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Intracellular localization of serine acetyltransferase in spinach leaves

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Abstract. Intact chloroplasts isolated from spinach leaves by a combination of differential and Percoll density gradient centrifugation and free of mitochondrial and peroxisomal contamination contained about 35% of the total leaf serine acetyltransferase (EC 2.3.1.30) activity. No appreciable activity of the enzyme could be detected in the gradient fractions containing broken chloroplasts, mitochondria, and peroxisomes. L-cysteine added to the incubation mixture at 1 mM almost completely inhibited serine acetyltransferase activity, both of leaf and chloroplast extracts. D-cysteine was much less inhibitory. L-cystine up to 5 mM and O-acetyl-L-serine up to 10 mM had no effect on the enzyme activity. When measured at pH 8.4, the enzyme extracted from the leaves had a K_m for L-serine of 2.4, the enzyme from the chloroplasts a K_m of 2.8 mM.

Key words: Chloroplast (serine acetyltransferase) – Cysteine – Serine acetyltransferase – *Spinacia.*

Introduction

Serine acetyltransferase (SATase) (EC 2.3.1.30) catalyzes the synthesis of O-acetyl-L-serine (OAS) from L-serine and acetyl-CoA. It is well established in microorganisms (Kredich and Tomkins 1966; Siegel 1975) that OAS is the substrate for the formation of cysteine, according to O-acetyl-L-serine $+ S^{2-} \rightarrow$ L-cysteine + acetate. This reaction is catalyzed by O-acetyl-L-serine sulfhydrylase (OAS-Sase) (EC 4.2.99.8). Evidence that OAS is also the substrate for cysteine formation in higher plants is indicated by the following evidence: i) both SATase and OASSase have been demonstrated in plants (Smith and Thompson 1971; Giovanelli and Mudd 1968); ii) OAS has been found in cultured tobacco cells (Smith 1977); iii) SATase from *Phaseolus vulgaris* is inhibited by L-cysteine (Smith and Thompson 1971). In addition, OAS could have a function in the reaction sequence for the synthesis of diacylsulfoquinovosylglycerol, the sulfolipid associated with chloroplasts (Douce et al. 1973). According to a proposal of Harwood and Nicholls (1979), this sequence begins with the formation of cysteic acid from OAS and sulfite.

SATase was detected in a mitochondrial fraction obtained from primary leaves of *Phaseolus vulgaris* by differential centrifugation (Smith 1972) and in an uncharacterized particulate fraction of extracts of wheat leaves (Ascaño and Nicholas 1977). In none of these studies were intact chloroplasts assayed for SATase. The following findings prompted us therefore to examine whether the enzyme could be found in chloroplasts: i) isolated spinach chloroplasts can form [^{35}S]cysteine from $^{35}SO_4^{2-}$ Trebst and Schmidt 1969); ii) spinach chloroplasts contain all the enzymes necessary for serine formation from D-3-phosphoglycerate (3-PGA) (Larsson and Albertsson 1979); iii) a concentration of about 8 nmol mg^{-1} chlorophyll of [^{14}C]acetyl-CoA can be estimated to be present during fatty acid synthesis from [1-^{14}C]acetate in isolated spinach chloroplasts (Roghan et al. 1980); iv) OASSase was localized in the chloroplasts of spinach, peas, and clover in appreciable activities (Fankhauser et al. 1976; Fankhauser and Brunold 1978a; Ng and Anderson 1978). Since we could not prepare pure intact chloroplasts from *Phaseolus vulgaris,* we turned to spinach. In this paper

Abbreviations: NAS = N-acetyl-L-serine; NADP-GPD = NADP-dependent glyceraldehyde-3-phosphate dehydrogenase; OAS = O-acetyl-L-serine; OASSase = O-acetyl-L-serine sulfhydrylase; 3-PGA = D-3-phosphoglycerate; SATase = serine acetyltransferase

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we present evidence that spinach chloroplasts contain appreciable amounts of SATase and we report on some properties of the enzyme.

