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Irreversible Inactivation of Aspartate Aminotransferase by 2-Oxoglutaconic Acid and Its Dimethyl Ester[1]

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Incubation of pig heart cytosolic aspartate aminotransferase (pyridoxal 5′-phosphate form) with 10 mM 2-oxoglutaconic acid dimethyl ester for 2 h at 25°C (pH 7.0) results in slight inactivation (~15%). However, incubation of the enzyme with glutamate, or prior conversion of the enzyme to the pyridoxamine 5′-phosphate form, results in more extensive inactivation. The inactivation of the enzyme by 2-oxoglutaconic acid dimethyl ester is most pronounced in the presence of both glutamate and α-ketoglutarate. *N*-Ethylmaleimide was previously shown to alkylate two surface cysteine residues (I and II) and to react syncatalytically with a third cysteine residue (III) of cytosolic pig heart aspartate aminotransferase [Birchmeier *et al.* (1973) *J. Biol. Chem.* 248, 1751-1759]. Alkylation of cysteine III results in inactivation of the enzyme, despite the fact that this residue is not essential for catalysis. The present results suggest that 2-oxoglutaconic acid dimethyl ester reacts with the enzyme in a similar fashion to that exhibited by *N*-ethylmaleimide. Some inactivation by alkylation of a susceptible group at the active site cannot be ruled out. However, the rate of inactivation of cytosolic pig heart aspartate aminotransferase is proportional to the concentration of 2-oxoglutaconic acid dimethyl ester up to a concentration of at least 40 mM, suggesting that the compound binds very poorly to the active site or that alkylation at the active site is slow compared with syncatalytic alkylation of cysteine III. The $t_{1/2}$ for inactivation of pig heart cytosolic aspartate aminotransferase by 40 mM 2-oxoglutaconic acid dimethyl ester (in the presence of 10 mM L-glutamate, pH 7.2, 25°C) is 9 min. Incubation of cytosolic pig heart aspartate aminotransferase with 10 mM 2-oxoglutaconate for 2 h (25°C, pH 7.2) results in significant inactivation (~30%). The enzyme is protected against inactivation by the presence of α-ketoglutarate, but glutamate enhances the inactivation. These findings suggest that 2-oxoglutaconate is an active site-directed inhibitor. The binding of 2-oxoglutaconate to the enzyme exhibits saturation kinetics (K_I ~2 mM), but the rate of inactivation is slow (limiting rate constant for inactivation in the presence of L-glutamate ~0.01 min^{-1}; pH 6.0, 25°C; $t_{1/2}$max ~70 min). This finding suggests that 2-oxoglutaconate does not readily react in a syncatalytic fashion with cysteine III. Possibly, the two negative charges of 2-oxoglutaconate do not allow ready approach to cysteine III. Rather, the findings suggest that 2-oxoglutaconate binds at the active site of the pyridoxal 5′-phosphate form of the enzyme as an affinity labeling reagent. However, the increased rate of 2-oxoglutaconate-induced inactivation in the presence of glutamate suggests that this unsaturated α-keto acid also exhibits the properties of a k_{cat} inhibitor. 2-Oxoglutaconate inactivates aspartate aminotransferase in cytosolic and mitochondrial fractions of rat kidney and purified pig heart alanine aminotransferase. Injection of 2-oxoglutaconate into mice results in inhibition of kidney aspartate aminotransferase. 2-Oxoglutaconate is a substrate of glutamate dehydrogenase. The kinetic constants are similar to those obtained with α-ketoglutarate. The results suggest that unsaturated α-keto acids and their esters may be useful probes for the study of α-keto acid-utilizing enzymes.

Key words: aspartate aminotransferase, glutamate dehydrogenase, 2-oxoglutaconate, 2-oxoglutaconic acid dimethylester, syncatalytic inactivation.

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Abbreviations: AlaAT, alanine aminotransferase; AMB, L-2-amino-4-methoxy-*trans*-3-butenoate; AOA, aminooxyacetate; AspAT; aspartate aminotransferase; cyt, cytosolic; DTT, dithiothreitol; GDH, glutamate dehydrogenase; LDH, lactate dehydrogenase; βMA, β-methylene-D,L-aspartate; MAS, malate-aspartate shuttle; MDH, malate dehydrogenase; mit, mitochondrial; NEM, *N*-ethylmaleimide; OG, 2-oxoglutaconate; OGDM, 2-oxoglutaconic acid dimethyl ester; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; TLC, thin layer chromatography.

