Phosphonate Analogues of α-Ketoglutarate Inhibit the Activity of the α-Ketoglutarate Dehydrogenase Complex Isolated from Brain and in Cultured Cells†

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Received February 19, 2005; Revised Manuscript Received May 12, 2005

ABSTRACT: The α-ketoglutarate dehydrogenase complex (KGDHC), a control point of the tricarboxylic acid cycle, is partially inactivated in brain in many neurodegenerative diseases. Potent and specific KGDHC inhibitors are needed to probe how the reduced KGDHC activity alters brain function. Previous studies showed that succinyl phosphonate (SP) effectively inhibits muscle and *Escherichia coli* KGDHC [Biryukov, A. I., Bunik, V. I., Zhukov, Yu. N., Khurs, E. N., and Khomutov, R. M. (1996) *FEBS Lett. 382*, 167−170]. To identify the phosphonates with the highest affinity toward brain KGDHC and with the greatest effect in living cells, we investigated the ability of SP and several of its ethyl esters to inhibit brain KGDHC, other α-keto acid-dependent enzymes, and KGDHC in intact cells. At a concentration of 0.01 mM, SP and its phosphonoethyl (PESP) and carboxyethyl (CESP) esters completely inhibited isolated brain KGDHC even in the presence of a 200-fold higher concentration of its substrate [α-ketoglutarate (KG)], while the diethyl (DESP) and triethyl (TESP) esters were ineffective. In cultured human fibroblasts, 0.01 mM SP, PESP, or CESP produced 70% inhibition of KGDHC. DESP and TESP were also inhibitory in the cell system, but only after preincubation, suggesting the release of their charged groups by cellular esterases. Thus, SP and its monoethyl esters target cellular KGDHC directly, while the di- and triethyl esters are activated in intact cells. When tested on other enzymes that bind KG or related α-keto acids, SP had minimal effects and its two esters (CESP and TESP) were ineffective even at a concentration (0.1 mM) 1 order of magnitude higher than that which inhibited cellular KGDHC activity. The high specificity in targeting KGDHC, penetration into cells, and minimal transformation by cellular enzymes indicate that SP and its esters should be useful in studying the effects of reduced KGDHC activity on neuronal and brain function.

KGDHC[1] is a member of the family of α-keto acid dehydrogenase complexes, which also includes the pyruvate dehydrogenase complex (PDHC) and the branched-chain α-keto acid dehydrogenase complex. KGDHC is a control point of the tricarboxylic acid cycle in different tissues (*1*−*3*). The overall transformation catalyzed by KGDHC (reactions 1−5 in Scheme 1) involves the sequential actions of α-ketoglutarate dehydrogenase (E_1k), dihydrolipoamide succinyltransferase (E_2k), and dihydrolipoamide dehydrogenase (E_3). As a result, the energy of the C_1-C_2 bond of the KG substrate is preserved in succinyl-CoA and NADH.

Brain KGDHC activity is reduced in many neurodegenerative diseases (*2*, *3*). Potent and specific inhibitors are needed to probe how a decrease in KGDHC activity alters brain function. To achieve this goal, targeting the first component of the complex, the ThDP-dependent α-ketoglutarate dehydrogenase (E_1k) is preferred because this enzyme catalyzes the rate-limiting step of the overall process (*4*, *5*) and largely determines the substrate specificity of KGDHC (*6*). The branched-chain α-keto acids that are elevated in maple syrup urine disease [e.g., α-keto-β-methylvalerate (KMV)] are known inhibitors of cellular KGDHC (*7*). However, millimolar concentrations of KMV are required to inhibit KGDHC (*7*, *8*), and at these concentrations, KMV

† Supported by NIH Grants AG14930, AG19589, and AG11921 to G.E.G. C.M.T. acknowledges support from the National Science Foundation (Grants MCB9808372 and EPS0091995) and the NIH (Grant RO1 NS-38248).

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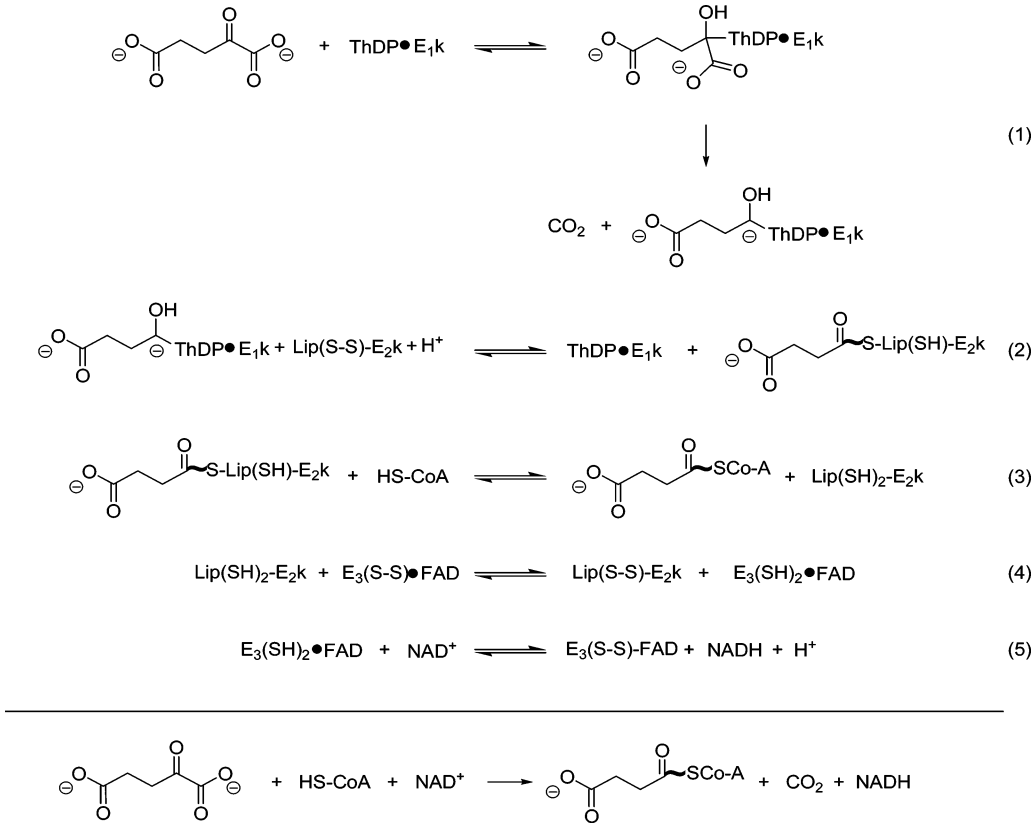
[1] Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; CESP, carboxy ethyl ester of SP; DESP, diethyl (carboxy and phosphono) ester of SP; DTPA, diethylenetriaminepentaacetic acid; EtOAc, ethyl acetate; E_1k, α-ketoglutarate dehydrogenase (EC 1.2.4.2); E_2k, dihydrolipoamide succinyltransferase (EC 2.3.1.61); E_3, dihydrolipoamide dehydrogenase (EC 1.8.1.4); KG, α-ketoglutarate; KGDHC, α-ketoglutarate dehydrogenase complex; HBSS, Hank's Balanced Salt Solution; HRMS, high-resolution mass spectrometry; KMV, α-keto-β-methylvalerate; PDHC, pyruvate dehydrogenase; PEG, polyethylene glycol; PMSF, phenylmethanesulfonyl fluoride; PESP, phosphono ethyl ester of SP; SP, succinyl phosphonate; TESP, triethyl ester of SP; ThDP, thiamine diphosphate.