Materials and methods

Spinach (*Spinacia oleracea* L., var. Glares) was purchased from the local market. Leaf extracts were prepared by grinding deribbed leaves in 50 mM Hepes-KOH, pH 7.0, in a glass homogenizer cooled with ice. Five milliliters buffer were used per g of tissue. The homogenate was centrifuged for 10 min at 20,000 *g* and 0° C. The supernatant fluid was used for SATase determination.

For chloroplast isolation, 25 g deribbed leaves were homogenized with an Omni-mixer (Sorvall, Norwalk, Conn., USA) in 50 mM Hepes-KOH, pH 6.8, containing 0.33 M sorbitol, 0.1% (w/v) bovine serum albumin (BSA), 2 mM ethylene diaminetetraacetate (Na_2 EDTA), and 1 mM $MgCl_2$. Two strokes of 5 and 2 s were applied. The homogenate was passed through miracloth. The chloroplasts were sedimented by centrifugation for 2 min at 1,000 *g* and 0° C. Chloroplast extracts were prepared by resuspending the 1,000 *g* pellet in 10 ml 50 mM Hepes-KOH, pH 7.0. After centrifuging this suspension for 30 min at 30,000 *g* and 0° C, the supernatant fluid was used for SATase determination.

Chloroplast suspensions were prepared by resuspending the 1,000 *g* chloroplast pellet in 50 mM Hepes-KOH, pH 7.0, containing 0.45 M sorbitol and 0.1% (w/v) BSA.

A suspension enriched with mitochondria and peroxisomes was prepared by homogenizing 25 g deribbed leaves in 100 ml 10 mM 2-(N-morpholino)ethane sulfonic acid (MES), pH 7.5, containing 0.3 M mannitol, 1 mM EDTA, 0.2% (w/v) BSA, and 0.6% (v/v) polyvinylpyrrolidone (PVP). Homogenization was as indicated for chloroplast preparation. The homogenate was passed through miracloth, and centrifuged for 5 min at 1,000 *g*. The supernatant was centrifuged for 30 min at 10,000 *g* and 0° C. The resulting pellet was resuspended in 2 ml homogenizing buffer. The resuspended 1,000 and 10,000 *g* pellets were analyzed after separation on 10–80% (v/v) continuous Percoll gradients containing 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid (Hepes)-KOH, pH 7.0, 0.45 M sorbitol, and 0.1% (w/v) BSA. One hundred percent Percoll means undiluted preparation of Pharmacia. Aliquots of the resuspended pellets were layered on top and centrifuged for 5 min at 3,000 *g* and 0° C in a Sorvall SS-90 vertical superspeed rotor. Fractions consisiting of 25 drops were collected.

SATase was measured either at pH 7.0 or 8.4. At pH 7.0, the assay system routinely consisted of 3.3 mM L-[^{14}C]serine, 0.83 mM acetyl-CoA, 8% (w/v) BSA, and 30 µl extract in a total volume of 120 µl. The reaction was started by addition of the extract. Incubation was for 60 min at 37° C. The reaction was stopped with 10 µl 98% formic acid, and 10 µl of a 136 mM OAS-hydrochloride solution were added. An aliquot of 20 µl of the assay mixture was subjected to paper electrophoresis at 225 V cm^{-1} using formic acid: acetic acid: H_2O= 91.2:59.2:1,000 as a buffer system. OAS was visualized with ninhydrin (Krebs et al. 1967). The spot containing OAS was cut out and radioactivity was counted using a scintillation spectrometer. For measuring SATase activity at pH 8.4, 10 µl 1 M tris-HCl, pH 9.0, were added to the assay system at pH 7.0. The L-serine concentration was increased to 15 mM. Incubation was identical to the measurement at pH 7.0. The reaction was terminated by placing the assays in a boiling water bath for 3 min. Ten microliters of a 136 mM OAS-hydrochloride solution, 5 µl 0.1% (w/v) phenolphthalein in 96% ethanol, and, finally, 1 M NaOH were added until a strong red color developed (pH about 10.0). The assay was kept at room temperature for 30 min to convert OAS to N-acetyl-L-serine (NAS) (Nagai and Flavin 1967). Then the pH was adjusted to 6.0 using 0.73 M H_3PO_4. An aliquot of 20 µl of the assay mixture was subjected to paper electrophoresis, using 67 mM phosphate buffer, pH 6.0. Electrophoresis was carried out for 15 min at 150 V cm^{-1}. The paper was then dried and exposed to an atmosphere of Cl_2 in a covered glass tank for 20 min. Cl_2 was produced in the tank by adding 1 ml 1 M HCl to 1 ml $KMnO_4$ (1.5%, w/v). The paper was kept at 80° C for 5 min in a ventilated oven to remove unreacted Cl_2. NAS was visualized by spraying with an o-tolidine solution (Krebs et al. 1967). The spot containing NAS was cut out and its radioactivity determined. For SATase determination in the Percoll gradient fractions, BSA was omitted, but 0.01% (w/v) Brij was included. The counting fluid was toluene with 4 g 2,5-diphenyloxaxole (PPO) and 120 mg 1,4-bis-2-(5-phenyloxyzoyl)-benzene (POPOP).

