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Purification and Comparative Characterization of an Enolase from Spinach[1]

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ABSTRACT

An enolase has been purified to apparent homogeneity, as measured by gel electrophoresis, some 400-fold from spinach (*Spinacia oleracea*). This is the first plant enolase that has been purified to homogeneity. At moderate ionic strengths, the 5,5-dithio-bis-2-(nitrobenzoate) (DTNB)- or parachloromercuribenzoate-reacted enzyme elutes from a Bio-Gel P-200 column with somewhat greater volumes than the yeast enzyme (M_r = 93,000) indicating a greater size. Its elution volume from Ultrogel in 50% ammonium sulfate, however, suggests it exists as an active monomer (M_r = 47,000). Sodium dodecyl sulfate-gel electrophoresis indicates the subunit molecular weight is 50,000 ± 3,000, like that of yeast enolase.

The enzyme contains 23 ± 4 half-cystines per mole of subunit. Titrations with DTNB in guanidine hydrochloride or nondenaturing media indicate that most of these, if not all, are in the reduced state. Reaction of one or more of the sulfhydryls with DTNB or parachloromercuribenzoate stabilizes the enzyme.

The kinetic parameters of the reaction catalyzed by spinach enolase, as well as the inhibitions by transition metal ions and fluoride, are similar to those properties of the yeast and rabbit muscle enzymes.

As an integral component of glycolysis, enolase (2-phospho-D-glycerate hydrolyase, EC 4.2.1.11) is probably ubiquitous. Enolases have been isolated and purified to some degree from at least twenty sources (24). The only plant enolase claimed to have been purified to homogeneity is that from potato tubers in 1959. Boser (3) purified the enzyme in low yield 67-fold from the crude, and demonstrated its homogeneity by paper electrophoresis. He found a turnover number for potato enolase of only 21% of that of yeast enolase. He also found significant activity if Cu(II) replaced Mg(II). Yeast enolase shows no activity with Cu(II) (24). Boser (3) also mentioned the high sensitivity of the potato enolase to sulfhydryl reagents, especially the rapid loss of activity in the presence of pCMB[2]. This is of interest because of the suspected importance of sulfhydryl compounds in regulating enzyme activities in plants (2). We have purified to apparent homogeneity an enolase from spinach and have examined many of its properties. We have also attempted the purification of potato enolase by the same method.

MATERIALS AND METHODS

Fresh spinach (*Spinacia oleracea*) was purchased from a local grocery. Ultra-pure (enzyme grade) ammonium sulfate was purchased from Schwarz-Mann, Mes from Aldrich, and the barium salt of 2-PGA and DTT from Calbiochem. The sodium salt of 2-PGA, DTNB (Ellman's reagent), and rabbit muscle enolase were from Sigma. 2-Mercaptoethanol and iodoacetic acid were obtained from JT Baker. Ampholyte and Ultrogel AcA 34 were purchased from LKB. DEAE-cellulose (DE 32) was from Whatman, and DEAE-Biogel A and Biogel P-2 from BioRad. All other chemicals were 'Analyzed Reagent' grade. Buffers and stock solutions were made up with deionized H_2O (Continental Services), and plasticware was used wherever possible.

The barium salt of 2-PGA was converted to the tricyclohexylammonium derivative by the method of Winstead and Wold (23). Yeast enolase was purified by a modification of the method described by Westhead (22).

Conductivity measurements were done on a CDM3 conductivity meter from Radiometer, Copenhagen, and pH measurements on a Corning model 10 pH meter. All assays of enzymic activity were done at ambient temperatures (22–25°C) on a Bausch and Lomb Spectronic 200 UV spectrophotometer equipped with digital readout. The yeast and rabbit muscle enolases were assayed as described by Westhead (22). Protein concentrations were determined using the method of Bradford (5), with BSA as standard.

Disc electrophoresis of native proteins were run in the 'standard' alkaline disc system (7). The gels were stained with 1% Amido Black. Sodium thioglycolate (0.1 μmol) was added to each protein sample to prevent oxidation in the gel (7). In order to locate the enolase activity of a gel, protein samples were run in duplicate. One gel, immediately after electrophoresis, was sliced into 0.1 to 0.15-cm pieces, and each piece was incubated in 2.5 ml of the standard assay medium. Each fraction was then, rapidly, successively transferred to a cuvette, and any increase in A at 230 nm monitored for 1 min. The duplicate gel was stained and destained as usual, and the enolase activity was assigned to any given band depending on the concurrence of the relative mobility to that of the slice(s) exhibiting enzymic activity.

SDS-PAGE was carried out as described (21) using 7.5% acrylamide gels in 0.5 cm i.d. by 12-cm tubes, stained with 0.25% Coomassie Blue R-250.

Stoke's radii of purified enolase and protein standards were determined by gel filtration (1). Absorption spectra were recorded on a modified on-line Cary 14 spectrophotometer, and fluorescence measurements on a modified on-line Spex spectrofluorimeter.

