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A 5,10-METHYLENETETRAHYDROFOLATE DEHYDROGENASE: 5,10-METHENYLTETRAHYDROFOLATE CYCLOHYDROLASE PROTEIN FROM *PISUM SATIVUM*

CAROLE D. KIRK, LIANGFU CHEN, HELENA C. IMESON* and EDWIN A. COSSINS†

Department of Biological Sciences, University of Alberta, Edmonton, Alberta, T6G 2E9, Canada; *Department of Biological Sciences, University of Lethbridge, Lethbridge, Alberta, T1K 3M4, Canada

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Key Word Index—*Pisum sativum*; Leguminosae 5,10-methylenetetrahydrofolate dehydrogenase; 5,10-methenyltetrahydrofolate cyclohydrolase; higher plant; purification; bifunctional enzyme.

Abstract—A cytosolic protein, that exhibits NADP-dependent 5,10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) and 5,10-methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9) activities but lacks 10-formyltetrahydrofolate synthetase (EC 6.3.4.3) activity, was isolated from extracts of pea (*Pisum sativum* L.) cotyledons and leaves. Dehydrogenase:cyclohydrolase activities co-purified when protein was fractionated by $(NH_4)_2SO_4$ precipitation, followed by chromatography on Sephacryl S-300, Matrex Green A and heparin agarose. The resulting protein (M_r 38 500) was apparently homogeneous after SDS–polyacrylamide gel electrophoresis and silver staining. Purified enzyme preparations lacked methylenetetrahydrofolate dehydrogenase activity when NAD replaced NADP in the reaction system. Attempts to sequence this protein suggested that it may be blocked at the *N*-terminus. Tryptic digestion of the purified protein resulted in coordinate losses of dehydrogenase and cyclohydrolase activity. The NADP provided significant protection of both enzyme activities during short-term treatments with trypsin but additions of 5,10-methylenetetrahydrofolate appeared to accentuate the proteolytic loss of dehydrogenase activity. The apparent Michaelis constants for NADP (11 μM) and methylenetetrahydrofolate (21 μM) in the dehydrogenase reaction were not changed significantly when the folylpentaglutamate substrate was provided (8 and 25 μM, respectively). The dehydrogenase and cyclohydrolase activities were competitively inhibited by dihydrofolates. The K_i values of the dehydrogenase reaction indicated that the pentaglutamate derivative was a more potent inhibitor than dihydrofolate monoglutamate. The ELISA and Western blot analyses, using rabbit polyclonal antibodies raised against the purified enzyme, revealed cross-reactivity with proteins of similar molecular size in leaf extracts of wheat, barley, corn, bean and pea. Chromatography of these leaf extracts on Matrex Green A, in the presence of protease inhibitors, showed that 10-formyltetrahydrofolate synthetase was readily separated from protein with dehydrogenase and cyclohydrolase activity.

INTRODUCTION

Plants, like other eukaryotic species, generate formylmethionyl-tRNA, purines, thymidylate, methionine and serine by pathways that include folate-dependent enzymes [2, 3]. Characteristically, each of these metabolic products is synthesized by the incorporation of one-carbon units that are donated by tetrahydrofolate polyglutamate ($H_4PteGlu_n$) derivatives. The folates of major physiological importance are 10-HCO-$H_4PteGlu_n$, 5,10-CH_2-$H_4PteGlu_n$ and 5-Me-$H_4PteGlu_n$ which donate formyl-, methylene- and methyl groups, respectively [3]. Plant cells also contain enzymes for the synthesis and interconversion of these C-1 substituted folate derivatives. Thus there is good evidence that the reactions catalysed by serine hydroxymethyltransferase (EC 2.1.2.1), (eqn 1), glycine synthase (EC 2.1.2.10), (eqn 2) and 10-HCO-$H_4PteGlu_n$ synthetase (EC 6.3.4.3), (eqn 3) represent routes for generation of such one-carbon units [2, 3].

$$\text{Serine} + H_4PteGlu_n = 5,10\text{-}CH_2\text{-}H_4PteGlu_n + \text{glycine} \quad (1)$$

$$\text{Glycine} + H_4PteGlu_n + NAD = 5,10\text{-}CH_2\text{-}H_4PteGlu_n + CO_2 + NH_3 + NADH \quad (2)$$

$$\text{Formate} + H_4PteGlu_n + ATP = 10\text{-}HCHO\text{-}H_4PteGlu_n + ADP + Pi. \quad (3)$$

†Author to whom correspondence should be addressed.

Abbreviations: folate derivatives are designated in accordance with the IUPAC-IUB Commission (see [1]); AICAR transformylase = phosphoribosyl aminoimidazolecarboxamide formyltransferase (EC 2.1.2.3).

