Respiratory energy requirements and rate of protein turnover in vivo determined by the use of an inhibitor of protein synthesis and a probe to assess its effect

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Protein turnover is generally regarded as a major maintenance process, but experimental evidence to support this contention is scarce. Here we quantify the component of dark respiration rate associated with overall protein turnover of tissues in vivo. The effect of an inhibitor of cytosolic protein synthesis (cycloheximide, CHM) on dark respiration was tested on a cell suspension from potato (*Solanum tuberosum* L.) and quantified on leaf discs of expanding and full-grown primary leaves of bean (*Phaseolus vulgaris* L.). The in vivo effect of CHM on protein biosynthesis was assessed by monitoring the inhibition of the induction of the ethylene-forming enzyme (EFE) activity. The present method yields the energy costs of turnover of the total pool of proteins irrespective of their individual turnover rates. Average turnover rates were derived from the respiratory costs and the specific costs for turnover.
Inhibition of respiration by CHM was readily detectable in growing-cell suspensions and discs of expanding leaves. The derived respiratory costs of protein turnover in expanding leaves were maximally 17–37% of total respiration. Turnover costs in full-grown primary leaves of bean amounted to 17–21% of total dark respiration. The maximum degradation constants (i.e. K_d-values) derived for growing and full-grown leaves were up to 2.42×10^{-6} and 1.12×10^{-6} s^{-1}, respectively.

Key words – Cycloheximide, ethylene, ethylene-forming enzyme, leaf protein turnover, maintenance respiration, protein biosynthesis.

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Introduction

The relation between respiration rate and protein content and its physiological basis are not fully understood. A positive correlation between the rate of leaf respiration and leaf protein content has been found (e.g. De Visser et al. 1992, Lambers et al. 1989, Pons et al. 1989), but not always (Byrd et al. 1992). It has been hypothesized that the energy costs of protein turnover, defined as a cycle of degradation and 're-synthesis', may explain the positive relationship between respiration and protein content (De Visser et al. 1992, Lambers et al. 1989, Pons et al. 1989), as both protein degradation (Vierstra 1993) and (re)-synthesis (De Visser et al. 1992) are energy dependent. The energy costs of protein turnover were estimated to 30–60% of leaf dark respiration, both by experimental and theoretical analyses (De Visser et al. 1992), whereas Byrd et al. (1992) concluded these costs to be of minor importance. A thorough testing of the hypothesis is hampered by the absence of a more direct method to deter-

Tab. 1. The expected effects of CHM on processes of protein turnover and on respiration rate [E_{CHM}; mol ATP (mol peptide bond)$^{-1}$]. The sum of the costs of all processes, or of synthesis processes only, are the specific energy costs [mol ATP (mol peptide bond)$^{-1}$] of protein turnover (E_{sp}), and protein synthesis (E_s), respectively. The listing of the processes involved is according to De Visser et al. (1992).

Process	CHM-effect	Specific energy costs [mol ATP (mol peptide bond)$^{-1}$]	
		Low	High
Protein biodegradation	–	1.0	2.0
Protein biosynthesis			
Amino acid activation	+	2.0	2.0
Error correction (by aminoacyl-tRNA synthetases)	+	5.0	8.0
Peptide bond formation and translocation	+	2.0	2.0
Tool maintenance	+	1.0	1.0
Signalling sequences	+	0.6	1.9
Amino acid turnover	–	0	3.5
Post-translational processing			
Methylation, acetylation, etc.	+	0.1	0.1
Phosphorylation	+	0.1	0.3
Protein turnover (E_{sp})		11.8	20.8
Protein synthesis (E_s)		10.8	18.8
Expected CHM effect (E_{CHM})		10.8	15.3

mine the energy costs of protein turnover in vivo. Also, in vivo estimates on the average protein turnover rates are hard to obtain. Our objective is to introduce an experimental method alternative to the approach of De Visser et al. (1992), for estimating the energy requirements associated with overall in vivo protein turnover in plant tissues, irrespective of the turnover rates of individual proteins. From this value the average protein turnover rate is derived.