The amino acid composition was determined using 100 μg samples of protein, dialyzed *versus* distilled H_2O, then hydrolyzed in 6 N HCl in a sealed, evacuated Pyrex tube for 24 to 72 h (12). Tryptophan determination presented problems (see text). To determine the half-cystine content, performic acid oxidation or reduction and carboxymethylation were performed according to published procedures (8, 12).

Titration with pCMB was carried out by the method of Boyer

[1] Supported by a grant from the National Institutes of Health, GM 27742.

[2] Abbreviations: pCMB, parachloromercuribenzoate; 2-PGA, 2-phospho-D-glycerate; DTNB, 5,5-dithio-bis-2-(nitrobenzoate).

(4), by the incremental addition of 10 μl of 1 mM pCMB to 1 ml of a 0.2 to 0.4 mg/ml protein solution, at pH 6 or 7.6, monitoring the increase in A at 250 nm. Since pCMB itself absorbs at this wavelength, equal amounts of reagent were added to the reference cuvette. Reaction with DTNB was monitored by adding 10 μl of a 10 mM DTNB solution to 1 ml of a 0.1 to 0.2 mg/ml solution of protein in a cuvette, at pH 6 or 7.6, in the presence of 2 mM EDTA (10).

The pH dependence of enzyme activity was determined using four different buffer systems. In the pH 6 to 8 range, 50 mM imidazole and 50 mM Pipes were used, while in the pH 7.5 to 9.5 region, 50 mM Tris and 50 mM Tricine were. Observed activities were corrected for the variation of extinction coefficient of phosphoenolpyruvate at 230 nm with pH (25).

The Michaelis parameters were determined at the optimal pH, in 0.05 ionic strength Tris-HCl (pH 7.8; 0.15 M in Tris), containing 1 mM Mg(II) and 0.1 to 2 mM 2-PGA or 1 mM 2-PGA and 0.1 to 2.5 mM Mg(II). The data were corrected for 2-PGA-Mg(II) association (25). The 2-PGA used was made free of endogenous Mg(II) contamination by passing through a Chelex-100 column.

Activity in the presence of divalent cations other than Mg(II) was measured after rapid desalting of the enzyme on a Biogel P-2 column equilibrated with 10 mM Mes (pH 6). Remaining activity was measured in an assay medium containing 0.05 ionic strength Tris-HCl (pH 7.8) and 1 mM 2-PGA. Less than 2% of the maximum rate was observed. To this, different divalent cations at different concentrations were added, and the activity was measured.

RESULTS

Purification of Spinach Enolase. One to 1.5 kg of fresh spinach was rinsed with distilled H_2O, dried with paper towels, and homogenized in a Waring Blendor with an equal volume of 'homogenization buffer' (10 mM Mes [pH 6.0], 0.2 M NaCl, 1 mM magnesium chloride, and 0.1 mM EDTA). The slurry was filtered through cheesecloth, and centrifuged at 5000 rpm in a HG-4L rotor in a Sorvall RC-3 centrifuge for 30 min at 4°C. The pellet was discarded.

The 'crude homogenate', the supernatant, was then made up to 50% saturation in ammonium sulfate by the addition of solid salt at 4°C and stirred for a further 15 min after all the salt dissolved. The slurry was then centrifuged as before and the pellet discarded.

DEAE-Cellulose Chromatography in Ammonium Sulfate (15). The supernatant was then applied to a 6.5-cm i.d. by 7.5 cm DEAE-cellulose (DE-32) column, preequilibrated with 10 mM Mes (pH 6), 1 mM magnesium chloride, 0.1 mM EDTA, 50% saturated with ammonium sulfate ('buffer A-50% ammonium sulfate'). Over 70% of the protein bound to the column, which was washed with equilibrating buffer, and then eluted with a reverse gradient of decreasing ammonium sulfate concentration, with 450 ml buffer A flowing into 450 ml buffer A-50% ammonium sulfate. The enolase activity eluting from the column was pooled.

Biogel P-2 Desalting. Eluted proteins were precipitated by adding ammonium sulfate to 90% saturation, redissolved in a minimum volume of 'buffer B' (10 mM Tris-HCl [pH 7.8], 1 mM Mg[II]), and rapidly desalted by chromatography on a 3.6-cm i.d. by 12-cm Biogel P-2 column, preequilibrated with buffer B. The conductivity across the protein peak was measured.

DEAE-Biogel A Chromatography. The pooled fractions of the desalted protein were then applied to a 3.6-cm i.d. by 12 cm DEAE-Biogel A column, pre-equilibrated with buffer B. The column was washed with the same buffer, and then eluted with a linear gradient of 0 to 200 mM KCl (250 ml each, in buffer B). The enolase activity eluted at around 90 mM KCl, and fractions with specific activities greater than 20 units/mg were pooled.

