mmol, 56%) of product **17** as a white solid: IR (neat, NaCl) 3638 (w), 3550 (w), 3478 (w), 3100-2870 (br), 1748 (s), 1440 (s), 1372 (s), 1163 (s) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.85 (ddd, J = 12, 12, 12 Hz, 1 H), 2.04 (s, 3 H), 2.08 (s, 3 H), 2.50 (ddd, J = 2, 5, 13 Hz, 1 H), 3.44 (dd, J = 6, 11 Hz, 1 H), 3.51 (dd, J = 3, 11 Hz, 1 H), 3.79 (s, 3 H), 4.18 (dd, J = 2, 12 Hz, 1 H), 4.95 (dd, J = 9, 9 Hz, 1 H), 5.03-5.09 (m, 1 H); ^{13}C NMR ($CDCl_3$) δ 20.7, 20.8, 30.8, 33.3, 52.5, 71.2, 73.9, 76.7, 77.0, 169.1, 169.6, 170.2; MS, m/e (relative intensity) 355 (9) M^+ + 2, 353 (9) M^+, 295 (91), 293 (91), 252 (87), 250 (100), 221 (65), 219 (65), 196 (61), 194 (61). Anal. ($C_{12}H_{17}O_7Br$) C, H.

β-(2,6-Anhydro-3-deoxy-D-*arabino*-heptulopyranosid)onate 7-Phosphonate (2a). Brominated 2,6-anhydropyranoside **17** (0.48 g, 1.4 mmol) was taken up in 5.5 mL of triethyl phosphite and refluxed 24 h under nitrogen. Excess triethyl phosphite was removed under reduced pressure at 50 °C and the resulting dark brown syrup subjected to flash chromatography (hexane/ethyl acetate, 1:2, v/v) to yield a white solid. This solid, dissolved in dichloromethane, was cooled to 0 °C under nitrogen and bromotrimethylsilane (1.21 g, 7.92 mmol) added dropwise. After the resultant mixture was stirred 1 h at 0 °C, excess reagent was removed under reduced pressure and the silyl ester hydrolyzed by vigorous stirring with 10 mL of water for 30 min. The solution was concentrated to an oil and treated with 30.5 mL of 0.5 M aqueous sodium hydroxide for 5 min at 0 °C. The mixture was loaded onto 30 mL of Dowex 50 (H^+ form) and the product eluted with 60 mL of water at 4 °C. The eluant was concentrated under reduced pressure, loaded onto 10 mL of AG 1 × 8 equilibrated with 0.20 M sodium acetate at 4 °C, and washed with 10 mL of 0.20 M sodium acetate, followed by elution of the phosphonic acid with a linear gradient of (100 mL + 100 mL, 0.20-2.0 M) sodium acetate, pH 4.75. The fractions were assayed and those containing phosphonic acid pooled, passed down 200 mL of Dowex 50 (H^+ form), and then eluted with 300 mL of water. The water was removed under reduced pressure, during which the temperature was kept below 30 °C to yield 0.041 g (0.16 mmol, 35%) of **2a** as a hygroscopic foam: ^1H NMR (D_2O) δ 1.64 (ddd, J = 12, 12, 12 Hz, 1 H), 1.94-2.08 (m, 1 H), 2.31-2.41 (m, 2 H), 3.16 (dd, J = 9, 9 Hz, 1 H), 3.60 (ddd, J = 10, 10, 10 Hz, 1 H), 3.73-3.79 (m, 1 H), 4.28 (dd, J = 2, 12 Hz, 1 H); ^{13}C NMR (D_2O) δ 32.3 (J_{PC} = 137 Hz), 38.5, 73.8, 76.5, 77.6 (J_{PCC} = 15 Hz), 77.9, 177.1; ^{31}P NMR (D_2O) δ 27.43. Anal. ($C_7H_{13}O_8P \cdot H_2O$) C, H.

Methyl α-(2,6-Anhydro-7-bromo-3-deoxy-D-*arabino*-heptulopyranosid)onate (19). An anomeric mixture of methyl α- and β-(2,6-anhydro-3-deoxy-D-*arabino*-heptulopyranosid)onates (**16** and **18**; 2.5 g, 12 mmol) was dissolved in 100 mL of dry dimethylformamide with *N*-bromosuccinimide (4.4 g, 25 mmol) and cooled in an ice bath under a nitrogen stream. Triphenylphosphine (6.4 g, 25 mmol) was added over the course of 30 min. The reaction was heated at 50 °C for 2 h and cooled and 25 mL of methanol added. After being stirred for 15 min, the solution was concentrated to a syrup and purified by flash chromatography (ethyl acetate) on silica gel to yield a mixture containing succinimide, triphenylphosphine oxide, and an anomeric mixture of methyl (2,6-anhydro-7-bromo-3-deoxy-D-*arabino*-heptulopyranosid)onate. The above mixture was stirred in 30 mL of dimethylformamide and 12 mL of acetic anhydride with catalytic 4-(dimethylamino)pyridine for 18 h. The solvents were removed under reduced pressure, and the resulting orange oil was subjected to flash chromatography (hexane/ethyl acetate, 3:1, v/v) to yield 0.22 g (0.68 mmol, 6% based on the anomeric mixture) of white, solid **19**: IR (neat, NaCl) 2951 (w), 1745 (s), 1433 (m), 1372 (m), 1235 (s), 1143 (m), 1050 (s) cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.02-2.11 (m, 1 H), 2.04 (s, 3 H), 2.06 (s, 3 H), 2.51 (ddd, J = 3, 5, 13 Hz, 1 H), 3.43 (dd, J = 5, 11 Hz, 1 H), 3.57 (dd, J = 3, 11 Hz, 1 H), 4.15-4.20 (m, 1 H), 4.61 (dd, J = 2, 6 Hz, 1 H), 4.97 (dd, J = 9, 9 Hz, 1 H), 5.00-5.06 (m, 1 H); ^{13}C NMR ($CDCl_3$) δ 20.7, 20.9, 31.1, 31.6, 52.5, 69.0, 70.9, 71.0, 73.1, 169.6, 170.0, 170.7; MS, m/e (relative intensity) 213 (10), 175 (16), 173 (16), 170 (21), 43 (100). Anal. ($C_{12}H_{17}O_7Br$) C, H.

