IN VITRO MODULATION OF RAT LIVER GLYOXALASE, II ACTIVITY

THESIS

Presented to the Graduate Council of the University of North Texas in Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

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August, 1988
Mbamalu, Godwin, E.  **In Vitro Modulation of Rat Liver Glyoxalase II Activity.** Master of Science (Biochemistry), August, 1988, 48 pp., 2 tables, references, 66 titles.

Glyoxylase II (Glo II, E.C. 3.1.2.6) catalyzes the hydrolysis of S-D-Lactoylglutathione (SLG) to D-Lactate and glutathione. This is the rate limiting step in the conversion of methylglyoxal to D-Lactate.

The purpose of the present study was to determine whether or not a relationship exists between some naturally occurring metabolites and *in vivo* modulation of Glo II. We have observed a non-competitive inhibition (~45%) of Glo II in crude preparation of rat liver by GTP (0.3 mM).

A factor (apparently protein), devoid of Glo II, when reconstituted with the purified Glo II, enhanced Glo II activity. This coordinate activation and inhibition of Glo II suggest a mechanism whereby SLG levels can be modulated *in vivo.*
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CHAPTER I

INTRODUCTION

In 1913, the discovery that phenylglyoxal could be converted to mandelic acid when rabbits were fed phenylglyoxal led to the discovery of the glyoxalase system (1). Subsequently, the glyoxalase system was demonstrated to effect the conversion of phenylglyoxal and methylglyoxal (pyruvaldehyde) to mandelic acid and lactic acid respectively in a variety of plant, yeast and animal tissues (2-5). Initial studies had tended to implicate the glyoxalase enzymes as an essential component of the fermentation process (glycolysis), since lactic acid is the final product of the glyoxalase system as well as in glycolysis. However, Lohman (6) demonstrated that the glyoxalase system was not an essential part of glycolysis and that the system required glutathione as a cofactor.

In 1951, Racker found that the glyoxalase system comprises two enzymes (7), namely glyoxalase I (E.C 4.4.1.5) and glyoxalase II (E.C. 3.1.2.6). The stereochemistry of the product was shown to be D-lactate when methylglyoxal was employed as the substrate (8-10). A kinetic mechanism of the reaction of glyoxalase I was proposed by Kermack and Matheson (11) who studied the steady state parameters of the reaction using free methylglyoxal and free glutathione as substrates. A second mechanism was subsequently proposed by Cliffe and Waley (12) which showed the true substrate for glyoxalase I to be a hemi-mercaptal adduct formed from methylglyoxal and glutathione in a non-enzymatic manner. Another mechanism was also proposed by Mannervik et al. (13) which utilized one or two alternative substrates. The one substrate process was
shown to involve the hemimercaptal adduct while the two substrate process involved glutathione as the first and methylglyoxal as the second substrate. The reaction catalyzed by the glyoxalase system, the conversion of methylglyoxal to D-lactate, is illustrated by the following processes.

\[
\begin{align*}
0 & \quad 0 \\
\text{CH}_3\text{-C-C-H} & + \quad \text{GSH} \quad \text{nonenzymatic} \\
\text{(methylglyoxal)} & \quad \text{(glutathione)}
\end{align*}
\]

\[
\begin{align*}
0 & \quad \text{OH} \\
\text{CH}_3\text{-C-CH-SG} & \quad \text{glyoxalase I} \\
\text{(hemimercaptal)} & \\
\text{CH}_3\text{-CH-C-SG} & + \quad \text{H}_2\text{O} \\
\text{(S-D-lactoylglutathione)} & \\
\text{OH} & \quad 0 \\
\text{CH}_3\text{-CH-C-SG} & + \quad \text{GSH} \quad \text{glyoxalase II} \\
\text{(D-lactic acid)} & \quad \text{(glutathione)}
\end{align*}
\]
The reaction involves an equilibrium formation of the hemi-mercaptal adduct from methylglyoxal and reduced glutathione. Glyoxalase I (Glo I) [S-D-lactoyl glutathione methyl lyase (isomerizing), E.C. 4.4.1.5] acts upon the hemi-mercaptal to form S-D-lactoyl glutathione (SLG), a thioester. This intermediate is then hydrolyzed by glyoxalase II (Glo II) [S-2-Hydroxyacylglutathione hydrolase, E.C. 3.1.2.6] to regenerate the reduced glutathione (GSH) and free D-lactic acid.