The folate derivatives produced by these reactions are interconvertible as evidenced by the widespread occurrence [2–4] of 5,10-CH_2-H_4PteGlu$_n$ dehydrogenase (EC 1.5.1.5), (eqn 4) and 5,10-CH^+-H_4PteGlu$_n$ cyclohydrolase (EC 3.5.4.9), (eqn 5). These latter reactions allow an equilibrium between the pools of one-carbon units at the formyl and methylene levels of oxidation. Consequently, serine and formate commonly serve as precursors of one-carbon units for the pathways of purine, thymidylate and methionine biosynthesis [2, 3].

$$5,10\text{-}CH_2\text{-}H_4PteGlu_n + NADP = 5,10\text{-}CH^+\text{-}H_4PteGlu_n + NADPH \quad (4)$$

$$5,10\text{-}CH_2\text{-}H_4PteGlu_n + H_2O = 10\text{-}HCO\text{-}H_4PteGlu_n + H^+ \quad (5)$$

The acknowledged role of these enzymes in one-carbon metabolism has prompted extensive study of their physical and catalytic properties [3–5]. The synthetase, dehydrogenase and cyclohydrolase activities of mammalian and yeast cells are associated with a trifunctional peptide often referred to as C_1-tetrahydrofolate synthase [4]. This complex is found in both the cytosolic and mitochondrial compartments of yeast cells where it plays a major role in one-carbon metabolism [6]. In many bacteria, these three folate-dependent activities are associated with separate, monofunctional proteins [4]. However, in *Escherichia coli* [7, 8] and *Clostridium thermoaceticum* [9] distinct polypeptides with combined dehydrogenase:cyclohydrolase activities have been isolated.

The structural organization of these folate-dependent enzymes in plants is less certain. In spinach leaves [10, 11] and pea cotyledons [12] the synthetase activity is associated with a discrete monofunctional protein. On the other hand, the dehydrogenase and cyclohydrolase activities of these tissues appeared to co-purify during the early stages of protein fractionation [10, 12], but homogeneous preparations with both activities have not been isolated. It is also noteworthy that earlier studies of these plant enzymes [2] did not employ protease inhibitors. Thus it is not clear whether the 5,10-CH_2-H_4PteGlu$_n$ dehydrogenase and 5,10-CH^+-H_4PteGlu$_n$ cyclohydrolase reactions in plants are catalysed by separate proteins or by a bifunctional complex. Furthermore, there is little information on the catalytic properties of these enzymes in plants.

In the present studies we have purified a cytosolic dehydrogenase:cyclohydrolase to apparent homogeneity from one-day-old pea cotyledons and 14-day-old pea leaves. Polyclonal antibodies, raised against the cotyledon protein, were used to assess the occurrence of this complex in leaf extracts of four other plant species.

RESULTS AND DISCUSSION

Distribution of enzyme activities after differential centrifugation

In an earlier study [12], we found that 10-HCO-H_4PteGlu synthetase and 5,10-CH_2-H_4PteGlu dehydrogenase were predominately cytosolic enzymes in pea

Table 1. Distribution of cyclohydrolase and dehydrogenase activities after differential centrifugation of six-day-old cotyledon extracts

Enzyme activity	Supernatant 1 (7000 *g*)	Supernatant 2 (21 000 *g*)	Mitochondrial pellet sonicated + Triton X-100
5,10-CH^+-H_4PteGlu cyclohydrolase			
Total activity (nkat)	14	11	n.d.
nkat mg^{-1} protein $\times 10^{-3}$	19	52	n.d.
Recovery (%)	100	83	0
5,10-CH_2-H_4PteGlu dehydrogenase			
Total activity (nkat)	39	38	2.5
nkat mg^{-1} protein $\times 10^{-3}$	52	171	77
Recovery (%)	100	98	6
Alcohol dehydrogenase			
Total activity (nkat)	5390	5690	52
nkat mg^{-1} protein	6.7	25	1.7
Recovery (%)	100	106	1
Catalase			
Total activity (μkat)	680	370	130
μkat mg^{-1} protein	0.9	1.7	4.3
Recovery (%)	100	54	20
Succinic dehydrogenase			
Total activity (nkat)	14	1.3	54
nkat mg^{-1} protein $\times 10^{-3}$	19	6.2	1660
Recovery (%)	100	10	377

n.d. = activity not detected.

cotyledon extracts. Table 1 shows that 5,10-CH^+-H_4PteGlu cyclohydrolase also occurs as a cytosolic protein in pea cotyledons. Attempts to detect this activity in sonicated, detergent-treated mitochondrial fractions were not successful. This is surprising as minor amounts of the synthetase [12] and the dehydrogenase (Table 1) were associated with pea mitochondria. An earlier study of this species [13] reported some cyclohydrolase activity in the mitochondrial fraction of six-day-old seedlings but these workers removed the cotyledons before making isotonic extracts. We also examined the distribution of these enzymes in extracts of 14-day-old pea leaves. Synthetase, dehydrogenase and cyclohydrolase activities were greatest in the cytosolic fraction with minor amounts of each being associated with the mitochondria (data not shown).

