GLUTAMATE SYNTHASE FROM *AZOSPIRILLUM BRASILENSE*

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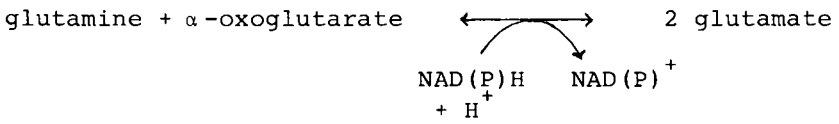
Introduction

Since the work of Tempest et al (1), glutamate synthase has been indicated as an enzyme partecipating in a new alternative pathway of ammonia assimilation. The enzyme (E.C.1.4.1.13) catalyzes the reductive transfer of the amino group from glutamine to α-oxoglutarate with the formation of two moles of glutamic acid, according to the following scheme:



In prokariotes and in the yeast cells the enzyme, purified to a different degree of homogeneity, has been identified as an iron-sulfur flavoprotein which utilizes NAD(P)H as electron donor. It seemed to us interesting to study glutamate synthase also in a free-living diazotroph, the *Azospirillum brasilense*, a microorganism commonly associated with the roots of grasses. In diazotrophs, the glutamate synthase pathway is the preferred route for nitrogen fixation. In *Azospirillum* the level of glutamate synthase seems correlated to the level of

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nitrogenase expression (2), indicating a role for the glutamate synthase in the regulation of the nitrogen fixation cycle. Thus, we attempted the isolation of glutamate synthase from *Azospirillum brasilense Sp6*; some physico-chemical properties of the enzyme, purified almost to homogeneity, are reported and briefly discussed.

Results and discussion

In order to obtain cells with maximum specific activity of glutamate synthase, *Azospirillum brasilense Sp 6* was grown on a minimal medium (2) under limiting concentrations of ammonia. The purification procedure consisted of heat treatment, ammonium sulphate fractionations, ion exchange chromatography, gel filtration and affinity chromatography. The enzyme thus obtained was about 95% pure with a purification factor of 300-fold and a yield of 25%. The purified enzyme has a specific activity of 17 μ mol NADPH oxidized.min^{-1}.mg^{-1}. If rapidly frozen in liquid nitrogen and stored at - 80°C, the enzyme is stable for several weeks. The molecular weight of the enzyme, as indicated by calibration of the Ultrogel AcA 22 column used at the fifth step of the purification procedure, was 740,000 + 10%. SDS gel electrophoresis of the enzyme gave two bands in equimolar amounts with a MW of 135,000 and 50,000, respectively. The glutamate synthase of *Azospirillum brasilense Sp6* thus seems an oligomer of four protomers, each being a dimer of non identical subunits.

HPLC analysis revealed the presence of one mole each of FAD and FMN for protomer; the iron and sulfur analyses gave about

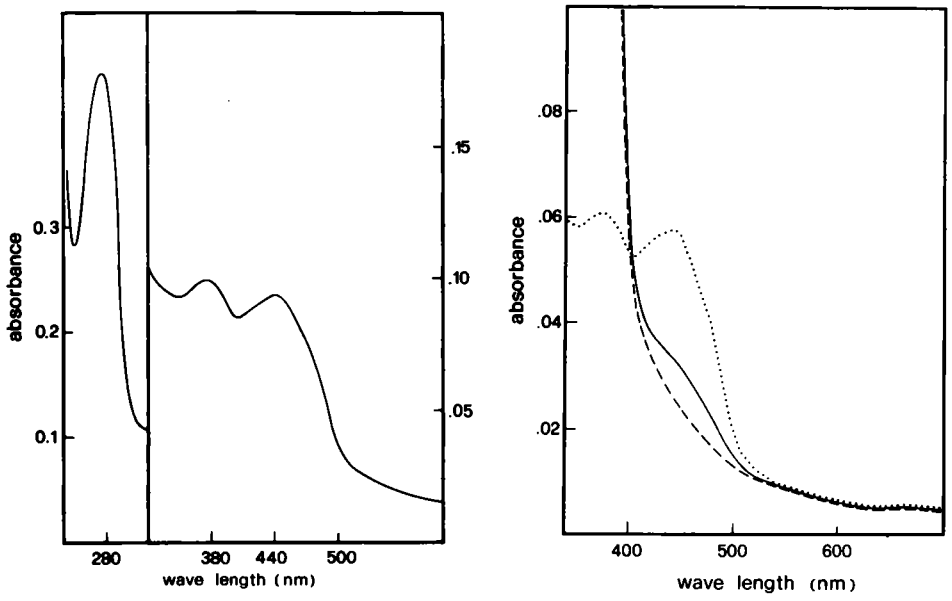

Fig. 1. - Absorption spectrum of glutamate synthase. Conditions: 0.388 $mg.ml^{-1}$ enzyme in 25 mM HEPES, pH 7.5, containing 1 mM *α*-oxoglutarate, 1 mM EDTA, 5 mM β-mercaptoethanol.