10.1021/bi0503100 CCC: $30.25
Published on Web 07/15/2005

Scheme 1



may have multiple targets in cells. Because structurally similar α-keto acids are metabolized by a number of enzymes, a strategy for targeting the respective α-keto acid dehydrogenase complexes in situ and in vivo may require mechanism-based inhibitors. This class of compounds is becoming increasingly useful in proteomic research by selectively inhibiting components in complex biological mixtures (*9* and references therein).

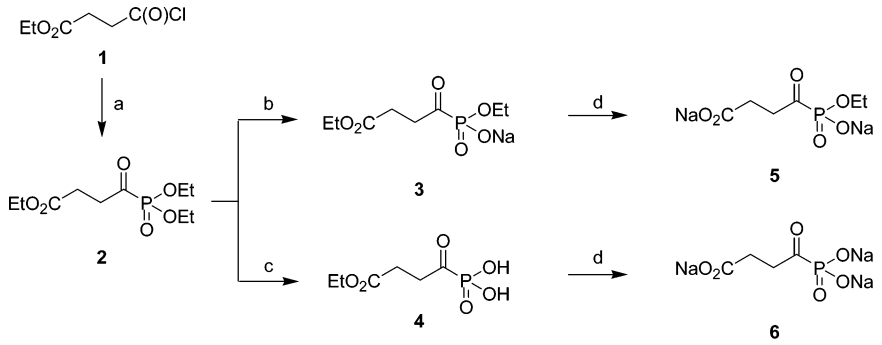
Mechanism-based inhibitors target specific enzymes because they mimic initial steps of catalysis, but are unable to complete the catalytic cycle. In many cases, the enzyme undergoes a covalent modification by the mechanism-based inhibitor. The inhibitory properties are often acquired or strengthened through conformational changes of the enzyme−ligand complex, analogous to those occurring during catalysis. In the α-keto acid dehydrogenases, conformational changes upon binding of α-keto acids contribute to the selectivity and catalytic transformation of specific substrates (*6*, *10*−*12*). Substitution of the carboxylate of α-keto acids with a phosphonate results in mechanism-based inhibitors of the α-keto acid dehydrogenases as first described in the pioneering work by Kluger and Pike on the inhibition of pyruvate dehydrogenase by methyl acetyl phosphonate and derivatives (*13*). The phosphonates enter reaction 1 and induce conformational transitions that both increase the specificity of the phosphonate reaction with ThDP and tighten binding of the resulting adduct but are unable to undergo cleavage analogous to that exhibited by the α-keto carboxylate substrates (*13*−*18*). Limited pathways for biodegradation of the C−P bond (*19*) ensure that phosphonates, upon entering cells, will not be quickly metabolized to ineffective species. Indeed, several phosphonate analogues of α-keto and α-amino acids have been successfully used to target specific pathways in intact cells and organisms (*20*, *21*). These considerations suggest that the phosphonate analogues of KG will specifically target cellular KGDHC.

Binding of the phosphonate analogues of α-keto acids to their target enzymes (*13*, *16*, *20*, *22*−*24*) and their permeability through biological membranes may depend on the inhibitor charge. Therefore, to develop efficient and specific inhibitors of cellular KGDHC, the KG phosphonate analogue, succinyl phosphonate (SP), and a number of its ethyl esters were tested in this work on both isolated brain KGDHC and KGDHC within living cells in culture.

EXPERIMENTAL PROCEDURES

Materials. Ethyl succinyl chloride and triethyl phosphite were purchased from Aldrich and used without additional purification. Alanine aminotransferase (pig heart), cytosolic aspartate aminotransferase (pig heart), glutamate dehydrogenase (bovine), lactate dehydrogenase (rabbit muscle), mitochondrial malate dehydrogenase (pig heart), leucine dehydrogenase (bacterial), and all substrates, buffers, cofactors, and protease inhibitors for the enzyme isolation and assays were from Sigma Chemical Co. (St. Louis, MO). Thiol-Sepharose and HiTrap desalting columns were from Amersham/Pharmacia (Uppsala, Sweden). The reagents for growing the fibroblasts were as previously described (*25*). Recombinant mitochondrial aspartate aminotransferase (rat) was a gift from A. Iriarte (Department of Molecular Biology and Biochemistry, School of Biological Sciences, University of Missouri, Kansas City, MO). Recombinant mitochondrial branched-chain aminotransferase (human) and cytosolic branched-chain aminotransferase (human) were from S. M. Hutson (Department of Biochemistry, Wake Forest University School of Medicine, Winston-Salem, NC).

Scheme 2 [a]



[a] (a) $(EtO)_3P$, (b) NaI, (c) TMS-Br, and (d) NaOH.

Synthesis of the α*-Ketoglutarate Phosphonate Derivatives.* The phosphonate analogues of KG were prepared by a modification of the procedure previously reported by Khomutov et al. (*26*) (Scheme 2). All ^1H, ^{13}C, and ^{31}P NMR spectra were recorded on a Varian Gemini spectrometer at 400, 100, and 161 MHz, respectively. Chemical shifts were reported in parts per million (ppm, δ) using residual solvent signals (^1H and ^{13}C) as internal standards and 35% H_3PO_4 in $CDCl_3$ as the ^{31}P external standard. Mass spectra were recorded on a MicroMass LCT ESI-MS instrument.

*Ethyl 4-(diethylphosphono)-4-oxobutanoate [triethyl ester of SP (TESP)] (**2**).* To triethyl phosphite (3.97 mL, 23.16 mmol) under argon was added ethyl succinyl chloride **1** (3.00 mL, 21.05 mmol) dropwise. The resultant solution was stirred at room temperature for 8 h. The byproducts were removed by distillation (below 85 °C, 0.050 mm Hg), and the ethyl 4-(diethylphosphono)-4-oxobutanoate was collected by distillation (98−100 °C, 0.050 mm Hg, 5.37 g, 95.6%) as a colorless oil: ^1H NMR ($CDCl_3$) δ 4.12 (dq, $J = 7.1$ Hz, 4 H), 4.03 (q, $J = 7.1$ Hz, 2 H), 3.04 (td, $J = 2.6$, 6.5 Hz, 2 H), 2.51 (t, $J = 6.5$ Hz, 2 H), 1.26 (t, $J = 7.1$ Hz, 6 H), 1.13 (t, $J = 7.1$ Hz, 3 H); ^{13}C NMR ($CDCl_3$) δ 210.0, 208.3, 63.6 (d, $J_{C-P} = 6.0$ Hz), 60.5, 37.9 (d, $J_{C-P} = 57.7$ Hz), 26.6 (d, $J_{C-P} = 6.1$ Hz), 16.1 (d, $J_{C-P} = 6.1$ Hz), 13.8; ^{31}P NMR ($CDCl_3$) δ 2.7; HRMS calcd for $C_{10}H_{20}O_6P$ 267.0998, found 267.0993 ($[M + H]^+$).