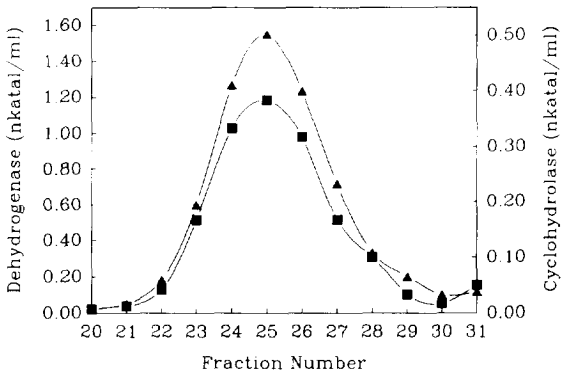

Fig. 1. Coelution of enzyme activities from heparin agarose. Protein was eluted from a heparin agarose column as summarized in Table 2. Fractions (3 ml) were collected and assayed for dehydrogenase (▲) and cyclohydrolase (■) activities.

Co-purification of dehydrogenase and cyclohydrolase activities

When PMSF-containing extracts of one-day-old pea cotyledons were fractionated as summarized in Table 2, dehydrogenase and cyclohydrolase activities co-purified and the ratio of these activities did not change appreciably through Steps 1–6 of the protocol. As noted earlier [12], 10-HCO-H_4PteGlu synthetase activity was removed by the Matrex Green A step and was, therefore, lacking in the protein isolated from heparin agarose. By Step 6, dehydrogenase activity was purified about 6800-fold whereas cyclohydrolase was purified about 7300-fold. This protocol gave recoveries of *ca* 8% and both activities were coeluted as a common peak from heparin agarose (Fig. 1). A similar co-purification of these activities occurred when extracts of 14-day-old pea leaves were fractionated by a protocol that included $(NH_4)_2SO_4$ precipitation followed by chromatography on heparin agarose, Matrex Green A, Sephadex G-75 and Matrex Orange A. The SDS–PAGE of protein from the final steps of these purifications revealed a single, silver-staining protein band with an average M_r of 38 500. The protein, therefore, appeared to be homogeneous and bifunctional. In this regard, our data are like those reported for *E. coli* [8] where these folate-dependent enzymes are associated with a bifunctional protein of subunit M_r 31 000.

The native M_r of Step 6 protein was examined by gel filtration. Calibrated columns of Sephadex G-75 and Sephacryl S-200 gave average M_r values of 58 000. Considering the average M_r value obtained after SDS–PAGE, it appears unlikely that the protein is monomeric. Our failure to detect subunit proteins of differing mass after SDS–PAGE suggests that the protein is probably not a heterodimer. Conceivably, differences between the shape of the dehydrogenase:cyclohydrolase protein and the standards used to calibrate the gel filtration columns could lead to inaccuracies in the calculation of native M_r [14]. D'Ari and Rabinowitz [8] have noted that all of the 5,10-CH_2-H_4PteGlu dehydrogenases characterized to date are homodimeric, irrespective of their mono-, di- or trifunctional nature. Further characterization of this plant dehydrogenase is needed to test the validty of this generalization. Attempts to determine the amino acid sequence of this protein were not successful due to an apparent block at the *N*-terminus.

Table 2. Co-purification of plant 5,10-methylenetetrahydrofolate dehydrogenase and 5,10-methenyltetrahydrofolate cyclohydrolase activities

Fractionation step	Volume (ml)	Protein (mg)	Dehydrogenase activity		Cyclohydrolase activity		Ratio
			Total*	Specific†	Total*	Specific†	
1. Crude homogenate	290.5	3664	0.3	0.1	0.10	0.03	2.8
2. Streptomycin SO_4	290.5	1737	0.3	0.2	0.07	0.04	n.d.
3. 55–70% $(NH_4)_2SO_4$	13.5	200	0.1	0.5	0.04	0.21	2.4
4. Sephacryl S-300	46.5	106	0.1	1.1	0.04	0.43	2.4
5. Matrex Green A	54.8	2	0.1	36.1	0.04	23	n.d.
6. Heparin agarose	10.4	0.04	0.02	542	0.01	208	2.3

*Expressed in μkat.
†nkat mg^{-1} protein.
n.d. = not determined.

Major properties of the dehydrogenase:cyclohydrolase protein

Stability of the purified protein was optimal at 4° in the presence of 25% glycerol. Under these conditions, 85% of the dehydrogenase and 60% of the cyclohydrolase activity remained after one month. Storage at −20° for the same period, gave residual activities of 50 and 20% for the dehydrogenase and cyclohydrolase, respectively. Under our assay conditions, maximal dehydrogenase activity occurred at 65° and at pH 6.5. Product formation in the dehydrogenase reaction was dependent on NADP and 5,10-CH_2-H_4PteGlu. Activity was not detected when NAD (1.4 μmol) and $MgCl_2$ (2.5 μmol) replaced NADP. Furthermore, the NADP-dependent activity was not inhibited when up to 0.3 μmol of NAD were included in the reaction system. This suggests that NAD does not bind to the active site of this protein. The earlier observation [15] that crude preparations of pea 5,10-CH_2H_4PteGlu dehydrogenase use NAD and NADP raises the possibility that plants, like other organisms [4,6,16–20], may also produce distinct, NAD-dependent forms of this enzyme.