Energy costs of protein turnover may be estimated from the decrease in respiration rate after complete inhibition of cytosolic protein synthesis, in combination with a test to quantify the in vivo action of the inhibitor. Such an approach seems adequate since (1) at least 80% of the energy costs of protein turnover is associated with the re-synthesis of proteins (Tab. 1, cf. De Visser et al. 1992), (2) plant proteins, including most of the mitochondrial (Leaver and Gray 1982) and chloroplast proteins (Ellis 1981, Mullet 1988), are predominantly encoded by the nucleus and synthesized on cytosolic ribosomes and (3) the method is independent of the turnover rates of individual proteins. Here we examine whether this approach can be used in vivo, assuming similar protein turnover rates in the light and dark. The application of the inhibitor is optimized and validated by examining the induction of ethylene-forming enzyme (EFE) activity as a probe. The respiratory efficiency (i.e. P/O$_2$-ratio) is determined both in the absence and presence of the inhibitor. Estimates of turnover costs in leaves are compared with the theoretical inhibitor-effect on respiration.

Abbreviations – ACC, 1-amino-cyclopropane-1-carboxylic acid; BA, N^6-benzyladenine (<=> 6 benzylaminopurine); CHM, cycloheximide; EFE, ethylene-forming enzyme; RER, relative ethylene production rate; SHAM, salicylhydroxamic acid.

Materials and methods

Plant material

Bean (*Phaseolus vulgaris* L. cv. Berna) was used because of the similar growth rate of its two primary leaves. Plants were grown from seeds on tap water in a climate room. Temperature was maintained during day and night at $20 \pm 2°C$, and the relative humidity at 75%. During germination, seeds were covered with humid filter paper and transparent plastic for 5 days. Seedlings received 165 μmol m^{-2} s^{-1} PAR fluorescent light (Philips TLD 36 W/33) during 16 h per day. After 7 to 9 days, the seedlings were transplanted to pots filled with 0.5 l aerated Hoagland solution [macro nutrients 1/2 strength according to Hoagland and Schnijder (1933) and micro nutrients 1/2 strength according to Lewis and Powers (1941) with iron as Fe(III)-EDTA]. Light conditions were 16 h 600 μmol m^{-2} s^{-1} PAR SON-T light (Philips, 400 W). The effect of cycloheximide on the activity of the alternative pathway, was, due to technical constraints, determined on plants grown at a daylength of 14 h at 170 μmol m^{-2} s^{-1} PAR fluorescent light (Philips TL-D-HF).

The time of maturity of the primary leaves was determined from the time course of dry and fresh weight and leaf area. To reduce variation, leaf expansion of individual primary leaves was also monitored non-destructively, with a video camera (SONY AVC 3250 CE, Japan) and monitor (SONY PVM 90 CE, Japan) connected to an integrator (TFDL nr 720314, Wageningen, The Netherlands).

A cell suspension was obtained from callus of leaves of potato (*Solanum tuberosum* L. cv. Alcmaria). The nutrient medium contained MS macro- and micro-elements (Murashige and Skoog 1962), MS vitamins, 88 mM sucrose, 555 μM inositol, 27 μM NAA, 444 nM BA and 5 g

l⁻¹ agar in the case of callus culture. Weekly, 9 ml of cells of a 7 days old growing suspension culture were transferred to 40 ml fresh medium in a 250-ml Erlenmeyer flask closed with a cotton plug. The flasks were kept on an orbital shaker at 20°C under continuous 10 μmol m⁻² s⁻¹ PAR fluorescent light (Philips TLD 36 W/33). Growth of the cells was measured after Gilissen et al. (1983).