*Ethyl 4-(sodium, ethylphosphono)-4-oxobutanoate [diethyl ester of SP (DESP)] (**3**).* To a solution of ethyl 4-(diethylphosphono)-4-oxobutanoate (0.72 g, 2.71 mmol) in acetone (15 mL) under argon was added NaI (0.43 g, 2.84 mmol) dropwise over the course of 10 min. The resultant solution was heated to reflux and stirred for 14 h. The solvent was removed in vacuo to afford the ethyl 4-(sodium, ethylphosphono)-4-oxobutanoate, as a pale yellow solid, which was >95% pure as determined by ^1H NMR analysis. This material was used in biological assays and additional reactions without further purification: ^1H NMR (d_6-acetone) δ 4.01 (q, $J = 7.1$ Hz, 2 H), 3.86 (t, $J = 7.1$ Hz, 2 H), 3.10 (br s, 2 H), 2.54 (b t, $J = 5.8$, 3.9 Hz, 2 H), 1.14−1.10 (m, 6 H); ^{13}C NMR (d_6-acetone) δ 208.6, 174.2, 62.6, 61.7, 38.2 (d, $J_{C-P} = 45.6$ Hz), 28.1, 17.3 (d, $J_{C-P} = 6.1$ Hz), 14.9; ^{31}P NMR (d_6-acetone) δ 4.7. Spectral data also recorded in D_2O: ^1H NMR (D_2O, 400 MHz) δ 4.05 (q, $J = 7.1$ Hz, 2 H), 3.85 (q, $J = 7.1$ Hz, 2 H), 3.07 (td, $J = 6.5$, 5.8, 1.9 Hz, 2 H), 2.54 (t, $J = 6.5$, 5.8 Hz, 2 H), 1.15 (qd, $J = 7.1$, 1.9 Hz, 6 H); ^{13}C NMR (D_2O, 100 MHz) δ 220.5 (d, $J_{C-P} = 165.1$ Hz), 176.2, 63.7 (d, $J_{C-P} = 6.1$ Hz), 63.0, 39.3, 38.8, 28.2 (d, $J_{C-P} = 4.1$ Hz), 17.0 (d, $J_{C-P} = 5.1$ Hz), 14.4; ^{31}P NMR (D_2O, 161 MHz) δ 1.35; HRMS calcd for $C_8H_{16}O_6P$ 239.0685, found 239.0684 ($[M + H]^+$).

*Ethyl 4-phosphono-4-oxobutanoate [carboxy ethyl ester of SP (CESP)] (**4**).* To ethyl 4-(diethylphosphono)-4-oxobutanoate **2** (1.27 g, 4.78 mmol) under argon was added bromotrimethylsilane (5.68 mL, 43.04 mmol) dropwise over the course of 5 min. The resultant solution was stirred at room temperature overnight. The excess bromotrimethylsilane was removed in vacuo, and the remaining residue was partitioned between water (50 mL) and EtOAc (50 mL). The aqueous layer was collected and washed with EtOAc (2 × 50 mL), and the combined organic fractions were back-extracted with water (2 × 20 mL). The combined aqueous portions were concentrated in vacuo to afford ethyl 4-oxo-4-phosphonobutyrate as a clear semisolid which was >90% pure as determined by ^1H NMR analysis. This material was used in biological assays and additional reactions without further purification: ^1H NMR (D_2O) δ 3.41 (q, $J = 5.2$ Hz, 2 H), 2.46 (br, 2 H), 1.93 (br, 2 H), 0.50 (br, 3 H); ^{13}C NMR (D_2O) δ 218.1 (d, $J_{C-P} = 167.1$ Hz), 175.6, 62.7, 38.3 (d, $J_{C-P} = 51.1$ Hz), 27.8, 14.0; ^{31}P NMR (D_2O) δ 3.97; HRMS calcd for $C_6H_{12}O_6P$ 211.0372, found 211.0364 ($[M + H]^+$).

*Sodium 4-(sodium, ethylphosphono)-4-oxobutanoate [phosphono ethyl ester of SP (PESP)] (**5**).* To a solution of ethyl 4-(sodium, ethylphosphono)-4-oxobutanoate (1.35 mmol, used directly without purification from the previous reaction) in deionized water (3 mL) was added 1 N NaOH (1.35 mmol), and the resultant solution was stirred at room temperature for 4 h. The solvent was removed in vacuo to afford the sodium 4-(sodium, ethylphosphono)-4-oxobutanoate, as a yellow oil, which was >95% pure as determined by ^1H NMR analysis. This material was used in biological assays without further purification: ^1H NMR (D_2O) δ 3.95 (m, $J = 7.1$ Hz, 2 H), 3.07 (t, $J = 7.1$ Hz, 2 H), 2.41 (m, 2 H), 1.25 (t, $J = 7.1$ Hz, 3 H); ^{13}C NMR (D_2O, 100 MHz) δ 221.8, 183.2, 63.9 (d, $J_{C-P} = 6.4$ Hz), 41.0 (d, $J_{C-P} = 46.7$ Hz), 31.4 (d, $J_{C-P} = 4.2$ Hz), 17.5 (d, $J_{C-P} = 6.4$ Hz); ^{31}P NMR (D_2O, 161 MHz) δ 0.6; HRMS calcd for $C_6H_{10}O_6P$ 209.0215, found 209.0203 ($[M - H]^-$).

*Trisodium 4-phosphono-4-oxobutanoate [succinyl phosphonate (SP)] (**6**).* To a solution of ethyl 4-phosphono-4-butanoate (0.29 g, 1.38 mmol) in deionized water (10 mL) was added NaOH (1 M, 3.2 mmol) in portions, and the resultant solution was heated to 50 °C and stirred overnight. The solvent was removed in vacuo to afford trisodium 4-phosphono-4-oxobutanoate, as a clear semisolid, which was >95% pure as determined by ^1H NMR analysis. This material was used in biological assays without further

purification: ^{1}H NMR (D_2O) δ 2.94 (t, J = 6.8 Hz, 2 H), 2.22 (t, J = 6.8 Hz, 2 H); ^{13}C NMR (D_2O) δ 228.6 (d, J_{C-P} = 156.6 Hz), 183.4, 40.4 (d, J_{C-P} = 42.8 Hz), 31.5 (d, J_{C-P} = 3.6 Hz); ^{31}P NMR (D_2O, 161 MHz) δ 0.5; HRMS calcd for $C_4H_6O_6P$ 180.9902, found 180.9904 ([M − H]$^-$.); HRMS calcd for $C_4H_5O_6NaP$ 202.9721, found 202.9713 ([M + Na − H]$^-$).