Apparent K_m values, obtained from kinetic studies of the bifunctional enzyme, are summarized in Table 3. Linear Lineweaver–Burk plots were obtained for the dehydrogenase reaction when systems containing varying concentrations of (6*S*)-H_4PteGlu$_n$ (n = 1 and 5) or NADP, were incubated with fixed saturating concentrations of the other components for 3 min at 37°. For the cyclohydrolase, systems containing varying amounts of (6*R*,*S*)-5,10-CH^+-H_4PteGlu$_1$ were incubated for up to 5 min at 20°. The dehydrogenase activity displayed similar affinities for the mono- and pentaglutamate forms of the folate substrate. This observation is similar to that reported for the dehydrogenase domain of yeast C_1-tetrahydrofolate synthase where affinity for the folate substrate is not greatly affected by polyglutamylation [21]. This is in contrast, however, to the greater affinities shown for polyglutamates by the dehydrogenase domain of mammalian C_1-tetrahydrofolate synthase [22] and the cyclohydrolase domain of the avian trifunctional enzyme [23]. Table 3 also shows that the plant dehydrogenase activity had similar affinities for NADP when the reaction systems contained the mono- or pentaglutamate forms of (6*S*)-H_4PteGlu$_n$. The apparent K_m value of the cyclohydrolase for (6*R*,*S*)-5,10-CH^+-H_4PteGlu (Table 3) is similar to earlier values reported for the *E. coli* and pea seedling enzyme but lower than those of 250 and 500 μM for the enzymes of bovine liver and yeast respectively [4].

In further studies (Table 4) it was clear that the dehydrogenase (Step 6 protein) was inhibited by H_2PteGlu$_n$ (n = 1 and 5) and PteGlu. Lineweaver–Burk plots showed that both pteroylglutamates competed with 5,10-CH_2-H_4PteGlu without changing the V_{max} of the reaction. Based on the calculated K_i values, the dihydrofolates were more potent inhibitors than PteGlu. The effectiveness of H_2PteGlu$_n$ appeared to increase with polyglutamylation. Cyclohydrolase activity was also inhibited when variable amounts of PteGlu, H_2PteGlu$_1$ and (6*R*,*S*)-H_4PteGlu$_1$ were included in the reaction system (Fig. 2). Lineweaver–Burk plots of data for H_2PteGlu$_n$ (n = 1 and 4) indicated the inhibition was competitive with (6*R*,*S*)-5,10-CH^+H_4PteGlu (data not shown). Cyclohydrolase activity was also inhibited by about 70 and 80% when NADP (0.3 and 0.6 μmol) was included in the standard reaction system. This pyridine nucleotide inhibits the cyclohydrolase activities of liver C_1-tetrahydrofolate synthases [23–25]. On the other hand, (6*R*,*S*)-5-HCO-H_4PteGlu$_1$ at up to 150 μM, did not inhibit either the cyclohydrolase or dehydrogenase activities of the pea cotyledon protein when assayed under our standard conditions.

Treatment of purified dehydrogenase:cyclohydrolase protein with trypsin

Trypsin digestion has previously been used to elucidate the structural organization of the yeast and mammalian trifunctional C_1-tetrahydrofolate synthases [4]. In the present work, limited trypsin treatments were initially used in an attempt to isolate peptides with either dehydrogenase or cyclohydrolase activity. These attempts were not successful as incubations with trypsin (Fig. 3) resulted in losses of both activities. In the pres-

Table 3. Apparent Michaelis constants for substrates of the dehydrogenase and cyclohydrolase reactions

Substrate of varying concentration (μM)	Polyglutamate chain length of folate provided	
	Monoglutamate	Pentaglutamate
1. Dehydrogenase reaction		
(6*S*)-H_4PteGlu$_n$	21	25
NADP	11	8
2. Cyclohydrolase reaction		
(6*R*,*S*)-5,10-CH^+-H_4PteGlu$_1$	7	n.d.

Reaction systems containing Step 6 protein and variable amounts of the indicated substrate were incubated for 3 min with saturating amounts of the other substrates (see Experimental).

n.d. = not determined.

Table 4. Inhibitors of plant 5,10-CH_2-H_4PteGlu dehydrogenase activity

Competitive inhibitor (μM)	Folate substrate of varying concentration: (6*S*)-H_4PteGlu$_1$ K_i (μM)	(6*S*)-H_4PteGlu$_5$ K_i (μM)
PteGlu (500 μM)	610	n.d.
H_2PteGlu$_1$ (80 μM)	35	n.d.
H_2PteGlu$_1$ (87 μM)	n.d.	194
H_2PteGlu$_5$ (6.1 μM)	3	n.d.
H_2PteGlu$_5$ (5.3 μM)	n.d.	6

n.d. = not determined.