α-(2,6-Anhydro-3-deoxy-D-*arabino*-heptulopyranosid)onate 7-Phosphonate (2b). Intermediate **19** was converted to **2b** by using the conditions and reagents described for anhydro phosphonate **2a** synthesis from **17**. ^1H NMR (D_2O) δ 1.88 (ddd, J = 6, 12, 14 Hz, 1 H), 2.02-2.15 (m, 1 H), 2.32-2.41 (m, 1 H), 2.47 (ddd, J = 2, 5, 14 Hz, 1 H), 3.24 (dd, J = 9, 9 Hz, 1 H), 3.60-3.66 (m, 1 H), 3.80 (ddd, J = 3, 9, 21 Hz, 1 H), 4.64 (dd, J = 2, 6 Hz, 1 H); ^{13}C NMR (D_2O) δ 32.4 (J_{PC} = 138 Hz), 35.7, 71.7, 74.7, 75.7 (J_{PCCC} = 6 Hz), 77.4 (J_{PCC} = 13 Hz), 177.6; ^{31}P NMR (D_2O) δ 27.40. Anal. ($C_7H_{13}O_8P \cdot H_2O$) C, H.

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Probing Lethal Metabolic Perturbations in Plants with Chemical Inhibition of Dehydroquinate Synthase

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Abstract: Plant dehydroquinate synthase is purified from *Pisum sativum*. Inhibition of substrate 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate binding to enzyme is determined with 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphonate (**1a**), 3-deoxy-D-*arabino*-heptulosonic acid 7-homophosphonate (**1b**), β-(2,6-anhydro-3-deoxy-D-*arabino*-heptulopyranosid)onate 7-phosphonate (**2a**), and α-(2,6-anhydro-3-deoxy-D-*arabino*-heptulopyranosid)onate 7-phosphonate (**2b**). Competitive inhibition of *Pisum* dehydroquinate synthase is observed with phosphonate **1a** (K_i = 0.8 μM) and anhydro phosphonate **2b** (K_i = 296 μM). Phosphonate **1b** and anhydro phosphonate **2a** do not inhibit substrate binding to *Pisum* dehydroquinate synthase. A range of plant species (*P. sativum*, *Echinochloa crusgalli*, *Setaria viridis*, *Sorghum halepense*, and *Avena fatua*) is then treated with the organophosphonate (**1a**) found to be the most potent inhibitor of plant dehydroquinate synthase. An increase in dephosphorylated substrate of dehydroquinate synthase serves as a marker of in vivo enzyme inhibition, while visual and growth indexes are used to gauge herbicidal activity. Both DAH buildup and herbicidal effects are observed for several of the plant species upon postemergent exposure to phosphonate **1a**.

Elaboration of the mechanism of action of the broad-spectrum herbicide *N*-phosphonomethylglycine (glyphosate)[1] has played an important role in the ongoing evolution of strategies employed in herbicide identification. There can be little doubt that EPSP synthase, an enzyme of the common pathway of aromatic amino acid biosynthesis (Scheme I), is the primary target of glyphosate.[2] Yet it is not simply enzyme inhibition but rather the impact of

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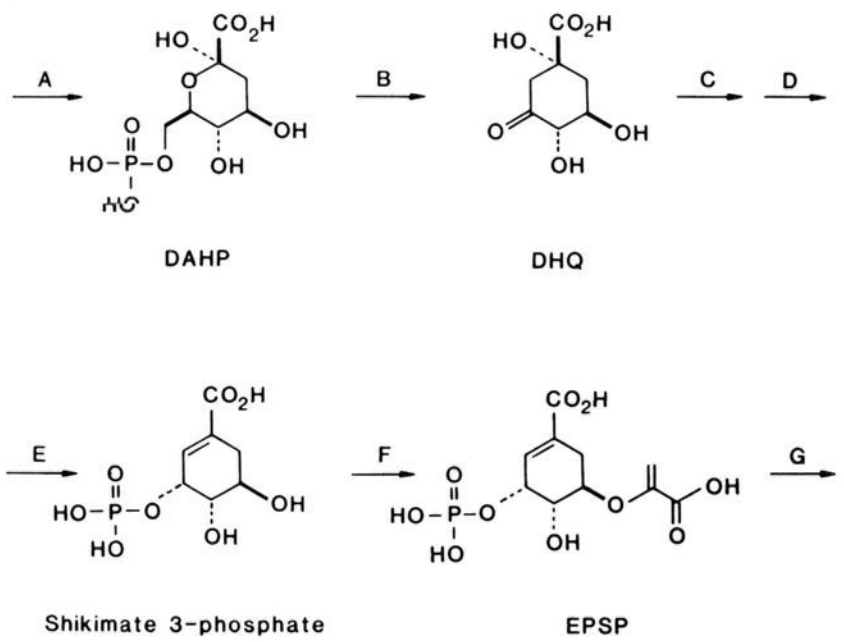
(1) Grossbard, E.; Atkinson, D. *The Herbicide Glyphosate*; Butterworths: Boston, 1985.

(2) Three lines of evidence support this view. These include shikimate buildup,[3] EPSP synthase inhibition,[4] and mutation and overproduction of EPSP synthase which result in resistance to glyphosate.[5]

0002-7863/89/1511-1866$01.50/0

Scheme I[a]



[a] (A) 3-Deoxy-D-*arabino*-heptulosonic acid 7-phosphate synthase; (B) 3-dehydroquinate (DHQ) synthase; (C) 3-dehydroquinate dehydratase; (D) 3-dehydroshikimate reductase; (E) shikimate kinase; (F) 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase; (G) chorismate synthase.

Table I. Purification of Dehydroquinate Synthase from *Pisum sativum*

step	total units[a]	specific activity[b]	total protein[c]	yield[d]	*x*-fold purified
crude homogenate	2.0	0.00012	17000	100	1.0
ammonium sulfate	2.5	0.00031	8200	130	2.6
DE-52	3.3	0.0023	1400	170	19
DE-52	2.7	0.0095	290	140	79
G-75 Superfine	1.1	0.020	50	53	170
Blue Sepharose	1.6	0.058	27	78	480
Dyematrex Green	0.18	0.59	0.30	19	4900

[a] Unit = 1 μmol of DAHP consumed/min at 15 °C. [b] Units/milligram. [c] Milligrams. [d] Units after a purification step/units in crude homogenate.

enzyme inhibition on the plant's matrix of metabolites that determines plant survival. The generality of amino acid biosynthetic enzyme inhibition as a vehicle for enzyme-targeted herbicide action is ultimately a function of which metabolic perturbations dominate the linkage between biosynthetic enzyme inhibition and plant death.[6] Specifically, will herbicidal activity result from inhibition of a common pathway aromatic amino acid biosynthetic enzyme other than EPSP synthase?