Partial purification of brain KGDHC and PDHC was performed by a modification of published methods (*27*, *28*). Cow brains, obtained from a local slaughterhouse, were stored frozen at −80 °C. Except where indicated, the purification procedures were performed at 4 °C. The pH of the buffers was adjusted at room temperature. The buffers were then cooled to 4 °C. Frozen tissue (0.5 kg) was freed of white matter as much as possible, cut in pieces, and homogenized in 1 L of isolation buffer A [0.05 M MOPS (pH 7.0) containing 1.5 mM EDTA, 1.5 mM diethylenetriaminepentaacetic acid (DTPA), 0.5 mM ThDP, 1 mM benzamidinium chloride, 8 μM leupeptin, and a 1:300 diluted mammalian AEBSF protease inhibitor cocktail that included AEBSF, pepstatin A, E-64, bestatin, leupeptin, and aprotinin]. Homogenization was carried out in a Waring blender at maximal speed for 5 min. The homogenate was diluted with 1 L of isolation buffer A (pH 6.8) containing 12% (V/V) Triton X-100 and 2 mM PMSF and centrifuged for 30 min at 28000*g*. The supernatant was filtered through cotton and adjusted to pH 6.45 with 10% (v/v) acetic acid followed by addition of 0.15 volume of a 35% (w/v) PEG-6000 solution. After stirring for 50 min, the suspension was centrifuged for 30 min at 28000*g*. The pellet was dissolved in 400 mL of isolation buffer B [buffer A (pH 6.8) containing 0.05 mM AEBSF instead of the complete mammalian protease inhibitor cocktail]. Insoluble material was removed by centrifugation at 28000*g* for 40 min. $MgCl_2$ (1 M) was added to the supernatant to a final concentration of 13 mM. The ionic strength was increased by addition of 1 M potassium phosphate buffer (pH 6.3) to a final concentration of 50 mM. The pH was adjusted to 6.45 with 10% (v/v) acetic acid followed by addition of 0.15 volume of a 35% (w/v) PEG-6000 solution. After the suspension had been stirred for 30 min, it was centrifuged for 40 min at 28000*g*. The resulting pellet was suspended in 100 mL of isolation buffer B (pH 6.8). The suspension was stirred at room temperature for 1 h and centrifuged at 20 °C for 60 min at 41000*g*. The supernatant was subjected to pH fractionation with 10% (v/v) acetic acid. Pellets were collected by centrifugation at 40000*g* for 10 min. The precipitate which formed at pH 6.0 was discarded. The highest KGDHC specific activity (0.1−0.2 μmol min^{-1} mg^{-1}) was obtained upon precipitation in the pH interval from 6.0 to 5.7. The precipitate from pH 5.7 to 5.4 contained PDHC activity. The pellets were dissolved in 5−10 mL of 0.1 M potassium phosphate buffer (pH 7.0) containing the mammalian AEBSF protease inhibitor cocktail at a dilution of 1:5000. The preparations were stable upon freezing and storage. Further attempts to purify increased the specific activity, but resulted in a significant loss of total activity and instability of the preparation. Because protein instability interferes with studies of inhibition, the less pure but more stable preparation was used in most of this work.

Cell Culture. Fibroblasts were grown according to our published protocol in a growth medium of Dulbecco's Modified Eagle's Medium (DMEM) (*25*).

Assays of Enzymatic Activities. Activity of the partially purified brain KGDHC was measured spectrophotometrically by monitoring NADH production in 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$, 1 mM ThDP, 2.5 mM dithiothreitol, and saturating concentrations of substrates (2 mM KG, 0.1 mM CoA, and 2.5 mM NAD^+). The same reaction mixture at pH 7.6 was used for the PDHC assay, except that 2 mM pyruvate was added instead of KG. Reactions were started by addition of the enzymes. No reaction was observed when any of the substrates of the complexes was omitted.

Glutamate dehydrogenase, lactate dehydrogenase, mitochondrial malate dehydrogenase, leucine dehydrogenase, alanine aminotransferases, mitochondrial and cytolic aspartate aminotransferase (*29*), and the branched-chain cytosolic and mitochondrial aminotransferases (*30*) were assayed spectrophotometrically by monitoring NADH consumption according to our published methods except that the concentration of the α-keto acid substrate was fixed at 2 or 5 mM for estimation of SP inhibition or substrate properties, respectively.

Histochemical Assay of KGDHC. To estimate relative KGDHC activity in intact cells, the formation of a dark blue formazan product, produced from an electron transfer from NADH to nitroblue tetrazolium, was assessed (*31*). The in situ staining for KGDHC was assessed after subtracting the background in the absence of substrates KG and CoA. Human fibroblasts were seeded on 24-well plates at a seeding density of 5×10^3 cells/well for 6 days in 0.5 mL of growth medium (DMEM with 10% fetal calf serum). On the day of each experiment, the medium was aspirated, and the cells were washed once with balanced salt solution (BSS) [140 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES (pH 7.4)]. The cells were then treated with 0.01 mM SP or its various ethyl esters in 0.5 mL of BSS for 0, 60, or 120 min at 37 °C. At the end of the treatment, the buffer was aspirated and the well was washed with 200 μL of Hank's Balanced Salt Solution (HBSS) containing 0.05% (v/v) Triton X-100. Wells were incubated with 200 μL of either complete KGDHC assay mixture or KGDHC assay mixture without substrates KG and CoA. The reaction mix contained 50 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.05 mM EDTA, 0.3 mM ThDP, 0.5 μg/mL rotenone (in 100% ethanol; final ethanol concentration of 0.1%), 0.2% Triton X-100, 3.5% polyvinyl alcohol (MW = 3000−7000), 3 mM KG, 3 mM NAD^+, 1 mM CoA, 0.75 mM nitroblue tetrazolium, and 0.05 mM phenazine methosulfate. Nitroblue tetrazolium and phenazine methosulfate were added immediately before the reaction. In experiments in which SP and its ethyl esters were examined, the assay mixture included the compound of interest (0.01 mM). Samples were incubated with the assay mixture for 40 min. After incubation, the treatment medium was aspirated, and the cells were washed with Ca^{2+}- and Mg^{2+}-free HBSS. The dark blue formazan product was solubilized with 10% (w/v) SDS in 0.01 N HCl overnight in a CO_2 incubator (37 °C).The absorbance was read at 570 nm with a Spectra Max 250 model plate reader (Molecular Devices, Sunnyvale, CA).

