0026-895X/88/030395-06$02.00/0

MOLECULAR PHARMACOLOGY, 34:395-400

Inhibition of Glucokinase by Alloxan through Interaction with SH Groups in the Sugar-Binding Site of the Enzyme

SIGURD LENZEN, SVENJA FREYTAG,[1] and UWE PANTEN

Institute of Pharmacology and Toxicology, University of Göttingen, D-3400 Göttingen, Federal Republic of Germany

Received February 10, 1988; Accepted June 8, 1988

SUMMARY

Alloxan is known to inhibit pancreatic B cell and liver glucokinase and glucose protects the enzyme against inhibition. The dithiol 1,4-dithiothreitol (1,4-DTT) protected against and reversed the inhibition of glucokinase by alloxan. An investigation into the structure-activity relationship using a variety of different dithiols demonstrated that the ability of the dithiols to protect against and to reverse the inhibition of glucokinase by alloxan was dependent on the spacing between the SH (thiol) groups of the various dithiols. Only 1,3-dimercaptopropane, 1,4-dimercaptobutane, 1,4-dithioerythritol, and 1,4-DTT, with intermediate spacing between the SH groups, reversed the inhibition of glucokinase induced by alloxan. Dithiols with two vicinal SH groups such as 1,2-dimercaptoethane and 2,3-dimercaptopropanol (BAL) were ineffective in the same way as dithiols with more widely spaced SH groups such as 1,5-dimercaptopentane and 1,6-dimercaptohexane. Except for 1,6-dimercaptohexane, all dithiols also protected glucokinase against the inhibition of alloxan. The monothiol cysteine, but not glutathione, a tripeptide monothiol, also protected glucokinase against alloxan inhibition but both were unable to reverse the inhibition. Like alloxan, other dithiol reagents such as ninhydrin, *N*-ethylmaleimide, and maleimide inhibited glucokinase. Glucose and 1,4-DTT protected glucokinase against this inhibition. 1,4-DTT partially reversed this inhibition. It is concluded, therefore, that the mechanism of inhibition of glucokinase by alloxan is a reaction of alloxan with two adjacent SH groups in the depth of the sugar-binding site of the glucokinase, with formation of a disulfide bond and concomitant inactivation of the enzyme. Because glucokinase can couple changes in the blood glucose concentration to changes in the glycolytic flux rate and corresponding changes in the rate of insulin secretion, it may function as a glucose signal recognition enzyme in the pancreatic B cell. This mechanism of interaction of alloxan with glucokinase may thereby provide an explanation for the ability of alloxan to inhibit glucose-induced insulin secretion.

Alloxan (1) may inhibit glucose-induced insulin secretion and might be pancreatic B cell toxic by virtue of its ability to react with SH-containing cellular structures (2–4). The pancreatic B cell glucokinase may be such a structure, because alloxan inhibits this enzyme (5–8).

Glucokinase in pancreatic B cells has the characteristics that qualify it as a signal recognition enzyme that can couple changes in the blood glucose concentration to corresponding changes in the glycolytic flux rate and hence in insulin secretion (for review, see Ref. 9). However, whereas Meglasson *et al.* (7) proposed that the reactive site of the glucokinase for alloxan was not the active site of the enzyme, Lenzen *et al.* (8, 9) concluded that alloxan inhibited glucokinase through interaction with the sugar-binding site of the enzyme, because not only the substrates glucose and mannose (8) but also the enzyme inhibitors mannoheptulose (6, 8) and *N*-acetylglucosamine (7, 9) provide protection of the glucokinase against alloxan inhibition, when bound to the active site of the enzyme (for discussion, see Ref. 9). In order to elucidate the mechanism underlying the interaction of alloxan with thiol groups of the glucokinase, we compared the effects of the thiol reagent alloxan (10, 11) with those of its more stable analogue ninhydrin and with other thiol reagents, such as *N*-ethylmaleimide (12), maleimide (12), and iodoacetamide (13). In addition we tested the ability of a variety of monothiols and dithiols to protect glucokinase against alloxan inhibition as well as to reverse the inhibition of the enzyme after interaction with alloxan. These studies provide a model of the molecular mechanism underlying the inhibition of glucokinase by alloxan through interaction with SH groups in the sugar-binding site of the glucokinase.

Due to the limited availability of pancreatic islet tissue for studies on pancreatic B cell glucokinase, we also used liver glucokinase, which is functionally and immunologically identical with pancreatic B cell glucokinase (8, 9). Some of the results have been presented in abstract form (14).

This work was supported by the Deutsche Forschungsgemeinschaft.
[1] Some of the results have been obtained during medical thesis work by S.F.

ABBREVIATIONS: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GSH, glutathione; 1,2-DME, 1,2-dimercaptoethane; 1,3-DMP, 1,3-dimercaptopropane; 1,4-DMB, 1,4-dimercaptobutane; 1,5-DMP, 1,5-dimercaptopentane; 1,6-DMH, 1,6-dimercaptohexane; 2,3-DMP, 2,3-dimercaptopropanol (BAL); 1,4-DTT, 1,4-dithiothreitol; 1,4-DTE, 1,4-dithioerythritol.