Mitochondrial and cytosolic aspartate aminotransferases (mitAspAT and cytAspAT) [EC 2.6.1.1] are integral components of the malate-aspartate shuttle (MAS). The MAS is one of several shuttles that transport reducing equivalents across the mitochondrial membrane in lieu of NADH. A method frequently used to assess the importance of the MAS in tissue preparations is to determine the metabolic consequences of inhibition of AspAT. Commonly used inhibitors of AspAT include aminooxyacetate (AOA) and cycloserine. However, AOA is a general carbonyl reagent that reacts with the pyridoxal 5′-phosphate (PLP) cofactor of many enzymes and with α-keto acids to yield relatively stable oximes (*1*). Cycloserine dimerizes to a compound that also forms stable oximes with α-keto acids (*2*). Therefore, we have sought a more selective and generally less reactive inhibitor of AspAT. Several strategies are available for the design of inhibitors of PLP-containing enzymes. One strategy is to incorporate a carbonyl reagent into a substrate analog. Thus, *N*-hydroxyglutamate (*3*), *N*-aminoaspartate (L-hydrazinosuccinate) (*4*), and aminooxysuccinate (*5*) irreversibly inhibit AspAT by forming a nitrone, hydrazone, and oxime, respectively, with the PLP cofactor. Inhibitors containing carbonyl reagents generally have high affinities for PLP enzymes. Indeed, both L-hydrazinosuccinate ($K_i = 0.5$ nM) and L-aminooxysuccinate ($K_i = 160$ nM) are slow-binding, but potent, inhibitors of AspAT. L-Hydrazinosuccinate inhibits mouse liver and kidney cytAspAT *in vivo*. However, the compound also inhibits alanine aminotransferase (AlaAT) [EC 2.6.1.2] *in vivo* (*4*). Moreover, because *N*-hydroxyglutamate, L-hydrazinosuccinate, and aminooxysuccinate are carbonyl reagents, they are likely to be quite reactive toward a number of PLP enzymes and toward α-keto acids and other carbonyl-containing biomolecules. Another strategy for the design of inactivators of PLP enzymes is to construct k_{cat} (=suicide or mechanism-based) inhibitors. The inhibition constants of these compounds are often in the mM range (*5*), but they are less reactive than compounds that are carbonyl reagents. The k_{cat} inhibitors become reactive, however, during processing at the active site, resulting in modification of active site residues or of the cofactor. Several β,γ-unsaturated amino acids are known to be k_{cat} inhibitors of AspAT. Thus, Rando and colleagues showed that vinylglycine (*6*) and L-2-amino-4-methoxy-*trans*-3-butenoate (AMB) (*7*) are k_{cat} inhibitors of AspAT. These inhibitors are, however, not ideal for studies of the consequences of inhibition of AspAT *in vivo*. Vinylglyoxylate is a relatively poor inhibitor of AspAT (*6*) and AMB is a strong inhibitor of methionine adenosyltransferase (*8*). We previously showed that the β,γ-unsaturated amino acid β-methylene-D,L-aspartate (βMA) is also a k_{cat} inhibitor of AspAT (*9*). This compound, although it inhibits some other PLP-containing enzymes (*9*) and inhibits brain glutamate transporter(s) (*10*), has proved useful in studies of the MAS in rat brain slices (*11*) and in cultured neural cells (*9, 12*). Kidney and liver AspAT activities were significantly decreased (by 40 and 23%, respectively) within 6 h after βMA was injected intraperitoneally into mice (*9*). AspAT activity in the brain was not affected, presumably because βMA does not cross the blood-brain barrier (*9*). Other β,γ-unsaturated amino acids, such as β-ethylidene-D,L-aspartate and β-methylene-D,L-glutamate, are largely ineffective as k_{cat} inhibitors of AspAT (*13*). On continuing our search for selective inhibitors of AspAT we discovered that the unsaturated α-keto acid 2-oxoglutaconate and its dimethyl ester are novel inhibitors of AspAT.

MATERIALS AND METHODS

Enzymes and Reagents—Cytosolic pig heart AspAT (~200 U/mg; suspension in 3.2 M ammonium sulfate), pig heart alanine aminotransferase (AlaAT) (~80 U/mg; suspension in 3.2 M ammonium sulfate), and beef liver glutamate dehydrogenase (GDH) [EC 1.4.1.3] (~120 U/mg in 50% glycerol) were obtained from Boehringer Mannheim (Indianapolis, IN). Pig heart malate dehydrogenase (MDH) [EC 1.1.1.37] (~400 U/mg, suspension in 2.8 M ammonium sulfate) and rabbit muscle type II lactate dehydrogenase (LDH) [EC 1.1.1.27] (cystalline suspension in 3.2 M ammonium sulfate) were obtained from Sigma (St. Louis, MO). Before use, the enzyme suspensions were centrifuged and the pellets were dissolved in 10 mM potassium phosphate buffer, pH 7.2. AspAT and AlaAT were assayed spectrophotometrically by coupling oxaloacetate formation with MDH and pyruvate formation with LDH, respectively (*14*). 2-Oxoglutaconic acid dimethyl ester [*Z*(=*trans*) configuration] (OGDM) was purchased from Fluka (Buchs, Switzerland). In some experiments the commercial cytAspAT (PLP form) was converted to its pyridoxamine 5′-phosphate (PMP) form by treatment with cysteine sulfinic acid as described (*15*). OG was prepared by hydrolyzing 250 mM OGDM in 1 M KOH (or NaOH), followed by neutralization with 1 M HCl. The solution was used directly without removal of NaCl/KCl or methanol. In preliminary experiments it was shown that 50 mM OGDM and 50 mM OG slowly inactivate both LDH and MDH (possibly by acting as non-specific alkylating agents) (data not shown). However, this inactivation did not interfere with the AspAT and AlaAT assays because the coupling enzymes were present in the assay mixtures in large excess, and in most cases the final concentration of OGDM or OG in the transaminase assay mixtures was 0.5 mM or less.

Characterization of OGDM by NMR—A JEOL JNM-EX400 spectrometer (JEOL, Tokyo) was used to obtain ^{1}H- and ^{13}C-NMR spectra. Solvents were D_2O and $CDCl_3$. 3-(Trimethylsilyl) propionate 2,2,3,3-*d*4 (TSP-*d*4) and tetramethylsilane (TMS) were used as internal standards in D_2O and $CDCl_3$, respectively.

Other Procedures—Adult male Sprague Dawley rats weighing ~300 g each were killed by decapitation. The kidneys were removed and fractionated into mitochondria and cytosol as described (*16*). Protein determinations were carried out essentially according to the method of Lowry *et al.* (*17*) using bovine serum albumin as the standard. Experiments to determine the effect of OG *in vivo* were carried out as follows: Adult male Swiss Webster mice (~30 g each, Charles River, New York) were injected intraperitoneally with 175 μmol of OG. This preparation of OG was obtained by hydrolysis of OGDM with NaOH followed by neutralization with HCl and contained 700 μmol of NaCl and 350 μmol of methanol. Controls were injected with 175 μmol of α-ketoglutarate, 700 μmol of NaCl, and 350 μmol of methanol. After 4 h the mice were killed by cervical dislocation. The brains, livers, and

kidneys were removed and separately homogenized in 10 volumes of ice-cold 10 mM potassium phosphate buffer (pH 7.2). The homogenates were freeze-thawed twice. After a low-speed centrifugation, an aliquot (1-2 μl) of the supernatant was assayed for AspAT activity. Results are expressed as mean ± SEM. Significance was evaluated by use of the Mann-Whitney *U* test.