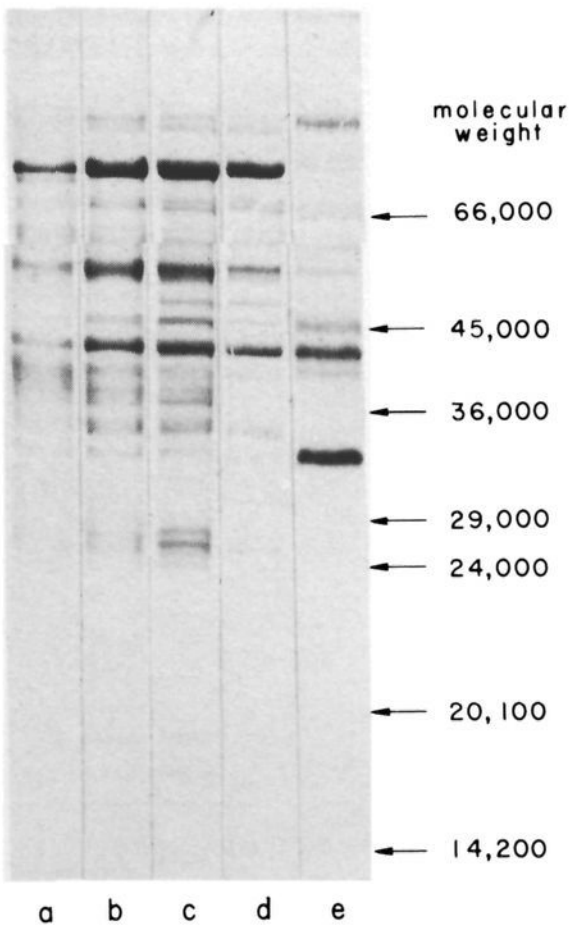


Figure 1. Isolation of DHQ synthase from *Pisum sativum* seedlings: (lane a) after DE-52 with step gradient elution; (lane b) after DE-52 with linear gradient elution; (lane c) after G-75 superfine; (lane d) after blue Sepharose; (lane e) after Dyematrex Green.

Organophosphonate inhibition of nonplant (*Escherichia coli*) dehydroquinate synthase examined in the preceding account provides the chemical inhibition used in this study to gauge the impact of dehydroquinate (DHQ) synthase inhibition on plant survival. DHQ synthase is now purified from a plant source (*Pisum sativum*) and its inhibition examined with 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphonate (**1a**), 3-deoxy-D-*arabino*-heptulosonic acid 7-homophosphonate (**1b**), β-(2,6-anhydro-3-deoxy-D-*arabino*-heptulopyranosid)onate 7-phosphonate (**2a**), and α-(2,6-anhydro-3-deoxy-D-*arabino*-heptulopyranosid)-onate (**2b**). *P. sativum* and four species of weeds are then treated with the organophosphonate ascertained to be the most potent inhibitor of *Pisum* DHQ synthase. The buildup of dephosphorylated enzyme substrate, 3-deoxy-D-*arabino*-heptulosonic acid (DAH), in plant tissue exposed to phosphonate **1a** is exploited as a molecular marker of DHQ synthase inhibition in the intact plant. Herbicidal activity is evaluated according to qualitative growth and visual indexes.

Results

Isolation of Dehydroquinate Synthase from a Plant Source. Purification (Table I) of DHQ synthase from *P. sativum* (pea) seedlings began with mechanical grinding of stems and leaves. Roots also contained DHQ synthase activity but for convenience were not used. Ammonium sulfate fractionation reduced the liters of crude extract to a manageable volume and separated all chlorophyll-containing material from the DHQ synthase activity. Elution from DE-52 with phosphate buffer resulted in extract that could be concentrated by ultrafiltration to the small volumes required for effective purification by size exclusion chromatography (G-75 Superfine). Subsequent filtration through Blue

(3) (a) Steinrücken, H. C.; Amrhein, N. *Biochem. Biophys. Res. Commun.* **1980**, *94*, 1207. (b) Amrhein, N.; Schab, J.; Steinrücken, H. C. *Naturwissenschaften* **1980**, *67*, 356. (c) Holländer, H.; Amrhein, N. *Plant Physiol.* **1980**, *66*, 823. (d) Amrhein, N.; Deus, B.; Gehrke, P.; Steinrücken, H. C. *Plant Physiol.* **1980**, *66*, 830. (e) Amrhein, N.; Holländer, H. *Naturwissenschaften* **1981**, *68*, 43. (f) Berlin, J.; Witte, L. *Z. Naturforsch.* **1981**, *36C*, 210. (g) Amrhein, N.; Johänning, D.; Schab, J.; Schulz, A. *FEBS Lett.* **1983**, *157*, 191. (h) Holländer-Czytko, H.; Amrhein, N. *Plant Sci. Lett.* **1983**, *29*, 89.

(4) (a) Boocock, M. R.; Coggins, J. R. *FEBS Lett.* **1983**, *154*, 127. (b) Mousdale, D. M.; Coggins, J. R. *Planta* **1984**, *160*, 78. (c) Duncan, K.; Lewendon, A.; Coggins, J. R. *FEBS Lett.* **1984**, *165*, 121. (d) Rubin, J. L.; Gaines, C. G.; Jensen, R. A. *Plant Physiol.* **1984**, *75*, 839. (e) Steinrücken, H. C.; Schulz, A.; Amrhein, N.; Porter, C. A.; Fraley, R. T. *Arch. Biochem. Biophys.* **1986**, *244*, 169.

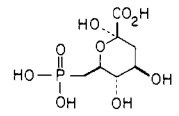
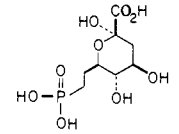
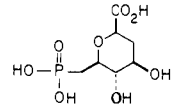
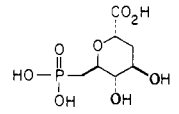
(5) (a) Comai, L.; Sen, L. C.; Stalker, D. M. *Science* **1983**, *221*, 370. (b) Stalker, D. M.; Hiatt, W. R.; Comai, L. *J. Biol. Chem.* **1985**, *260*, 4724. (c) Comai, L.; Facciotti, D.; Hiatt, W. R.; Thompson, G.; Rose, R. E.; Stalker, D. M. *Nature* **1985**, *317*, 741. (d) Nafziger, E. D.; Widholm, J. M.; Steinrücken, H. C.; Killmer, J. L. *Plant Physiol.* **1984**, *76*, 571. (e) Smart, C. C.; Johänning, D.; Müller, G.; Amrhein, N. *J. Biol. Chem.* **1985**, *260*, 16338. (f) Duncan, K.; Lewondon, A.; Coggins, J. R. *FEBS Lett.* **1984**, *165*, 121. (g) Rogers, S. G.; Brand, L. A.; Holder, S. B.; Sharps, E. S.; Brackin, M. J. *Appl. Environ. Microbiol.* **1983**, *46*, 37. (h) Schulz, A.; Sost, D.; Amrhein, N. *Arch. Microbiol.* **1984**, *137*, 121. (i) Sost, D.; Schulz, A.; Amrhein, N. *FEBS Lett.* **1984**, *173*, 238. (j) Smith, C. M.; Pratt, D.; Thompson, G. A. *Plant Cell Rep.* **1986**, *5*, 298.