The formulation of the chemical mechanism of the reaction has met with some controversy. Initial studies proposed that Glo I catalyzes the transfer of a hydride ion equivalent from C1 to C2 of α-ketoaldehyde, resulting in the formation of a thioester, to be the limiting step (14,15). This is the accepted mechanism although Hall et al. (16) suggested an enediol proton transfer process. The reaction mechanisms of the two glyoxalase enzymes were shown to differ markedly. Glo I from human, porcine erythrocytes, rat liver, and yeast were suggested to be zinc-metalloenzymes by Arronson et al. (17). Ball and Vander Jagt demonstrated that the chemistries involved nucleophilic catalysis by histidine residues in the active site as the mechanism for Glo II hydrolysis of SLG (18).

While the products and kinetic parameters of the glyoxalase system are known, there is still no consensus by investigators on the natural substrate for Glo I and the physiological significance of the glyoxalase system. Two recent reports provide evidence that methylglyoxal is most probably the true substrate for the Glo I (19,20). Methylglyoxal was isolated from beef liver as its 2,4-dinitrophenylhydrazone and identified by comparison to synthetic methylglyoxal (19). Sato et al. (20) concluded that methylglyoxal formation in rat liver cells is a metabolic process, and that it is mostly derived from
dihydroxyacetone phosphate. It was later shown that \textit{Escherichia coli} contains an enzyme which converts dihydroxyacetone phosphate (DHAP) to methylglyoxal and inorganic phosphate (9,21,22). This enzyme, methylglyoxal synthase, was proposed to constitute the first step in a glycolytic by-pass from triose phosphates to pyruvate via methylglyoxal and D-lactate. There has been an unsubstantiated report on the presence of methylglyoxal synthase in goat liver (23). It has been suggested, however, that non enzymatic formation of methylglyoxal also occurs in glycolyzing tissues with triose phosphates serving as the immediate precursors.

Methylglyoxal was once thought to be involved in glucose metabolism as a component of the so-called non-phosphorylating glycolysis pathway but since this mode of methylglyoxal formation was considered to be a non-enzymatic side reaction, it was judged to be of no importance (21,24). Methylglyoxal, apparently the natural substrate of the glyoxalase system, and possibly some other \(\alpha\)-keto-aldehydes, has been postulated to function as a regulatory molecule in cell division (25-27). It was demonstrated that methylglyoxal exhibits a retarding effect on the development of several experimental tumors. Conditions which could lead to the accumulation of methylglyoxal, i.e., the observed decrease or absence of Glo II in neoplastic tissues, could have a negative effect on tumor growth (27,28). Further roles ascribed to the glyoxalase enzyme system include:

1) The production of methylglyoxal from aminoacetone deamination and the possibility that the glyoxalase system may therefore function in the degradation cycle of certain amino acids, namely glycine and threonine (29,30). This enzymatic conversion of amino acetone to
methylglyoxal was found in ox plasma and a few other species (31).

2) There is also the possibility that the glyoxalase system, together with glutathione, regulates porphyrin biosynthesis, since Glo I has been shown to convert γδ-dioxovalerate in the presence of reduced GSH to a product which is hydrolyzed by Glo II to form D-α-hydroxyglutamate (32).

3) Methylglyoxal, the natural substrate, and some glyoxalase inhibitors may function as possible antitumor agents (28, 29).

4) Gillespie et al. (33) have also shown that conditions where SLG could accumulate endogenously enduces histamine release from leukocytes.

5) The glyoxalase system has also been linked to microtubule aggregation (33, 34).

Over the years, Glo I has been the more studied enzyme of the glyoxalase system. It is a more stable enzyme than Glo II and with stands extensive dialysis without considerable loss of activity. It has been purified from a number of sources, such as calf liver, mouse lymphomasarcoma, porcine erythrocytes, mouse liver and rat liver (35-41).

Glo II, on the other hand, is less stable than Glo I, with some crude preparations losing activity rapidly at -30°, and upon dialysis (42, 43). The existence of polymeric forms of Glo II derived from a native enzyme in rat liver has been reported using sodium dodecyl sulfate electrophoresis (44). A single form of Glo II was found in rat erythrocytes but fractionation by isoelectric focusing was not performed (45).
Purification studies including isoelectric focusing suggested a single form of Glo II in both human liver (46) and calf liver (47). In contrast, Uotila (48), using similar techniques, separated Glo II activity in human erythrocytes into two peaks, showing different substrate specificities and different PI values.

Glo II has been purified from many sources (46,47). Rat liver Glo II has been purified to homogeneity in this laboratory using a rapid two-step affinity chromatographic procedure (44). Homogeneous Glo II has been separated into two forms (a, pI= 8.0; b, pI= 7.4) from both liver and brain of Wistar rats by column isoelectric focusing (50). These a and b forms were shown to have similar molecular weights and kinetic constants with three substrates. Chemical modification studies also show a close similarity. Whether these results indicate distinct structures (sequences) for a and b forms still remains to be demonstrated.