Experimental Procedures

Methods. Pancreatic islets from *ob/ob* mice (50–60 g body weight) were isolated from the pancreas after collagenase digestion (15). The pancreas from *ob/ob* mice has large islets that contain more than 90% B cells. Liver was obtained from fed Wistar rats (200–250 g body weight). Pancreatic islets and liver were homogenized in ice-cold homogenization medium (20 mM HEPES, 210 mM mannitol, 70 mM sucrose, pH 7.4) (16), supplemented with 0.1–0.5 mM 1,4-L-DTT and 5% glycerol. Cytoplasmic supernatant was obtained by three successive centrifugation steps at 4° and at 700 × *g*, 8000 × *g*, and 100,000 × *g*, respectively (16). This procedure kept all glucose-phosphorylating enzyme activity in the active state (8, 9). Rates of glucose phosphorylation in 100,000 × *g* soluble cytoplasmic fractions were assayed at 37° and pH 7.4 by recording the increase in absorbance at 340 nm in 250 μl of a reaction mixture containing 20 mM HEPES (pH 7.4), 125 mM KCl, 7.5 mM $MgCl_2$, 5 mM ATP, 0.5 M NADP, 0.35–0.70 units/ml glucose-6-phosphate dehydrogenase from baker's yeast, 0.005–0.010 units/ml 6-phosphate-gluconate dehydrogenase plus soluble cytoplasmic supernatant from pancreatic islets (15–20 μg of protein in 50 μl) or liver (15–20 μg of protein in 10 μl) (8, 17). Glucokinase activity was assayed in this test medium in the presence of 100 mM D-glucose. Hexokinase activity was assayed in the presence of 1 mM D-glucose and subtracted from the total activity recorded at 100 mM D-glucose to give glucokinase activity. None of the inhibitors tested inhibited hexokinase activity significantly at concentrations that yielded half-maximal inhibition of glucokinase. Blank values of the increases of absorbance were obtained for hexokinase and glucokinase activities in the absence of cytoplasmic supernatant and were subtracted from the increases of absorbance in the presence of cytoplasmic supernatant before calculation of enzyme activities. Protein was determined according to the method of McKnight (18). In the experiments on glucokinase inhibition, cytoplasmic fractions were preincubated for 5 min with the inhibitors in the absence of glucose before measurement of the glucose phosphorylation rate. Alloxan and ninhydrin were dissolved in 0.01 M HCl and kept on ice before the experiments.

1,2-DMP increased blank values in the enzymatic glucokinase test, which were subtracted from the increases of absorbance in the presence of cytoplasmic supernatant before calculation of enzyme activities. The D-isomer of 1,4-DTT was unable to protect glucokinase against alloxan inhibition in control experiments. Thus, as 1,4-DTE was commercially available only in the DL-form, the concentration of DL-1,4-DTE was doubled, to allow comparison of the L-form of 1,4-DTE with other L-dithiols tested at the same concentrations. 1,4-DTT in its oxidized form, as well as cystine, was also unable to protect glucokinase against alloxan inhibition in control experiments.

Materials. 1,2-DME, 1,3-DMP, and 1,6-DMH were purchased from Aldrich, Steinheim, Germany; ATP (disodium salt), NADP (disodium salt), 6-phosphogluconate dehydrogenase, and collagenase were from Boehringer, Mannheim, Germany; 1,4-DMB and 1,5-DMP were from K & K Fine & Rare Chemicals, Plainview, NJ; D-glucose, D-mannitol, alloxan (monohydrate), ninhydrin, GSH (reduced), and all other reagents of chemical grade were from Merck, Darmstadt, Germany; sucrose, glycerol, and 2,3-DMP were from Serva, Heidelberg, Germany; DL- and L-1,4-DTT (reduced) (thyreo-1,4-dimercapto-2,3-butandiol), DL-1,4-DTE (reduced) (erythro-1,4-dimercapto-2,3-butandiol), glucose-6-phosphate dehydrogenase (type XV from baker's yeast), HEPES, N-ethylmaleimide, maleimide, iodoacetamide, L-cysteine, and 1,1-dimethylurea were from Sigma Chemical Co., St. Louis, MO.

Results

When cytoplasmic fractions from *ob/ob* mouse pancreatic B cells or rat liver were preincubated for 5 min with alloxan or ninhydrin in the absence of glucose, glucokinase activities were inhibited in a concentration-dependent manner with a half-maximal inhibitory concentration around 5 μM (Tables 1 and 2). A concentration of 50 μM of either alloxan or ninhydrin resulted in a virtually complete inhibition of the glucokinase activity. Residual enzyme activities with alloxan (50 μM) were 4 ± 1% (18 determinations) for pancreatic B cell and 4 ± 1% (36 determinations) for liver glucokinase and with ninhydrin (50 μM), 1 ± 1% (seven determinations) for pancreatic B cell and 0% (14 determinations) for liver glucokinase. Concentrations of ninhydrin and alloxan necessary for half-maximal inhibition of pancreatic B cell hexokinase were 20 and 100 times higher, respectively, than required for half-maximal inhibition of glucokinase (data not shown).