(6) In addition to aromatic amino acid biosynthesis, disruption of histidine biosynthesis by amitrole[7] and imidazolinone and sulfonylurea interference with branched-chain aliphatic amino acid biosynthesis[8] are precedented to result in plant death. See also: Kishore, G. M.; Shah, D. M.; *Annu. Rev. Biochem.* **1988**, *57*, 627.

(7) Hilton, J. L.; Kearney, P. C.; Ames, B. N. *Arch. Biochem. Biophys.* **1965**, *112*, 544.

(8) (a) LaRossa, R. A.; Schloss, J. V. *J. Biol. Chem.* **1984**, *259*, 8753. (b) Chaleff, R. S.; Mauvais, C. J. *Science (Washington, D.C.)* **1984**, *224*, 1443. (c) Ray, T. B. *Plant Physiol.* **1984**, *75*, 827. (d) Rost, T. L.; Reynolds, T. *Plant Physiol.* **1985**, *77*, 481. (e) LaRossa, R. A.; Smulski, D. R. *J. Bacteriol.* **1984**, *160*, 391. (f) Schloss, J. V.; Van Dyk, D. E.; Vasta, J. F.; Kutny, R. M. *Biochemistry* **1985**, *24*, 4952. (g) Shaner, D. L.; Anderson, P. C.; Stidham, M. A. *Plant Physiol.* **1984**, *76*, 545.

Table II. Inhibition of *Pisum sativum* Dehydroquinate Synthase with Organophosphonate Analogues of Substrate DAHP

organophosphonate	inhibition	K_i, μM	K_i/K_m
1a 	competitive	0.8	0.025
1b 	no inhibition		
2a 	no inhibition		
2b 	competitive	296	9.3

Sepharose was followed by chromatography on a second type of affinity material (Dyematrex Green) using buffer containing 1 mM of substrate DAHP.[9] An increase in total units observed through the first portion of the purification likely reflects the removal of phosphatases. DHQ synthase after the final purification step was analyzed by polyacrylamide gel electrophoresis under denaturing conditions with Coomassie Brilliant Blue staining (Figure 1). The dominant band of the purified extract migrates with a mobility equal to a molecular weight of 33 500. Size exclusion chromatography of 4900-fold purified, native DHQ synthase indicated a molecular weight of 66 000.

Inhibition of DAHP Binding to *Pisum* DHQ Synthase. The colorimetric determination[10] of substrate DAHP loss used throughout the purification could not be employed for the inhibition studies due to the interference of the inhibitors with the assay. Instead, determining enzyme inhibition relied on quantitation of product inorganic phosphate.[11] This put strict constraints on the purity of DHQ synthase needed for the inhibition studies since nonspecific phosphatases would interfere by converting DAHP to DAH and inorganic phosphate. Phosphatase contamination had been removed from the DHQ synthase at its highest level of purification. Unfortunately, the enzyme was found to be unstable in the phosphate-free medium demanded by inhibition studies. Inhibition was superimposed on the kinetics of irreversible enzyme denaturation.

Extract at each stage of the purification (Table I) was subsequently evaluated for phosphatases and the stability of DHQ synthase. Polyacrylamide gel electrophoresis under nondenaturing conditions was used in tandem with visualization[12] for inorganic phosphate generating activity. Although multiple inorganic phosphate generating bands were evident after size exclusion chromatography on G-75 Superfine, only one band was evident in extract after filtration through Blue Sepharose. DHQ synthase at this stage of purification retained catalytic activity in phosphate-free solution. Phosphonate **1a**, **1b**, **2a**, and **2b** inhibition was evaluated (Table II) with *Pisum* DHQ synthase purified through Blue Sepharose. No reduction in initial velocity of inorganic phosphate formation was observed with phosphonates **1b** and **2a** even at inhibitor concentrations of 500 μM.

In Vivo Enzyme Inhibition and Herbicidal Activity. Evaluating the impact of phosphonate **1a** on plant survival relied on postemergent treatment of *P. sativum*, *Echinochloa crusgalli* (barnyard grass), *Setaria viridis* (foxtail), *Sorghum halepense* (johnson grass), and *Avena fatua* (wild oat) (Table III). Phosphonate **1a** and glyphosate were applied as an aerosol formulated to ensure wetting of the plant leaf surface. A single concentration of enzyme-targeted agent was applied to all plants. Herbicidal effects were gauged according to the weight of freshly harvested seedlings as well as the extent of leaf discoloration and desiccation. Glyphosate-treated *E. crusgalli*, *S. viridis*, *S. halepense*, and *A. fatua* experienced a reduction in harvested weight, some level of tissue discoloration and desiccation, as well as an increase in shikimate concentration (Table IV). *P. sativum* differed only in that tissue discoloration and desiccation was minimal. While every plant treated with glyphosate experienced an increase in substrate concentration and herbicidal effects, exposure of the same plant species to phosphonate **1a** resulted in a more diverse range of responses. Postemergent application of phosphonate **1a** (Table III) resulted in tissue discoloration and desiccation in *E. crusgalli*, *S. viridis*, and *A. fatua*. Growth inhibition was detected in *S. halepense* and *A. fatua*. Increases in the concentration of DAH were detected for *P. sativum*, *S. viridis*, *S. halepense*, and *A. fatua*. The plant experiencing the largest increase in DAH in plant tissue upon postemergent application of phosphonate **1a** was *A. fatua*.

Table III. Postemergent Evaluation of the Impact of Phosphonate **1a** on the Survival of *Pisum sativum*, *Echinochloa crusgalli*, *Setaria viridis*, *Sorghum halepense*, and *Avena fatua*

	nmol of DAH/mg of plant tissue[a]		growth index[b]	visual index[c]
	untreated	treated		
P. sativum	0.42	0.62	1.2	–
E. crusgalli	0.28	0.25	1.1	+
S. viridis	0.17	0.33	0.97	++
S. halepense	0.37	0.70	0.41	–
A. fatua	0.22	0.80	0.32	+

[a] Plants were desiccated prior to weighing. [b] Weight of harvested treated plant/weight of harvested untreated plant. *Pisum* cotyledons were removed prior to weighing. [c] (+++) complete discoloration and desiccation of plant tissue, (++) extensive but not complete discoloration and desiccation, (+) detectable discoloration and desiccation, (–) no detectable discoloration or desiccation.