Recently, Glo II has been shown to be present in rat liver mitochondria and to comprise 10-15% of the total rat liver Glo II activity. The results implicated mitochondrial Glo II species which are different from the cytosolic Glo II in substrate specificity. Isoelectric focusing gave five separate forms of the enzyme with PI values ranging from 6.6 to 8.1 (51).

Recent evidence suggests that the glyoxalase system functions through the regulation of cellular SLG concentrations and indirectly by affecting the SLG mediated functions. The cellular concentrations of SLG, the Glo II substrate, have been quantitated in resting and activated human neutrophils (52). SLG concentrations range from 0.2-0.4 nMol/10^6 cells in human neutrophils; however, when neutrophils are activated, the SLG concentration increases 100%; concomitantly the activity of Glo I increases by 20-40% while that of Glo II decreases by 20-40%. This Glo II
decrease was suggested to be the result of a non-competitive inhibition by an unknown inhibitor (34,54). Microtubule assembly, phagocytosis and increased respiration have been shown to be associated with reduced Glo II activity (53-55).

Gillespie et al. (56) demonstrated that while SLG potentiates microtubule assembly, Glo II inhibits the same in vitro in neutrophils. Since Glo II catalyzes the rate-limiting step of the glyoxalase system in the resting cells, where the activity of Glo II is approximately 16% of the activity of Glo I, the mechanism of microtubule induction and inhibition has been proposed to be mediated mostly by Glo II (57,58).

In the present study, we will demonstrate that some naturally occurring metabolites, e.g. guanosine and adenosine triphosphates, inhibit Glo II in a non-competitive manner and in a limiting fashion. We will propose that conditions which increases the cellular concentrations of these nucleotides could induce increased SLG concentrations in vivo by affecting Glo II inhibition. The subsequent results of which will be microtubule elongation (assembly), histamine release, etc., by as yet an undetermined mechanism. Since the inhibition pattern from Glo II observed in this laboratory closely resembles that reported by Thornally et al. (52), it is possible that the in vivo concentrations of these or similar metabolites are involved in the modulation of Glo II activity and thereby SLG concentrations in vivo.

The presence of a Glo II stimulating factor in crude rat liver preparations will also be demonstrated. The factor will be partially purified using chromatographic techniques. The stiochiometric requirement for maximum activation of Glo II by this factor will be determined. The effect of some nucleotides and their analogues on Glo II stimulation by this factor will also be shown. A proposal as to how the factor
and the nucleotides could co-ordinately modulate Glo II activity \textit{in vivo} will also be given.
CHAPTER II

METHODS

**General.** Swiss mice (24-27g) were purchased from Timco, Houston, Tx. Reduced glutathione, Sepharose 4B-200, Blue Dextran, Sephadex G-100, sodium acetate, guanosine 5' triphosphate agarose and phosphate buffer were purchased from Sigma Chemical Company St. Louis, Mo. Diamide, 5,5'-dithio-bis (2-nitro benzoic acid), DTNB; and Mops were obtained from Fisher Scientific Company, Fair Lawns, NJ. Nucleotides, nucleosides and structurally related compounds were purchased either from Sigma Chemical Company, or from Calbiochem, Los Angeles, CA. S-D-Lactoyl glutathione was prepared and purified by the procedure of Uotila (59). All other chemicals were of highest purity obtainable from commercial sources. All chromatographic columns were run at 4° and enzyme preparations stored at -30°.

**Crude enzyme preparation.** Young rats under normal dietary conditions were anesthetized with ether and then sacrificed by decapitation. After bleeding for approximately one minute, the livers were removed, weighed, and homogenized with a Potter-Evehjem tissue homogenizer at 0° in 2.5 volumes of medium A (5mM Mops buffer, pH 6.8, containing 25% glycerol). The homogenate was then centrifuged at 20,000 x g for one and one half hours. The supernatant solution obtained was approximately 2.5 ml per gram wet weight of liver. Further centrifugation was performed 85,000 x g for one and one quarter hours. The supernatant solution, after removal of lipid material, was then stored at -30°. This solution
served as the crude glyoxalase II enzyme source.

**Purification of Glyoxalase II by affinity chromatography.** A carboxbenzoxyl glutathione CBG-Sepharose affinity column (49) was used for the purification of Glo II. The column was washed and equilibrated with medium A overnight at 4\(^\circ\). The crude enzyme preparation was diluted 1:1 with medium A and 1.0ml of the diluted enzyme was added for each 0.4ml column volume of CBG-Sepharose. The column was then washed with two volumes each of increasing concentration of Mops buffer pH 6.8 (5, 10, 20, 30, and 40mM containing 25% glycerol). The washings were continued until no protein was detected in the effluent. The bulk of the protein in the crude preparation passed through the column in the early washings.