The concomitant presence of D-glucose during the 5-min preincubation of cytoplasmic fractions from pancreatic B cells or liver protected glucokinase enzyme activity against an inhibition by alloxan (50 μM) as well as by ninhydrin (50 μM). However, the protective effect of glucose was greater against

TABLE 1

Half-maximal effective concentrations for the inhibition of pancreatic B cell glucokinase activities by alloxan or ninhydrin, for the protection by glucose against inhibition, and for the reversal of inhibition by 1,4-DTT

Experiments were performed as described in Table 2, using the same concentrations of the test agents. The glucokinase activities were 5.5 ± 0.1 mU/mg of protein (10 experiments). The hexokinase activities were 2.2 ± 0.1 mU/mg of protein (10 experiments). Values shown are the means ± standard error with the numbers of experiments given in parentheses.

Half-maximal effective concentrations for	Alloxan	Ninhydrin
Inhibition (μM)	4.2 ± 0.7 (6)	5.4 ± 0.2 (4)
Glucose protection against inhibition (mM)	8.2 ± 0.7 (6)	25.0 ± 0.7 (3)*
1,4-DTT reversal of inhibition (mM)	0.31 ± 0.03 (4)	1.55 ± 0.59 (4)

*$p < 0.01$ compared with alloxan (Student's *t* test).

TABLE 2

Half-maximal effective concentrations for the inhibition of liver glucokinase activities by alloxan or ninhydrin in the absence or presence of 100 mM glucose, for the protection by glucose or by 1,4-DTT against inhibition, and for the reversal of inhibition by 1,4-DTT

For determination of the half-maximal effective concentrations, rat liver cytoplasmic fractions were preincubated for 5 min with increasing concentrations of alloxan (0, 1, 5, 10, or 50 μM) or ninhydrin (0, 1, 5, 10, or 50 μM) in the absence or presence of glucose (100 mM). Protection against inhibition of glucokinase activities was obtained by addition of increasing concentrations of glucose (0, 1, 5, 10, 20, and 100 mM) or 1,4-DTT (0, 10, 50, 100, 500, and 1000 μM) immediately before addition of alloxan (50 μM) or ninhydrin (50 μM). Reversal of inhibition was obtained immediately when 1,4-DTT (0, 0.5, 1, 5, or 10 mM) was added 5 min after alloxan (50 μM) or ninhydrin (50 μM) and just before the beginning of the enzyme activity measurement. Then measurement of enzyme activities was started. The glucokinase activities were 6.3 ± 0.5 mU/mg of protein (11 experiments). The hexokinase activities were 0.9 ± 0.1 mU/mg of protein (11 experiments). Values shown are the means ± standard error, with the numbers of experiments given in parentheses. Inhibition by alloxan and ninhydrin was lower ($p < 0.01$) in the presence than in the absence of glucose (100 mM). Half-maximal effective concentration for protection by 1,4-DTT was lower ($p < 0.01$) than for reversal in the case of alloxan.

Half-maximal effective concentrations for	Alloxan	Ninhydrin
Inhibition (μM)	4.8 ± 0.3 (9)	5.6 ± 0.3 (10)
Inhibition in the presence of glucose (100 mM) (μM)	875 ± 180 (4)	15.0 ± 1.0 (4)*
Glucose protection against inhibition (mM)	9.0 ± 0.4 (9)	25.7 ± 1.6 (6)†
1,4-DTT protection against inhibition (mM)	0.045 ± 0.004 (13)	0.80 ± 0.0 (3)†
1,4-DTT reversal of inhibition (mM)	0.93 ± 0.11 (8)	0.88 ± 0.08 (4)

*$p < 0.05$ compared with alloxan (Student's *t* test).
†$p < 0.01$ compared with alloxan (Student's *t* test).

alloxan than against ninhydrin inhibition, as demonstrated by the significantly lower half-maximal protective concentrations of glucose against alloxan inhibition (Tables 1 and 2). When 100 mM D-glucose was present during the 5-min preincubation period of the liver cytoplasmic fraction, the half-maximal glucokinase inhibitory concentration of alloxan was increased by a factor of nearly 200, whereas the half-maximal glucokinase inhibitory concentration of ninhydrin was increased only by a factor of 3 (Table 2).

Addition of 1,4-DTT after a 5-min preincubation of cytoplasmic fractions from pancreatic B cells or liver with alloxan (50 μM) or ninhydrin (50 μM) resulted in a reversal of the inhibition of glucokinase activity by half-maximal 1,4-DTT concentrations around 1 mM (Tables 1 and 2). At 50 μM alloxan or ninhydrin and at 1,4-DTT concentrations of 5 or 10 mM, the inhibition of pancreatic B cell and liver glucokinase was completely reversed. With increasing concentrations of alloxan, however, the ability of 1,4-DTT to reverse the enzyme inhibition continuously vanished. For liver glucokinase the alloxan concentration at which 10 mM 1,4-DTT was only able to restore 50% of the enzyme activity was 250 ± 20 μM (four experiments). Addition of 1,4-DTT before the beginning of the 5-min preincubation of cytoplasmic fractions from rat liver with either alloxan (50 μM) or ninhydrin (50 μM) protected glucokinase activity against inhibition by these agents (Table 2).

The monothiol cysteine protected glucokinase against inhibition by alloxan (50 μM) and ninhydrin (50 μM) with half-maximal protective concentrations far below 1 mM (Table 3), when added before the beginning of the 5-min preincubation of cytoplasmic fractions from rat liver with the inhibitor. The monothiol GSH, a tripeptide, was only a very weak protector of glucokinase against alloxan (50 μM) as shown by its high half-maximal protective concentration, well above 10 mM (Table 3); there was no protection at all against inhibition by ninhydrin (50 μM) (Table 3). Reversal of glucokinase inhibition by cysteine and GSH after a 5-min preincubation of cytoplasmic fractions from liver with alloxan (50 μM) was virtually impossible. Half-maximal effective concentrations for reversal were >20 mM in the case of cysteine, whereas GSH did not provide any reversal (Table 3).