Statistical Analysis. All values for cells were expressed as means ± the standard deviation. SPSS (SPSS Co., Chicago, IL) was used for statistical analysis. A P of <0.05

Trivial name	Systematic name	Structure	Number in scheme 1
Succinylphosphonate (SP)	4-Phosphono 4-oxobutanoate	$^{-}OP(O)(O^{-})\text{-}C(O)\text{-}(CH_2)_2\text{-}COO^{-}$	6
Carboxy ethyl ester of SP (CESP)	Ethyl 4-phosphono 4-oxobutanoate	$^{-}OP(O)(O^{-})\text{-}C(O)\text{-}(CH_2)_2\text{-}C(O)\text{-}O\text{-}C_2H_5$	4
Phosphono ethyl ester of SP (PESP)	4-Ethyl phosphono-4-oxobutanoate	$^{-}OP(O)(O\text{-}C_2H_5)\text{-}C(O)\text{-}(CH_2)_2\text{-}COO^{-}$	5
Diethyl (carboxy and phosphono) ester of SP (DESP)	Ethyl 4-(ethyl phosphono) 4-oxobutanoate	$^{-}OP(O)(O\text{-}C_2H_5)\text{-}C(O)\text{-}(CH_2)_2\text{-}C(O)\text{-}O\text{-}C_2H_5$	3
Triethyl ester of SP (TESP)	Ethyl 4-(diethyl phosphono)-4-oxobutanoate	$H_5C_2\text{-}OP(O)(O\text{-}C_2H_5)\text{-}C(O)\text{-}(CH_2)_2\text{-}C(O)\text{-}O\text{-}C_2H_5$	2

FIGURE 1: Succinyl phosphonate and its esters.

was considered significant. The statistical significance of group differences was tested by one-way analysis of variance (ANOVA) followed the Student−Newman−Keuls post hoc test.

RESULTS

The structures of the phosphonate analogues of KG (described in the Experimental Procedures) that were used in studies of isolated enzymes and in cell studies are provided in Figure 1. The ability of the analogues to act as enzyme inhibitors was first tested with our preparation of brain KGDHC. To assess the specificity of the inhibition in cells, the phosphonates were examined with enzymes that bind KG or its structural analogues. The substrate properties of the phosphonates were also tested with these enzymes. Finally, the ability of the compounds to inhibit KGDHC activity in living cells in culture was studied.

Brain KGDHC was assayed in the presence of different concentrations of SP and its various ethyl esters (Figure 2). SP and the monoethyl esters of its phosphonate (PESP) or carboxyl (CESP) groups efficiently inhibited KGDHC (Figure 2A,C) even at a high concentration of KG (2 mM). In view of the complex kinetic dependence of the reaction rate on KG concentration, the affinity of the brain KGDHC for KG was assessed by the value of $S_{0.5}^{KG}$. It was determined to be 0.12 mM, which is within the range of K_m^{KG} values from 0.1 to 0.2 mM obtained for KGDHC from different sources under similar conditions (*6*, *32*−*34*). At 2 mM KG, the rate of the reaction catalyzed by brain KGDHC was ∼80−90% of the maximal observed reaction rate. Together with the value of $S_{0.5}^{KG}$, this suggested an almost complete saturation of brain E_1k with KG at 2 mM. Remarkably, at this nearly saturating substrate concentration, the IC_{50} for substrate analogues SP, PESP, and CESP was less than 0.0005 mM (Figure 2A), indicating an extreme sensitivity of brain KGDHC to the phosphonates. The strength of the inhibition exhibited by the three derivatives did not differ significantly, but additional ester groups led to the loss of the inhibition. The diethyl and triethyl esters of SP were very poor inhibitors, even at KG concentrations (0.05 mM) significantly lower than the $S_{0.5}^{KG}$ (Figure 2B). Thus, decreasing the SP charge by conversion to the monoethyl esters of either the phosphonate or carboxyl group does not significantly alter the ability of the phosphonate analogues to inhibit brain KGDHC relative to unesterified SP; however, esterification of both the phosphonate and carboxyl groups of SP in the di- or triethyl esters abolishes the inhibition of the isolated brain enzyme.

The observed inhibition by phosphonates was not altered by further purification of KGDHC. The purification was achieved by means of the substrate-induced affinity binding of α-ketoglutarate dehydrogenase complexes to thiol-Sepharose (*35*). A significant portion of impurities were removed with 0.1 M potassium phosphate before the bound KGDHC was eluted by the same buffer supplemented with 1 mM DTT. As a result, the maximal specific activity of KGDHC was increased 10-fold (from 0.05 to 0.5 μmol min^{-1} mg^{-1}). Like the enzyme before the binding to thiol-Sepharose (Figure 2A,B), the affinity-purified KGDHC was nearly completely inhibited by 0.005 mM SP and its mono ethyl esters, while the di- and triethyl esters of SP were inefficient (Figure 2C). Thus, the relative efficiencies and acting concentrations of the phosphonates found in our inhibition studies with partially purified KGDHC were not dependent on the accompanying proteins in the preparation.

The high efficiency of SP inhibition (Figure 2) is in good accord with the known mechanism of the α-keto phosphonates forming covalent adducts with the ThDP at the active sites of the thiamine-dependent α-keto acid dehydrogenases (*13*−*18*, *20*, *22*−*24*). Because no chemical transformation of the adduct is possible, such inhibition is expected to be reversible. Indeed, we observed full restoration of the KGDHC activity after removal of SP from KGDHC through gel filtration on a HiTrap desalting column. The experimentally observed reversal of the SP inhibition rejects the alternative explanation of its high efficiency, as due to the irreversibility of some other modification under our conditions.

As a prerequisite for testing SP and its various esters on the activity of KGDHC in intact cells, the possibility that these compounds substantially interfere with, or are transformed by, other α-keto acid-utilizing enzymes was tested.