Glo II activity was then eluted by the addition of 5mM CBG in Medium A. Generally, all the Glo II activity was eluted by use of 4 volumes of CBG solution (as compared to the volume of crude enzyme added to the column). All fractions were assayed for Glo II activity and the most active fractions were pooled. The pooled fractions were then diluted 1:5 with Medium A. Further purification was achieved by passing the diluted, pooled Glo II fractions through a second CBG-Sepharose affinity column of the same volume as in the previous affinity step. The column was washed and Glo II activity eluted as in the previous affinity step above. Active fractions were pooled and utilized in activation and inhibition studies. This method of purification is similar to the previously described procedure (49) and it has been shown to yield a homogenous preparation of rat liver Glo II.

**Routine Glyoxalase II assay.** The reaction mixture contained 0.4 mM S-D-lactoyl glutathione (SLG); 0.2 mM 5,5'-dithio bis (2-nitro benzoic acid; DTNB) in 5 mM Mops pH
6.8; and a rate limiting amount of Glo II activity. The total reaction volume was 1.0 ml. Enzyme activity was measured by following the increase in absorbance at 412 nm resulting from the interaction of DTNB with the enzymatically produced GSH ($\varepsilon_{412}^{\text{MM}} = 13.6 \text{ mM}^{-1}\text{cm}^{-1}$). The reaction was followed for 2 to 4 minutes on a varian DMS 70 UV-visible spectrophotometer. A blank without enzyme was always included and the reaction was usually initiated by the addition of enzyme (0.04 to 0.09 I.U.).

The initial enzymatic rate of SLG hydrolysis was measured from the linear portion of the registered absorption increase. A unit of Glo II activity is defined as the amount of enzyme catalyzing the hydrolysis of 1 μmole of S-β-lactoylglutathione per minute under standard assay conditions. Specific activity is expressed in Units/mg protein.

Effects of some nucleotides on Glo II activity. The nucleotides tested were GTP, GDP, ATP, ADP, AMP, CDP, CMP, UMP, NADH, NADPH, and the GTP analogue, [5' guanylylimidodiphosphate, also called guanosine 5' (β,δ-imido) triphosphate; GppNHp]. Solutions (10 mM) were prepared in medium A and the final concentration in the assay ranged from 0.1 to 0.5 mM. Enzymatic activity was followed (employing crude and purified Glo II preparations) by measuring the absorbance increase at 412 nm as described above. Percent inhibition was determined by measuring the ratio of Glo II activity in the presence and absence of the nucleotides.

Synthesis of glutathione-Affi gel 10 affinity column. Glutathione, 350 mg, was dissolved in 50 ml of cold sodium acetate, pH 5.5. The pH was then adjusted to 5.0 with 10% sodium hydroxide. Affi Gel 10 (Bio Rad), 25 ml wet volume, was washed with three volumes of cold 10 mM sodium acetate,
pH 5.0, to remove isopropyl alcohol, and then added to the 
GSH solution. Slow stirring of the resulting suspension was 
continued overnight at 4^0. The white solid material, after 
washing several times with 10 mM phosphate buffer, pH 6.5, 
gave a positive ninhydrin test in water suspension, but was 
negative for sulfhydryl groups when tested with Ellman's 
reagent (DTNB). This indicated that the glutathione was 
attached to Affi gel 10 by a thioester linkage. This 
affinity material, useful for purification of Glo II from a 
variety of sources, is stable in aqueous suspension at 4^0 for 
at least six months

Preparation of crude Glyoxalase II stimulating factor. 
The initial fractions obtained from the first CBG - Sepharose 
column, (used for Glo II purification), see Methods section, 
contained most of the Glo II stimulating activity but only 
traces of Glo II activity. These early fractions were 
combined and utilized as starting material for the 
purification of the stimulating factor. The last traces of 
Glo II activity were removed by passing the combined 
fractons through a small glutathione-Affi gel 10 affinity 
column (0.1ml column volume/ml combined fraction). The 
effluent, which contained no detectable Glo II activity, was 
stored at -30^0. This served as the crude stimulating factor, 
and was also called the Glo II factor for ease of 
identification.

Partial purification of Glyoxalase II stimulating 
factor. The fractions obtained from the glutathione-Affi gel 
10 affinity column were employed. A one ml portion of the 
combined fractions was added to a one ml column of GTP 
agarose (GTP attached through ribose hydroxy to epoxy 
activated, 4% cross linked agarose [Sigma]), which had been 
equilibrated with medium A.
The column was washed with 5 volumes of medium A and one ml fractions of effluent were collected. Elutions were carried out by the additions of three volumes of 1.0 mM GTP. The one ml elution fractions obtained were stored at -30° and assayed later for Glo II stimulating activity.