Ultrogel AcA 34 Gel Filtration in 50% Ammonium Sulfate. The pooled eluate was reprecipitated with 90% ammonium sulfate, centrifuged, and the pellet redissolved in a minimum volume of buffer A. It was then chromatographed on a 2-cm i.d. by 60 cm Ultrogel AcA 34 column, preequilibrated with buffer A-50% ammonium sulfate, flowing at a constant 15 to 20 ml/h and eluted with the same buffer. Five- to 6-ml fractions were collected and fractions with specific activities above 80 were pooled.

Second DEAE-Cellulose-Ammonium Sulfate Chromatography. The pooled fractions were immediately applied to a second (2 cm i.d. by 10 cm) DEAE-Cellulose column in buffer A-50% ammonium sulfate, washed with the same buffer, and eluted as before with a linear gradient of decreasing ammonium sulfate concentration, 125 ml each, in buffer A. Fractions with specific activities above 110 were pooled.

Ammonium Sulfate Extraction of Enolase. In the final step, the protein from the previous step was precipitated with 90% ammonium sulfate, and the pellet successively extracted with small volumes of buffer A containing 79%, 76%, 73%, 70%, etc. ammonium sulfate. One to 1.5 ml of the extracting solution was added per 3 to 4 mg of protein. Each time, the suspended pellet was incubated at 0°C for 15 min, and then centrifuged at 10,000 rpm for 15 min at 0°C in a Sorvall SS-34 rotor. The supernatant was decanted. Fractions with specific activities above 230 were pooled, diluted to 50% ammonium sulfate concentration with buffer A, and stored at 4°C.

A purification chart for the spinach enolase is given in Table I. There was always a large increase in total enolase activity after addition of ammonium sulfate to 50% saturation or more. The potato enzyme behaved similarly.

In the case of the spinach enzyme, the overall apparent purification obtained was 685-fold, using the crude homogenate value as starting point. The crude homogenate activity is probably low because of the ammonium sulfate effect, so 400-fold is a more realistic estimate. The overall yield is low, only 5%, though yields of up to 10% have been obtained. The specific activity of the purified enzyme is 240 units/mg, a value within a factor of two of the yeast enzyme and higher than that of the rabbit muscle enzyme.

The enzyme as isolated is extremely unstable in the absence of ammonium sulfate. This severely limited both the methods available for purification and the methods we could employ for analysis of its properties afterwards. Preparative isoelectric focusing, for example, resulted in large losses of enzymic activity.

Purification of Potato Enolase. The same protocol was used to purify enolase from potatoes, except 0.1% mercaptoethanol was added to the homogenization buffer. The overall apparent purification was about 90-fold, with a yield of 17%.

Boser purified potato enolase 67.5-fold, to a specific activity of 17.5, in 5% yield (3). In order to compare his results with ours, the enzyme activity and the protein concentration of our purified potato preparation were determined exactly as Boser had (3, 20). The specific activity of our purified potato enzyme preparation came out to be 24, 35 to 40% higher than that obtained by Boser (3). Our protocol purifies the potato enolase to a higher specific activity with higher yield. Also, since our enzyme preparation was not homogeneous, Boser probably did not have a homogeneous preparation.

Gel Electrophoresis of Purified Enolases. Analytical disc gels of the spinach and potato enolases, and an SDS-gel of the purified spinach enolase, are shown in Figure 1. The potato enzyme is clearly heterogeneous. The spinach enolase gives a single band on the disc gel. A gel slice *versus* enzyme activity graph identifies this band as the only enzymically active species present (not shown). On the SDS-gel, the spinach enolase also migrates as a single band. There are two lower mol wt contaminants that are

Table I. *Purification of Enolase from Spinach*

Purification Step	Total Protein	Total Units[a]	Specific Activity[a]	Yield
	mg			%
Crude homogenate	7059	2470	0.35	
0–50% Ammonium sulfate	1355	4200	3.1	100[b]
DEAE-Cellulose ammonium sulfate	546	3110	5.7	74
Biogel P-2		1865		44
DEAE-Biogel A	69	1585	23	38
Ultrogel AcA 34	14.7	1265	86	30
DEAE-Cellulose ammonium sulfate II	6.6	820	125	19.5
Ammonium sulfate extraction	0.85	205	240	5

[a] Units as defined by Westhead (22).
[b] See text.