Catalase activity was assayed using the method of Lueck (1962), and cytochrome c oxidase by the method of Rocha and Ting (1970). NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (NADP-GPD) was measured according to Frosch et al. (1973).

Chlorophyll was determined by its absorption at 652 nm (Arnon 1949). Proteins were measured using the Bio-Rad assay (Bio-Rad, Richmond, Cal., USA) and BSA as a standard.

L-[^{14}C]serine was purchased from the Radiochemical Centre, Amersham, U.K., acetyl-CoA, cytochrome c, D- and L-cysteine, L-cystine, methionine, NAS and OAS-hydrochloride from Sigma, Saint Louis, Mo., USA, and Percoll from Pharmacia, Uppsala, Sweden.

Results

Table 1 presents the requirements for the measurement of serine acetyltransferase (SATase) activity at pH 7.0 and 8.4 in crude extracts from spinach leaves. PH 7.0 was chosen to approximate the pH of the cytoplasm, pH 8.4 is a compromise between the pH optimum of the enzyme, which is above 8.5, and the pH of the stroma of chloroplasts in the light (Werdan et al. 1975). At pH 7.0, OAS is the major product with only traces of NAS formed in a nonenzymatic reaction from OAS (Kredich and Tomkins 1966). This is substantiated by the results in Table 1: after treatment of an aliquot of the incubation mixture at pH 7.0 with NaOH, NAS exceeds the OAS originally present in the aliquot by only a small amount (170.3 and 189.2 pkat mg^{-1} protein). The complete nonenzymatic conversion of OAS to NAS could be demonstrated in an experiment, where OAS formed by SATase is eluted from the paper after electrophoresis at pH 2.0 and treated with NaOH. The total radioactivity originally determined in OAS could then be recovered as NAS after electrophoresis at pH 6.0 (data not shown).

A more alkaline pH during incubation favors the nonenzymatic conversion of OAS to NAS to

Table 1. Requirements for the assay of L-serine acetyltransferase activity at pH 7.0 and 8.4. The complete assay system contained, in a total volume of 120 µl, 15 mM L-[^{14}C] serine (2.11 Bq nmol^{-1}), 0.83 mM acetyl-CoA, 8% (w/v) bovine serum albumine (BSA), and 30 µl extract from spinach leaves in 50 mM Hepes-KOH, pH 7.0. The assay at pH 7.0 was analysed for O-acetyl-L-serine. An aliquot of this assay was treated with NaOH to convert O-acetyl-L-serine to N-acetyl-L-serine and was then analysed for N-acetyl-L-serine. For the assay at pH 8.4, 10 µmol tris-HCl, pH 9.0, were included. Incubation was for 60 min at 37° C