**Reconstitution and other pre-incubation experiments.**

Reconstitution was performed by incubating purified Glo II and Glo II stimulating factor in a 1:1 ratio at 4° overnight. Putative association of Glo II and Glo II stimulating factor was measured by the increase in Glo II activity at various time intervals. A control containing purified Glo II alone and purified Glo II plus medium A (1:1, v:v) was employed. Enzyme activities were monitored by using equal aliquots from the incubation mixture as enzyme source and measuring the increase or decrease in activity with time. The assay method was described earlier. Purified Glo II was also reconstituted with the Glo II factor in the presence and absence of 1 mM GTP and the GTP analogue (GppNHp); the final concentration of GTP or GppNHp in the assay mixture was 0.04 mM. Activity changes in response to GTP or GppNHp were monitored as described earlier.

**Demonstration of Glyoxalase II stimulating factor complex using gel filtration chromatography.** The sample and control were prepared as described under reconstitution experiments in the absence of GTP or GppNHp. Sephadex G-100 was washed and equilibrated in medium A and packed in a 2 x 30 cm column. A control sample, 1.5 ml, containing no Glo II stimulating factor, was introduced to the column and eluted with medium A. The reconstituted sample (Glo II + Glo II factor), 1.5 ml, was then introduced to the column and similarly eluted as above. Fractions, 1.0 ml, were collected
and assayed for Glo II activity and the active fractions were pooled for use in studies of nucleotide effects.
CHAPTER III

RESULTS AND DISCUSSION

As part of our continuing study of the glyoxalase enzymes, we have sought in this investigation to explore the possible mode of in vivo, Glo II regulation. The elucidation of the regulatory mechanisms of these enzymes will help in understanding the role of Glo II in cellular metabolism, which is still obscure. There are several findings which suggest that the enzyme must be regulated in vivo.

a) The enzyme responds to certain cellular conditions, e.g. it is found at very low or lowered levels in proliferating tissues under certain pathological conditions, e.g., regenerating liver after heptectomy (60,61) and tumors (62).

b) Microtuble assembly is apparently induced by increased endogenous levels of the Glo II substrate, SLG. Such increases accrue under conditions where Glo II is inhibited (33). Associated events include increased respiration.

c) There is an increase in the activity of Glo II relative to Glo I during differentiation of many cell lines, e.g., N-methylformamide-treated human promyelocytic leukemia HL60 cells (64).

d) The activity of Glo II decreases by approximately 40% when human neutrophils are activated, allowing SLG concentrations to double, which may mediate microtubule assembly (65).
In the present study, it was decided to examine the influences of certain naturally occurring metabolites which are possible respiratory products. Thus, the effect of various nucleotides on Glo II activity in crude rat liver preparations were initially studied (Table 1). The results showed that some of these nucleotides inhibit Glo II. The purine nucleotides were better inhibitors than the pyrimidines and the guanosine nucleotides better than the adenosine nucleotides. Guanosine triphosphate at 0.3 mM concentrations gave a 45-50% inhibition, and this was consistently observed in different crude preparations. The diphosphate forms of all nucleotides tested were found to be generally less inhibitory. A GTP analogue, 5'-guanylylimido diphosphate (GppNHp), was also used and showed similar inhibition to that of GTP, thereby suggesting the absence of involvement of GTPase activity. Therefore, it seemed reasonable that Glo II inhibition in vivo could involve the transfer of purine nucleoside triphosphates to the active or regulatory site of Glo II or a Glo II regulatory protein. Though guanosine triphosphate is a better inhibitor of Glo II than adenosine triphosphate, there is reason to suggest that the latter nucleotide is more tightly bound to its binding site (Fig 1). Glo II inhibition, when both GTP and ATP are present in the assay, is no greater than that given by ATP alone. Thus, it appears likely that under conditions where ATP and GTP are present in saturating conditions for a common binding site, the observable inhibition of Glo II might not exceed that recorded for ATP alone (= 30-40%).

Unlike most of the synthetic analogues and derivates of the Glo II substrate, e.g., carbobenzoxy glutathione, which generally show a competitive inhibition pattern (63), the inhibition by these nucleotides namely ATP and GTP, appears to be non-competitive as shown in Fig 2. This is consistent with the findings of Gillespie et al. who showed that under
TABLE I

EFFECTS OF VARIOUS NUCLEOTIDES ON GLO II ACTIVITY IN CRUDE RAT LIVER PREPARATIONS.

<table>
<thead>
<tr>
<th>Nucleotide Added</th>
<th>% Inhibition of Glo II</th>
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<tbody>
<tr>
<td>GTP</td>
<td>49.0</td>
</tr>
<tr>
<td>GppNHp</td>
<td>47.0</td>
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<tr>
<td>GDP</td>
<td>42.0</td>
</tr>
<tr>
<td>GMP</td>
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<tr>
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<tr>
<td>dTTP</td>
<td>9.9</td>
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Nucleotide concentrations were 0.3mM in the assay mixture. The assay procedure is described in Methods section.
Figure 1

Inhibition of crude Glyoxylase II activity by GTP and ATP.