RESULTS

Characterization of OGDM—To understand the mechanism by which OGDM interacts with AspAT it was necessary to characterize fully the structure of the compound in solution. This was accomplished by means of ^{13}C-NMR. The peak assignments for OGDM in $CDCl_3$ (100 MHz) are as follows: dppm 182.1 (carbonyl), 165.1 and 160.9 (carboxyls), 135.4 and 134.1 (olefinic carbons), 53.4 and 52.6 (methyls). After 24 h incubation at room temperature, two new peaks appear at 138.3 and 129.2 (olefinic carbons) due to isomerization to the *cis* form. The ^{13}C spectrum in D_2O (pH ~4.0) is different and shows that the carbonyl is completely hydrated to a ketal. Assignments are as follows: dppm 92.1 (ketal), 174.4 and 171.4 (carboxyls), 147.5 and 126.4 (olefinic carbons), 53.8 and 52.7 (methyls). The hydrate (ketal) structure is also consistent with the H-C COSY two-dimensional spectrum (data not shown). In D_2O (pH ~4.0), OGDM ketal is a mixture of ~90% *trans* isomer and ~10% *cis* isomer as judged by the presence of smaller peaks at 137.7 (olefinic), 137.6 (olefinic), and 53.1 (methyl). No change in the spectrum occurs over 24 h and no peaks attributable to methanol (51.0) are observed. The properties of OGDM in various buffers in D_2O are interesting. In 0.5 M potassium phosphate buffer (pH 6.0) the ^{13}C-NMR spectrum shows OGDM to be completely in the ketal form. In 0.5 M potassium phosphate buffer (pH 7.2) the predominant form is also the ketal, but within 36 h, complete conversion to the free carbonyl (keto) compound occurs. The free carbonyl compound is also the preferred form in potassium phosphate buffer (pH 8.0). Thus, the chemical shift values of OGDM in D_2O at pH 7.0 (after 36 h) and pH 8.0 (within minutes) are 197.5, 172.2, 170.4, 139.3, 137.3, 55.8, and 51.7. These values are similar to those observed for the carbonyl compound in $CDCl_3$. In contrast to its stability in $CDCl_3$ and D_2O (pH ≤8.0), OGDM is hydrolyzed to a mixture of *trans* and *cis* isomers (~60 : 40) of 2-oxoglutaconic acid (OG) in strongly alkaline solution. Chemical shift assignments of the potassium salt in D_2O (after hydrolysis in 1 M KOH followed by neutralization with HCl) are as follows: 200.1 [carbonyl (*trans*)], 198.8 [carbonyl (*cis*)], 175.7 (carboxyl adjacent to the carbonyl), 173.8 [carboxyl adjacent to an olefinic carbon (*trans*)], 171.1 [carboxyl adjacent to an olefinic carbon (*cis*)], 146.4 and 134.4 [olefinic carbons (*trans*)], 139.4 and 137.4 [olefinic carbons (*cis*)], 51.8 (methanol by-product). Thin layer chromatographic (TLC) analysis is consistent with the above interpretation of the ^{13}C-NMR data. After incubation for 6 h in 0.5 M potassium phosphate buffer (pH 6.0), OGDM yielded one spot (visible as a yellow spot or by quenching under UV light) (R_f 0.7) in a solvent system consisting of ethyl acetate : hexane [60 : 40 (v/v)] on a silica gel TLC plate (Merck). No spot attributable to OG (R_f 0.3) (obtained from alkaline hydrolysis of OGDM) was visible on the TLC plate under these conditions.

Most α-keto acids exist predominantly as the ketal (hydrate) at low pH values, but at pH values >4.0 these α-keto acids exist predominantly in the keto form (*18*). OG appears to behave similarly in that the predominant form at neutral pH is also the keto form. On the other hand, OGDM which is an α-keto acid ester exists in the hydrate at pH 6.0 and is only converted to the keto form at higher pH values.

The structures of OGDM in various solutions and its

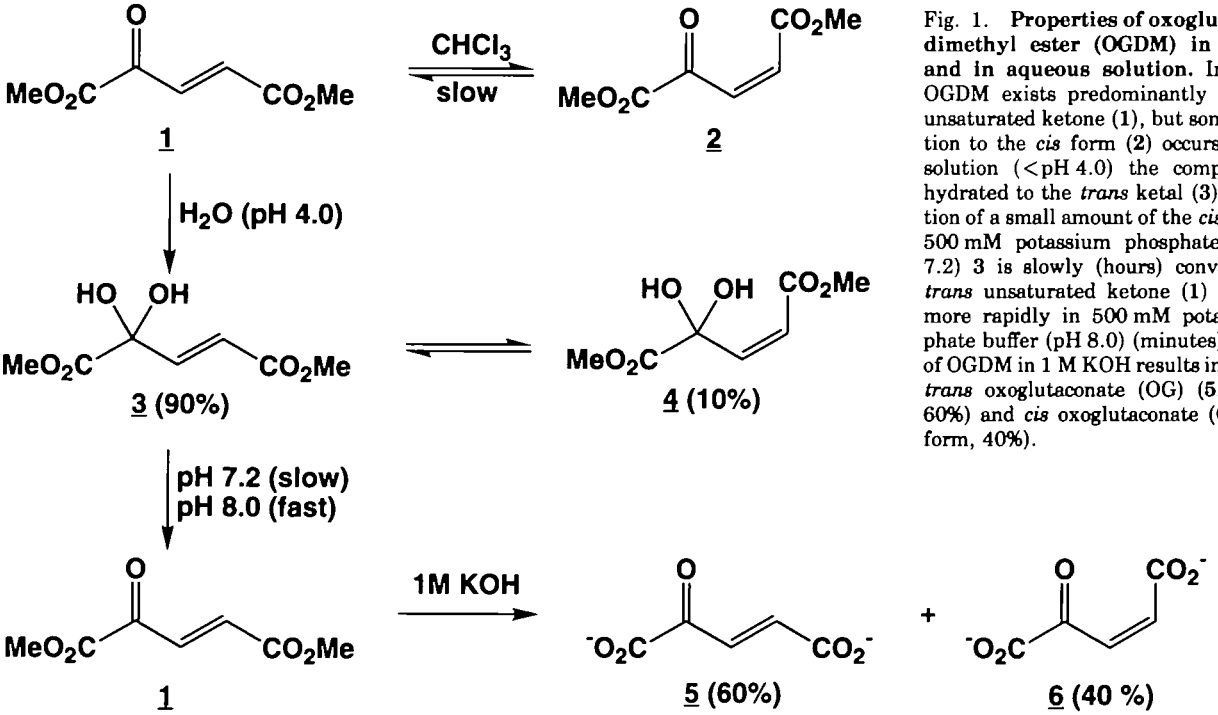

Fig. 1. **Properties of oxoglutaconic acid dimethyl ester (OGDM) in chloroform and in aqueous solution.** In chloroform OGDM exists predominantly as the *trans* unsaturated ketone (**1**), but some isomerization to the *cis* form (**2**) occurs. In aqueous solution (<pH 4.0) the compound (**1**) is hydrated to the *trans* ketal (**3**) with formation of a small amount of the *cis* ketal (**4**). In 500 mM potassium phosphate buffer (pH 7.2) **3** is slowly (hours) converted to the *trans* unsaturated ketone (**1**) at 25°C, but more rapidly in 500 mM potassium phosphate buffer (pH 8.0) (minutes). Hydrolysis of OGDM in 1 M KOH results in a mixture of *trans* oxoglutaconate (OG) (**5**, keto form, 60%) and *cis* oxoglutaconate (OG) (**6**, keto form, 40%).