Assessment of the in vivo action of cycloheximide

Cycloheximide (CHM), is known to block total cytosolic protein synthesis (Lewin 1990). CHM also inhibits the induction of ethylene-forming enzyme (EFE) activity by its substrate, 1-amino-cyclopropane-1-carboxylic acid (ACC; De Visser and Spencer 1986, Philosoph-Hadas et al. 1986). This induction of EFE activity was used as a probe to establish the in vivo response of protein synthesis to CHM. Whether this inhibition of EFE activity is due to blocked de novo synthesis of either EFE itself (De Visser and Spencer 1986, Philosoph-Hadas et al. 1986) or some activator or regulatory protein, is not relevant to the present objective. Generally, ACC production limits in vivo ethylene biosynthesis, whereas EFE is largely constitutive (Yang 1980, Yang and Hoffman 1984). Therefore, EFE activity was measured as the ethylene production rate at saturating ACC concentrations.

We determined the lowest concentrations of ACC and CHM yielding maximal ethylene production and inhibition of protein synthesis, respectively. The optimal ACC concentration was determined from ethylene production during 8 h by both leaf discs and detached intact leaves of bean (17 to 20 days old). This ACC concentration was used to establish the dose-response relationship of CHM and protein synthesis, with 11 and 13 days old leaf discs and 10 to 12 days old detached leaves and CHM concentrations up to 80 μM. Both ACC and CHM were dissolved in demineralized water. To minimize variation, various sets of leaf discs were made up from the same set of leaves. Ethylene production rates were expressed as percentage of the control without ACC and CHM (relative ethylene production rate; RER).

Directly after some of the respiration measurements on 10, 12 and 19 days old discs (see next section), the effect of CHM on protein biosynthesis was assayed on line, using the induction of EFE activity as a probe. Ethylene production by EFE at saturating ACC concentrations was determined from 2 to 21 h. CHM was added at the start of the respiration measurements, but no CHM was present in the medium during the following ethylene production measurements. Therefore, the effects of CHM on the induction of EFE activity were solely due to CHM taken up during the respiration measurements.

Ethylene was collected in a vessel, placed upside down in a petri dish with two concentric compartments. The outer compartment was filled with water, to obtain a gas-tight seal under atmospheric pressure. The center compartment contained either leaf discs floating on the solution or detached leaves incubated with the basal end

of the petiole submerged. Samples were taken with a 1-ml syringe from the headspace, through a septum in the glass vessel (500 ml gas volume). Ethylene was measured by gas chromatography (Packard model 427, Canberra, Australia) with a flame ionization detector (FID). Oven temperature was 220°C. The stainless steel column (length 2 m, outer diameter 3.2 mm) contained aluminium oxide (80 mesh). The carrier gas was N_2.

Respiration and inhibition of protein synthesis

The activity of the alternative, non-phosphorylating pathway was determined both in the absence and in the presence of 80 μM CHM. Titration curves for salicylhydroxamic acid (SHAM, 1 M stock in 2-methoxyethanol) were made with a Clark-type oxygen electrode (Yellow Springs Instruments, OH, USA), to assess possible side effects such as stimulation of peroxidases or inhibition of the cytochrome path (Møller et al. 1988). Slices of primary leaves of bean of approximately 2 mm² were incubated in a buffer (pH 6.6; 20°C) containing 10 mM MES, 50 mM HEPES and 0.2 mM CaCl₂ (Azcón-Bieto et al. 1983).

The effect of CHM on the oxygen uptake of discs of growing (10–12 days old) and full-grown (17–19 days old) leaves was measured manometrically during the normal dark period using a Warburg apparatus at 22°C. Discs (9.5 mm in diameter) were floating on demineralized water with 0 to 80 μM CHM. To avoid induction of senescence, ethylene accumulation was prevented by the presence of KMnO₄ in the side arm of the vessel. The activity of the alternative path was determined manometrically, on 2 mm² slices of 12–16 days old leaves floating on MES/HEPES buffer (pH 6.6), either half-way or at the onset of the light period. SHAM was added from the side arm after approximately 3 h; there was no KMnO₄ present in these short-term measurements. The stock solutions of SHAM and the buffer were the same as those used for the titrations. The CHM effect on the respiration rate of growing (2, 3 or 7 days after transfer to a new medium) cell-suspensions of potato was also measured manometrically. CHM (20 μM) was added to one half of the cells, 5 h after start of the measurement. All respiration rates on leaves were expressed on initial leaf area.