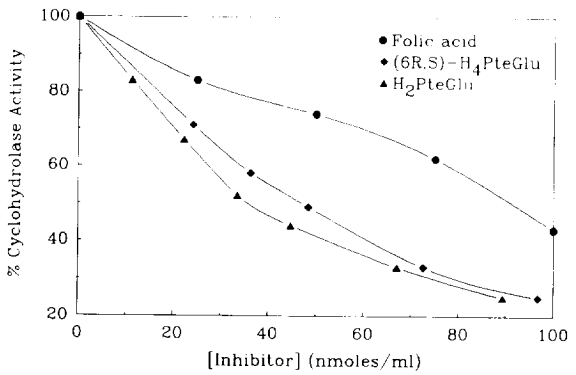

Fig. 2. Inhibition of cyclohydrolase activity. Reaction systems containing Step 6 protein (0.02 nkat of cyclohydrolase activity) and varying concentrations of the inhibitor were assayed under standard conditions (see Experimental).

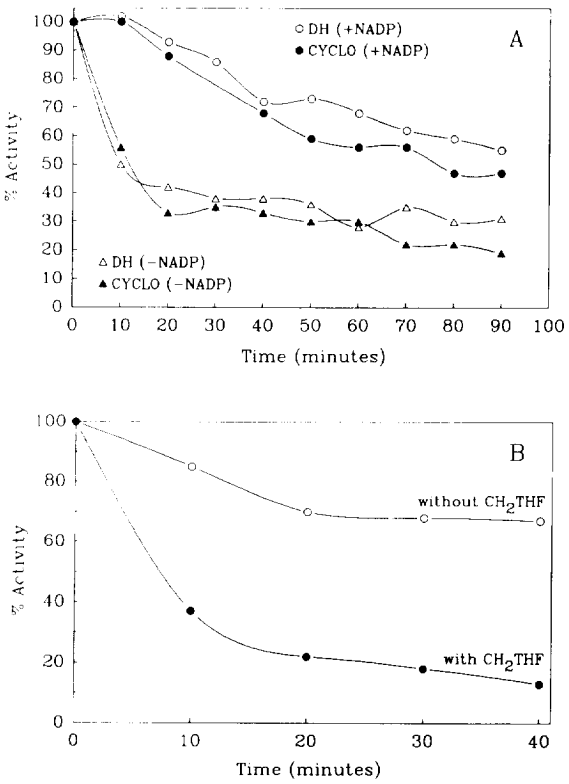

Fig. 3. Trypsin digestion of dehydrogenase:cyclohydrolase protein. (A) Step 6 protein (0.06 nkat of dehydrogenase activity, 0.02 nkat of cyclohydrolase activity) was incubated with trypsin in the presence or absence of NADP (see Experimental). After addition of trypsin inhibitor, residual enzyme activities were assayed under standard conditions. (B) Assay of dehydrogenase (0.03 nkat of activity). Conditions were as in (A) but trypsin digestion occurred in the presence or absence of (6*R*,*S*)-5,10-CH_2-H_4PteGlu (4 mM).

ence of NADP, the losses of dehydrogenase and cyclohydrolase activity were both noticeably delayed although still coordinated (Fig. 3(A)). This coenzyme protects the dehydrogenase:cyclohydrolase domains of liver C_1-tetrahydrofolate synthase from trypic digestion [26] and stabilizes these activities during heat inactivation [24]. In further experiments (Fig. 3(B)), purified protein was incubated with trypsin in the presence of (6*R*,*S*)-5,10-CH_2-H_4PteGlu. Under these conditions, the rate of loss of dehydrogenase activity appeared to be enhanced compared with the controls. Unfortunately, we were unable to determine whether this folate affected the trypsin-induced loss of cyclohydrolase activity. Clearly H_4PteGlu, arising from the dissociation of 5,10-CH_2-H_4PteGlu, would inhibit cyclohydrolase under these conditions to give erroneous results.

Cross-reactivity of polyclonal antibodies with proteins of other plants

Rabbit polyclonal antibodies were raised against the native, pea cotyledon dehydrogenase:cyclohydrolase protein (see Experimental). These antibodies were used to survey extracts of other plants for protein that was structurally related. In these studies (Table 5), leaf extracts of pea, bean, wheat, corn and barley were fractionated with $(NH_4)_2SO_4$, heparin agarose and Matrex Green A. During the latter step, the bulk of the applied 10-HCO-H_4PteGlu synthetase activity was not retained by the column (Table 5). In contrast, cyclohydrolase and dehydrogenase activities coeluted in reponse to the chloride gradient. These fractions were pooled and concentrated by ultrafiltration. The results of indirect, competitive ELISA assays of these extracts are summarized in Table 6. Significant cross-reactivity occurred with samples of purified, homogeneous cyclohydrolase:dehydrogenase protein from pea leaves. The antibodies also cross-reacted with extracts containing these activities from the other four plant species and with the $(NH_4)_2SO_4$-fractionated protein of the two *Neurospora* strains. Western blot analyses of the pea, bean, wheat and barley leaf extracts revealed a single cross-reacting protein band for each species that had an M_r of *ca* 40 000.