Percent inhibition of Glo II in crude preparation by GTP, ATP and ATP + GTP. Initial enzymatic rates were measured at various nucleotide concentrations. When ATP + GTP were both present, the concentration of each nucleotide was at the level depicted on the figure. (●) % inhibition by GTP; (◇) % inhibition by both GTP and ATP; (◆) % inhibition by ATP.
PERCENT INHIBITION

NUCLEOTIDE CONCENTRATIONS (mM)
Figure 2

Double reciprocal plot of GTP and ATP inhibitions of crude Glo II.

The substrate was SLG. Assay details are given in the methods section. (■) 0.2mM GTP; (●) 0.2mM ATP; (■) no nucleotide addition.
conditions where SLG concentrations increase, leading to microtubular induction, Glo II is non-competetively inhibited (33). More recently, it has been shown that microtubule induction is associated with an approximate 40% decrease in Glo II activity (52), which is also in agreement with our results involving GTP inhibition in vitro. Since most of the recent suggested functions of Glo II are through the modulation of its substrate concentration (SLG), it is logical to suggest that Glo II regulates SLG concentration and its related functions through a cellular response which increases purine nucleotide levels. These increased nucleoside triphosphate levels could inhibit Glo II in vivo, in a non-competetive manner and thus allow SLG to accumulate. Since non-competetive inhibition is not generally reversed by excess substrate, SLG accumulation may not by itself reverse this process but could be channeled to microtuble assembly, histamine release, etc. (33).

The most studied Glo II inhibitor is carbobenzyo glutathione (CBG). This compound was first synthesized in this laboratory (63). Like most other studied synthetic substrate analogues of Glo II, it inhibits Glo II competetively and thus differs markedly from the nucleotide inhibition. To explore further the combined influences of these inhibitors, their effects on Glo II in crude rat liver preparations were studied. The results (Table II) indicate that under conditions where Glo II is maximally inhibited by GTP, addition of CBG will cause a further inhibition of Glo II. This suggests that:

1) CBG and GTP have separate binding sites on the same protein or,

2) CBG and GTP have binding sites on different proteins:
TABLE II

EFFECTS OF aCBG AND GTP ON GLO II ACTIVITY IN CRUDE RAT LIVER PREPARATIONS

<table>
<thead>
<tr>
<th>Nucleotide Additions to Assay</th>
<th>Activity (I.U./Min.)</th>
<th>Percent Inhibition of Glo II</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONE</td>
<td>0.052</td>
<td>0.00</td>
</tr>
<tr>
<td>0.3 mM GTP</td>
<td>0.029</td>
<td>45.0</td>
</tr>
<tr>
<td>0.5 mM GTP</td>
<td>0.028</td>
<td>46.0</td>
</tr>
<tr>
<td>0.1 mM CBG</td>
<td>0.024</td>
<td>54.0</td>
</tr>
<tr>
<td>0.3 mM GTP + 0.1 mM CBG</td>
<td>0.018</td>
<td>65.0</td>
</tr>
</tbody>
</table>

Assay conditions are described in the Methods section.
aCBG is S-Carbobenzoxyglutathione.
To explore further the possibility that GTP binds to a different protein which in turn affects Glo II activity, crude rat liver preparations were preincubated with 1.5 M sodium chloride in medium A at 4°C. The rationale for this was that a high salt concentration might cause a dissociation of any non-covalent complex. Two effects were observed:

1) There is a time dependent loss (3-24 hours) of Glo II activity.

2) There is also a concomitant desensitization of Glo II to GTP inhibition.

These effects suggest a slow dissociation of a Glo II-complex (protein-protein complex?) which imparts GTP sensitivity. In order to study this possibility in more detail, purified Glo II was prepared and studied for GTP sensitivity. GTP was found not to inhibit pure Glo II. (Fig. 3). This is in agreement with the above proposal of a regulating factor which, when complexed with Glo II, imparts GTP sensitivity. This also suggests that the nucleotide binding site is on a dissociable factor molecule or possibly is an "induced" site on Glo II resulting from complexation with a factor molecule.

When purified Glo II was pre-incubated with a protein fraction (see Factor preparation, Methods section) from which all Glo II activity had been removed, GTP inhibition was restored in a time-dependent process (Fig 4). It was also noted that during these pre-incubations with the protein

a) CBG binds on the active site of Glo II

b) GTP binds on a different protein that affects the activity of Glo II.
Figure 3

Effect of GTP on rat liver Glo II activity in crude and purified preparations.