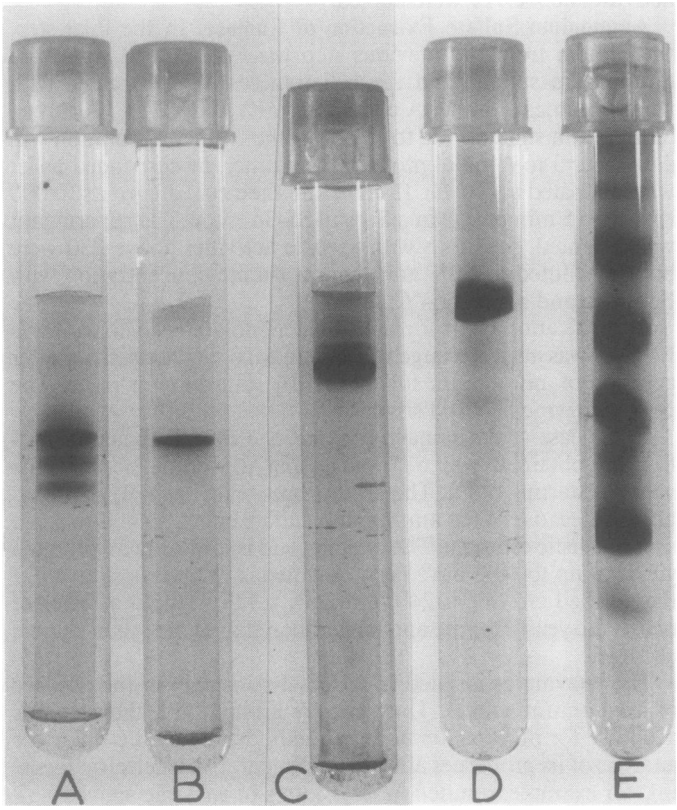

FIG. 1. Gel electrophoresis of spinach enolase. Samples A, B, and C are 'standard' (pH 9.5) disc gels of 20 to 30 μg of spinach enolase, specific activity 120 (gel A) or 240 (gel B) or 30 μg of potato enolase (gel C), specific activity 100. Sodium thioglycolate (0.1 μmol) was added before electrophoresis. Gels D and E were from SDS gels, carried out in 0.05 M sodium phosphate (pH 7.0) with 0.1% SDS present. These were preelectrophoresed for 2 h after overlayering with 1 μmol of sodium thioglycolate. Gel D shows 30 μg of spinach enolase originally of specific activity 240 and gel E shows protein standards, 25 μg each of BSA, ovalbumin, carbonic anhydrase, and lysozyme.

barely discernible, but they constitute much less than 5% of the major band. The spinach enolase has been obtained in a highly purified state.

Some preparations of the spinach enolase, during the later stages of the purification process, gave rise to three electrophoretic species (Fig. 1, gel A). All were found to have enzymic activity (not shown). These species may be artefacts of the purification process, or true 'isozymes'. Enolase isozymes have been demonstrated in castor oil plant (17).

Stokes Radius of Purified Enolase by Gel Filtration. Gel filtration of various proteins was carried out on an Ultrogel AcA 34 column, in 50% saturated ammonium sulfate, since the unmodified spinach enzyme is very unstable during gel filtration in the absence of the salt. K, defined as $(V_e - V_o)/V_t$, where V_e is the elution volume of protein, V_o the void volume of the column, and V_t the volume of the stationary phase (1), is plotted against the Stokes radii of the standards, as shown in the inset to Figure 2. The spinach enolase migrates with a K of 1.17. This is compared to 0.8 for the dimeric yeast enolase, and 1.43 for carbonic anhydrase. Spinach enolase appears to have a Stokes radius of 26 Å, suggesting that it is a monomer: it elutes nearly coincidentally with ovalbumin (M_r = 45,000 and a Stokes radius of 27.6 Å). The presence of 1 mM substrate does not change the elution volume.

The yeast enzyme can dissociate into monomers which retain full activity (13). The 'dissociated' spinach enzyme also has full activity. The high K value, however, suggests adsorption to the column may be occurring: the elution volumes of carbonic anhydrase and spinach enolase were greater than the elution volume of ferricyanide, used to calibrate the column. On the other hand, the spinach enolase profiles did not suggest adsorp-

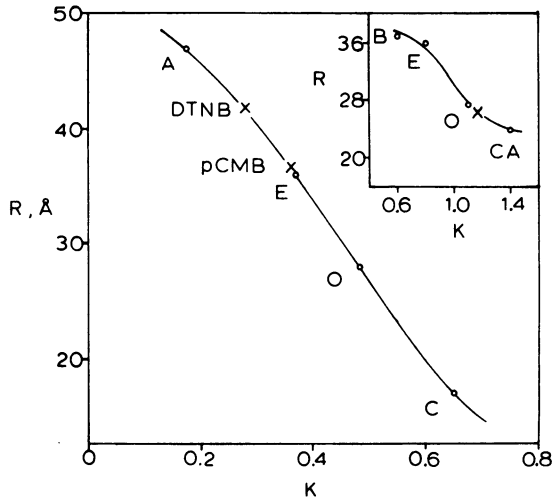

FIG. 2. Gel filtration of spinach enolase on molecular sieving columns. Fifty μg of DTNB- or pCMB-modified spinach enolase was chromatographed on a 1.6- × 74-cm Bio-Gel P-200 column in 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, and 0.1 M NaCl. The figure also shows the elution volumes of protein standards. Blue dextran and ferricyanide were used to establish the void and total column volumes, respectfully. In the figure, A is aldolase, E is yeast enolase, O is ovalbumin, CA (inset) is carbonic anhydrase, and C is Cyt *c*. Inset, The column (2 × 60 cm) contained Ultro-gel AcA 34 in 50% ammonium sulfate in 10 mM Mes (pH 6.0) and 1 mM $MgCl_2$. The unmodified enzyme was chromatographed.

tion was occurring. The high elution volumes may be an effect of the high salt concentrations used: Cyt *c* emerged with a volume almost identical to that of BSA.