Table IV. Postemergent Evaluation of the Impact of Glyphosate on the Survival of *Pisum sativum*, *Echinochloa crusgalli*, *Setaria viridis*, *Sorghum halepense*, and *Avena fatua*

	nmol of shikimate/mg of plant tissue[a]		growth index[b]	visual effects[c]
	untreated	treated		
P. sativum	1.4	15	0.72	+
E. crusgalli	5.6	52	0.46	+++
S. viridis	7.4	35	0.49	+++
S. halepense	2.0	22	0.34	++
A. fatua	0.8	9.2	0.70	++

[a] Plants were desiccated prior to weighing. [b] Weight of harvested treated plant/weight of harvested untreated plant. *Pisum* cotyledons were removed prior to weighing. [c] (+++) complete discoloration and desiccation of plant tissue, (++) extensive but not complete discoloration and desiccation, (+) detectable discoloration and desiccation, (–) no detectable discoloration or desiccation.

Discussion

Possible Lethal Metabolic Perturbations. The traditional view of the common pathway of aromatic amino acid biosynthesis has been that of a biosynthetic bottleneck which can be plugged by enzyme inhibition. In other words, inhibition of EPSP synthase by glyphosate causes a decline in the concentration of aromatic amino acids which leads to plant death.[13] A number of obser-

(9) DAHP was obtained by immobilized enzyme synthesis: Reimer, L. M.; Conley, D. L.; Pompliano, D. L.; Frost, J. W. *J. Am. Chem. Soc.* **1986**, *108*, 8010.

(10) Gollub, E.; Zalkin, H.; Sprinson, D. B. *Methods Enzymol.* **1971**, *17A*, 349.

(11) Ames, B. N. *Methods Enzymol.* **1966**, *8*, 115.

(12) Nimmo, H. G.; Nimmo, G. A. *Anal. Biochem.* **1982**, *121*, 17.

(13) (a) Hoagland, R. E.; Duke, S. O. In *Biochemical Responses Induced by Herbicides*; Moreland, D. E., St. John, J. B., Hess, F. D., Eds.; ACS Symposium Series 181; American Chemical Society: Washington, DC, 1982; Chapter 10, p 180. (b) Cole, D. J. In *The Herbicide Glyphosate*; Grossbard, E., Atkinson, D., Eds.; Butterworths: Boston, 1985; p 56.

vations central to the identification of aromatic amino acid biosynthesis as the target of glyphosate action have been responsible for this view. These include the reversal of glyphosate phytotoxicity upon application of aromatic amino acids and the decrease in concentration of aromatic amino acids in plant tissue exposed to glyphosate.[14] However, the impact of aromatic amino acid supplementation need not be restricted to amelioration of deficits in the supply of phenylalanine, tyrosine, or tryptophan. These externally applied organics may by direct or indirect feedback inhibition prevent unrestrained drainage of vital metabolites into aromatic amino acid biosynthesis. A variety of organic molecules other than aromatic amino acids have been reported to be capable of reversing the toxic effects of glyphosate in plant tissue.[15] Furthermore, not all studies that have examined the pools of free aromatic amino acids after exposure of plant tissue to glyphosate have reported a decrease in the concentration of these amino acids.[13b]

Consideration must be given to other interpretations of the metabolic perturbations caused by EPSP synthase inhibition that lead to plant death. Concentrations of aromatic amino acids may decrease below normal concentrations due to chemically induced stress, but the supplies may not dwindle to below life-sustaining levels. Plastidal concentrations of phosphoenolpyruvate (PEP) and shikimate 3-phosphate could be increased by regulatory responses to decreased availability of aromatic amino acids caused by inhibition of EPSP synthase. Such a response is best viewed in light of the competitive and uncompetitive inhibition of EPSP synthase by glyphosate relative to PEP and shikimate 3-phosphate, respectively.[4] Increases in the concentration of PEP could reverse the reduction in velocity caused by glyphosate competitive inhibition. Similar increases in shikimate 3-phosphate levels would be far less effective at relieving the impact on velocity of the uncompetitive inhibition of EPSP synthase. Over time, the sheer number of phosphorylation equivalents required to maintain the elevated levels of PEP and shikimate 3-phosphate may constitute an irreparable energy drain. This view of glyphosate action has been used as the basis for explaining growth inhibition in microbial models of plant systems.[16]

Alternatively, the elevated levels of substrates may themselves be toxic to the plant. Studies in microbes of the impact of herbicides that disrupt a biosynthetic enzyme involved in isoleucine and valine biosynthesis have led to the conclusion that accumulation of the substrate of the inhibited enzyme is toxic to the organism and likely an important component of herbicide action.[17] Tremendous increases in the concentration of shikimate have been observed in plant tissue exposed to glyphosate.[3] Accumulated shikimate and shikimate 3-phosphate could bind to proteins or enzymes remote to EPSP synthase. If such interactions disrupt metabolic processes, the effects could be lethal to the plant. Shikimate and shikimate 3-phosphate are also Michael acceptors and 1,4 addition to the α,β-unsaturated carboxylate[18] could occur with nucleophilic residues in plant cells.

Chemical Inhibition of DHQ Synthase as a Probe. Given the potential roles of energy drain and toxicity associated with substrate buildup in the mechanism of action of glyphosate, whether inhibition of common pathway enzymes other than EPSP synthase can lead to plant death is an open question.[19] DHQ synthase was chosen as the focus of attention due to the availability in phosphonate **1a** of a potent inhibitor for the enzyme. If starvation for amino acids is the primary metabolic perturbation responsible for phytotoxicity, in vivo inhibition of DHQ synthase is likely to result in plant death. However, should energy drain or toxicity associated with elevated concentrations of substrate dominate, the burden of the plant to break through enzyme inhibition will vary from enzyme to enzyme. This suggests that not all and potentially only a subset of common pathway enzymes would, upon in vivo reversible enzyme inhibition, lead to plant death. Indeed, the relevant subset of enzymes might consist of only EPSP synthase.

Table V. Comparison of Phosphonate **1a** Inhibition of DHQ Synthase with Glyphosate Inhibition of EPSP Synthase

enzyme	K_i, μM	K_M, μM	K_i/K_M
DHQ synthase[a] (*P. sativum*)	0.8	32	0.025
EPSP synthase[b] (*P. sativum*)	0.08	5.2	0.015

[a] See Experimental Section for assay conditions. [b] Literature values.[4b]

Although phosphonate **1a** was identified to be a potent inhibitor of DHQ synthase isolated from *E. coli*, the relevance of this inhibition to a plant system was not possible to ascertain prior to purification of DHQ synthase from a plant source. Chemical inhibition of an enzyme of microbial origin may not hold for the same enzyme isolated from a plant. However, like the *E. coli* enzyme, *Pisum* DHQ synthase was not inhibited by homophosphonate **1b** or by β-anhydro phosphonate **2a**. Phosphonate **1a** and α-anhydro phosphonate **2b** were competitive inhibitors for both enzymes with similar inhibition constants. On the basis of this inhibitory pattern, the more readily[20] obtained *E. coli* DHQ synthase appears to be a good model for the same enzyme derived from *Pisum*.