hydrolysis to OG are summarized in Fig. 1.

Interaction of Glutamate Dehydrogenase (GDH) with OG—OG may be regarded as an analog of α-ketoglutarate that possesses an unsaturated double bond β,γ to the keto group. Therefore, the possibility was considered that OG might be an irreversible inactivator of GDH by binding to the active site and alkylating a susceptible residue. However, this was not found to be the case. Rather, the compound was shown to be a good substrate of the enzyme. The K_m, V_{max}, V_{max}/K_m values [conditions: 300 mM Tris-HCl (pH 8.9), 100 mM ammonium chloride, 0.1 mM NADH, 25°C; absence of activating ADP] are 5.26 mM, 296 μmol/min/mg, 56 μmol/min/mg/mM, respectively, for α-ketoglutarate and 3.33 mM, 133 μmol/min/mg, 40 μmol/min/mg/mM, respectively for OG. As noted above, OG prepared from the hydrolysis of OGDM exists as a mixture of *trans* (60%) and *cis* (40%) isomers. No attempt was made to determine whether the *cis* and *trans* forms of OG interact differently with GDH.

Inactivation of Pig Heart CytAspAT by OGDM—A slow but significant inactivation of AspAT (PLP form) occurs when the enzyme is incubated with 10 mM OGDM (Table I). L-Glutamate (10 mM) enhances the rate at which the enzyme is inactivated by OGDM. The PMP form of the enzyme is also more rapidly inactivated by 10 mM OGDM than is the PLP form of the enzyme (Table I). The most rapid rate of OGDM-induced inactivation occurs in the presence of both glutamate and α-ketoglutarate (Table I). The OGDM-induced inactivation in the presence of glutamate can be almost completely prevented by the addition of 10 mM dithiothreitol (DTT) (Table I).

The kinetics of the OGDM-induced inactivation of cytAspAT were investigated. For this experiment the enzyme was incubated with variable concentrations of OGDM (5, 10, 20, 30, and 40 mM) in the presence of 100 mM potassium phosphate buffer, pH 7.2, and 10 mM L-glutamate (25°C). At intervals (2, 4, 10, 15, and 20 min), aliquots were withdrawn and residual activity was determined. In each case the rate of inactivation followed pseudo-first-order kinetics. A secondary plot of the data (*i.e.*, reciprocal of concentration *versus* rate constant for inactivation) is shown in Fig. 2A. No evidence was obtained that the enzyme can be saturated with OGDM at concentrations below 40 mM. From the plot shown in Fig. 2A one can calculate that the maximal rate constant for inactivation of pig heart cytAspAT by high concentrations of OGDM (in the presence of glutamate) is $\gg 0.1$ min^{-1}. Dialysis does not restore activity to enzyme inactivated by treatment with 20 mM OGDM (or with 20 mM OGDM plus 10 mM glutamate); activity is also not restored by addition of 0.1 mM PLP.

The structure of the coenzyme after inactivation of the PMP form of the enzyme by OGDM was investigated. In this experiment, the PMP form of the enzyme (5 mg, ~0.1 μmol of enzyme subunit) was incubated for 10 h with 10 mM OGDM in 0.5 ml of 100 mM potassium phosphate buffer (pH 7.2); 25°C. The inactivated enzyme was dialyzed and analyzed for PLP by the phenylhydrazone method of Wada and Snell (*19*). Less than 0.003 μmol of PLP was detected.

Inactivation of Pig Heart CytAspAT by OG—Incubation of the enzyme for 2 h at 25°C with 10 mM OG in the presence of 100 mM potassium phosphate buffer (pH 7.2)

TABLE I. **Inactivation of pig heart cytosolic aspartate aminotransferase (cytAspAT) by 2-oxoglutaconic acid dimethyl ester (OGDM).** Pig heart cytAspAT (~3 μg; PLP or PMP form as indicated) was incubated at 25°C in a solution (0.1 ml) containing 100 mM potassium phosphate buffer (pH 7.2) and, where indicated, 10 mM OGDM (keto form), 10 mM L-glutamate (Glu), 2 mM α-ketoglutarate (αKG), and 10 mM DTT. At intervals, 10-μl aliquots were withdrawn and assayed for enzyme activity. $n=6$-8 for each determination.

Addition	% Activity remaining		
	20 min	40 min	60 min
PLP form of the enzyme			
None	100±3	98±1	99±1
+L-Glu	101±3	99±1	98±1
+OGDM	95±2	90±1[a]	85±2[a]
+OGDM+L-Glu	49±4[a]	25±5[a]	13±2[a]
+OGDM+L-Glu+DTT	100±3	93±2	87±2[a]
+OGDM+L-Glu+αKG	20±2[a]	8±2[a]	4±1[a]
PMP form of the enzyme			
None	100±4	99±4	96±3
+OGDM	40±3[a]	20±3[a]	8±2[a]
+OGDM+DTT	98±2	93±4	88±2[a]
+OGDM+L-Glu+αKG	18±3[a]	6±1[a]	3±2[a]

[a]Significantly different from the values of the controls (*i.e.*, no addition) with $p \leq 0.025$.