Fig. 2. - Dithionite reduction of glutamate synthase. Conditions: (·····), oxidized enzyme, after the anaerobiosis was established; (——), immediately after Na_2SO_4 addition; (----), 30 min later.

8 atoms of Fe and 8 atoms of acid-labile S for protomer. The spectrum of the purified enzyme is reported in Fig. 1; the best preparations had a 278/440 absorbance ratio of 4.8. The enzyme is highly specific for NADPH as electron donor, as well as for glutamine, ammonia being less than 1% active as amino group donor. The pH optimum, with the standard substrates, is around 7.7. Catalytic activity was competiteve-

TABLE 1

KINETIC PARAMETERS OF GLUTAMATE SYNTHASE FROM *AZOSPIRILLUM BRASILENSE Sp6*

Substrate	Activity ($U.mg^{-1}$)	Substrate	K_M (μM)	K_I (mM)
NADPH + L-Glutamine	16.9	NADPH	6.04	
NADH + L-Glutamine	-	L-Glutamine	450.7	
NADPH + NH_4Cl	0.14	α-oxoglutarate	28.8	
		L-Methionine (a)		1.05

(a) Under standard substrates conditions.

ly inhibited by L-methionine with L-glutamine as substrate. A summary of the enzyme kinetic parameters is reported in Table 1: the data are very similar to values reported for glutamate synthase from other sources.

Earlier experiments of Miller et al (3) on anaerobic reduction of the *E. Coli* enzyme by NADPH, showed only partial reduction of the flavin spectrum, which was interpreted as the FAD being the species catalytically active. Using a NADPH regenerating system, we observed with our enzyme a complete reduction of the flavin spectrum (Fig. 3) similar to that observed with dithionite (Fig. 2) or by light irradiation

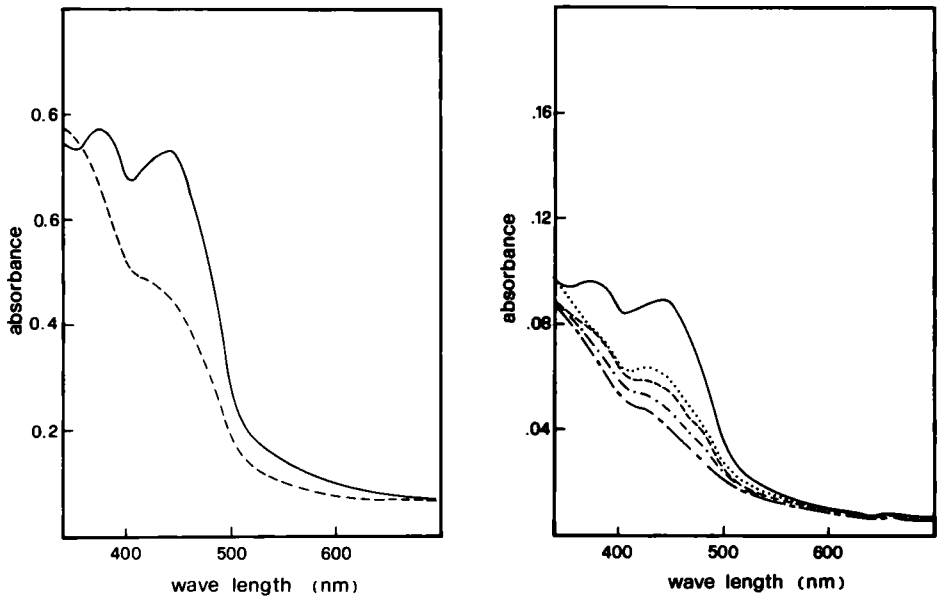

Fig. 3. - Reduction of glutamate synthase by a NADPH regenerating system. Conditions: 4.5 µM NADPH, 2 mM glucose--6-phosphate and 10 µg glucose-6-phosphate dehydrogenase. (——), oxidized enzyme; (- - -), after NADPH addition.

Fig. 4. - Photochemical reduction of glutamate synthase. Conditions: 8 mM EDTA, 0.5 µM 5-deazaflavin. (——), oxidized enzyme; (- - -), 60 sec irradiation; (- · - · -), 90 sec; (- ·· -), 210 sec; (·····), after adding 50 µl 0.1 mM $NADP^+$.

in the presence of EDTA + 5-deazaflavin (fig. 4). Thus NADPH is catalytically competent in reducing both the flavins. In the case of the NADPH reduction, full oxidized spectra were obtained only by addition of substrates after air admission to the spectrophotometric cell. In all cases, stable long wavelength intermediates were not observed along the reduction or the reoxidation path. Stopped flow and EPR

experiments are required in order to ascertain the presence of semiquinone forms of the enzyme during catalysis.

References

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