These immunological studies are in many respects similar to the earlier work of Staben and Rabinowitz [27]. These workers showed that antiserum to yeast

Table 5. Separation of 10-HCO-H_4PteGlu synthetase from dehydrogenase and cyclohydrolase activities in plant leaf extracts

Species	Enzyme	Activity applied to column (nkat)	Activity recovered from Matrex Green A (%)	
			Wash buffer	Elution buffer
Pea				
	Synthetase	98	60	n.d.
	Dehydrogenase	165	n.d.	58
	Cyclohydrolase	55	n.d.	51
Bean				
	Synthetase	18	72	n.d.
	Dehydrogenase	10	n.d.	59
	Cyclohydrolase	0.3	n.d.	54
Wheat				
	Synthetase	83	68	n.d.
	Dehydrogenase	55	14	53
	Cyclohydrolase	62	13	51
Corn				
	Synthetase	12	65	n.d.
	Dehydrogenase	22	15	66
	Cyclohydrolase	10	12	64
Barley				
	Synthetase	15	55	n.d.
	Dehydrogenase	33	17	55
	Cyclohydrolase	18	18	58

n.d. = activity not detected.

Table 6. Cross-reactivity of dehydrogenase:cyclohydrolase polyclonal antibodies. Indirect competitive ELISA assays of plant and *Neurospora* extracts (data are absorbance readings at 405 nm)

Extract dilution	Corn	Pea	Wheat	Bean	Barley	*Neurospora*	
						Wild type	Formate mutant
1:5	0.31	0.43	0.25	0.38	0.29	0.14	0.13
1:50	0.65	0.62	0.46	0.65	0.58	0.23	0.21
1:200	0.64	0.64	0.59	0.71	0.71	0.43	0.41
1:1000	0.75	0.69	0.65	0.75	0.84	0.82	0.71
1:3000	0.74	0.79	0.74	0.82	0.79	1.00*	0.94*
Controls	0.76	0.75	0.78	0.80	0.81	1.08	1.03

*1:5000 dilution of *Neurospora* extract.

ELISA assays were conducted as described in the Experimental. Higher plant leaf extracts were fractionated (see Table 5) and concentrated by ultrafiltration. *Neurospora* mycelial protein [50–65% saturation with $(NH_4)_2SO_4$)] was desalted prior to assay.

C_1-tetrahydrofolate synthase cross-reacted with the corresponding synthase protein of several other eukaryotic species and with the monofunctional 10-HCO-H_4PteGlu synthetase protein of *Clostridium acidi-urici*. They also reported that antibodies raised against the purified bacterial synthetase cross-reacted with C_1-tetrahydrofolate synthase proteins of several eukaryotic species. Immunoblot analyses confirmed the specificity of this cross-reaction and the authors concluded that all of these enzymes have common structural features. Based on the present data (Tables 5 and 6) and the results of our Western blot analyses, it appears likely that a dehydrogenase:cyclohydrolase protein of similar structure to that of pea cotyledons occurs in other plants species. The cross-reaction of these polyclonal antibodies with partially purified *Neurospora* extracts (Table 6) raises the possibility that the fungal trifunctional synthase may have dehydrogenase/cyclohydrolase regions that are structurally like those of the cotyledon protein.

Studies of eukaryotic C_1-tetrahydrofolate synthases provide strong evidence [4, 28–32] that the active sites of the dehydrogenase:cyclohydrolase region are kinetically interdependent, have a significant degree of overlap, support channelling of 5,10-CH^+-H_4PteGlu, and probably use a common folate binding site. The present studies suggest that the bifunctional protein of pea cotyledons

may have some of these properties. In this regard, the two activities of the plant protein, like those of eukaryotic enzyme [4], may be in close proximity. Thus trypsin, which causes a coordinate loss of both activities in the yeast enzyme [4] had a similar effect on the plant protein (Fig. 3). NADP, which is required in the dehydrogenase reaction (Table 3), partially protected both activities from proteolysis (Fig. 3) and this effect has been noted for the mammalian synthase protein [26]. The NADP and H_4PteGlu, which inhibit the cyclohydrolase activity of avian liver C_1-tetrahydrofolate synthase [23] also had an inhibitory effect in the present work. Both activities of the pea protein were also inhibited competitively by folic acid and dihydrofolate although it remains to be determined whether these inhibitors bind at a single, common site that is usually occupied by the folate substrates of the dehydrogenase and cyclohydrolase reactions. The proximity and possible interaction of these active sites could be determined by chemical modification of this protein [28] and by assessing the possible channelling of 5,10-CH^+-H_4PteGlu [4, 23, 31].