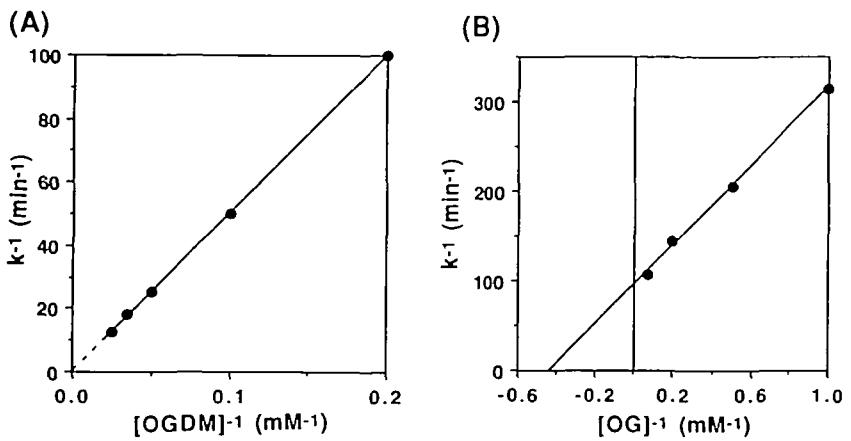

Fig. 2. **Plot of (A) reciprocal of rate constant for inactivation of pig heart cytAspAT *versus* reciprocal of the concentration of 2-oxoglutaconic acid dimethyl ester (OGDM), and (B) reciprocal of rate constant for inactivation of pig heart cytAspAT *versus* reciprocal of concentration of 2-oxoglutaconate (OG).** (A) The enzyme (5 μg; PLP form) was incubated in a reaction mixture (0.2 ml) containing 10 mM L-glutamate, 100 mM potassium phosphate buffer (pH 7.2), and varying concentrations of OGDM; 25°C. At intervals, aliquots (10-20 μl) were withdrawn and assayed for enzyme activity. The rate constant for inactivation (k) at each concentration of inhibitor was estimated from a plot of log activity remaining *versus* time. (B) As for A, except that OGDM was replaced by OG (obtained by hydolysis of OGDM with KOH followed by neutralization with HCl) and the pH of the phosphate buffer was 6.0.

results in some inactivation of the enzyme (~30%) (Table II). The extent of inactivation is enhanced by the presence of 10 mM L-glutamate. On the other hand, both α-ketoglutarate and DTT protect the enzyme against inactivation by OG (Table II). The PMP form of the enzyme is more strongly inactivated by OG than is the PLP form (Table II). The rate of inactivation of the PLP form of the enzyme (in the presence of glutamate) is slower with OG than with OGDM, even at high concentrations of OG. Thus, the $t_{1/2}$ values for inactivation of pig heart cytAspAT [PLP form; 30 μg/ml in 100 mM potassium phosphate buffer (pH 7.2) containing 10 mM L-glutamate; 25°C] in the presence of 40 mM OGDM and 50 mM OG are ~9 and ~80 min, respectively.

The kinetics of the OG-induced inactivation of cytAspAT were also investigated. For this experiment the enzyme was incubated with variable concentrations of OG (1.0, 2.0, 5.0, and 10 mM) in the presence of 100 mM potassium phosphate buffer, pH 6.0, and 10 mM L-glutamate (25°C). At intervals (20, 40, 60, 90, and 120 min), aliquots were withdrawn and residual activity was determined. In each case the rate of inactivation followed pseudo-first-order kinetics. A secondary plot of the data (*i.e.*, plot of reciprocal of concentration *versus* rate constant for inactivation) is shown in Fig. 2B. Unlike the case with OGDM, evidence was found for saturation of the enzyme with OG. The apparent K_I and the limiting rate constant for inactivation were found to be ~2 mM and ~0.01 min^{-1}, respectively (Fig. 2B). Similar results were obtained when pig heart cytAspAT (PLP form) was incubated with L-glutamate and varying concentrations of OG in the presence of 100 mM potassium phosphate buffer, pH 7.2 (data not shown). Inactivation by OG at both pH 6.0 and 7.2 is presumably due to the predominance of the keto form at these pH values. The activity of OG-inactivated enzyme could not be restored by dialysis or by addition of 0.1 mM PLP.

The similarity of OG to α-ketoglutarate suggests that turnover of OG to the corresponding L-amino acid (β,γ-dehydroglutamate) at the active site of cytAspAT might occur in the presence of glutamate or if the enzyme is in the PMP form. The partitioning ratio between turnover to dehydroglutamate and inactivation in the presence of OG and glutamate was not determined, but it was shown that incubation of PMP-enzyme with OG results in formation of PLP-enzyme. In this experiment, pig heart cytAspAT (5 mg, PMP form; ~0.1 μmol of cofactor) was incubated for 10 h in a solution (0.5 ml) containing potassium phosphate buffer, pH 7.2, and 10 mM OG at 25°C. PLP (0.07 μmol) was detected. An attempt was made to determine the partitioning ratio between transamination of aspartate with OG and inactivation. Enzyme (5 mg, PLP form) was incubated with 20 mM L-aspartate and 2 mM OG for 2 h in 0.5 ml of 100 mM potassium phosphate buffer, pH 7.2; 25°C. After 2 h the enzyme was 50% inactivated. The reaction mixture was then dialyzed against 4 volumes of distilled water at 4°C for 10 h and the protein-free fraction was analyzed for oxaloacetate. To the protein-free fraction (~2 ml) was added 0.1 ml of 1 M potassium phosphate buffer (pH 7.2), 0.01 ml of 10 mM NADH, and 0.01 ml of a solution containing MDH (1 mg/ml) and the absorbance decrease at 340 nm was measured; 25°C. No oxaloacetate could be detected by this procedure. Oxaloacetate is known to be unstable and in a control study, it was shown that the conditions of the experiment will result in 50% loss of oxaloacetate. Allowing for this loss, it is estimated that two turnover events of aspartate to oxaloacetate per inactivation event could have been detected.

Inactivation of Pig Heart CytAlaAT by OG—Some inactivation (~30%) of pig heart AlaAT was noted after 2

TABLE II. **Inactivation of pig heart cytosolic aspartate aminotransferase (cytAspAT) by 2-oxoglutaconate (OG).** Pig heart cytAspAT (~3 μg; PLP or PMP form as indicated) was incubated at 25°C in a solution (0.1 ml) containing 100 mM potassium phosphate buffer (pH 7.2) and, where indicated, 10 mM OG, 10 mM L-glutamate (Glu), 10 mM DTT, 20 mM methanol, 40 mM KCl, 10 mM α-ketoglutarate (αKG). At intervals, 10-μl aliquots were withdrawn and assayed for enzyme activity. $n=3$.