The spinach enzyme is stabilized by reaction with the sulfhydryl reagents DTNB and pCMB (see below). Hence, the reacted enzyme could be chromatographed at moderate ionic strengths without loss of activity (Fig. 2). At 0.1 ionic strength, the spinach enzyme modified with pCMB and DTNB elutes earlier than the yeast enzyme, suggesting a somewhat greater Stokes' radius. The yeast enzyme is believed to be highly symmetrical (6), so a greater Stokes radius of the spinach enzyme is reasonable. The difference in Stokes radii of the two derivatives seems too great to be experimental error, but we were reluctant to draw further conclusions based on work with a chemically modified enzyme.

Electrophoresis of the spinach enzyme in SDS-gels gave an apparent subunit mol wt of 50,000 ± 3,000 (Fig. 1; graph not shown), identical to that of the yeast enzyme (6, 24) (M_r = 46,700). We conclude that while the subunit mol wt of spinach enolase is about 47,000, similar to those of other enolases, the enzyme has a higher mol wt at moderate ionic stengths, also like other enolases. In 50% ammonium sulfate, it behaves more like an active monomer.

The absorption spectrum of the enzyme indicated tryptophan was present. The near UV maximum A was at 279 nm. We calculated an extinction coefficient for the enzyme of 1.95 ml/mg·cm at 280 nm, using the Bradford assay (5) to measure protein content.

The fluorescence emission spectrum of the protein also indicated tryptophan was present. The emission maximum was at 345 nm (excitation at 279 nm). We found no major effect of Mg(II), EDTA, or substrate on the emission spectrum, in contrast to their effects on the yeast enzyme (6).

Chemical Properties: Amino Acid Composition. Two complete sets (24, 48, and 72 h hydrolysis time) of amino acid analysis data were obtained. The average or extrapolated values, in residues per 47,000 g protein, are presented in Table II. A value of 42 mol residue/mol subunit was assumed for aspartate. Values for serine and threonine were extrapolated to zero time, and values for valine and isoleucine to infinite time.

Table II. *Comparison of Amino Acid Compositions of Enolases*

Residues are to nearest integral value. Compositions of the rabbit muscle and *E. coli* enzymes are from Reference 24; the composition of the yeast enzyme is from Reference 6. Average % SD of some randomly chosen spinach enolase residue values are given in parentheses. See the text for the origin of the tryptophan and half-cystine values.

Amino Acid	Spinach	Yeast	Rabbit Muscle	*E. coli*
	mol residue/mol N-terminus			
Asparate	42	50	39	43
Threonine	22	20	17	21
Serine	24	31	17	21
Glutamate	43 (3.5)	34	34	46
Proline	16	15	13	11
Glycine	41 (1.7)	37	36	45
Alanine	38 (2.1)	55	38	58
Half-cystine	23	1	6	4
Valine	31	35	29	24
Methionine	7	5	6	12
Isoleucine	19	22	22	32
Leucine	29 (4.0)	40	34	29
Tyrosine	11 (5.8)	9	9	11
Phenylalanine	14	16	13	12
Histidine	7	11	10	7
Lysine	29 (6.2)	36	34	38
Tryptophan	11	5	5	2
Arginine	13 (4.4)	14	15	11
Totals	420	436	377	431

The value for tryptophan was obtained fluorometrically. We were unable to resolve the tryptophan peak from that due to ammonia after hydrolysis in the presence of thioglycolic acid (14). The value obtained by the spectrophotometric method of Goodwin and Morton (11), at alkaline pH, was 4 mol of tryptophan/mol subunit. Because of the large number of cysteines in the enzyme (see below), whose absorbance would contribute, and because the extinction coefficient was 1.95 ml/mg·cm, much higher than that of the yeast enzyme (0.895 ml/mg·cm), we measured the integrated fluorescence of the spinach, yeast, and rabbit muscle enzymes in 6 M guanidine-HCl. These measurements gave a tryptophan content of 11 ± 2 residues for the spinach enzyme, which agrees with the extinction coefficient at 280 nm.

The half-cystine content was obtained after acid hydrolysis of performic acid oxidation of three preparations of the enzyme. Values of 41, 27, and 22 mols half-cystine/mol of subunit were obtained. Cysteic acid values tend to be biased upward by the occasional presence of extraneous ninhydrin-positive material eluting at or near the place the authentic acid emerges. Consequently, we also reduced and carboxymethylated a sample of spinach enolase. The carboxymethylcysteine content was 19 residues/mol. The carboxymethylcysteine content would tend to be low, owing to the possibilities of incomplete reduction or reaction. If we exclude the value of 41 residues as being clearly anomalous and average the other values, we obtain a half-cysteine content of 23 ± 4 residues/mol of subunit. This is the value reported in Table II.