There is evidence, from study of animal cells, that dihydrofolate polyglutamates are potent inhibitors of thymidylate synthase [33], AICAR transformylase [34] and methylenetetrahydrofolate reductase [35]. Inhibition of the reductase may spare 5,10-CH_2-H_4PteGlu$_n$ for thymidylate and purine synthesis [35]. The central role of 5,10-CH_2-H_4PteGlu$_n$ in one-carbon metabolism implies that the conversion of this folate to 10-HCO-H_4PteGlu$_n$ will also be regulated. This control would be of special relevance in cells that can generate 10-formyltetrahydrofolate, for purine biosynthesis and the formylation of methionyl-tRNA, via the 10-HCO-H_4PteGlu$_n$ synthetase reaction. The observed inhibiton of dehydrogenase and cyclohydrolase activity by H_2PteGlu$_n$ (Table 4; Fig. 2) suggests that changes in the 5,10-CH_2-H_4PteGlu$_n$/H_2PteGlu$_n$ ratio might affect the rate of 10-HCO-H_4PteGlu$_n$ production. This ratio would tend to decrease when thymidylate synthase activity was high because this reaction consumes 5,10-CH_2H_4PteGlu$_n$ and produces H_2PteGlu$_n$. Under these conditions we visualize a need to conserve 5,10-CH_2-H_4PteGlu$_n$ for pyrimidine biosynthesis. Our earlier study of pea cotyledon 10-HCO-H_4PteGlu synthetase showed that H_2PteGlu$_5$ was not inhibitory even when supplied at 0.7 mM [12]. Thus conditions that spare 5,10-CH_2-H_4PteGlu$_n$ for thymidylate synthesis may not curtail the folate-dependent activation of formate.

EXPERIMENTAL

Chemicals. PteGlu$_n$ (n = 2–5) were obtained from Dr B. Schircks Laboratories (Jona, Switzerland). PteGlu, (6*R*,*S*)-H_4PteGlu, (6*R*,*S*)-5-HCO-H_4PteGlu (Ca leucovorin) were supplied by Sigma. *Lactobacillus casei* DHFR was purchased from Biopure, Boston. Chromatographic media were from the following sources: heparin agarose, Sephacryl S-200, and S-300 (Sigma), Matrex Green A and Matrex Orange A (Amicon), Biogel P-2 and P-6 (Bio-Rad). Bradford's reagent was from Bio-Rad; all other general biochemicals were from either Sigma or Fisher Scientific.

Plant material. Seeds of pea (*Pisum sativum* L. cv Homesteader), from a local supplier were germinated as previously described [36]. Fourteen-day-old seedlings of pea, bean (*Phaselous vulgaris* L.), barley (*Hordeum vulgare*), wheat (*Triticum aestivum* L.) and corn (*Zea mays* L. var. Gills Early Market) were produced in growth chambers (16 hr days, 25°, 8 hr nights, 20°, 50% R.H.).

Assay of enzyme activity. 10-HCO-H_4PteGlu synthetase was assayed spectrophotometrically [37] using the reaction conditions of ref. [12]. Product formation is expressed in katals of synthetase activity (mol sec^{-1} product) based on an extinction coefficient of 24 900 M^{-1} cm^{-1} [38]. 5,10-CH^+-H_4PteGlu cyclohydrolase was measured by changes in A at 355 nm [38]. The reaction system (1 ml), containing 200 μmol K maleate (pH 8), 75 nmol (6*R*,*S*)-5,10-CH^+-H_4PteGlu, 100 μmol 2-mercaptoethanol (2-ME) and up to 100 μl of enzyme extract was incubated at 20°. After correction for non-enzymic substrate hydrolysis, reaction rates were calculated using the above extinction coefficient, and expressed as substrate hydrolysed (katals). 5,10-CH_2-H_4PteGlu dehydrogenase [37] was assayed spectrophotometrically using 1 ml reaction systems containing 25 μmol BES (pH 6.5), 1 μmol (6*R*,*S*)-H_4PteGlu, 10 μmol HCHO, 0.6 μmol NADP, 100 μmol KCl and up to 50 μl plant extract. After incubation at 37° for 5–15 min, 2 ml of 1 M HCl was added and 5,10-CH^+-H_4PteGlu was measured at 350 nm using the above extinction coefficient. Activity is expressed as product formed (katals).

Differential centrifugation of cotyledon extracts. Pea seeds were imbibed and germinated for four and six days at 20° in darkness. The cotyledons were then excised and homogenized in isotonic buffer followed by differential centrifugation to obtain mitochondrial and cytosolic fractions [39]. Biochemical purity of the fractions was assessed by assay of marker enzymes [40].

Purification of 5,10-CH_2-H_4*PteGlu dehydrogenase and* 5,10-CH^+-H_4*PteGlu cyclohydrolase.* Cotyledons (wt:vol. ratio of 1.6) were ground in 50 mM Tris–HCl (pH 7.5) containing 25% v/v glycerol, 10 mM 2-ME and 1 mM PMSF (Buffer A). After filtering through cheesecloth, the homogenate was centrifuged (6000 *g* for 15 min) to give Step 1 protein. Nucleic acids were removed using streptomycin sulphate [41] to yield Step 2 protein. Fractionation of these extracts with $(NH_4)_2SO_4$ (55–70% range of satn) gave Step 3 protein. This protein was dissolved in *ca* 10 ml of 100 mM K-Pi (pH 7.5) containing 50 mM NaCl, 25% v/v glycerol, 10 mM 2-ME and 1 mM PMSF (Buffer B) and chromatographed on Sephacryl S-300 [12] using the same buffer to give Step 4 protein. This protein was applied to a 1.5 × 10 cm column of Matrex Green A that had been pre-equilibrated with Buffer B. The column was washed with 25 ml of Buffer B containing 0.5 M NaCl and enzyme activity was eluted by applying a linear gradient of 0.5–1 M NaCl in Buffer B (50 ml of each). Fractions containing dehydrogenase/cyclohydrolase activity were designated Step 5 protein. After desalting (BioGel P-6) into 10 mM K-Pi (pH 7)