Addition	% Activity remaining	
	60 min	120 min
PLP form of the enzyme		
None	98±2	99±3
+Methanol+KCl[a]	98±3	95±2
+L-Glu	98±2	98±0
+OG	83±1[b]	68±1[b]
+OG+αKG	95±3	90±5
+OG+L-Glu	70±2[b]	50±1[b]
+OG+L-Glu+αKG	93±4	92±4
+OG+L-Glu+DTT	98±3	95±3
PMP form of the enzyme		
None	98±2	95±2
+Methanol+KCl[a]	96±2	94±4
+OG	56±3[b]	30±5[b]
+OG+αKG	96±3	92±1
+OG+DTT	97±2	92±3

[a]Control for the amount of KCl and methanol present when the enzyme was incubated with OG. [b]Significantly different from the values of the controls (*i.e.*, no addition) with $p=0.05$.

TABLE III. **Inactivation of aspartate aminotransferases by 2-oxoglutaconate (OG).** The various enzyme preparations (~3 μg pig heart cytAspAT; rat kidney cytosol containing 9 mg of protein; rat kidney mitochondria containing 5 mg of protein) were separately incubated at 25°C in solutions (0.1 ml) containing 100 mM potassium phosphate buffer (pH 7.2) and, where indicated, 50 mM OG (prepared from the alkaline hydrolysis of OGDM), 10 mM L-glutamate (Glu), 10 mM α-ketoglutarate (αKG). At time zero and at 1 h or 2 h, 10-μl aliquots were withdrawn and assayed for AspAT activity. $n=3$ for each determination. In all cases, the activities in the presence of OG and of OG+Glu after 1 or 2 h are significantly different from the control values with $p=0.05$. In a separate experiment it was found that the enzyme activity in each of the three preparations was not significantly decreased after a 2 h period by the presence of 200 mM KCl plus 100 mM methanol in 100 mM potassium phosphate buffer, pH 7.2 (=a control for the amount of methanol and KCl present when the enzyme preparations were incubated with OG).

Addition	Incubation time (h)	% Activity remaining		
		Purified pig heart cytAspAT	AspAT in rat kidney cytosol[a]	AspAT in rat kidney mitochondria[a]
None	2	100±0	98±1	100±0
+OG	1	87±1	86±1	93±2
+OG	2	72±1	71±2	81±2
+OG+Glu	1	76±3	68±2	78±1
+OG+Glu	2	54±1	56±2	66±3
+OG+αKG	2	95±2	92±3	94±4
+Glu	2	100±0	99±1	98±2

[a]The specific activities of AspAT (25°C) in the cytosolic and mitochondrial fractions were 0.21 and 0.80 μmol/min/mg of protein, respectively.

h of incubation in the presence of 50 mM OG (25°C; pH 7.0), but no additional experiments were carried out.

Inactivation of AspAT in Rat Kidney Cytosol and Mitochondrial Homogenates by OG—AspAT in rat kidney cytosol and mitochondrial homogenates is inactivated by OG in a time-dependent fashion (Table III). As was found for purified pig heart cytAspAT (Table II), the extent of OG-induced inactivation after 2 h was increased in the presence of glutamate (Table III).

Inactivation of Mouse Kidney AspAT In Vivo by OG—Four hours after intraperitoneal injection of OG into mice (5 μmol/g), the activity of total (*i.e.*, cytosolic plus mitochondrial) AspAT was significantly reduced ($p=0.0025$). The activity was 0.54 ± 0.06 μmol/min/mg of protein ($n=5$) in the controls *versus* 0.30 ± 0.03 ($n=6$) μmol/min/mg of protein in the animals injected with OG. No significant inhibition of AspAT was noted for liver or brain. Intraperitoneal injection of OGDM (5 μmol/g) into mice had no effect on the levels of AspAT in brain, liver, or kidney at 4 h (data not shown).

DISCUSSION

OG as a Possible Precursor of β,γ-Dehydroglutamate—OGDM has previously been used for the introduction of the third fused ring in the synthesis of the bacterial cofactor methoxatin (4,5-dihydro-4,5-dioxo-1*H*-pyrrolo[2,3]-quinoline [2,3-f]quinoline-2,7,9-tricarboxylic acid; PQQ) (*20*). The free α-keto acid form of OGDM (OG; 2-oxoglutaconate) has apparently not been synthesized. We reasoned that this dehydro analog of α-ketoglutarate may have some interesting biochemical properties and this proved to be the case. OGDM is readily hydrolyzed in base and the resulting OG was found to be a good substrate of GDH. Adaption of the reaction to the preparation of β,γ-dehydroglutamate should be relatively straightforward. Such a compound may prove interesting in studies of glutamate-utilizing enzymes and of glutamate receptors.

Inactivation of Pig Heart CytAspAT by OGDM—Birchmeier *et al.* showed that, of the five cysteine residues in the enzyme monomer, only two are rapidly alkylated by *N*-ethylmaleimide (NEM) (*21*). Alkylation of these two surface cysteines (cysteines I and II) does not result in inactivation; rather, alkylation of these two residues leads to a slight activation. Two deeply buried cysteine residues (cysteines IV and V) are completely resistent to alkylation by NEM. Another cysteine residue (residue III) is only very slowly alkylated by NEM when the enzyme is in the PLP form. This residue is more rapidly alkylated with NEM (and concomitantly inactivated) when the enzyme is in the PMP form or when the PLP-enzyme is incubated with the substrate analogs α-methylaspartate and *erythro*-β-hydroxyaspartate (*21*). The maximal rate of NEM-induced inactivation of the PLP-enzyme occurs in the presence of the substrate pair L-glutamate/α-ketoglutarate (*21*). Complete modification of cysteine III by NEM results in an enzyme with some residual activity (*21*). Modification of the -SH of cysteine III by disulfide bond formation with a variety of agents also results in an enzyme with residual activity (<5-17%, depending on the size of the disulfide) (*21*). On the other hand, modification of the -SH of cysteine III with the relatively small cyanide ion results in an enzyme with 60% activity relative to the native enzyme (*21*). Thus, the -SH of cysteine III is not absolutely required for active enzyme conformation or as a participant in the catalytic process (*21*). Indeed, X-ray crystallographic analyses of AspATs from several sources show that no cysteine residue occurs in the vicinity of the active site (see Ref. *22* for a review). Moreover, the PMP and PLP forms of chicken heart mitAspAT have almost identical crystal structures (*23*). Birchmeier *et al.* concluded that the alterations in the reactivity of cysteine residue III reflect conformational changes in the enzyme-coenzyme-substrate complex occurring "syncatalytically" (*21*). In other words, despite the fact that the PMP and PLP forms of the enzyme have similar configurations, movement occurs during the actual catalytic processing of substrates that can open the enzyme to attack by NEM at cysteine III, especially at the ketimine stage of the transamination cycle (*21*). All AspATs thus far investigated by X-ray crystallography have two domains in each of the two identical subunits—a large domain and a small domain, separated by a linkage region (*24*). The active site lies in a cavity close to the interface of the large and small domains and to the interface between the two subunits (*24*). In the absence of substrate, the enzyme active site is in an "open" conformation. During binding of substrates the two domains close in together (*24*). Torchinsky has suggested that cysteine III may be the cysteine that resides at the small domain interface and that its change in reactivity may be caused by motion of the small domain during the catalytic cycle (*25*).