Impact of DHQ Synthase Inhibition on Plant Survival. Comparison (Table V) of the kinetic parameters of glyphosate inhibition of *Pisum* EPSP synthase to phosphonate **1a** inhibition of *Pisum* DHQ synthase provides an indication of the potential of phosphonate **1a** as a disruptor of aromatic amino acid biosynthesis. The ratios K_i/K_m are similar for phosphonate **1a** competitive inhibition of DAHP binding and glyphosate competitive inhibition of PEP binding, while the K_i values indicate that higher concentrations of phosphonate **1a** might be required in vivo to achieve disruption of aromatic amino acid biosynthesis. Unfortunately, only the most qualitative of data are currently available as to the structural features of a molecule that facilitate or retard systemic translocation in plants. The concentration of targeted enzyme in vivo as well as the plastidal concentration of the native substrate of the inhibited enzyme will also be important contributors to whether phosphonate **1a** will function as an effective disruptor of the common pathway.

While prediction of in vivo enzyme inhibition is problematic, detection of such inhibition can follow from an increase in the concentration of the substrate of the enzyme targeted for inhibition. Accumulation of shikimate in plant tissue due to glyphosate treatment was one of the key observations that led to the identification of the enzyme target of this herbicide. Shikimate accumulation is interpreted as plant-mediated hydrolysis of excess shikimate 3-phosphate arising from inhibition of EPSP synthase followed by storage of the dephosphorylated metabolite in vacuoles.[3] DAH buildup can likewise serve as a molecular marker of in vivo enzyme inhibition. The increase in DAH concentration in plant tissue observed for *P. sativum*, *S. viridis*, *S. halepense*, and *A. fatua* indicates that sufficient levels of phosphonate **1a** are penetrating to the chloroplast to inhibit DHQ synthase.

What remains is consideration of herbicidal activity. Of five plant species treated with phosphonate **1a**, four suffered herbicidal effects as indicated by either growth or visual indexes. The one plant that was not affected (*Pisum*) was also the plant that suffered the least herbicidal effects when treated with glyphosate. DAH buildup in three of the four plants to suffer herbicidal effects is consistent with in vivo inhibition of DHQ synthase being the basis of phosphonate **1a** herbicidal activity. There is the possibility that inhibition of an enzyme remote to DHQ synthase contributes to the herbicidal action of phosphonate **1a**.[21] Furthermore, while

(14) Jaworski, E. G. *J. Agric. Food Chem.* **1972**, *20*, 1195.
(15) Killmer, J.; Widholm, J.; Slife, F. *Plant Physiol.* **1981**, *68*, 1299.
(16) Fischer, R. S.; Berry, A.; Gaines, C. G.; Jensen, R. A. *J. Bacteriol.* **1986**, *168*, 1147.
(17) LaRossa, R. A.; van Dyk, T. K.; Smulski, D. R. *J. Bacteriol.* **1987**, *169*, 1372.
(18) Plieninger, H., Schneider, K. *Ber. Dtsh. Chem. Ges.* **1959**, *92*, 1587.
(19) Inhibitors of shikimate dehydrogenase do not lead to herbicidal activity. See: Baillie, A. C.; Corbett, J. R.; Dowsett, J. R.; McCloskey, P. *Pestic. Sci.* **1972**, *3*, 113.
(20) Frost, J. W.; Bender, J. L.; Kadonaga, J. T.; Knowles, J. R. *Biochemistry* **1984**, *23*, 4470.

phosphonate **1a** is herbicidal, its activity (in terms of growth and visual indexes) is significantly less pronounced than glyphosate. Each of these points requires further investigation. Nonetheless, the data thus far collected—inhibition of purified target enzyme, substrate buildup in treated plant tissue, and herbicidal activity—suggest that herbicidal activity due to enzyme inhibition is not limited in the common pathway of aromatic amino acid biosynthesis to inhibition of EPSP synthase.

Experimental Section

General Procedures. DHQ synthase activity was measured by the rate of disappearance of substrate DAHP or the rate of formation of product inorganic phosphate at 15 °C. Enzyme was assayed[20] in MOPS buffer (50 mM, pH 7.5) containing cofactors NAD^+ (0.25 mM) and $CoCl_2$ (0.25 mM) and substrate DAHP. Aliquots (0.2 mL) were withdrawn at intervals and quenched with 10% trichloroacetic acid (0.1 mL). Precipitated protein in the quenched aliquots was removed by centrifugation. Remaining substrate DAHP was assayed after periodate oxidation by reaction with thiobarbituric acid.[10] Alternatively, product inorganic phosphate in the time points was visualized.[11] One unit of enzyme activity catalyzes the consumption of 1 μmol of DAHP or the production of 1 μmol of inorganic phosphate per minute at the assay temperature. Protein was quantitated for specific activity determinations by Coomassie dye binding.[22] Diethylaminoethyl cellulose (DE-52) was purchased from Whatman, Sephadex G-75 Superfine from Pharmacia, and Dowex 50 (H^+ form, 100–200 mesh) from Sigma; Dyematrex Green A and Dyematrex Blue A were from Amicon.

Extraction buffer consisted of 100 mM phosphate, pH 7.6, phenylmethylsulfonyl fluoride (1.0 mM), dithiothreitol (1.0 mM), and benzamidine hydrochloride (1.0 mM). All other solutions contained 0.25 mM $CoCl_2$ and varied only in the concentration of phosphate buffer: 50 mM phosphate, pH 7.5 (buffer A); 250 mM phosphate, pH 7.5 (buffer B); 100 mM phosphate, pH 7.5 (buffer C). All enzyme manipulations were performed at 4 °C. Protein solutions were concentrated by ultrafiltration (PM-10 Diaflo ultrafiltration membranes from Amicon). Dialysis tubing (12 000–14 000 molecular weight cutoff from Spectrapor) was cleaned prior to use. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to Laemmli.[23] The standards (from Sigma) were α-lactalbumin (MW 14 200), trypsin inhibitor (MW 20 100), trypsinogen (MW 24 000), carbonic anhydrase (MW 29 000), glyceraldehyde 3-phosphate dehydrogenase (36 000), egg albumin (MW 45 000), and bovine serum albumin (MW 66 000). Gel permeation standards used to calibrate the Sephadex G-75 Superfine column included cytochrome *c* (MW 12 400), carbonic anhydrase (MW 29 000), bovine serum albumin (MW 66 000), and blue dextran (MW 2 000 000).

