Characterization of the Metamitron Deaminating Enzyme Activity from Sugar Beet (*Beta vulgaris* L.) Leaves

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Enzymology of Herbicide Detoxification, *as*-Triazinone Herbicides

The enzymatic activity from sugar beet leaves which is responsible for the detoxification of the herbicide metamitron (4-amino-4,5-dihydro-3-methyl-6-phenyl-1,2,4-triazin-5-one, trade name Goltix®) has been characterized *in vitro*. The detoxification occurs by rapid deamination *in vivo* as well as *in vitro*. However, the deamination *in vitro* is only maximal under reducing conditions, *i. e.* with an electron donor and in a nitrogen atmosphere. The electron donor may be cystein, glutathione, dithionite or ascorbate. The enzymatic deamination further requires the addition of cytochrome c and a "supernatant factor", which may be replaced by FMN, FAD or DCPIP. However, in the presence of FMN or DCPIP cytochrome c is not essential but only stimulatory. The particulate as well as the soluble metamitron deaminating enzyme preparations obtained take up oxygen when supplied with cysteine and FMN. The particulate enzyme appears in the peroxysome-fraction.

It is therefore suggested, that the enzymatic deamination of metamitron in sugar beet leaves is mediated by a proxisomal membrane bound electron transport system which alternatively may reduce oxygen or metamitron (deaminating).

Introduction

Metamitron (subsequently referred to as MAT) is a photosynthesis inhibiting herbicide for weed control in sugar beet [1]. Its high compatibility in sugar beet has led to detailed investigations which have shown that the rate of detoxification is the prime cause for the selective action [2, 3]. The present investigations where undertaken in order to further characterize the MAT-deaminating enzyme from sugar beet leaves [2].

Materials and Methods

Sugar beet leaves (50 g) were homogenized in 50 mM tricin-NaOH pH 8.0 (150 ml) containing 1 mM cystein. The homogenate was filtered through perlon gaze and centrifuged (20 min at 50,000 × *g*) in order to yield a pellet (P). P was routinely suspended in its own supernatant (S) (10% of its original volume) to yield a System I preparation.

For obtaining a purified soluble enzyme preparation (System II) S was loaded on a 100 × 1 cm Sephadex G 50 column and eluated with 50 mM tricin pH 7.5. The first high molecular weight peak was taken for further tests, but metamitron deaminating activity was also found in other fractions (Fig. 3).

The standard enzyme test contained for System I/System II in 1.5/0.3 ml : 1.5/0.9 mg cytochrome c, 10^{-5} M MAT-2-^{14}C containing 0.01 μCi and 4/0.4 mg protein. For the System II 0.1 mM cystein and 0.1 mM FMN were additionally included. The standard incubation was 120 min at 25 °C in a nitrogen atmosphere.

After the incubation 200 μg each of [^{12}C]MAT and DA-MAT were added. The samples were then heated for 5 min 70 °C, centrifuged and taken to dryness at 70 °C. MAT (R_f 0.63) and DA-MAT (R_f 0.28) were separated on silica gel in chloroform : methanol = 9 : 1. The DA-MAT band was detected in UV-light, extracted at 90 °C with water and counted by liquid scintillation.

Results and Discussion

The MAT deaminating activity in a sugar beet leaf extract occurs in the particulate (P) as well as in the supernatant (S) fraction (Table I). The deamination rate in N_2 varies to some extent as indicated but always is considerably higher than in air. Deamination of MAT with the supernatant fraction (S) may only be detected in N_2. Further

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Abbreviations: FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; DCPIP, 2,6-dichlorophenolindophenol; cyt. c, cytochrome c; P, pellet obtained at 50 000 × *g*; S, supernatant at 50 000 × *g*; MAT, metamitron; DA-MAT, desamino-metamitron.

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Table I. Deamination of metamitron by sugar beet leaf extracts.

Preparation	% Deamination		
		in N_2	
	in air	test I	test II
P in buffer	2.7	20.7	2.1
P in S	9.5	40.4	27.2
S	0.4	17.8	13.0

work was done either with P suspended in its own S (System I) or with S purified by Sephadex gel filtration (System II). The results obtained support the idea that the enzyme proteins of both systems are derived from the same *in vivo* complex which appears to be located in the peroxisomes.

System I (particulate enzyme preparation)

The properties of the pelletable enzyme protein may be taken from Table II. A "supernatant factor" appears to exist which may in part be replaced by the cofactors FMN, FAD, DCPIP. The cofactors differ however in that only FMN and DCPIP are able to replace cytochrome c to some extent. Cystein is present in these assay systems with the preparation. The pH-dependency of the particulate enzyme is given in Fig. 1.

In order to localize the particulate enzyme at the subcellular level a homogenate was obtained as usual but including 0.4 M mannitol. This homogenate was run for 180 min on a sucrose gradient and subsequently fractionated (Fig. 2). Whereas the protein is concentrated in the chloroplast-peak, the MAT-deaminating activity shows a prominent maximum in the peroxisomal band below the chloroplasts.

Table II. Conditions for the *in vitro* deamination of metamitron with either P suspended in its own S (System I) or a pellet suspended in the original buffer.