Table II also gives a comparison of amino acid contents of the enolases from spinach, yeast, rabbit muscle, and *E. coli*. Major differences in the tryptophan content exist, but the greatest difference is in the high half-cystine content of the spinach enzyme.

Cysteine Reactivity of the Spinach Enolase: The total cysteine content was measured by adding excess DTNB to protein solution, and adding ultra-pure guanidine-HCl to 4.4 M. Immediate reaction of 10 residues was observed, followed by a slower further reaction over 3 to 5 h. A total of 17 cysteinyl residues was detected. Spinach enolase has a high cysteine content. Denatured protein dialyzed against low ionic strength buffer (pH 7.8) did not recover activity or reactivity towards DTNB (below).

We then examined the reactivity of sulfhydryls in the native enzyme. pCMB reacted slowly (3–5 h) with 2.4 residues at pH 6, in the presence of 50% ammonium sulfate, and with 2.7 residues of −SH at pH 8, in 10 mM Tris buffer. There was no loss of activity during or after the reaction. Indeed, there was routinely a 10 to 15% increase in the enzyme activity, and subsequent stabilization of the activity of the modified enzyme, at room temperatures even in the absence of 50% ammonium sulfate.

The reaction with DTNB was examined in greater detail. In the presence of a 25-fold excess of DTNB, one −SH residue per 46,000 g protein reacted in the first 20 min. The protein reacted more slowly with more DTNB, with 2.4 residues modified at the end of 6 h of reaction. DTNB continued to react with the protein for as long as 90 h, to 15.8 residues/mol protein (Fig. 3). The enzymic activity was constant.

That the 'exposed' residue was probably the residue that, on DTNB modification, resulted in the stabilization, was demonstrated by reacting the protein with DTNB for about 30 min, checking to confirm that only one sulfhydryl had reacted. It was then passed through a small Biogel P-2 column to remove excess reagent. This 'desalted' modified protein sample was then monitored for 24 h, along with an unmodified control. Again, no change in activity occurred with the modified enzyme. After 24

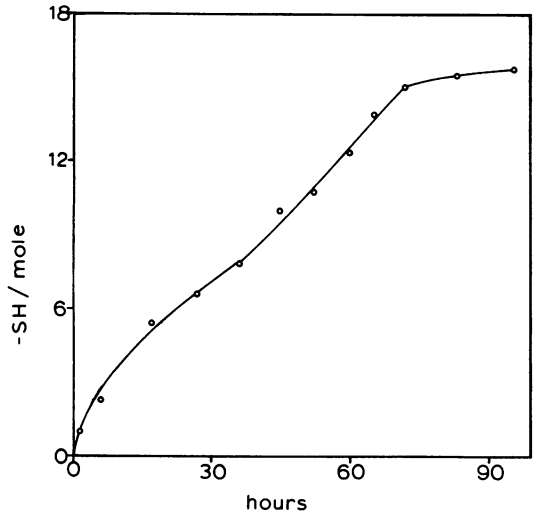

FIG. 3. Reaction of spinach enolase with DTNB. A sample (2 ml) of spinach enolase (3.3 μM or 0.33 mg/ml) in 10 mM Tris-HCl (pH 7.6), with 1 mM $MgCl_2$, was titrated with μl aliquots of 0.1 M DTNB at 23°C over several days. Equal volumes of DTNB were added to a reference cuvette. The cuvettes were kept covered between additions. Values of sulfhydryl/mol of subunit were calculated, assuming a mol wt of 50,000.

h, the unmodified enzyme had 40% less activity (see "Discussion").

The reactivity of the free −SH groups on the potato enolase was also examined. All of them were very slow to react with DTNB, overnight reaction being necessary. There was a proportionate loss of activity with the reaction of the first, second, and third residue, respectively (not shown). This confirms Boser's observations (3). However, in light of the results obtained with the spinach enolase, it seems reasonable to conclude that the modification is accompanied by structural changes in the protein, which in turn lead to the loss of activity.

Enzymic Properties of Spinach Enolase: pH Optimum. The pH dependence of the activity of the spinach enolase was 'bell-shaped' with an optimum at pH 7.8 to 8.0 (not shown). The buffers used (Tris, Tricine, Pipes, and imidazole) had no dramatic effect on enolase activity. The pH optimum is very close to that of the *E. coli* and yeast enzymes, and to those obtained from crude extracts of pea and soybean (24). It is higher by about 1 pH unit than the optima for the vertebrate and mammalian enolases, as expected (24).