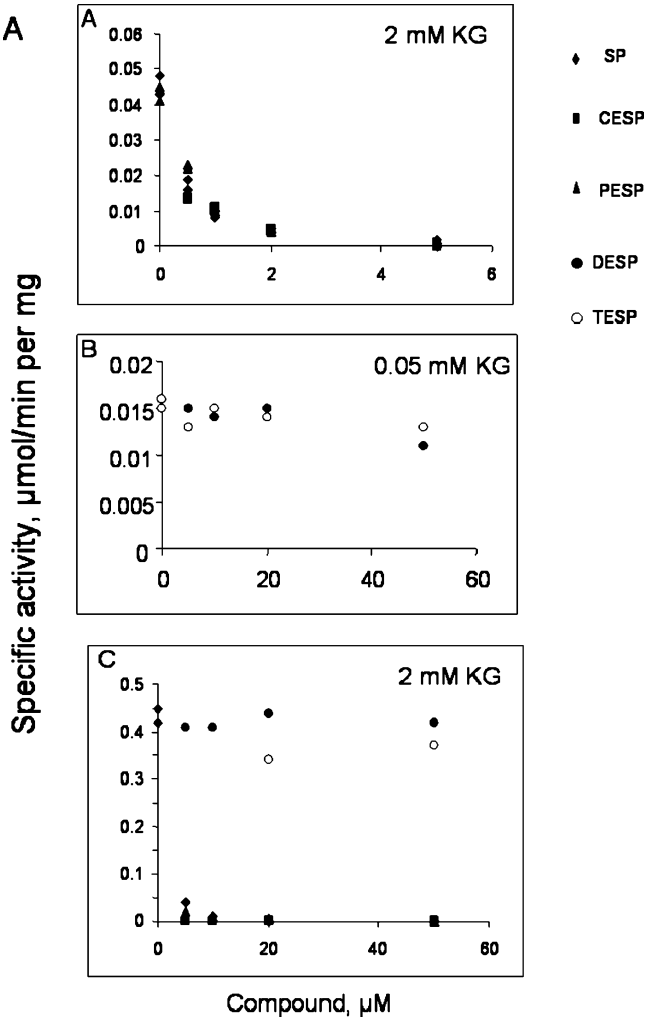


FIGURE 2: Dependence of the specific activity of KGDHC on the concentration of SP and SP esters. Steady-state reaction rates in the presence of varied concentrations of phosphonates were determined in 0.1 M potassium phosphate (pH 7.0) containing 2.5 mM NAD^+, 0.1 mM CoA, 2.5 mM DTT, 1 mM ThDP, 1 mM $MgCl_2$, and either 2 or 0.05 mM KG. Panels A and C show inhibition at a high level (2 mM, ~20-fold greater than K_m^{KG}) of KG saturation. The effects of the phosphonates, which are not efficient inhibitors of the KGDHC reaction under these conditions (DESP and TESP), were also investigated at a low level (0.05 mM, approximately half of K_m^{KG}) of KG saturation (B). The experiment in panel C was performed after the preparation used in panels A and B was purified a further 10-fold. See the text for the purification details.

SP was examined both as a substrate and as an inhibitor of a series of representative enzymes that use KG or other α-keto acids as substrates (Table 1). Inasmuch as SP is a structural analogue of KG, several aminotransferases and glutamate dehydrogenase, which utilize KG as a substrate, were tested because they have the potential to bind SP as a substrate and/or inhibitor. The pyruvate dehydrogenase complex (PDHC) is included in the list because its catalytic mechanism resembles that of KGDHC. Since the pyruvate analogues acetylphosphinate and methyl acetylphosphonate are potent, tight-binding inhibitors of this enzyme complex ($K_d \sim 10^{-7}-10^{-6}$ M) (*15*, *17*, *20*, *36*), the possibility that SP also interacts with PDHC existed. Lactate, malate, and leucine dehydrogenases could bind and transform SP because they work on structural analogues of KG (i.e., pyruvate, oxalacetate, and α-ketoisocaproate, respectively), and the phosphonate analogue of pyruvate is known to be a substrate of lactate dehydrogenase (*37*). Interaction of SP with these enzymes could complicate cellular studies and distort in vitro studies with purified aminotransferases, as the lactate, malate, and leucine dehydrogenases are coupling enzymes for assaying alanine, aspartate, and branched-chain amino acid aminotransferases, respectively.

For the determination of whether SP is an inhibitor of the enzymes listed in Table 1, the α-keto acid substrate and SP concentrations were 2 and 0.1 mM, respectively. α-Keto acid substrates were KG (glutamate dehydrogenase and aminotransferases), pyruvate (lactate dehydrogenase and PDHC), oxaloacetate (malate dehydrogenase), and α-ketoisocaproate (leucine dehydrogenase). The K_m values exhibited for KG by alanine aminotransferase (*38*), the aspartate aminotransferases (*39*), branched-chain aminotransferases (*40*), and glutamate dehydrogenase (*41*) are in the range of 0.2−2.0 mM. Thus, the enzymes are not fully saturated with KG under the conditions in which SP was tested as an inhibitor. Yet only two of the enzymes or enzyme complexes were slightly inhibited by SP at a concentration (0.1 mM) 10 times higher than that which completely inhibited KGDHC (0.01 mM) (Table 1). Lactate, malate, and leucine dehydrogenases were not inhibited by 0.1 mM SP under the assay conditions, which provides evidence that any inhibition noted with the aminotransferases was not due to the loss of activity of the coupling enzymes. In addition, inhibition of PDHC was not observed. The results are consistent with a high degree of SP specificity in inhibiting KGDHC.

To determine whether SP is a substrate of the enzymes listed in Table 1, the reaction rate with 0.1 mM SP was compared against the rate exhibited with 5 mM "natural" α-keto acid substrate. At a concentration of 0.1 mM, SP served as a poor substrate of two of the 10 enzymes that were tested, and could not be detected as a substrate in the remainder. That is, mitochondrial aspartate and cytosolic branched-chain amino acid aminotransferases transformed 0.1 mM SP at a rate which was less than 1% of their maximal velocities determined with 5 mM KG (Table 1).

Similar studies with CESP and TESP showed no detectable inhibition by or transformation of these compounds in the presence of the α-keto acid-utilizing enzymes listed in Table 1.

SP and monoethyl esters CESP and PESP produced up to 70% inhibition of KGDHC in living human fibroblasts at a concentration of 0.01 mM (Figure 3). Upon addition of the phosphonates directly into the assay mixture, the KGDHC inhibition in the cells paralleled that observed with the isolated brain enzyme complex (i.e., SP, CESP, and PESP are strong inhibitors, while DESP and TESP are ineffective). However, when the cells were preincubated with DESP or TESP prior to addition of the assay mixture, these compounds also became inhibitors. This finding suggests intracellular activation by esterases which may remove the ethyl residues, transforming DESP and TESP to CESP, PESP, and SP. An opposite effect of preincubation was observed with strong inhibitors SP, CESP, and PESP. In these cases, preincubation led to a slow reversal of KGDHC inhibition which becomes significant in 2 h (Figure 3). A small reversal could also be observed with DESP and TESP if the exposure at 2 h is compared to that at 1 h, although during 1 h the difference did not become significant. It is worth noting that CESP