Effects of GTP on purified and crude Glo II. Initial enzyme rates were measured in the presence and absence of various concentrations of GTP. Data are plotted as percent of inhibited activity. (○) 0.04 I.U. purified Glo II; (►) 0.04 I.U. crude Glo II.
fraction, the activity of Glo II was greatly enhanced. To further demonstrate this apparent activation process, and to study the effect of some nucleotides on this factor mediated Glo II activation, Glo II was pre-incubated with the factor in the presence and absence of GTP or GppNHp. Under our laboratory conditions, Glo II was generally activated ($\approx 2.5x$) over its original activity in a time-dependent manner by such preincubations (Fig 5). Since the purification technique employed in this study (49) results in only a 25% to 30% recovery of Glo II, much of the activity losses reported can be explained by the separation of the activating factor from Glo II. Note that when Glo II is pre-incubated with the protein fraction in the presence of GTP or GppNHp, Glo II activation is significantly decreased.

The protein fraction containing the activating and sensitizing components, when heated at 70$^\circ$ for 10 minutes, loses its ability to either activate or impart GTP sensitivity to Glo II. Further, a dialysis experiment showed that the factor is retained within the dialysis membrane. Partially purified protein fraction (See Methods section) shows a saturation activation at $\approx 2.8$ mg protein per 0.04 units purified of Glo II (Fig.6a). When preincubated in this ratio, there is a time-dependent Glo II activation (presumably a reassociation) and a restoration of GTP inhibition comparable with that of crude Glo II preparations (Fig. 6b). Metal ions, e.g., magnesium, had no influence in this activation and inhibition in either crude or purified preparations.

The GTP effect on the Glo II complex was utilized in an attempt to purify further the protein factor by affinity chromatography. GTP was employed as the ligand (see Methods section). The results obtained (Fig. 7) showed that a second peak having a GTP-sensitization capacity is eluted from the
Figure 4

Time dependent restoration of GTP inhibition of Glo II by Glo II factor.

Catalytic amount of Glo II was pre-incubated with the saturating amounts of the protein factor. Fractions were then assayed for GTP sensitivity. Activity was obtained by measuring the initial enzyme rate. Assay conditions are described in the Methods section.
Figure 5

Time dependent re-association of purified Glo II with activating factor in the presence and absence of GTP and GppNHp.

The assay methods are described in the methods section. The GTP and GppNHp concentrations in the assay mixture were 0.04mM. Pre-incubations were performed at 4°C over night. About 0.04 units of Glo II was used. (►) pure Glo II + medium A 1:1; (▲) pure Glo II + medium A in 0.3mM GppNHp (●) pure Glo II + Activating Factor + GppNHp; (▲) pure Glo II + Activating Factor + GTP; (■) pure Glo II + Activating Factor 1:1.
Figures 6a and 6b

Effects of Pre-incubation of partially purified Glo II activating factor on activation of Glo II and the effects of GTP present in the assay mixture.

Different amounts of the activating factor ranging from 0 to 6 mg were pre-incubated with 0.04 units of purified Glo II over night. The Procedure was described in the methods section. (○) activity changes of pure Glo II-factor complex at different factor concentrations; (●) percent GTP inhibition of pure Glo II-factor complex. The GTP concentration in the assay mixture was 0.3mM.
PERCENT ACTIVATION OF GLO II

PARTIALLY PURIFIED ACTIVATING FACTOR (mg Protein)

PERCENT INHIBITION OF ACTIVATED COMPLEX BY GTP

PERCENT ACTIVATION
column by 1 mM GTP. The first peak, which also showed activation/GTP sensitization capacity, is obviously unbound material passing through the very low capacity affinity column. The elution of the activating/sensitizing factor by GTP suggests that the GTP binding site resides on the factor (probably protein) which binds to Glo II. The lack of GTP effect on pure Glo II (above) indicates that no such binding site exists and that the activation/inhibition phenomenon is an induced process on the Glo II protein by the factor. The factor contained in the GTP fractions was assayed for protein by the Bradford method (66). Results indicate that the factor was purified approximately fourfold by passage through the GTP-affinity column.

To demonstrate further the existence of a Glo II-stimulating factor complex, gel permeation chromatography was carried out (Fig. 8). Studies in this laboratory had previously shown the existence of both monomeric and multimeric forms of Glo II in vitro (44). These forms were also shown to interconvert among the monomeric, dimeric, trimeric, and tetrameric forms. It was suggested that the formation of multimeric forms of Glo II may be a regulated process in vivo (44). Whether any of these multimeric forms is capable of adopting a GTP sensitive conformation is very questionable. It is possible however that since Glo II probably predominates as a monomer in vivo (as observed in vitro, 44), the protein factor might also function in the regulation of the formation of these multimeric forms in vivo. These multimeric forms in the presence of the factor might adopt GTP inhibition sensitivity. This is because conditions leading to the formation of these multimeric forms of Glo II in vitro, in the absence of the protein factor showed no GTP response in the purified extract. Observed results in Figure 8 clearly showed a highly activated enzyme in the presence of the factor, with an apparently higher
Figure 7

Elution profile from GTP agarose column of a Glo II stimulating factor.