Assay system	L-serine acetyltransferase (pkat mg^{-1} protein)		
	pH 7.0		pH 8.4
	O-acetyl-L-serine	N-acetyl-L-serine	N-acetyl-L-serine
Complete	170.3	189.2	356.1
Complete -acetyl-CoA	2.5	5.3	21.1
Complete -BSA	122.5	132.5	243.6
Complete extract boiled	0	14.4	24.7

the extent that NAS is the major product (Kredich and Tomkins 1966). Therefore, the OAS remaining after incubation at pH 8.4 was converted to NAS and the NAS formed served as a measure for SATase activity. The L-serine concentration of 15 mM was optimal for measuring SATase activity at pH 8.4, since a K_m for L-serine of 2.4 ± 0.60 mM (mean $\pm$ S.D. of six determinations) was found and no substrate inhibition by L-serine was observed. At pH 7.0, the K_m for L-serine was 26.9 mM (mean of three determinations). At both pH values the assay was linear with time up to 60 min and with protein added up to at least 100 µg. The enzyme activity was stable up to at least 7 h after extraction. BSA caused an increase in SATase activity of about 50%.

Table 2 shows the effect of different amino acids on SATase activity. The product of the reaction, OAS, did not inhibit the activity in concentrations of up to 10 mM. L-cysteine, however, almost completely inhibited SATase at 1 mM. D-cysteine at the same concentration had a much smaller effect, and L-cystine up to 5 mM was ineffective. High concentrations of methionine had to be included in the assay mixture to demonstrate an inhibitory effect. Taken together, the results from

Table 2. Inhibitors of L-serine acetyltransferase activity. The inhibitors were added to the assay systems described in Materials and methods. The incubation was under N_2

Inhibitor	Concentration (mM)	Inhibition (%)	
		pH 7.0	pH 8.4
O-acetyl-L-serine	10	0	0
L-cysteine	1	95	96
L-cystine	5	0	0
D-cysteine	1	14	21
L-methionine	10	25	10

Table 2 show that the inhibitors tested reacted very similarly at both ph 7.0 and 8.4.

Figure 1 illustrates the distribution of organelle markers and SATase ativity of resuspended 1,000- and 10,000 *g* pellets from homogenates of spinach leaves on a 10 to 80% Percoll gradient. The activities of the marker enzymes for peroxisomes and mitochondria, catalase and cytochrome c oxidase, respectively, were very prominent in the upper fractions of the gradients, when, in addition to the 1,000-*g* resuspended pellet, a suspension of the 10,000-*g* pellet was included (Fig. 1 a). It is clear, however, from Fig. 1 b that appreciable activities of catalase and cytochrome c oxidase were present in the 1,000-*g* pellet. Fig. 1 a and b clearly shows that mitochondria and peroxisomes were well separated from the intact chloroplasts, which can be localized on the gradients by chlorophyll and NADP-GPD. Broken chloroplasts, however, were not separated from mitochondria and peroxisomes, as estimated by chlorophyll and the corresponding marker enzymes. SATase activity, measured at pH 7.0 and 8.4, banded with the markers for whole chloroplasts. SATase activity was also detected in the fractions above the Percoll gradient, originating from organelles broken during resuspension of the pellets or gradient centrifugation. Figure 1 a shows that we could detect no appreciable SATase activity in the fractions containing mitochondria, peroxisomes, and broken chloroplasts. More than 90% of the SATase activity applied to the gradients could routinely be recovered, indicating that the enzyme is stable on the Percoll gradients and that Percoll does not inhibit the enzyme activity. This was substantiated by the fact that when an aliquot from the peak gradient fraction containing intact chloroplasts was mixed with an aliquot from a leaf homogenate, the SATase activity of the mixture corresponded to the sum of the SATase activity determined individually in the two aliquots.