All dithiols tested, with the exception of 1,6-DMH, were able to inactivate alloxan, thereby protecting glucokinase against alloxan inhibition, when cytoplasmic fractions from rat liver were preincubated for 5 min with the respective dithiol (Table 4). 1,6-DMH was ineffective, apparently because the spacing between the two SH groups in the molecule was too large. Half-maximal concentrations for protection were significantly lower for all effective dithiols than those required for reversal of inhibition. Addition of dithiols after a 5-min preincubation of cytoplasmic fractions from liver with alloxan (50 μM) resulted in a reversal of the inhibition of glucokinase activity only, when dithiols with an intermediate spacing between the two SH groups in the molecule were selected, i.e., the alcohols 1,4-DTT and 1,4-DTE and the hydrocarbons 1,3-DMP and 1,4-DMB (Table 4). The half-maximal concentrations for reversal by 1,4-DTT and 1,4-DTE, as well as by 1,3-DMP and 1,4-DMB, were not significantly different (Table 4); dithiols with two SH groups in the immediate vicinity in their molecular structure, 2,3-DMP and 1,2-DME, did not reverse the alloxan-induced inhibition of glucokinase activity (Table 4), as shown here by the half-maximal concentrations for reversal of >10 mM.

In a direct comparison, the ability of the three alcohol dithiols 2,3-DMP, 1,4-DTT, and 1,4-DTE at a concentration of 0.5 mM each, as well as of the hydrocarbons 1,3-DMP, 1,4-DMB, and 1,5-DMP at a concentration of 1 mM each, was compared with respect to their ability to reverse the inhibition of glucokinase after a 5-min preincubation with alloxan (50 μM) (Fig. 1). The ability of 2,3-DMP, a dithiol with two vicinal SH groups, to reverse the alloxan-induced enzyme inhibition was minimal compared with that of 1,4-DTT and 1,4-DTE (Fig. 1). The ability of 1,4-DTE, with its two OH groups in *cis*-position, to reverse the glucokinase inhibition by alloxan in cytoplasmic fractions from both pancreatic B cells and liver was slightly greater than that of 1,4-DTT, which contains two OH groups in *trans*-position (Fig. 1). The ability of 1,3-DMP to reverse

TABLE 3

Half-maximal effective concentrations of various monothiols for protection against and reversal of inhibition of liver glucokinase activities by alloxan and ninhydrin

For determination of the half-maximal effective concentrations of the monothiols cysteine or GSH for protection against inhibition of glucokinase activities by alloxan (50 μM) or ninhydrin (50 μM), rat liver cytoplasmic fractions were preincubated for 5 min with increasing concentrations of the respective monothiol (0, 0.1, 0.5, 1.0, 5.0, or 10.0 mM) in the reaction medium containing either alloxan or ninhydrin. Thereafter glucokinase activities were measured in the presence of 100 mM D-glucose. For determination of the half-maximal effective concentrations of the monothiols cysteine or GSH for reversal of inhibition of glucokinase activities by alloxan (50 μM), increasing concentrations of the respective monothiol (0, 5.0, 10.0, or 20.0 mM) were added to the reaction medium after a 5-min preincubation of rat liver cytoplasmic fractions in a reaction medium containing alloxan, before measurement of enzyme activities was started. The glucokinase activities were 6.7 ± 0.4 mU/mg of protein (10 experiments). The hexokinase activities were 0.9 ± 0.0 mU/mg of protein (10 experiments). Values shown are the means ± standard error with the numbers of experiments given in parentheses.

	Half-maximal effective concentrations for protection or reversal by	
	Cysteine	GSH
	mM	
Protection against alloxan (50 μM) inhibition	0.63 ± 0.05 (4)	11.68 ± 2.85 (5)*
Protection against ninhydrin (50 μM) inhibition	0.39 ± 0.04 (4)	No protection (4)
Reversal after alloxan (50 μM) inhibition	>20 (4)	No reversal (4)

* $p < 0.05$ compared with cysteine (Student's t test).

TABLE 4

Half-maximal effective concentrations of various dithiols for protection against and reversal of inhibition of liver glucokinase activities by alloxan

Experiments were performed as described in Table 3. The concentrations of the respective dithiol were 0, 0.01, 0.05, 0.10, 0.50, or 1.0 mM for protection against (left column) and 0, 0.5, 1.0, 5.0, 10.0, or 20.0 mM for reversal (right column) of alloxan (50 μM) inhibition. The glucokinase activities were 5.3 ± 0.6 mU/mg of protein (11 experiments). The hexokinase activities were 0.9 ± 0.1 mU/mg of protein (11 experiments). Values shown are the means ± standard error with the numbers of experiments given in parentheses.

