants (Fig. 6B), not only protects the glucose-6-phosphatase of native microsomes but also the partial purified enzyme against inhibition by MTAS at 37 °C and 0 °C (Table III). Suggesting that the low molecular thiol compound would reduce the sulfoxide to an inefficient sulfide derivative of MTAS, while glutathion disulfide is formed, glutathione thereby may prevent oxidation of protein thiol groups of the glucose-6-phosphatase. Thus, it is reasonable to conclude that inhibition of partially purified glucose-6-phosphatase by MTAS is induced by the reagent itself. Although we do not exclude that with native microsomes MTAS may initiate lipid peroxidation generating products which could react with the phosphohydrolase. The close correlation between the data obtained with the partial purified glucose-6-phosphatase and the enzyme of MTAS treated and Triton-modified microsomes, however, favors the conclusion that inhibition of microsomal glucose-6-phosphatase by MTAS is primarily induced by the agent itself.

Conclusions

In our present study the chemical probe MTAS has been employed as an instrument in resolving the question of an intimate relationship between the conformational state and the catalytic behavior of rat liver microsomal glucose-6-phosphatase. The rationale of our experimental findings obtained on native and Triton-modified microsomes and the partial purified glucose-6-phosphatase give rise to the conclusion that MTAS acts on the integral phosphohydrolase itself even in the native microsomal membrane. As the results from centrifugation-dilution experiments clearly indicate, glucose 6-phosphate : phosphohydrolase must have been affected by MTAS before the native membrane has been permeabilized by Triton-modification. Since glucose-6-phosphatase inhibited by MTAS cannot be regenerated by Triton-modification of the membrane this would exclude that distinct, separately operating transport proteins have been the target of the inhibitory agent. Therefore, interpretation of our data does not require participation of putative translocases as postulated by the 'translocase-catalytic unit concept' [1,2]. In analyzing the effect of MTAS on the kinetic parameters of microsomal glucose 6-phosphate hydrolysis, it is suggested that MTAS does not act on the catalytic area but most obviously modifies an enzyme domain responsible for maintaining the native, catalytically active conformation form.

Among the reactive groups of amino acid side chains of the enzyme protein, we would favor the sulfhydryl groups as prominent candidates for the modification reaction induced by MTAS, because (i) highly reactive sulfhydryl groups of glucose-6-phosphatase easily accessible from the cytoplasmic membrane surface are essential for maintaining enzyme catalysis [11,47]; (ii) at 0 °C MTAS completely prevents the inhibitory effect of DIDS and DASS which both are suggested to inhibit microsomal glucose 6-phosphate hydrolysis by chemical modification of sulfhydryl groups of the integral phosphohydrolase [12,13]; (iii) thiol compounds as glutathione, cysteine and dithiotreithol can easily block the inhibitory effect of MTAS on glucose-6-phosphatase by possibly scavenging the inhibitor and thereby preventing the protein sulfhydryl groups against oxidative attack. Moreover, our present experimental findings show that glucose 6-phosphate hydrolysis of MTAS-modified glucose-6-phosphatase is dependent on the temperature during glucose-6-phosphatase assay which implicates the presence of different conformational states of the enzyme at 0 °C and 37 °C exhibiting distinct catalytical properties. Therefore, these important results closely correlate with the findings obtained from studies on the effect of the sulfhydryl-group specific reagent *p*-mercuribenzoate on microsomal glucose 6-phosphate hydrolysis [11]. As elucidated from these studies cytoplasmic accessible sulfhydryl groups of the phosphohydrolase are a specific requirement for the native enzyme conformation. Interaction of these sulfhydryl groups with the mercurial promotes profound changes of the native, catalytically active behavior of the integral enzyme. The *p*-mercuribenzoate-modified hybrid membrane protein is found to exhibit kinetic properties which depend on the temperature upon microsomal glucose 6-phosphate hydrolysis. Subsequent Triton-modification of the microsomes is efficient in changing the kinetic properties of the *p*-mercuribenzoate-modified glucose 6-phosphate additionally. As the kinetic analysis of the inhibition of microsomal glucose-6-phosphatase in native microsomes by *p*-mercuribenzoate reveals that enzyme catalysis is inhibited competitively, whereas upon subsequent treatment of the microsomes with detergent the kinetic type of inhibition changes to a noncompetitive inhibition.

Thus, from these important observations and with regard to our findings from previous enzyme kinetic studies analyzing the effects of Triton X-114 on microsomal glucose-6-phosphatase [14] it is indicated that covalent modification of the integral protein and alterations within the native microenvironment of the microsomal membrane by detergent, are both efficient in generating an enzyme conformation with kinetic properties differing from those of the native form. While we were summarizing our present work independently van de Werve and coworkers have made an important contribution to explain the molecular mechanism of microsomal glucose 6-phosphate hydrolysis. Investigating the rapid kinetics of glucose 6-phosphate uptake and hydrolysis of rat liver microsomes it has been suggested that a hysteretic transition which is a major conformational change in the enzyme protein may account for latent activity and substrate specificity of glucose-6-phosphatase [48].

Taken together, several lines of evidence substantiate the conclusion that activity of hepatic microsomal glucose-6-phosphatase indeed is modulated by important changes of a flexible enzyme conformation induced by external (cytosolic) modifiers and internal factors of the surrounding microsomal membrane as it has been suggested recently by the 'conformation-substrate transport concept' [11]. A closer insight into the mechanism of the conformational transition, however, would be possible after successfull isolation of the pure and active glucose-6-phosphatase and concomitant insertion of the enzyme into liposomal membranes.

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