Preparation	Addition 10^{-3} M	% Deamination	
		−Cyt. c	+Cyt. c
P in S	—	<1	41.5
P in S	FMN	15.3	20.0
P in S	FAD	<1	17.5
P in S	DCPIP	9.5	18.0
P in buffer	—	1.2	6.8
P in buffer	FMN	6.6	18.2
P in buffer	FAD	5.4	17.2
P in buffer	DCPIP	4.4	21.6

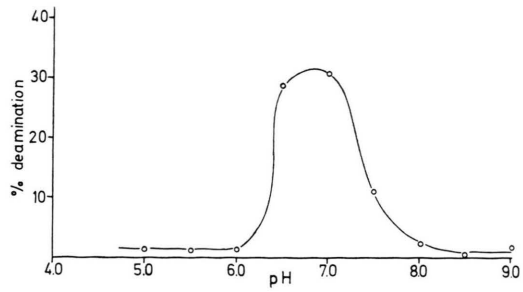

Fig. 1. Dependency of the rate of the *in vitro* deamination of metamitron on the pH. P was suspended in buffers of different pH and 0.1 mM FMN was included for replacement of the supernatant factor.

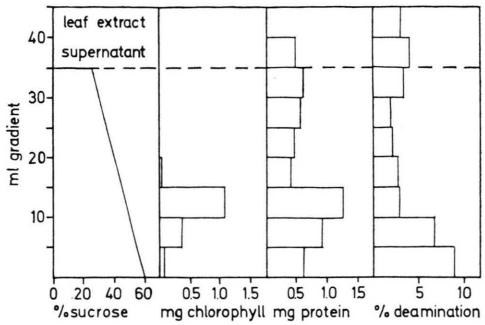

Fig. 2. Zentrifugation of a sugar beet leaf homogenate in a sucrose density gradient.

System II (soluble enzyme preparation)

For a better characterization of the MAT-deaminating enzyme reaction the supernatant fraction was purified by Sephadex gel filtration (Fig. 3). Enzyme activity again occurs in all protein fractions as in original centrifugation studies. However, the first and second peak display a very clear FMN-dependency in contrast to later fractions which appear to contain the "supernatant factor". This first peak has been taken for further experiments which have shown a pH-optimum around 8.5 and a Michaelis konstant of $km = 5.4 \times 10^{-5}$ M below and $km = 8.3 \times 10^{-4}$ M above 10^{-4} M MAT. The enzyme displays a very sharp activation at 16 °C beeing nearly inactive below 15 °C. The optimum cofactor concentrations are 10^{-4} M for FMN and cytochrome c which is identical with System I. However, only FMN and FAD but not DCPIP are accepted by the soluble enzyme. These properties are interpreted to mean that the soluble enzyme contains only a smaller part of the original enzyme complex than the

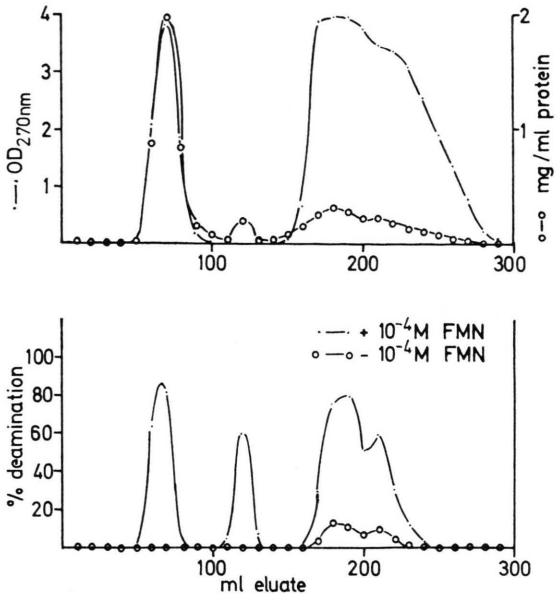

Fig. 3. Gel filtration of a 150 000 g supernatant from sugar beet leaves through a 100×1 cm sephadex G 50 column.

particulate enzyme whose properties are more close to these found *in vivo*. This includes the deamination of MAT in the presence of oxygen which is not obtained with the soluble enzyme.

It is concluded from the data, that the deamination is accompanied by reduction. The System I – as well as the System II – preparations take up oxygen in the presence of cystein and FMN.

Table III. Comparison of different reducing agents in the *in vitro* deamination of metamitron by System II.

Reductant	M	Cytochrome c	% Deamination
Cystein	10^{-5}	+	32.6
Cystein	10^{-4}	+	63.6
Cystein	10^{-3}	+	26.9
Cystein	10^{-3}	–	1.4
Glutation	10^{-3}	+	74.6
Glutation	10^{-3}	–	1.8
$Na_2S_2O_4$	10^{-3}	+	45.0
$Na_2S_2O_4$	10^{-3}	–	17.0
Ascorbate	10^{-4}	+	38.2
Ascorbate	10^{-3}	+	87.2
Ascorbate	10^{-2}	+	22.7
Ascorbate	10^{-3}	–	1.2
–	–	–	2.1

It has already been stated that a reductant is required for the *in vitro* deamination. It is interesting that the reductant with the most negative redox potential, $Na_2S_2O_4$, also deaminates MAT in the absence of cytochrome c, although with a lower rate (Table III). The enzyme complex responsible for the reductive deamination of MAT therefore appears to be able to accept electrons from various donors depending on the redox potential and presumably also on endogenous redox-cofactors.

Acknowledgements

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