Dithiol	Half-maximal effective concentrations for	
	Protection	Reversal
	mM	
2,3-DMP	0.090 ± 0.009 (4)	>10 (4)*
1,4-DTT	0.053 ± 0.006 (7)	1.43 ± 0.29 (6)*
1,4-DTE	0.043 ± 0.008 (4)	1.53 ± 0.22 (7)*
1,2-DME	0.353 ± 0.074 (4)	>10 (12)*
1,3-DMP	0.230 ± 0.057 (4)	2.68 ± 0.45 (5)*
1,4-DMB	0.235 ± 0.071 (4)	3.16 ± 1.04 (5)*
1,5-DMP	0.485 ± 0.053 (4)	>10 (4)*
1,6-DMH	>10 (4)	>10 (4)

* $p < 0.01$ compared with protection (Student's t test).

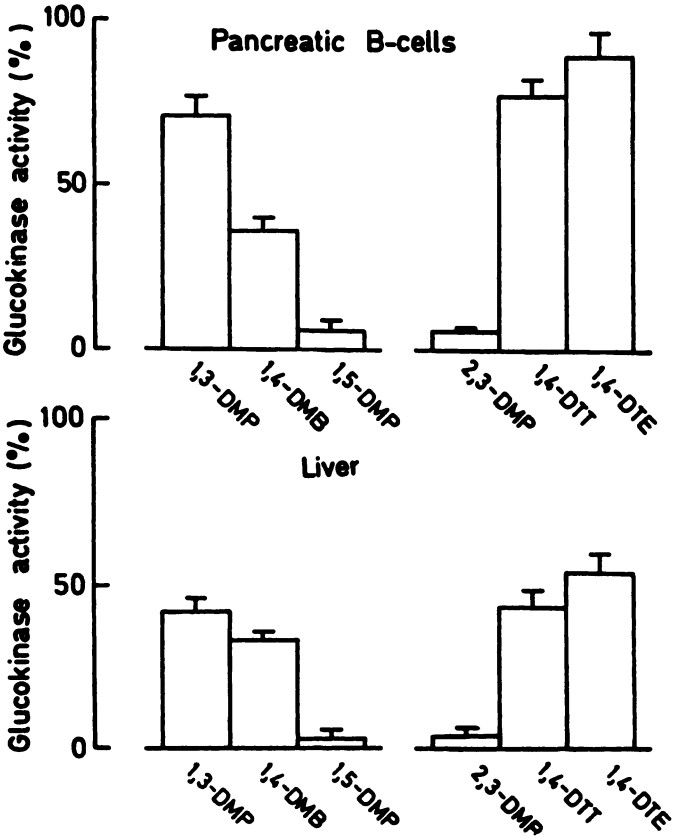

Fig. 1. Reversal by various dithiols of inhibition of glucokinase activities induced by alloxan in cytoplasmic fractions of *ob/ob* mouse pancreatic B cells or rat liver. During a 5-min preincubation period, the cytoplasmic fractions were exposed to alloxan (50 μM). Residual enzyme activities with alloxan (50 μM) were 4 ± 2% for pancreatic B cell and 1 ± 1% for liver glucokinase in the absence of any dithiol. Thereafter, 1,3-DMP (1 mM), 1,4-DMB (1 mM), 1,5-DMP (1 mM), 2,3-DMP (0.5 mM), 1,4-DTT (0.5 mM), or 1,4-DTE (0.5 mM) were added to reverse inhibition of glucokinase. Measurement of glucokinase activities was started by addition of 100 mM D-glucose. The 100% glucokinase activities were 5.5 ± 0.1 mU/mg of protein for pancreatic B cell and 6.3 ± 0.7 mU/mg of protein for liver cytoplasmic fractions. The hexokinase activities were 2.2 ± 0.1 mU/mg of protein for pancreatic B cell and 0.9 ± 0.1 mU/mg of protein for liver cytoplasmic fractions. Values shown are the means ± standard error for six experiments and are expressed as percentage of the glucokinase activities measured in the absence of alloxan and dithiol. For both pancreatic B cell and liver glucokinase, all differences between 1,3-DMP, 1,4-DMB, and 1,5-DMP, on the one hand, and 2,3-DMP, 1,4-DTT, and 1,4-DTE, on the other hand, were significantly different ($p < 0.01$ with Student's paired *t* test).

the glucokinase inhibition by alloxan in cytoplasmic fractions from pancreatic B cells and liver was significantly greater than that of 1,4-DMB (Fig. 1). 1,5-DMP, a dithiol with even wider spacing between the two SH groups of the dithiol, was nearly completely unable to reverse the glucokinase inhibition by alloxan (Fig. 1).

Dimethylurea (50 mM), a hydroxyl radical scavenger, neither inhibited nor protected liver glucokinase activity against inhibition by alloxan (10 or 50 μM) (data not shown).

Other agents known to react with SH groups are the dithiol reagents *N*-ethylmaleimide and maleimide and the monothiol reagent iodoacetamide. When cytoplasmic fractions from liver were preincubated for 5 min in the absence of glucose with one of these agents, glucokinase activities were inhibited in a concentration-dependent manner. The half-maximal inhibitory concentrations of the dithiol reagents *N*-ethylmaleimide and maleimide were about 1 decimal power higher than those of alloxan or ninhydrin but nevertheless well in the micromolar concentration range (Table 5). Residual enzyme activities were 20 ± 2% (24 experiments) for 100 μM and 2 ± 2% (six experiments) for 500 μM *N*-ethylmaleimide and 20 ± 3% (23 experiments) for 100 μM and 1 ± 1% (six experiments) for 500 μM maleimide. The half-maximal inhibitory concentrations of the monothiol reagent iodoacetamide, however, were nearly 10 mM (Table 5). Residual enzyme activities with iodoacetamide (20 mM) were 8 ± 2% (11 experiments).