Isolation of DHQ Synthase. Seeds of *P. sativum* L. cv. Progress Number Nine (Kilgore Seed Co., Sanford, FL) were soaked in water overnight and then planted in moist vermiculite. Plants were grown at 25 °C with a 16-h light (Sylvania, GroLux F40-GRO) and 8-h dark regime. Seedlings were watered once every 48 h. After 14 days, approximately 2000 shoots (1.3 kg fresh weight) were excised above the first leaf pair and homogenized in extraction buffer (1.5 mL/g of shoots) for 1–2 min in a Waring blender (Model CB-6) at 20 000 rpm. After the brei was squeezed through four layers of cheesecloth, the filtrate was centrifuged at 20000*g* for 20 min to provide the crude homogenate and powdered ammonium sulfate was added slowly to give a final concentration of 242 g/L (40% saturation). After being stirred for 1 h, the precipitated protein was removed by centrifugation at 15000*g* for 15 min. Ammonium sulfate was added to the supernatant to a final concentration of 430 g/L (65% saturation) and stirred for 1 h, and precipitated protein was collected by centrifugation. The pellet was redissolved in and dialyzed against buffer A.

Dialyzed enzyme solution was layered onto DE-52 15 cm diameter column, 5 mg of protein applied/mL of gel) equilibrated with buffer A. Washing with two column volumes of buffer A was followed by a two column volume wash with buffer B. The eluant from buffer B elution was collected and concentrated by addition of ammonium sulfate to 70% saturation. After the protein was pelleted by centrifugation (20000*g* for 15 min), the enzyme was dissolved and dialyzed against buffer A. This dialyzed enzyme solution was layered onto clean DE-52 5 cm diameter column, 0.5 mg of protein applied/mL of gel). After two column volumes of buffer A, enzyme eluted with a linear gradient (eight column volumes) from buffer A to buffer B. Active DHQ synthase fractions were pooled and concentrated by ultrafiltration to 1.5 mL and then applied to a gel filtration column (Sephadex G-75, two 1 cm × 90 cm columns in tandem) equilibrated with buffer C. The column was eluted with buffer C, and 0.5-mL fractions were collected. Active DHQ synthase fractions were pooled and concentrated by ultrafiltration.

Without dialysis, the concentrated solution was loaded onto a Dyematrex Blue A (1.5 cm diameter column, 1 mg of protein applied/mL gel) which had been equilibrated with buffer C. DHQ synthase did not bind to the column under these conditions. After the enzyme solution was loaded, the column was eluted with buffer C, and active fractions were combined, concentrated by ultrafiltration, and then loaded onto Dyematrex Green (1 cm diameter column, 0.5 mg of protein applied/mL of gel). The enzyme was allowed to bind to the column for 30 min, washed with two column volumes of buffer C, and then eluted with buffer C containing 1 mM DAHP. Fractions containing DHQ synthase activity were concentrated by ultrafiltration. If enzyme was not to be used immediately, it was quickly frozen in liquid nitrogen and stored at −80 °C.

Determination of the Kinetic Parameters of Phosphonate 1a, Homophosphonate 1b, Anhydro Phosphonate 2a, and Anhydro Phosphonate 2b Inhibition of DHQ Synthase. DHQ synthase isolated from *Pisum* that had been purified through blue dye, homogeneous *E. coli* DHQ synthase, and phosphate-free solutions were used for all kinetic studies. Contaminating phosphate in the purified enzyme solutions was removed by desalting into phosphate-free buffer with prepacked Sephadex G-25 (PD-10 columns, from Pharmacia). The Michaelis constant, K_m, was calculated from a nonlinear least-squares fit of substrate concentration–initial velocity ([s]–*v*) data to the Michaelis–Menten equation.[24] Substrate DAHP concentrations were varied from 25 to 200 μM. Assays of DHQ synthase preparations for the rate of substrate loss and the rate of product formation provided values that agreed to within 10%.

The assay for time-dependent formation of inorganic phosphate was used exclusively for the studies of DHQ synthase inhibition by the organophosphonates. To avoid errors associated with nonlinear optical response, assay volumes were adjusted such that optical density values for the visualized product inorganic phosphate remained between 0.1 and 0.7. Measurements of initial velocity were made for each of five inhibitor concentrations (0, 2, 4, 7, and 10 μM for phosphonate **1a** and 0, 10, 100, 250, and 500 μM for homophosphonate **1b**, anhydro phosphonate **2a**, and anhydro phosphonate **2b**) and then plotted as $[s]/v$ versus [s] (Hanes–Woolf plot) for each [i] to determine the type of inhibition.[25] The inhibition constants, K_i, were determined from least-squares fitting of apparent (K_m/V) versus [i].

Postemergent Evaluation of Herbicidal Activity. Measured amounts (1 g) of *P. sativum*, *E. crusgalli*, *S. viridis*, *S. halepense*, and *A. fatua* seeds were planted in vermiculite-filled containers (4 in. diameter, 4 in. deep). There were two plantings for each species. Plants were grown at constant temperature (26 °C) with 16-h light and 8-h dark cycles. Once the plants emerged from the vermiculite (4–5 days), one planting of each species was sprayed daily with 1.3 mL of enzyme-targeted agent (10 mM) dissolved in a surfactant-containing solution (by volume: 80% water, 10% methanol, 10% acetone, 0.01% Triton X-100). The second planting of each species was sprayed daily with 1.3 mL of the surfactant-containing solution lacking enzyme-targeted agent. Plants were grown for 14 days after the initial treatment.

Analysis of Dephosphorylated Substrate Buildup. Plants exposed to phosphonate **1a** and glyphosate were desiccated under high vacuum and stored at −80 °C. Analysis for shikimate buildup in glyphosate-treated plant tissue began with addition of uniformly ^{14}C-labeled shikimate (purchased from NEN) to a weighed portion of the desiccated plant tissue. Metabolites were extracted[3d] with refluxing 80:20 ethanol/water, filtered through a Centriflo CF25 Ultrafiltration membrane cone (Amicon), and separated by anion-exchange high-pressure liquid chromatography[26] (semiprep Whatman Partisil-10 SAX; 80:20 water/acetonitrile, 75 mM sodium acetate buffer, pH 4.75). Fractions containing ^{14}C radiolabel were collected and concentrated, and the amount of shikimate present was determined by colorimetric assay.[27]

Analysis for DAH buildup in phosphonate **1a** treated plant tissue required enzymatic conversion of uniformly ^{14}C-labeled D-fructose (purchased from NEN) to [4,5,6,7-^{14}C]DAH.[9] Metabolite extraction, separation, and quantitation[11] of DAH paralleled the procedures ex-

(21) Rigorous proof that in vivo inhibition of DHQ synthase is the primary cause of plant death requires demonstration that mutation and/or overproduction of DHQ synthase results in resistance to phosphonate **1a** herbicidal activity. See ref 5.