Kinetic Parameters of Spinach Enolase. The Michaelis constant for free substrate, 2-PGA, for the spinach enolase (at pH 7.8) was determined from a double-reciprocal plot (not shown) to be 55 ± 1 μmol. This is close to the Michaelis constant for 2-PGA (70 μM) for yeast enolase (24) under identical conditions. The maximum velocity was 110 enzyme units/mg (110 μmol product formed/min · mg), which was 55% of that of yeast enolase (200 enzyme units/mg), measured under identical conditions. The maximum velocity and Michaelis constant for 2-PGA for the rabbit muscle enzyme under optimal conditions for its activity are 100 enzyme units/mg (22) and 45 μM (24).

Like other enolases (24), activity of the spinach enzyme is absolutely dependent on metal ion. This could be shown by titrating away the activity with EDTA, then restoring the activity with excess Mg(II). Determining the Michaelis constant for Mg(II) was more complex. Residual metal ion carried over with the enzyme produced greater than 10% of maximal activity. By performing a careful EDTA titration of the residual metal ion, and using Mg(II)-free 2-PGA, we found that the activity is inhibited by Mg(II) at concentrations above 0.6 to 0.7 mM. The optimal concentration range was 0.35 to 0.5 mM, lower than that of most other enolases (1 mM) (24) (not shown). Extrapolation of the linear portion of the double-reciprocal plot gave an apparent Michaelis constant for free Mg(II) of 60 μM. This is similar to values obtained with yeast enolase (150 μM) and rabbit muscle enolase (80 μM) (24).

Divalent Cation Activation and Inhibition of Spinach Enolase. Spinach enolase is activated by several divalent cations, and the levels of activity obtained, at the metal ion concentrations noted, are presented in Table III. Mg(II) is by far the most effective activator of the spinach enolase. It is almost certainly the activator *in vivo*. Cu(II), Mn(II), Zn(II), and Cd(II) are less effective. Co(II) and Ni(II) provide under 10% of the activity obtained with Mg(II) (not shown). Ca(II), as expected, did not activate the enzyme at all, and even eliminated the low basal rate (due presumably to contaminating Mg[II]) obtained without added divalent cation.

It was surprising to obtain a relatively high level of activity with Cu(II), since yeast enolase is not activated at all by Cu(II). However, this agrees with Boser's results with potato enolase (3).

In the presence of 1 mM Mg(II), all of the above activating cations inhibit the activity of the spinach enolase. Such inhibition of the yeast enzyme by Zn(II), Co(II), Cd(II), Mn(II), and Ni(II) results from the binding of these metal ions to 'inhibitory' sites, not by displacing Mg(II) from the catalytic site, as does Ca(II) (9). If this type of inhibition occurs with other enolases, it suggests that those enzymes have similar sites, lending credence to the view that there are remarkable similarities between the various enolases in their catalytic and possibly regulatory properties (24).

We measured spinach enolase activity in the presence of 1 mM Mg(II) in a noninteracting buffer. We plotted the logarithm of the transition metal ion concentrations against per cent activity relative to a noninhibited control (9). The transition metal ion concentrations producing 50% inhibition were determined by interpolation using both spinach and rabbit muscle enolases

Table III. *Effect of Varying Levels of Several Metal Ions on Spinach Enolase Activity*

Assays were carried out in 2.5 ml of 0.05 ionic strength Tris-HCl (pH 7.8) with 1 mM substrate (sodium salt) present, at 23°C. Reactions were initiated by addition of spinach enolase (about 10 μg) and mixing.

Metal Ion Added	Final Concn.	Relative Activity
	μM	%
None		2.5
Mg^{2+}	10	12
	50	55
	500	(100)
Cu^{2+}	5	6
	10	20
	25	10
Mn^{2+}	10	4
	50	19
	100	12.5
Cd^{2+}	10	5
	100	14
	200	9
Zn^{2+}	50	7
	100	12
	200	9
Ca^{2+}	10	0
	100	0

(Table IV).

The concentrations for 50% inhibition for the spinach enzyme are more similar to those for yeast, though all values are in the 1 to 80 μM range. The order of the values for rabbit muscle enolase correlates with the doses of intraperitoneal metal ion injections producing 50% fatality in mice (19), except for Ni(II). Enolase might be a target enzyme for metal ion poisoning and not only in mammalian systems.

Substrate-Dependent Fluoride Inhibition of Spinach Enolase. In the absence of Pi, fluoride inhibits yeast enolase in a substrate- and time-dependent fashion (18). Figure 4 shows the time courses of inhibition by fluoride of the forward reaction catalyzed by spinach and rabbit muscle enolases in the standard assay medium.

The inhibition of spinach enolase has two components, a fast (0–1 min), and a second, slower rate of increase (1–10 min). The early rate of increase of the inhibition was faster for the spinach than the yeast enzyme. The rabbit muscle enzyme shows a similar pattern, although the inhibition develops more slowly. The inhibition patterns with all three enolases suggest similar mechanisms.