Glucose protected glucokinase against inhibition by all three agents with half-maximal protective concentrations between 12 and 17 mM (Table 5). Addition of 1,4-DTT before the beginning of the 5 min preincubation of the cytoplasmic fractions with the dithiol reagents *N*-ethylmaleimide (100 μM) or maleimide (100 μM) protected glucokinase activity against inhibition by these agents, with half-maximal concentrations in the micromolar range (Table 5). 1,4-DTT did not protect glucokinase against inhibition by iodoacetamide (20 mM). Addition of 1,4-DTT after a 5-min preincubation of cytoplasmic fractions from liver resulted only in a partial reversal of the inhibition of glucokinase activity, which accounted for maximally 50% in the case of *N*-ethylmaleimide (100 μM) and 40% in the case of maleimide (100 μM) (Table 5). Inhibition of glucokinase by iodoacetamide (20 mM) could not be reversed at all by 1,4-DTT.

Discussion

The theory that alloxan is B cell toxic by virtue of its ability to react with SH-containing cellular structures such as GSH (19) or an essential SH-containing enzyme or coenzyme (2–4) has been the most widely held view on its mechanism of action (20). The present results provide support for the view that the site of action of alloxan is two vicinal SH groups (21–25), which are situated in the depth of the sugar-binding site of the pancreatic B cell glucokinase (8, 9).

Both alloxan and ninhydrin are dithiol reagents that inhibit glucokinase at very low concentrations and glucose protects the enzyme against the inhibition. However, the reason why only alloxan is selectively B cell toxic whereas ninhydrin is systemically toxic resides in their different stabilities (11, 19). Alloxan is very labile, with a half-life at physiological pH of around 1 min (19), whereas the more lipophilic ninhydrin is chemically stable. This is documented by the ability of glucose at a concentration of 100 mM to increase the concentration of alloxan necessary for half-maximal glucokinase inhibition by a factor of nearly 200, whereas the corresponding factor for ninhydrin was only 3.

Reaction of alloxan with two adjacent SH groups results in the formation of a disulfide bond in the depth of the sugar-binding site of the glucokinase, with concomitant permanent but reversible inactivation of the enzyme and reduction of alloxan to dialurate (10, 11). Glucose hinders the access of alloxan to these SH groups through binding to the sugar-binding site of the enzyme. This conclusion is supported by several findings as follows.

1. As alloxan is long decomposed to yield alloxanate and other decomposition products (10, 11) after a 5-min preincubation with glucokinase in test medium at 37° with a pH of 7.4 (19), reversal of alloxan-induced glucokinase inhibition by di-

TABLE 5

Half-maximal effective concentrations for the inhibition of liver glucokinase activities by *N*-ethylmaleimide, maleimide, or iodoacetamide for the protection by glucose or by 1,4-DTT against inhibition, and for the reversal of inhibition by 1,4-DTT

For determination of the half-maximal effective concentrations, rat liver cytoplasmic fractions were preincubated for 5 min with increasing concentrations of *N*-ethylmaleimide (0, 10, 25, 50, 100, and 500 μM), maleimide (0, 10, 25, 50, 100, and 500 μM), or iodoacetamide (0, 0.5, 1, 5, 10, and 20 mM) in the absence of glucose. Protection against inhibition of glucokinase activities was obtained by addition of increasing concentrations of glucose (0, 1, 10, 20, 50, and 100 mM) or 1,4-DTT (0, 10, 50, 100, and 500 μM) immediately before addition of *N*-ethylmaleimide (100 μM), maleimide (100 μM), or iodoacetamide (20 mM). Reversal of inhibition was obtained immediately when 1,4-DTT (0, 0.5, 1, 5, and 10 mM) was added 5 min after *N*-ethylmaleimide (100 μM), maleimide (100 μM), or iodoacetamide (20 mM), just before the beginning of the enzyme activity measurement. Then measurement of enzyme activities was started. The glucokinase activities were 5.8 ± 0.6 mU/mg of protein (six experiments). The hexokinase activities were 0.9 ± 0.1 mU/mg of protein (six experiments). Values shown are the means ± standard error with the numbers of experiments given in parentheses.

Half-maximal effective concentrations for	*N*-Ethylmaleimide	Maleimide	Iodoacetamide
Inhibition (μM)	43.0 ± 8.7 (6)	38.0 ± 5.7 (6)	9510 ± 2420 (9)*
Glucose protection against inhibition (mM)	14.0 ± 1.4 (4)	12.3 ± 0.9 (6)	17.0 ± 2.5 (4)
1,4-DTT protection against inhibition (μM)	28.0 ± 3.0 (3)	35.0 ± 8.0 (5)	No protection (4)
1,4-DTT reversal of inhibition (mM)	max. 50% at 0.5–10 mM (9)	max. 40% at 0.5–10 mM (8)	No reversal (4)

* $p < 0.01$ compared with *N*-ethylmaleimide and maleimide (Student's *t* test).

thiols can only result from reduction of two S atoms of the S—S bond in the depth of the glucokinase sugar-binding site, thereby restoring the alloxan-modified sugar-binding site to its original state. Reaction with free or bound alloxan and removal from a presumed site of attachment (23) is an unlikely alternative explanation.