Calculations of costs of protein turnover and synthesis

Maintenance and synthesis of the protein pool involves various processes, most of which are inhibited by CHM (Tab. 1). CHM affects the energy utilization in cytosolic turnover and synthesis of all proteins, irrespective of their individual rates and pathways of turnover and synthesis. The theoretically expected CHM-inhibition of the respiration rate for (1) cytosolic protein turnover (m_{CHM}; mol O_2 m⁻² s⁻¹), (2) net cytosolic protein synthesis for growth (s_{CHM}; mol O_2 m⁻² s⁻¹) and (3) total protein synthesis (r_{CHM}; mol O_2 m⁻² s⁻¹), were calculated by Equation 1 (after De Visser et al. 1992), 2 and 3, respectively. Note

Tab. 2. List of symbols and abbreviations used in the calculations of expected CHM effects on the respiration rate and overall respiratory costs of protein synthesis and turnover, with dimensions.

Abbreviation	Description	Unit
K_d	degradation 'constant' of protein turnover	s^{-1}
m_{CHM}	CHM-inhibited (maintenance) respiration rate for cytosolic protein turnover	mol O_2 m^{-2} s^{-1}
r_{CHM}	CHM-affected respiration rate for the total of cytosolic protein synthesis and turnover	mol O_2 m^{-2} s^{-1}
From literature		
E_{CHM}	sum of the specific energy costs of the processes blocked by CHM	mol ATP (mol peptide bond)$^{-1}$
E_s	specific energy costs of protein biosynthesis	mol ATP (mol peptide bond)$^{-1}$
E_{sp}	specific energy costs of protein turnover	mol ATP (mol peptide bond)$^{-1}$
F_{org}	fraction of protein turnover or synthesis in organelles	–
N_p	average nitrogen content of leaf protein	mol N-protein (mol peptide bond)$^{-1}$
Measured		
N_t	protein-N content of the leaf	mol N-protein (g DW)$^{-1}$
P/O_2	ATP/O_2 ratio of oxidative phosphorylation	mol ATP (mol O_2)$^{-1}$
RGR_l	relative growth rate of a leaf	g DW (g DW)$^{-1}$ s^{-1}
RN_tC	relative change rate of leaf protein-N	mol N-protein (g DW)$^{-1}$ s^{-1}
SLA	specific leaf area	m^2 (g DW)$^{-1}$
Calculated		
m_p	overall (maintenance) respiration rate for protein turnover, in cytosol and organelles	mol O_2 m^{-2} s^{-1}
r_p	overall respiration rate for the total of protein synthesis and turnover in cytosol and organelles	mol O_2 m^{-2} s^{-1}
s_{CHM}	CHM-inhibited respiration rate for net cytosolic protein synthesis	mol O_2 m^{-2} s^{-1}
s_p	overall respiration rate for net protein synthesis, in cytosol and organelles	mol O_2 m^{-2} s^{-1}

that in full-grown leaf, s_{CHM} equals zero so that r_{CHM} is equal to m_{CHM}.

$$m_{CHM} = (E_{CHM} \times K_d \times N_t) / [(P/O_2) \times N_p \times SLA] \qquad (1)$$

$$s_{CHM} = [E_{CHM} \times (RGR_l \times N_t + RN_tC)] / [(P/O_2) \times N_p \times SLA] \qquad (2)$$

$$r_{CHM} = m_{CHM} + s_{CHM} \qquad (3)$$

where E_{CHM} is the sum of the energy costs of the processes blocked by CHM [mol ATP (mol peptide bond)$^{-1}$], K_d is the degradation constant of protein turnover (s^{-1}), N_t is the protein-N in the leaf [mol N-protein (g DW)$^{-1}$], P/O_2 is the ATP/O_2 ratio of oxidative phosphorylation [mol ATP (mol O_2)$^{-1}$], N_p is the average nitrogen content of leaf protein [1.26 mol N-protein (mol peptide bond)$^{-1}$; De Visser et al. 1992], SLA is the specific leaf area [m^2 (g DW)$^{-1}$], RGR_l is the relative growth rate of the primary leaf [g DW (g DW)$^{-1}$ s^{-1}] and RN_tC is the rate of change of the protein-N in the leaf [mol N-protein (g DW)$^{-1}$ s^{-1}]. The resultant of $(RGR \times N_t) + RN_tC$ gives an estimate of net protein synthesis.