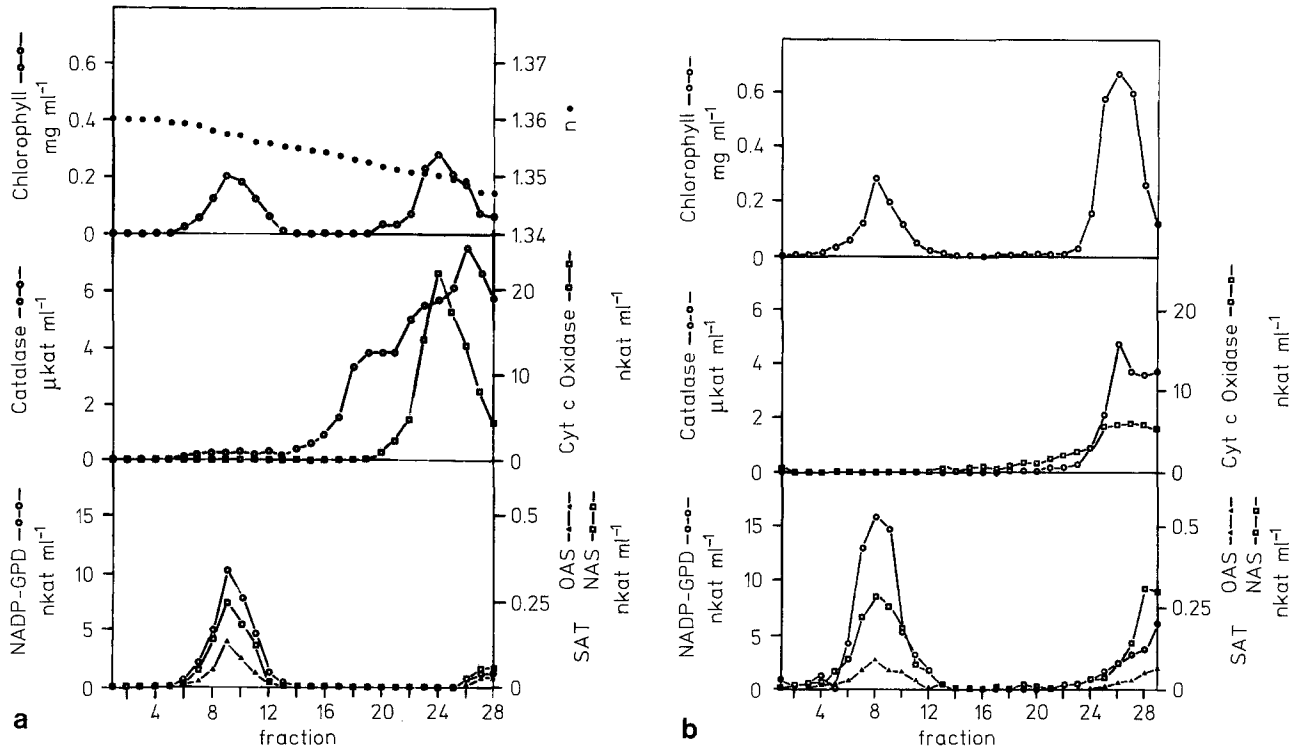

Fig. 1 a, b. Distribution of chlorophyll (Chlorophyll –o–o–o–) and activities of catalase (Catalase –o–o–o–), cytochrome c oxidase (Cyt c Oxidase –□–□–□–), NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (NADP-GPD –o–o–o–), and L-serine acetyltransferase, measured at pH 7.0 (SAT OAS –△–△–△–) and pH 8.4 (SAT NAS –□–□–□–) on a 10 to 80% (v/v) Percoll gradient. **a** 1.6 ml resuspended 1,000 *g* chloroplast pellet and 1.6 ml resuspended 10,000 *g* mitochondrial and peroxisomal pellet applied to the gradient (n ● = refractive index). **b** 2.0 ml resuspended 1,000 *g* chloroplast pellet applied to the gradient

Table 3 shows the estimation of the distribution of SATase activity between the chloroplasts and the other compartments of spinach leaf cells. As a control for the intactness of the chloroplasts in the peak fraction, NADP-GPD was included which is located exclusively in the chloroplasts.