2. Cysteine and GSH did not reverse alloxan-induced glucokinase inhibition, whereas 1,4-DTT and a variety of other dithiols reversed the inhibition. The explanation for this ineffectiveness is that monothiols apparently do not reduce the S—S bond formed in the sugar-binding site of the glucokinase through reaction with alloxan. Both monothiols and dithiols were able to protect islet tissue against an alloxan-induced permeability increase (22, 23) and animals against the diabetogenic action of alloxan (25), but only dithiols could reverse these actions of alloxan (22, 23, 25).

3. Not all dithiols were equally well suited to reverse alloxan-induced inhibition of pancreatic B cell and liver glucokinase. Their potency was critically dependent on the distance between the two SH groups in the molecule. Dithiols such as 1,2-DME and 2,3-DMP were ineffective, because the distance was too short to reduce under ring formation the S—S bridge in the sugar-binding site of the glucokinase. 1,5-DMP and 1,6-DMH were apparently molecules with spacing between the two SH groups too wide to reduce the S—S bridge in the sugar-binding site. Only dithiols with intermediate spacing between the two SH groups of the molecule were effective reversers, with 1,3-DMP being somewhat more effective than 1,4-DMB.

4. The two residues in the sugar-binding site of the glucokinase that carry the SH groups are likely to be two vicinal cysteines in the protein chain of the enzyme (26–29). Because both the dithiols, 1,3-DMP, 1,4-DMB, 1,4-DTT, and 1,4-DTE, and the protein chain of the glucokinase in the depth of the sugar-binding site, where these two cysteines are situated, are flexible, this provides some liberalism to the system, which it requires as a prerequisite for dithiols to react with the S—S bridge in the sugar-binding site under ring formation in order to reactivate the enzyme.

In contrast to cysteine and all dithiols tested, which react with alloxan and ninhydrin and thereby protect glucokinase against inhibition by these agents, GSH, a more bulky tripeptide molecule, reacts only at very high concentrations with alloxan and not at all with ninhydrin. Thus, the pancreatic B cell toxicity of alloxan and the general toxicity of ninhydrin appear not to result primarily from oxidation of GSH. Dimethylurea, a scavenger of cytotoxic hydroxyl radicals, did not protect glucokinase against alloxan inhibition. This does not support the view that the effects of alloxan on glucokinase activity and function of the pancreatic B cell are due to peroxide sensitivity of the system (30, 31).

Other dithiol reagents such as *N*-ethylmaleimide and maleimide also inhibited glucokinase through oxidation of the SH groups in the sugar-binding site, albeit with somewhat higher half-maximal inhibitory concentrations, but glucose, as well as 1,4-DTT, also protected glucokinase against this inhibition. However, as tools they are less suitable than alloxan because they are not pure thiol reagents in this system but also alkylating agents (12, 13). For this reason, only a partial reversal of glucokinase inhibition by *N*-ethylmaleimide or maleimide could be achieved with 1,4-DTT, which did not surpass 40–50% even in the presence of high DTT concentrations; 1,4-DTT at concentrations above 5 mM completely reversed glucokinase inhibition by alloxan or ninhydrin. The high concentrations of iodoacetamide required for inhibition of glucokinase, as well as the inability of 1,4-DTT to protect against or reverse the inhibition of the enzyme, indicate that such an alkylating monothiol reagent (13) is not a suitable reversible inhibitor of glucokinase through reaction with the two vicinal SH groups in the depth of the sugar-binding site (26–29).

The present results do not support the alternative contention that alloxan might interact with sites other than the sugar-binding site of the glucokinase (7) in order to inhibit this enzyme. However, the present study supports the view that the mechanism of inhibition of glucokinase is a reaction of alloxan with two vicinal SH groups in the depth of the sugar-binding site of the glucokinase (26–29), with formation of a disulfide bond and concomitant inactivation of the enzyme. This mechanism of interaction of alloxan with the glucokinase can provide an explanation for the ability of alloxan to inhibit glucose-induced insulin secretion.

References

1. Wöhler, F., and J. Liebig. Untersuchungen über die Natur der Harnsäure. *Ann. Pharm.* **26**:241–340 (1838).
2. Lazarow, A. Protective effect of glutathione and cysteine against alloxan diabetes in the rat. *Proc. Soc. Exp. Biol. Med.* **61**:441–447 (1946).
3. Lazarow, A., and J. W. Patterson. The mechanism of cysteine and glutathione protection against alloxan diabetes. *Science (Wash. D. C.)* **108**:308–309 (1948).
4. Lazarow, A. Factors controlling the development and progression of diabetes. *Physiol. Res.* **29**:48–74 (1949).