(22) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.

(23) Laemmli, U. K. *Nature* **1970**, *227*, 680.

(24) Marquardt, D. W. *J. Soc. Ind. Appl. Math.* **1963**, *11*, 431.

(25) (a) Segel, I. H. *Biochemical Calculations*; Wiley: New York, 1976. (b) Cornish-Bowden, A. *Fundamentals of Enzyme Kinetics*; Butterworths: Boston, 1979.

(26) (a) Boudet, A. M. *Plant Cell Physiol.* **1980**, *21*, 785. (b) Mousdale, D. M.; Coggins, J. R. *J. Chromatogr.* **1985**, *329*, 268.

(27) Millican, R. C. *Methods Enzymol.* **1971**, *17A*, 352.

ploited for shikimate analysis except for the HPLC conditions employed (semiprep Whatman Partisil-10 SAX; 80:20 water/acetonitrile, 7.5 mM phosphate buffer, pH 2.4). Typically the recovery of radiolabeled shikimate or DAH was 75-80% after extraction, workup, and separation by anion-exchange high-pressure liquid chromatography. Values for DAH and shikimate accumulation were corrected for radiolabel lost during the analysis.

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Studies of Protein Hydration in Aqueous Solution by Direct NMR Observation of Individual Protein-Bound Water Molecules†

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Abstract: Proton nuclear magnetic resonance was used to study individual molecules of hydration water bound to the protein basic pancreatic trypsin inhibitor (BPTI) in aqueous solution. The experimental observations are nuclear Overhauser effects (NOE) between protons of individual amino acid residues of the protein and those of sufficiently tightly bound water molecules. These NOEs were recorded by two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) in the laboratory frame, and by the corresponding experiment in the rotating frame (ROESY). The detection of NOEs with water protons was enabled by a solvent suppression technique which provides a uniform excitation profile in NOESY and ROESY, except at the ω_2 frequency of the water signal. From NOESY and ROESY spectra recorded at 5, 36, 50, and 68 °C, intermolecular ^1H-^1H NOEs between the protein and four water molecules buried in its interior were individually assigned, and additional NOEs between surface residues of the protein and labile protons with the chemical shift of the bulk water were identified. For the hydration waters that can be observed by NOEs at 36 °C, an upper limit for the proton-exchange rate with the bulk water is estimated to be 3×10^9 s^{-1}. These NOE-observable water molecules account only for a small percentage of the hydration waters seen in the crystal structure of BPTI. This observation supports the independently established picture of increased disorder near the molecular surface in protein structures in solution.

Three-dimensional protein structures can nowadays be determined either by X-ray diffraction in single crystals or by nuclear magnetic resonance (NMR)[1] in solution.[2] High-resolution crystal structures of globular proteins include typically numerous water molecules in defined hydration sites.[3,4] In aqueous solution, evidence for the presence of protein-bound hydration water could also be established, but the experiments used so far could not provide observations on individual molecules of hydration water. For example, measurements of the relaxation enhancement on the water signal in aqueous protein solutions led to the conclusion that the observed magnetization transfer is not only a consequence of chemical exchange between water protons and labile protons of the protein, but occurs also via cross relaxation at the water-protein interface.[5] In different experiments, pH-independent transfer of magnetization to slowly exchanging protein protons was observed upon irradiation of the water resonance in protein solutions.[6] In these experiments the observation of negative nuclear Overhauser effects (NOE)[6] showed that there must be hydration water bound with lifetimes that are comparable with the overall rotational correlation time of the hydrated protein, which is of the order of nanoseconds. Evidence for the presence, in certain proteins, of a small number of hydration waters that exchange with the bulk water on a time scale of seconds was deduced from ^{18}O tracer experiments.[7] None of these solution experiments could, however, provide information on the location of the hydration sites in the protein. The present paper describes a NMR technique that is capable of identifying individual molecules of hydration water and characterizing their binding sites on the protein molecule.

Our interest in detailed studies of protein hydration in solution was revived by recent observations with the protein Tendamistat, for which high-resolution structures were determined by NMR in solution and in single crystals.[8] Even though the global molecular architectures in the two states are very nearly identical, significant structural differences were identified near the protein surface. There is also evidence that compared to the core of the protein, the increase of structural disorder near the molecular surface is more pronounced in solution than in the crystals.[8] The experiments described in the present paper now demonstrate by observations with individual water molecules that in solution the hydration network on the protein surface is kinetically highly labile. Evidence for mobile hydration waters in water-protein systems

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(1) Abbreviations and symbols used: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; ROE, rotating-frame Overhauser enhancement; 2D, two-dimensional; NOESY, two-dimensional nuclear Overhauser enhancement spectroscopy in the laboratory frame; ROESY, two-dimensional nuclear Overhauser enhancement spectroscopy in the rotating frame; ω_0, Larmor frequency; τ_c, rotational correlation time.

(2) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; Wiley: New York, 1986.

(3) Deisenhofer, J.; Steigemann, W. *Acta Crystallogr., Sect. B* **1975**, *31*, 238.

(4) (a) Blundell, T. L.; Johnson, L. N. *Protein Crystallography*; Academic Press: New York, 1976. (b) Teeter, M. M. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 6014.

(5) Koenig, S. H.; Bryant, R. G.; Hallenga, K.; Jacob, G. S. *Biochemistry* **1978**, *17*, 4348.

(6) Stoesz, J. D.; Redfield, A. G.; Malinowksi, D. *FEBS. Lett.* **1978**, *91*, 320.

(7) Tüchsen, E.; Hayes, J. M.; Ramaprasad, S.; Copie, V.; Woodward, C. *Biochemistry* **1987**, *26*, 5163.

(8) (a) Kline, A. D.; Braun, W.; Wüthrich, K. *J. Mol. Biol.* **1986**, *189*, 377. (b) Kline, A. D.; Braun, W.; Wüthrich, K. *J. Mol. Biol.* **1988**, *204*, 675. (c) Pflugrath, J.; Wiegand, E.; Huber, R.; Vertesy, L. *J. Mol. Biol.* **1986**, *189*, 383.

0002-7863/89/1511-1871$01.50/0