The evidence presented herein suggests that OGDM, in a similar fashion to NEM, alkylates cysteine III of pig heart cytAspAT in a syncatalytic fashion. The possibility that OGDM also inactivates the enzyme by binding at the active site and alkylating a nearby residue seems unlikely, but cannot be ruled out completely. Evidently, if OGDM does bind to the active site the K_i is very high ($\gg$40 mM) or the alkylation of an active site residue is slow relative to alkylation of cysteine III. Inactivation of cytAspAT by OGDM is not likely to be due to some hydrolysis of OGDM to the free α-keto acid. The ^{13}C-NMR experiments showed that the sample of OGDM is not contaminated with methanol and that OGDM is quite stable in various phosphate buffers over a 24 h period. Moreover, 50 mM OG inactivates AspAT more slowly than does 40 mM OGDM (see below). Finally, when pig heart cytAspAT (PLP form, 3 μg) was incubated for 2 h (25°C) in a solution (0.1 ml) containing 100 mM potassium phosphate buffer (pH 6.0), 20 mM OGDM and 10 mM L-glutamate, essentially no inactivation occurred. This finding suggests that OGDM in the free carbonyl form, but not in its ketal (hydrate) form (the favored species at pH 6.0) is the inactivating species. The keto form is expected to be an excellent Michael acceptor, whereas the hydrated form is expected to be a poor acceptor. The idea that OGDM acts toward cytAspAT as an alkylating agent is supported by the finding that the rate of OGDM-induced inactivation (in the presence of glutamate) is greatly reduced in the presence of DTT (Table I). DTT is expected to form a Michael adduct with the reactive double bond of OGDM (keto form) and thereby prevent attack of OGDM on cysteine III of AspAT.

Inactivation of Pig Heart CytAspAT by OG—OG inactivates the enzyme by a somewhat different mechanism from that observed with OGDM. Thus, the glutamate-enhanced inactivation of cytAspAT in the presence of high

concentrations of OG is slower than that in the presence of high concentrations of OGDM (Fig. 2). Inactivation of cytAspAT with OG (in the presence of glutamate) exhibits saturation kinetics (K_I ~2 mM), whereas saturation kinetics with OGDM cannot be demonstrated (Fig. 2). The enzyme is protected against OG-induced inactivation by α-ketoglutarate and by glutamate/α-ketoglutarate (Table II), whereas glutamate/α-ketoglutarate stimulates the rate of inactivation by OGDM (Table I). Finally, inactivation of the PMP form of cytAspAT by OG results in the recovery from denatured enzyme of coenzyme mostly in the form of PLP, whereas inactivation of the PMP form with OGDM results in recovery of only a small amount of PLP from the denatured enzyme.

Because OG inactivates the PLP form of cytAspAT in the absence of glutamate it is possible that OG alkylates cysteine residue III. However, because the maximal rate of inactivation is much less with OG than with OGDM, OG must react with cysteine III (if at all) much less effectively than does OGDM. Possibly, the two carboxylates of OG preclude its efficient binding at the site of cysteine III. The present findings are consistent with the idea that OG binds at the active site of the PLP form of the enzyme and slowly alkylates a susceptible residue at the active site. This idea is in accord with the fact that α-ketoglutarate, which has a size and charge similar to that of OG, can bind at the active site of the PLP-enzyme as an abortive complex (*26*). Inactivation of the PLP form of the enzyme by OG suggests that this compound behaves as an affinity labeling agent. On the other hand, other evidence is consistent with the role of OG as a k_{cat} inhibitor. Thus, the rate of OG-induced inactivation of the PLP-enzyme is enhanced in the presence of glutamate or if the enzyme is converted to the PMP form. Evidently, Michael addition to a susceptible active site base is more favored with the OG-ketimine than with free OG. Therefore, in this sense OG is a k_{cat} inhibitor (Fig. 3).

Many k_{cat} inhibitors of AspAT thus far described possess a double bond β,γ to a carbon attached to an amino group. Turnover at the active site produces the unsaturated α-keto acid or the unsaturated α-keto acid bound in a ketimine linkage to the cofactor. Both of these are much stronger Michael acceptors than is the parent amino acid. As a result, processing of the β,γ-unsaturated amino acid at the active site leads to alkylation of a nearby residue, or possibly, in the case of vinylglycine, to a PLP-aldol adduct (*27*). OG possesses a double bond β,γ to a keto group and, as noted above, is poised either directly or indirectly (following ketimine linkage to cofactor) to act as a Michael acceptor for the alkylation of a susceptible residue at the active site of cytAspAT. The present findings are reminiscent of those of Rando *et al.* (*7*). These authors showed that the α-keto analog of L-2-amino-4-methoxy-*trans*-3-butenoate (AMB) (generated *in situ* by incubation of AMB with L-amino acid oxidase) is an affinity labeling reagent of the active site of pig heart cytAspAT (*7*). The α-keto analog of AMB can act on the PLP form of the enzyme, but the rate of inactivation is enhanced in the presence of aspartate (or if the enzyme is converted to the PMP form). The enzyme is protected by 2-mercaptoethanol, presumably because the thiol adds to the activated double bond of "keto" AMB, preventing it from adding to a susceptible enzyme residue (*7*). Exactly analogous results were found in the present study with OG (Table II).