5. Miwa, I., H. Hara, H. Matsunaga, and J. Okuda. Inhibition of glucokinase in hepatocytes by alloxan. *Biochem. Int.* **9**:595–602 (1984).
6. Hara, H., I. Miwa, and J. Okuda. Inhibition of rat liver glucokinase by alloxan and ninhydrin. *Chem. Pharm. Bull. (Tokyo)* **34**:4731–4737 (1985).
7. Meglasson, M. D., P. T. Burch, D. K. Berner, H. Najafi, and F. M. Matschinsky. Identification of glucokinase as an alloxan-sensitive glucose sensor of the pancreatic β-cell. *Diabetes* **35**:1163–1173 (1986).
8. Lenzen, S., M. Tiedge, and U. Panten. Glucokinase in pancreatic B-cells and its inhibition by alloxan. *Acta Endocrinol.* **115**:21–29 (1987).
9. Lenzen, S., and U. Panten. Signal recognition by pancreatic B-cells. *Biochem. Pharmacol.* **37**:371–378 (1988).
10. Labes, R., and H. Freisburger. Das Alloxan als Oxydationsmittel für Thiolgruppen, als Kapillargift und als Krampfgift. *Naunyn-Schmiedeberg's Arch. Pharmakol. Exp. Pathol.* **156**:226–252 (1930).
11. Webb, J. L. Alloxan, in *Enzyme and Metabolic Inhibitors* (J. L. Webb, ed.). Academic Press, New York, 367–419 (1966).
12. Webb, J. L. *N*-Ethylmaleimide, in *Enzyme and Metabolic Inhibitors* (J. L. Webb, ed.). Academic Press, New York, 337–365 (1966).
13. Webb, J. L. Iodoacetate and iodoacetamide, in *Enzyme and Metabolic Inhibitors* (J. L. Webb, ed.). Academic Press, New York, 1–283 (1966).
14. Lenzen, S., S. Freytag, and F.-H. Brand. Mechanismus der Hemmung der Glucokinase in B-Zellen des Pankreas durch Alloxan. *Aktuel. Endokrinol. Stoffwechsel* **8**:96 (1987).
15. Lernmark, A. The preparation of, and studies on, free cell suspensions from mouse pancreatic islets. *Diabetologia* **10**:431–438 (1974).
16. Lenzen, S., W. Schmidt, and U. Panten. Transamination of neutral amino acids and 2-keto-acids in the pancreatic B-cell mitochondria. *J. Biol. Chem.* **260**:12629–12634 (1985).
17. Walker, D. G., and M. J. Parry. Glucokinase. *Methods Enzymol.* **9**:381–391 (1966).
18. McKnight, G. S. A colorimetric method for the determination of submicrogram quantities of protein. *Anal. Biochem.* **78**:86–92 (1977).
19. Patterson, J. W., A. Lazarow, and S. Levey. Alloxan and dialuric acid: their stabilities and ultraviolet absorption spectra. *J. Biol. Chem.* **177**:187–196 (1949).
20. Cooperstein, S. J., and D. Watkins. Action of toxic drugs on islet cells, in *The Islet of Langerhans* (S. J. Cooperstein, and D. Watkins, eds.). Academic Press, New York, 387–425 (1981).
21. Watkins, D., S. J. Cooperstein, and A. Lazarow. Effect of sulfhydril reagents on permeability of toadfish islet tissue. *Am. J. Physiol.* **219**:503–509 (1970).
22. Watkins, D., S. J. Cooperstein, and A. Lazarow. Effect of sulfhydryl-binding reagents on islet tissue permeability: protection and reversal by thiol compounds. *J. Pharmacol. Exp. Ther.* **176**:42–51 (1971).
23. Watkins, D., and S. J. Cooperstein. Effect of alloxan on islet tissue permeability: protection and reversal by dithiols. *J. Pharmacol. Exp. Ther.* **199**:575–582 (1976).
24. Cooperstein, S. J., and D. Watkins. Effect of sulfhydryl-binding reagents on islet tissue permeability: protection and reversal by D-glucose and phlorizin. *J. Pharmacol. Exp. Ther.* **204**:230–238 (1978).
25. Sen, P. B., and G. Bhattacharya. Reversal of the diabetogenic action of alloxan by sulhydryl compounds. *Science (Wash. D. C.)* **115**:41–43 (1952).
26. Anderson, C. M., R. E. Stenkamp, and T. A. Steitz. Sequencing a protein by X-ray crytallography. II. Refinement of yeast hexokinase B co-ordinates and sequence at 2.1 Å resolution. *J. Mol. Biol.* **123**:15–33 (1978).
27. Anderson, C. M., R. E. Stenkamp, R. McDonald, and T. A. Steitz. A refined model of the sugar binding site of yeast hexokinase. *B. J. Mol. Biol.* **123**:207–219 (1978).
28. Connolly, B. A., and I. P. Trayer. Reaction of rat hepatic glucokinase with substrate-related and other alkylating agents. *Eur. J. Biochem.* **99**:299–308 (1979).
29. Connolly, B. A., and I. P. Trayer. Affinity labelling of rat-muscle hexokinase type II by a glucose-derived alkylating agent. *Eur. J. Biochem.* **93**:375–385 (1979).
30. Heikkila, R. E., and F. S. Cabbat. Protection against alloxan-induced diabetes in mice by the hydroxyl radical scavenger dimethylurea. *Eur. J. Pharmacol.* **52**:57–60 (1978).
31. Malaisse, W. J. Alloxan toxicity to the pancreatic B-cell. *Biochem. Pharmacol.* **31**:3527–3534 (1982).

Send reprint requests to: Dr. Sigurd Lenzen, Institut für Pharmakologie und Toxikologie, Universität Göttingen, Robert-Koch-Str. 40, D-3400 Göttingen, Federal Republic of Germany.