Correspondingly, NADP-GPD activity was essentially equal in the homogenate and the chloroplast fraction, when expressed on a chlorophyll basis. This result indicates that broken chloroplasts did not appreciably contaminate the peak fraction of intact chloroplasts. When SATase activity of the homogenate and the chloroplast fraction measured at pH 7.0 and 8.4 are compared, we can estimate that 33.0% (pH 7.0) and 29.5% (pH 8.4) of the total SATase are localized in the chloroplasts. Applying the type of calculation performed in Table 3 to the Percoll gradients presented in Fig. 1a, 40.5% (pH 7.0) and 30.4% (pH 8.4) of the SATase activity can be attributed to the chloroplasts. The corresponding values for Fig. 1b are 23.5% (pH 7.0) and 35.1% (pH 8.4). From a total of 6 gradients, 34.6±6.1% (mean±S.D.) of leaf SATase activity measured at pH 8.4 were calcu-

Table 3. Chlorophyll content and activity of NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (NADP-GPD), and serine acetyltransferase, measured at pH 7.0 (SAT OAS) and 8.4 (SAT NAS) in a homogenate from spinach leaves and in a peak fraction of the intact chloroplasts prepared from this homogenate on a 10 to 80% Percoll gradient

	Homogenate	Peak fraction of intact chloroplasts
Chlorophyll (mg ml^{-1})	0.225	0.486
NADP-GPD (nkat ml^{-1})	6.07	14.0
SAT OAS (nkat ml^{-1})	0.367	0.262
SAT NAS (nkat ml^{-1})	1.033	0.658
NADP-GPD (nkat mg^{-1} chlorophyll)	26.978 = 100%	28.807 = 106.8%
SAT OAS (nkat mg^{-1} chlorophyll)	1.631 = 100%	0.539 = 33.0%
SAT NAS (nkat mg^{-1} chlorophyll)	4.591 = 100%	1.354 = 29.5%

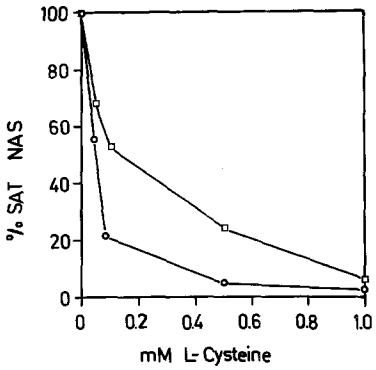

Fig. 2. Inhibition of L-serine acetyltransferase (SAT NAS) activity by L-cysteine in extracts from spinach leaves (-□-□-□-) and chloroplasts (-o-o-o-). Incubation was at pH 8.4 under N_2. 100% L-serine acetyltransferase was 314.2 pkat mg^{-1} protein for the leaf extract and 66.9 pkat mg^{-1} protein for the chloroplast extract

lated to be localized in the chloroplasts. The K_m of SATase for L-serine determined at pH 8.4 in the peak gradient fraction containing intact chloroplasts was 2.8 mM (mean of two determinations).

In view of the two proposed physiological functions of OAS, it was interesting to determine whether the chloroplast SATase was inhibited by cysteine. Figure 2 shows that the inhibitory effect of L-cysteine on SATase activity was more pronounced in the chloroplast extract than in the total leaf extract.

Discussion

The favorable characteristics of Percoll for obtaining pure organelle fractions have been presented by Mills and Joy (1980). In the present investigation these advantages of Percoll over sucrose have been combined with those of vertical rotor centrifugation, so that chloroplast preparations essentially free of contaminating peroxisomes were obtained by reduction of centrifugation time and centrifugal forces. At the same time, our preparations of intact chloroplasts were free of mitochondria and mitochondrial fragments, as estimated from the absence of cytochrome c oxidase. This last aspect was especially important in view of the findings of Smith (1972), that crude mitochondrial preparations from leaves and roots of *Phaseolus vulgaris* contained firmly bound SATase activity. Any contamination of the intact chloroplast fractions with mitochondria would make our results questionable.

The 35% of the total SATase estimated in the chloroplasts from spinach leaves are consistent with earlier findings which showed that isolated spinach chloroplasts are able to form [^{35}S]cysteine from $^{35}SO_4^{2-}$ (Trebst and Schmidt 1969). The results presented here, together with the demonstration of OASSase in spinach chloroplasts (Fankhauser et al. 1976), indicate that this [^{35}S]cysteine could have be formed from OAS. In this regard connection, it should be pointed out, that cysteine can also be formed from L-serine and H_2S (Thompson and Moore 1968). The rate of this reaction is so low, however, that it seems unlikely that it is physiologically important as long as OAS is present.