containing 25% v/v glycerol, 10 mM 2-ME and 1 mM PMSF (Buffer C), this protein was applied to a 1.5 × 10 cm column of heparin agarose that had previously been pre-equilibrated in Buffer C. Dehydrogenase and cyclohydrolase activities were coeluted by a linear Cl^- gradient (100 ml of Buffer C to 100 ml of Buffer C containing 500 mM KCl). Enzyme-active fractions were concd by ultrafiltration (Amicon Centriprep-10) to give Step 6 protein.

A modified protocol was used to purify these enzyme activities from leaves of 14-day-old pea seedlings. Leaves were homogenized in Buffer A and the extract was filtered, centrifuged and treated with streptomycin sulphate as described above. Protein was then fractionated with $(NH_4)_2SO_4$. The ppt. obtained in the 60–80% range of satn was dissolved in Buffer C. After desalting on BioGel P-6, this protein was chromatographed on heparin agarose and then on Matrex Green A (see above). Enzyme-active fractions were concd by ultrafiltration and applied to an 80 × 2.5 cm column of Sephadex G-75. Dehydrogenase and cyclohydrolase activities were coeluted from this column in Buffer C. These fractions were concd by ultrafiltration before chromatography on a 7 × 2 cm column of Matrex Orange A. Both activities were coeluted from this medium using a gradient of NaCl (0–0.4 M) in Buffer C. Protein was measured according to ref. [42] and SDS–PAGE was according to ref. [43] using a Mini Protean II system (Bio-Rad).

Trypsin digestion of Step 6 protein. Samples (2 ml) of Step 6 protein (6 nkat of dehydrogenase activity) in 10 mM K-Pi buffer (pH 7) containing *ca* 250 mM KCl, 10 mM 2-ME, 25% glycerol and either 0.6 mM NADP or 4 mM 5,10-CH_2-H_4PteGlu were incubated for up to 100 min at room temp. with 0.2 mg of trypsin (Sigma, bovine pancreas Type XIII, TPCK-treated). Controls lacked NADP or folate. At 10 min intervals, samples (200 μl) were withdrawn and mixed with 30 μg of trypsin inhibitor (Sigma, soybean, Type 1-S) in 200 μl of 10 mM K-Pi (pH 7). Aliquots (50 μl) of this mixture were then assayed for dehydrogenase and cyclohydrolase activities as described above. In the case of folate-treated samples, the final concn of 5,10-CH_2-H_4PteGlu was adjusted to 0.5 mM before assay of dehydrogenase activity with a 5 min reaction time.

Synthesis of (6S)-H_4*PteGlu polyglutamates.* $PteGlu_n$ (n = 2–5) were converted to $H_2PteGlu_n$ by dithionite reduction and purified on Biogel P-2 in the presence of argon [44]. After lyophilization, (6S)-H_4PteGlu polyglutamates were generated enzymatically using *L. casei* DHFR. Reaction systems (1.5 ml) contained DHFR (*ca* 0.6 units), glucose-6-phosphate dehydrogenase (*ca* 50 units), glucose-6-phosphate (15 mM), NADPH (0.33 μmol), and the dihydrofolate polyglutamate, dissolved in deoxygenated 20 mM 2-ME (pH 7.5). After purging with Ar, the tubes were sealed and incubated for 3 hr at 32°. (6S)-H_4PteGlu polyglutamate concns were determined by reaction with rabbit liver cytosolic serine hydroxymethyltransferase [45], using a molar extinction coefficient (A_{492} nm) of 40 000 [46].

Production of polyclonal antibodies and immunological assays. Step 6 cotyledon protein was concd by ultrafiltration, desalted and lyophilized prior to injection into rabbits. The resulting antisera were used, without purification, as a source of polyclonal antibodies. For indirect, competitive ELISA assays, wells were coated with antigen (homogeneous pea cotyledon dehydrogenase/cyclohydrolase protein) and blocked with BSA. Prior to assay, test proteins were diluted in K-Pi buffered saline, pH 7.2, containing 0.05% Tween 20 (PBST). Equal vols of these proteins and diluted rabbit antiserum were added to the wells. Sigma goat anti-rabbit IgG, linked to alkaline phosphatase, was used as a secondary antibody. Control wells lacked the plant test extract which was replaced by PBST. Colour development in the presence of *p*-nitrophenyl phosphate was determined at 405 nm using a Bio-Rad ELISA reader. Western blot analyses were carried out according to the manufacturers instructions in a Bio-Rad Mini Trans-Blot Electrophoretic transfer cell. Rabbit polyclonal antibodies to purified pea cotyledon dehydrogenase:cyclohydrolase (diluted 500-fold) were used as primary antibodies. The secondary antibodies were as for the ELISA assays.

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