The enhancement of the rate of OG-induced inactivation by glutamate is the opposite of the case for inactivation of cytAspAT by unsaturated amino acids such as βMA. In the latter case, the enzyme is protected by the presence of glutamate (*9*). Interaction of cytAspAT with glutamate will promote the conversion of the enzyme from the PLP form (lower affinity toward OG) to the PMP form (higher affinity toward OG) and promote the production of reactive OG-ketimine. Interestingly, the K_I exhibited by AspAT toward OG (~2 mM) is of the same order as that exhibited toward the β,γ-unsaturated amino acid βMA (3 mM for the L

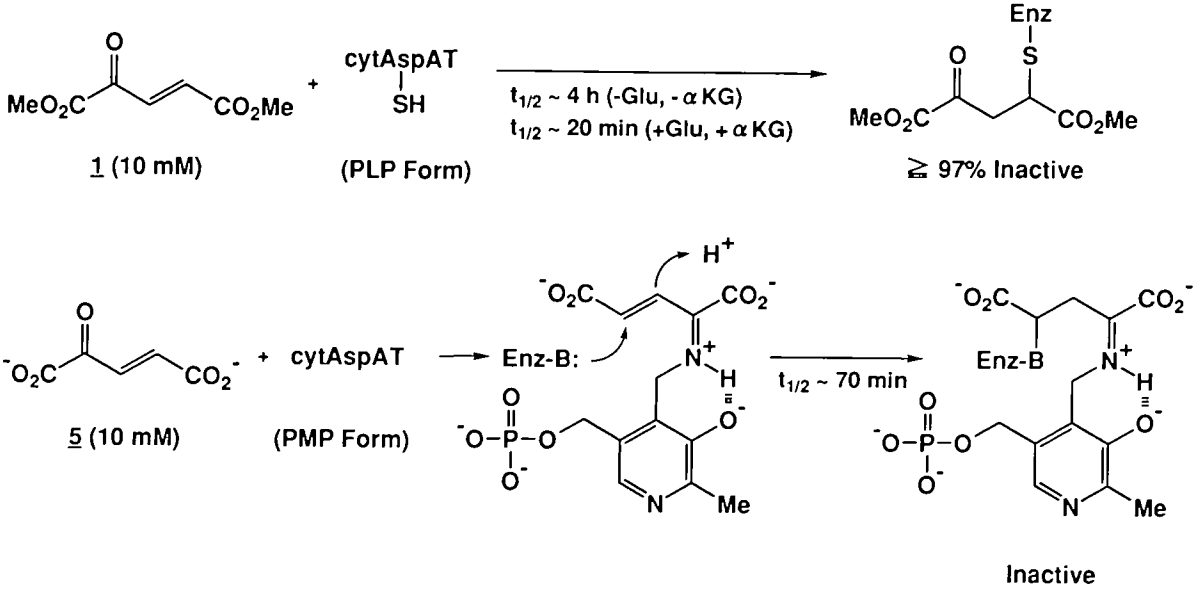

Fig. 3. **Proposed mechanism for inactivation of pig heart cytAspAT by 2-oxoglutaconic acid dimethyl ester (OGDM; 1) and by 2-oxoglutaconate (OG; 5).** The -SH group shown in the top sequence is part of a cysteine residue that is not located at the active site, but which is syncalytically modified by alkylating agents (see the text).

form) (*9*). However, the maximal $t_{1/2}$ values for inactivation of pig heart AspAT by OG (in the presence of glutamate) and by βMA are ~70 min (present work) and ~1.7 min (*9*) at 25°C, respectively. This contrast in maximal rates of inactivation may be due to differences in the positioning of the double bond within the active site. It is probable that both unsaturated compounds bind to the active site such that their carboxylates occupy roughly similar positions. Possibly, the γ-olefinic carbon of OG (either bound unchanged to the PLP form of the enzyme or bound in ketimine linkage with the PMP form of the enzyme) is more deeply buried within the active site than is the γ-olefinic carbon of the ketimine intermediate of βMA. Thus, the double bond of enzyme-bound βMA may be more accessible than that of OG for Michael addition to a susceptible active site residue. The limiting rate constant for inactivation of AspAT by AMB was not reported by Rando *et al.* (*7*), but the maximal $t_{1/2}$ must have been $\ll$20 min. The greater rate of inactivation with AMB than with OG may be due to the fact that AMB does not possess a group that binds to the second carboxylate binding site. In the case of AMB, lack of a second carboxylate will allow more freedom of movement of the ketimine intermediate within the active site and greater probability that the γ-olefinic carbon will come into contact with a susceptible active site group.

The partitioning ratio between events leading to transamination of OG (to β,γ-dehydro-L-glutamate) and events leading to inactivation at the active site of cytAspAT is low ($\leq$2). Similarly, a low partitioning ratio between transamination and inactivation was previously noted for the interaction of cytAspAT with βMA (*9*). It is also interesting to note that pig heart cytAspAT catalyzes transamination between *erythro*-β-hydroxyaspartate and α-ketoglutarate 500 times more slowly than it catalyzes transamination between L-aspartate and α-ketoglutarate, despite the fact that the K_m values exhibited by the enzyme for the two amino acids are comparable (*28*). In some cases, compounds that are closely related to the natural substrates can bind effectively to the active site of cytAspAT, but do not turn over readily because of the slowing of a key catalytic step (*29*). For a quantitative treatment of the reaction of *erythro*-β-hydroxyaspartate with cytAspAT see Ref. *29*.

Conclusions—OG is a slow irreversible inactivator of pig heart cytAspAT and of rat kidney cytAspAT and mitAspAT. *In vivo* inhibition of total (cyt-plus mit) AspAT by OG was demonstrated for mouse kidney. OG is a good substrate of GDH and therefore should be amenable to preparative enzymatic conversion to β,γ-dehydro-L-glutamate. Finally, the present results may suggest possible strategies for the design of more selective and effective inhibitors of AspAT *in vivo* than are currently available.

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