The K_m of chloroplast SATase for L-serine (2.8 mM) is comparable to the L-serine concentration of about 2 mM which can be calculated from the values presented by Aach and Heber (1967) for spinach chloroplasts.

The inhibition of SATase by cysteine both in the leaf and the chloroplast extract is consistent with the physiological function of the enzyme in catalyzing the first step in a reaction sequence for cysteine synthezis. In bacteria, Kredich and Tomkins (1966) demonstrated a 50% inhibition of SAT activity at a cysteine concentration of 1.1 μM. SAT extracted from *Phaseolus vulgaris* seems less sensitive to L-cysteine inhibition, since at 1 mM there was only a 65% inhibition (Smith and Thompson 1971). At this concentration there was almost complete inhibition of the SATase from spinach leaves and chloroplasts. Our results indicate that it is essential to prevent oxidation of cysteine by incubation in an inert atmosphere, since cystine had no effect on SATase activity. The other results from our inhibitor experiments with crude extracts from spinach leaves are comparable with findings from purified SATase from *Paracoccus denitrificans* (Burnell and Whatley 1977).

At the pH of chloroplast stroma in the light it seems inevitable that some NAS is formed from OAS in a nonenzymatic reaction (Werdan et al. 1975; Kredich and Tomkins 1966). NAS is not a substrate for OASSase (Smith and Thompson 1971) and we do not know the fate of the NAS if it is formed in the chloroplasts. In bacteria there exists an enzyme complex composed of SATase and OASSase (Kredich et al. 1969). Such a complex could help to prevent nonenzymatic NAS formation from OAS, since OAS could be used for cysteine synthesis immediately after formation. Such a complex has however not been found in plants (Smith and Thompson 1971; Smith 1972).

We could find no appreciable SATase activity on the Percoll gradient banding with the marker enzymes for mitochondria and peroxisomes. This

is in contrast to the findings with *Phaseolus vulgaris*, where in 14-day-old leaves approximately one-third of the SATase activity was present in the mitochondrial fraction (Smith 1972). This SATase activity appeared to be firmly associated with the mitochondria, since after osmotic shock the organelles retained two-thirds of the initial activity. These results do not exclude the possibility that a fraction of SATase activity is localized in the chloroplasts of *Phaseolus vulgaris* too. In spinach, SATase activity does not seem to be firmly bound to the chloroplast membrane, since no appreciable activity of the enzyme was detected in the fractions containing broken chloroplasts. This indicates that chloroplast SATase is a soluble stromal enzyme. Our results show that in spinach the major portion of SATase activity is localized outside the chloroplast. This is similar to OASSase (Fankhauser et al. 1976), but in contrast to adenosine 5′-phosphosulfate sulfotransferase, which was found almost exclusively in the chloroplasts (Frankhauser and Brunold 1978b). The function of the extrachloroplastic, most likely cytoplasmic SATase, is not clear. It has been shown that under certain conditions plants emit H_2S (Wilson et al. 1978). Since this is a light-dependent process, the chloroplasts are a likely source of this H_2S. The H_2S emission could mean that in the chloroplasts the flux of sulfur through the pathway of assimilatory sulfate reduction exceeds the formation of the C_3-acceptor for the sulfate reduced to the thiol level. Indeed, chloroplast O-phospho-L-serine aminotransferase (EC 2.6.1.5), which catalyzes one step in the synthesis of L-serine from 3-PGA, has a rather low activity of about 19 pkat mg^{-1} chlorophyll (Larsson and Albertsson 1979). It is tempting to speculate that the SATase in the cytoplasm together with cytoplasmatic OASSase (Fankhauser et al. 1976) could help to fix all or a portion of the H_2S escaping from the chloroplast. L-serine formed outside the chloroplast would be the substrate for this reaction sequence.

In combination with our earlier findings (Fankhauser et al. 1976), the results presented here show that spinach chloroplasts contain the enzymes necessary for the formation of cysteine via OAS.

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