DISCUSSION

Considerable difficulty was encountered in the enzyme purification, owing to the marked instability of spinach enolase, especially upon dialysis. A variety of stabilizing agents, including DTT and 2-mercaptoethanol, sucrose or glycerol and protease inhibitors such as PMSF, benzamidine, leupeptin, and soybean trypsin inhibitor were tried, with little or unreproducible effect.

Table IV. *LD_{50} and 50% Inhibition Concentrations*

Concentrations for 50% inhibition were determined in 0.033 M tetrapropylammonium-borate buffer (pH 7.9) in the presence of 1 mM $MgCl_2$ and 1 mM substrate, as described in "Materials and Methods" and in Reference 9. About 10 μg of spinach and 20 μg of dialyzed rabbit muscle enolase were used in each assay. Data for the yeast enzyme are from Reference 9; LD_{50} values are from Reference 19.

Metal Ion	Concn. for 50% Inhibition			LD_{50} (Mice)
	Spinach	Yeast	Rabbit muscle	
		μM		μM
Cu^{2+}	4.5	0.5	8	130
Zn^{2+}	6.5	2.5	19	180
Cd^{2+}	3.7	4	26	350
Co^{2+}	13	21	35	350
Mn^{2+}	16	19	42	800
Ni^{2+}	62	80	66	140

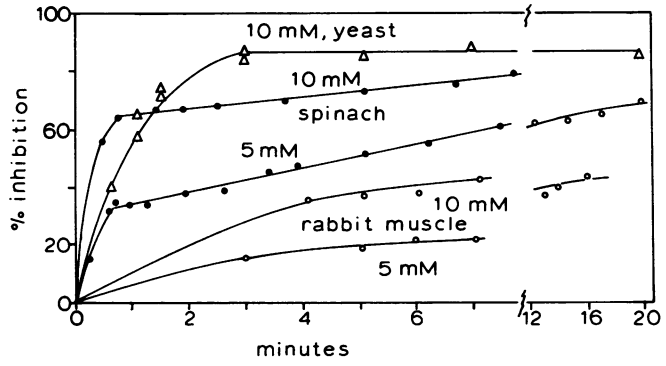

FIG. 4. Inhibition by fluoride of the enolase reaction catalyzed by enolases from spinach, rabbit muscle, and yeast. Spinach enzyme assays were carried out in the standard assay medium in the presence or absence of 5 or 10 mM fluoride. Rabbit muscle assays were carried out in 0.05 M imidazole-HCl (pH 7.0), 0.4 M KCl, 1 mM $MgCl_2$, and 1 mM substrate. Inhibitions were calculated from relative slopes of 230 mm *A versus* time graphs taken at equivalent midpoint 230 nm values, as described by Spencer and Brewer (18). The yeast enolase data (Δ) are taken from Reference 18 and are included for the sake of comparison. (○), rabbit muscle data; (●), the spinach enzyme.

The unconventional purification method described in "Results" was successful. Chromatography of proteins in concentrated ammonium sulfate solutions was first studied in detail by Mayhew and Howell (15) in 1971. This procedure probably separates proteins on the basis of nonionic interactions. The procedure is used for both the spinach and potato enolases twice with success.

The spinach enzyme appears to be essentially homogeneous on the basis of SDS-gel electrophoresis at pH 7.0 and disc electrophoresis at pH 9.5. The specific activity of the enzyme also is reasonable, between those of the yeast and rabbit muscle enzymes.

It is possible that the multiple electrophoretically distinct species observed are partially degraded enolase molecules, obtained by proteolytic cleaving. However, two enolase isozymes, one associated with chloroplasts and the other with the cytosol, have been found in the castor oil plant (17). Two yeast enolase isozymes also exist (16).

The Ultrogel chromatography data suggest that under the conditions of isolation of this enzyme, it exists as an active monomer. The yeast enzyme can exist as an active monomer (13), although it is generally accepted (24) that yeast and other enolases exist as dimeric proteins *in vivo* as well as under most experimental conditions. The high lability of the unmodified spinach enolase during dialysis and gel filtration in the absence of ammonium sulfate prevents a more complete investigation of this aspect of protein structure being carried out.

There is no obvious reason for the large number of sulfhydryl groups in this enzyme. Sulfhydryl oxidation and reduction seem to be involved in photosynthetic metabolism (2). However, it is hard to see how the sulfhydryls in spinach enolase can be involved in metabolism, in view of the large number of sulfhydryls, the sluggish reactions undergone by most of them, and the often irreversible inactivation accompanying oxidation of some of them. Again, this question must remain open.

Otherwise, the sulfhydryl data seem easy to interpret. There seems to be one, relatively 'exposed' cysteine residue, and blocking of this by either DTNB or pCMB leads to a stabilization of the enzyme activity. The bulk of the half-cysteines, perhaps all of them, react ultimately. Remarkably, even on reaction of as many as sixteen of them with the bulky DTNB group, no loss of activity is observed. The sulfhydryls are certainly not involved at the active site.

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