GTP agarose column elutions were preformed as described in the methods section. The column was eluted with several portions of medium A. The retained factor was then eluted with 1.0mM GTP. The eluted fraction were then pre-incubated with Glo II as described, and then assayed for sensitivity to GTP inhibition. The GTP concentration in the assay was 0.3mM. (+) percent inhibition by GTP (0.3mM); (++) protein content (mg).
PERCENT INHIBITION BY GTP

ACTIVATING FACTOR FACTOR (mg Protein)

FRACTION NUMBER
molecular weight, supporting the existence of a Glo II-factor complex. Since the purified Glo II eluted as a single peak, thereby indicating that the reference Glo II was a monomer, it would suggest that the factor probably combines with the predominating monomeric forms in vivo, in regulating Glo II activity.
Figure 8

Sephadex G-100 column chromatography of pure Glo II pre-incubated with and without Glo II stimulating factor.

Experimental details of chromatographic methods and enzyme assays were described in the methods sections. Stimulating factor and purified Glo II were pre-incubated over night at 4°. Fractions were collected at same time intervals in 1.0ml volumes and assayed immediately for Glo II activity. (▲) purified Glo II + activating factor; (▼) purified Glo II.
GLO II
ACTIVITY 0.02
(umoles/min)
Evidence has been presented in this study for a Glo II stimulating factor which might regulate Glo II activity in vivo. The stimulating factor which appears to be a protein in nature is present in crude Glo II preparations but lost during Glo II purification. Metallic ions such as magnesium were not essential for this stimulatory and inhibitory process. The stimulating factor is stable to dialysis but destroyed by heating at 70°C for five minutes.

Pure Glo II could be activated at least two fold and inhibited about 45 to 50% by the combined influence of the factor and GTP. Since the inhibition values observed in this study closely resemble those recently demonstrated by Thornally et al. (52), when neutrophils are functionally activated, it appears that the associated events - namely, increased respiration, resulting in increased purine nucleoside triphosphate concentrations, could cause inhibition (45-50%) of Glo II activity. These are also the conditions leading to higher substrate (SLG) concentrations and the other secondary effects mentioned earlier.

The possible mechanisms of these in vivo Glo II modulations might be the ability of Glo II to assume several conformations in the presence and absence of the activating factor. Since uncomplexed Glo II is less active and unaffected by nucleotides but stimulated in the presence of the factor, it is possible that:

a) The factor complexes with and stimulates the Glo II catalytic activity and also promotes the binding of GTP
to the Glo II protein.

b) Glo II complexes with the factor, and adopts a more catalytically active conformation which is inhibited by nucleotides binding with the factor portion of the complex.

c) It may be possible that the activating protein has a higher affinity for GTP than for Glo II such that at high GTP concentrations, GTP-protein complex is favored over protein-Glo II complex.

Our results would suggest that proposal (b) above would be a more likely mechanism for in vivo, modulation of Glo II activity. With respect to proposal a) the binding of GTP apparently occurs with the Glo II factor and not with Glo II (affinity chromatography experiment, Fig. 7). This finding does not support proposal a). While the factor-Glo II complex is dissociable, purine nucleoside triphosphates do not cause a complete dissociation of this complex. This is evident since the activity of factor-Glo II complex in the presence of saturating amounts of these nucleotides is higher than that of purified Glo II alone (Fig. 5). This finding would tend to rule out proposal c). The data presented herein are consistent with proposal b); a plausible regulatory process is presented in (Fig. 9). This proposed process is consistent with the finding that nucleotides do not bind with Glo II but rather bind with the Glo II factor. This binding results in an inhibition of the activity of the Glo II-factor complex.

Future interesting studies in this area would be to investigate further the cellular concentrations of GTP and ATP when neutrophils are activated, and when human HL60 cell lines are differentiating and compare changes in nucleotide concentration during both processes. Also, a complete
Proposed mechanism for the modulation (activation and inhibition) of Glo II. Details are given in the Methods Discussion and Summary Sections.

- **I** = Uncomplexed Glo II (Less Active)
- **II** = Protein (Activating) factor
- **III** = Activated Glo II
- **IV** = Inhibited Glo II
purification and study of the activating/inhibiting protein would supplement our understanding of this apparent regulatory system.
REFERENCES