Table 1: Succinyl Phosphonate (SP) as an Inhibitor and/or Substrate of Various α-Keto Acid-Utilizing Enzymes[a]

enzyme	inhibition by SP (%)	reaction rate with SP relative to the natural α-keto acid substrate (%)
alanine aminotransferase (pig heart)	ND[b]	ND[b]
pyruvate dehydrogenase complex (cow brain)	ND[b]	ND[b]
mitochondrial aspartate aminotransferase (rat)	ND[b]	0.016
cytosolic aspartate aminotransferase (pig heart)	ND[b]	ND[b]
mitochondrial branched-chain aminotransferase (human recombinant)	~10	ND[b]
cytosolic branched-chain aminotransferase (human recombinant)	~10	0.6
glutamate dehydrogenase (bovine)	ND[b]	ND[b]
lactate dehydrogenase (rabbit muscle)	ND[b]	ND[b]
mitochondrial malate dehydrogenase (pig heart)	ND[b]	ND[b]
leucine dehydrogenase (bacterial)	ND[b]	ND[b]

[a] For testing of SP as an inhibitor, the phosphonate compound and natural α-keto acid substrate were present in the assay mixture at concentrations of 0.1 and 2 mM, respectively. For testing of SP as a substrate, the natural α-keto acid substrate was replaced by the phosphonate compound at a concentration of 0.1 mM. The rate of SP utilization was compared to the reaction rate exhibited with 5 mM natural substrate. This concentration of α-keto acid is close to saturation for all of the enzymes listed here. For details of the assay procedure, see Experimental Procedures. [b] Not detectable under the conditions of the assay.

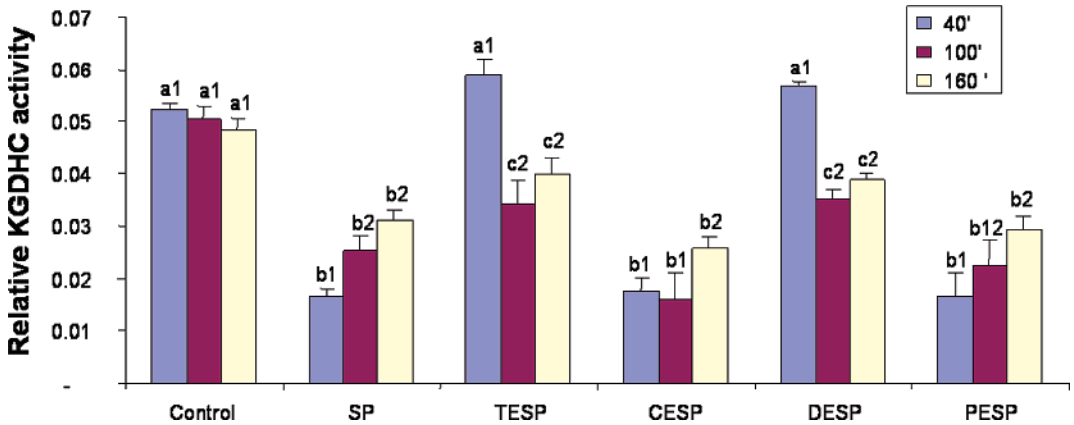


FIGURE 3: Effects of succinyl phosphonate (SP) and its derivatives on KGDHC activity in intact cells. The cells were treated with 10 μM phosphonates for 0, 60, or 120 min in BSS containing 5 mM glucose and buffered with HEPES. The concentration of the phosphono compound was maintained in the medium in the assay mixture described in Experimental Procedures. Incubation of cells for a further 40 min was required to determine the KGDHC activity. The time on the graph indicates the total time with the phosphonate derivative. The vertical axis provides relative values in OD. Values with different letters across all groups differ significantly ($p < 0.05$) from each other. Values with different numbers within a treatment group differ significantly from each other. b12 does not differ significantly from either b1 or b2. Values are means ± the standard error of the mean of nine measurements on three separate days.

appears to be the most stable inhibitor in situ following the 1 h preincubation (Figure 3).

DISCUSSION

Phosphonate analogues of α-keto acids are mechanism-based inhibitors that target the catalytic center of the enzyme-bound ThDP (i.e., the C_2 atom of the coenzyme thiazole ring). The resulting ThDP adducts can be detected by circular dichroism spectroscopy (*18*) and are similar to those formed between ThDP and α-keto acids, except that the C−P bond of the phosphonate analogue cannot be cleaved. This may explain the potent inhibition of the ThDP-dependent α-keto acid dehydrogenases by the phosphonates. However, the formation and stability of the phosphonate adducts may vary with the experimental conditions, substitutions on the phosphonate group, enzyme type (α-keto acid dehydrogenase, decarboxylase, or oxidase), and source (e.g., pyruvate decarboxylase from yeast vs that from wheat germ; KGDHC from pigeon breast muscle vs that from *Escherichia coli*) (*13*, *15*−*18*, *22*−*24*, *37*). The present results show that the phosphonate analogue of KG (SP) and its monoethyl esters, CESP and PESP, are powerful inhibitors of both isolated bovine brain KGDHC and KGDHC in living human fibroblasts. Apart from the high affinity of the phosphonates for the E_1k active site due to formation of covalent intermediates with tightly bound ThDP, the inhibition may be further strengthened by the cooperative action of the subunits in the E_1k dimer. The three-dimensional structures and site-directed mutagenesis of the α-keto acid dehydrogenases suggest that catalysis in the neighboring active sites is synchronized by a catalytically essential proton transfer through an intersubunit channel (*42*) and by intersubunit helix−helix interactions (*43*). The resulting half-of-the-sites reactivity, or the flip-flop action of the α-keto acid dehydrogenases, implies that the inability of one active site to turn over would compromise the competence of the other site. In particular, this has been observed in functional studies on KGDHC and its first component E_1k (*44*−*46*). Similarly, one molecule of SP or an analogue bound per dimer may switch off both subunits, which could contribute to the high potency of the α-keto-glutarate phosphonate mimics as KGDHC inhibitors.

These results show that KGDHC from brain (Figure 2) is more sensitive to SP than KGDHC preparations isolated from muscle or *E. coli*. Comparison of earlier findings (*24*) to those reported here shows that the most and least resistant to SP are KGDHCs from pigeon breast muscle ($I_{50} \sim 0.05$ mM) and bovine brain ($IC_{50} < 0.0005$ mM; Figure 2), respectively. In view of the possible species- and/or tissue-

specific differences, the similar sensitivity of the brain (Figure 2) and fibroblast (Figure 3) KGDHCs to the phosphonates is an important result of this work. Fibroblast cells provide a convenient tool for patient-oriented studies. In particular, fibroblasts from Alzheimer's disease patients have previously been used to study metabolic perturbations, including changes in KGDHC activity (*47*). Our results indicate that KGDHC in living fibroblasts is an appropriate model of brain KGDHC, as far as inhibition by phosphonates is concerned.

Since KG is a major intermediate and intramitochondrial KG concentrations may vary in the range of $10^{-3}-10^{-4}$ M (*48*, *49*), it is important that phosphonates were potent inhibitors even at a high level of saturation of KGDHC with KG (Figures 2 and 3). The substrate does not efficiently protect the enzyme from the phosphonates, although both KG and phosphonates interact with the ThDP catalytic site. Such a feature of the inhibition is explained by earlier studies on the mechanism of the interaction of E_1k with structural analogues of KG (*16*, *50*). The α-keto acid analogues may react with ThDP that is tightly bound at the E_1k active site. The resulting complexes, which are catalytically inactive due to blocking of the KG-binding pocket, dissociate slowly compared to the rate of catalysis. The kinetically slow dissociation of the phosphonates and some other α-keto acid analogues decreases the E_1k maximal reaction rate (*16*, *50*). Thus, as far as the steady-state kinetics is concerned, the inhibition should be considered quasi-irreversible, although it is fully reversible on the time scale of minutes after the enzyme is regenerated from the reaction medium. The structural similarity of an α-keto acid analogue to KG is crucial for the efficacy of this inhibition (*11*, *50*). Because substitution of the carboxylate of KG with the phosphonate group mimics KG the most closely, the phosphonate inhibition greatly surpasses that of naturally occurring α-keto acids.

Monoethylation of the phosphonate group of SP to PESP does not diminish the ability of the compound to inhibit bovine brain KGDHC (Figures 2 and 3), while weakened binding occurred with the analogous methyl ester (*11*, *16*, *24*). Although species- and/or tissue-specific differences cannot be excluded, weakened inhibition by the methyl phosphonate was observed with both pigeon breast and *E. coli* KGDHC, which suggests that the difference is due to general properties of the E_1k active site. The increased hydrophobicity of the ethylated versus methylated SP may contribute to the similar affinity of PESP and SP (Figure 2). Indeed, α-ketoadipate, which has an additional CH_2 group compared to KG, binds to KGDHC from different sources 2−5-fold more tightly than KG (*6*, *46*). Assuming a nonextended conformation of the α-keto acid substrate in the E_1k active site (*6*), there is a possibility that the length of the ethyl residue allows PESP, but not the methylated SP, to form additional hydrophobic interactions with the E_1k active site. This may improve the binding of the ethyl phosphonate compared to that of the methyl phosphonate.

A new phosphonate analogue of KG, CESP, in which the carboxylate group was ethylated, was studied in this work. The γ-carboxyl of KG, which is known to be important for binding of KG to E_1k from different sources (*6*, *46*, *50*), is modified in CESP. Because inhibition of KGDHC by CESP is as efficient as that by SP (Figures 2 and 3), the interaction of KG analogues with the active site may be effective even in the absence of a charge on the γ-carboxyl group. Obviously, the polarity of the esterified carboxylate contributes to its binding, distinguishing this type of compound from α-keto acids such as α-ketobutyrate or α-ketovalerate which do not efficiently bind to E_1k. With the polarity preserved, strengthened hydrophobic interactions of the carboxyethyl residue may partially compensate for the loss of the distal carboxyl charge, strengthening CESP binding in a manner analogous to that discussed above for PESP.

Further esterification of SP monoethyl esters to DESP and TESP leads to the loss of inhibition of the purified enzyme (Figure 2). This presumably results from a decrease in the total negative charge and/or increased bulkiness.

Thus, SP and its monoethyl esters, but not the di- and triethylated analogues, are efficient inhibitors of brain KGDHC in vitro. On the other hand, in vitro studies showed a limited ability of SP and its esters to inhibit other α-keto acid-utilizing enzymes (Table 1), indicating a high specificity of the inhibitory action of these compounds toward KGDHC. In particular, although SP and CESP are potent inhibitors of KGDHC, they do not affect brain PDHC. This is in good accord with the earlier data showing no inhibition by SP of PDHC from *E. coli* (*13*) and with the known structural requirements for the binding and transformation of the α-keto substrates by PDHC (*51*) and KGDHC (*6*). Indeed, PDHC cannot effectively accommodate α-keto acids bulkier than pyruvate or those with additional charges. A distal charge is also not tolerated by the branched-chain α-keto acid dehydrogenases (*52*, *53*). Although SP can slightly inhibit at least some aminotransferases (Table 1), it is likely that SP will not bind to these enzymes more efficiently than does KG itself. Typical K_m values reported for mitochondrial and cytosolic aspartate aminotransferases toward KG are 1.0 and 0.3 mM, respectively (*39*). These values are orders of magnitude higher than the concentrations of SP that effectively inhibit KGDHC in vitro and in situ (0.01 mM). The phosphonate analogues of the α-keto acids are not chemically reactive; hence, they are not expected to act as indiscriminate protein reagents. The specificity revealed in this work makes the phosphonates promising inhibitors of KGDHC in intact cells and possibly even in intact animals.

The data in Table 1 indicate that some aminotransferases can catalyze a slow transamination of SP to the corresponding phosphono glutamate analogue. This is in accord with the known transformation of the α-amino phosphinic and phosphonic acid analogues of alanine and glutamate to the corresponding pyruvate and KG analogues through aminotransferase reactions (*24*, *36*). The rate of the transaminase reaction with the phosphonates is very slow compared to rate of the native reaction (Table 1), but it nevertheless may be responsible for the slow reversal of the KGDHC inhibition by SP in cells (Figure 3). This explanation of the reversal is favored by the fact that inhibition of cellular KGDHC by CESP, which is not an aminotransferase substrate, was constant during the 1 h incubation, in contrast to that of SP. The combined action of esterases and aminotransferases on the SP esters and SP, respectively, may explain the reversal of the inhibition of the esterified phosphonates. Although, in general, one should take into account these reactions in situ and in vivo, their contribution in our experiments with fibroblasts is obviously limited to prolonged incubation times (Figure 3).

Earlier studies on the inhibition of KGDHC in cells used KMV. This α-keto acid inhibits brain KGDHC (*54*), kidney KGDHC, and KGDHC in brain slices and homogenates (*7*) much better than it inhibits PDHC. The difference is in good accord with the K_i^{KMV} value of 1.3 mM for KGDHC (*54*) versus the K_i^{KMV} value of 18 mM for PDHC (*51*), yet only ~50% inhibition of KGDHC is achieved with 3 mM KMV in cultured neuroblastoma cells (*31*). Enhanced inhibition would require KMV to be present at concentrations (10^{-2} M) at least 3 orders of magnitude higher than that required to inhibit KGDHC by SP and its derivatives (10^{-5} M). At these concentrations, KMV, like many α-keto acids, would also act as an antioxidant (*8*) or inhibit other α-keto acid-utilizing enzymes. Moreover, KMV is a substrate of the branched-chain α-keto acid dehydrogenases and may cause metabolic perturbations due to this reaction. For instance, accumulation of one of the reaction products, namely, the KMV-generated derivative of CoA, may affect metabolism by sequestering CoA and through inhibition of the CoA-dependent enzymes. Thus, use of KMV to reveal the consequences of the KGDHC inhibition in situ has serious limitations which are easily overcome by the application of much more efficient and specific KGDHC inhibitors, SP, and its monoethyl esters, characterized in this work.

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BI0503100