As CHM inhibits only part of the processes in Tab. 1, its effect on respiration is an underestimation of the overall costs of protein turnover and net protein synthesis. The actual overall respiratory costs of protein turnover (m_p) and net protein synthesis (s_p) were obtained from the CHM inhibition of the respiration rate (m_{CHM} and s_{CHM}) using Equation 4 and 5, respectively. The

overall respiratory cost of *total* synthesis (r_p) is the sum of m_p and s_p (Eqn 6).

$$m_p = m_{CHM} \times (E_{sp}/E_{CHM}) / (1 - F_{org}) \qquad (4)$$

$$s_p = s_{CHM} \times (E_s/E_{CHM}) / (1 - F_{org}) \qquad (5)$$

$$r_p = m_p + s_p \qquad (6)$$

where, E_{sp} is the specific energy costs of protein turnover (mol ATP [mol peptide bond]$^{-1}$; Tab. 1), E_s is the specific energy costs of synthesis (mol ATP [mol peptide bond]$^{-1}$; Tab. 1) and F_{org} is the fraction turnover or synthesis in the organelles, i.e. mitochondria and chloroplasts. In full-grown leaves, s_{CHM} is zero so that r_p equals m_p.

We used the range of K_d values given in the literature (i.e. 0.31×10^{-6} to 6.02×10^{-6} s^{-1}; Barneix et al. 1988, Davies 1982, Dungley and Davies 1982, Eising and Gerhardt 1987) as input for some of our calculations. Table 1 (after De Visser et al. 1992) shows the processes expected to be affected by CHM and the range of possible values of E_{CHM}. CHM inhibits the peptidyl transferase on the 60S subunit of the cytosolic ribosomes which blocks protein elongation (Lewin 1990) and, thus, any polypeptide processing. F_{org} was estimated to be about 30% of overall protein synthesis (see Discussion). Other parameters were derived from the present experiments. The symbols and abbreviations used are listed in Tab. 2.

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Characteristic	Unit	Growing leaf	Full-grown leaf
Age plant	day	9 to 12	17 to 19
N_t	mmol N-protein $(g\ DW)^{-1}$	2.93 ± 0.11	2.02 ± 0.11
SLA	$m^2\ (g\ DW)^{-1}$	0.046 ± 0.006	0.023 ± 0.002
RGR_1	$g\ DW\ (g\ DW)^{-1}\ day^{-1}$	$0.312\ (r^2 = 0.96;\ n = 16)$	
RN_tC	mmol N-protein $(g\ DW)^{-1}\ day^{-1}$	$-0.299\ (r^2 = 0.78;\ n = 18)$	

Chemical determinations

Total nitrogen and NO_3^--nitrogen content [mg N $(g\ DW)^{-1}$] were determined by CHN analysis on a Heraeus CHN-rapid (Hanau, Germany) and a TRAACS 800 continuous flow system (Bran and Luebbe Analyzing Technologies, Elmsford, NY, USA), respectively. The organic N content, i.e. the difference between the total nitrogen content and NO_3^--nitrogen content, was taken as upper estimate of protein-N content in the leaf (N_t).

Statistics

Differences were tested by ANOVA, using the statistical package GENSTAT 5 (Payne 1987). LSD values were calculated only if the corresponding F-tests of ANOVA were significant (protected LSD method). LSD values were calculated for $P < 0.05$. All experiments (except those for the SHAM titrations) had a block design.

Results

Characteristics of primary leaves of bean

The primary leaves of bean grew exponentially from day 9 up to day 16 after germination, and started yellowing after day 20. Therefore, we selected leaves of 17 to 19 days old plants as full-grown primary leaves. The rate of change of the protein-N concentration in the leaf (RN_tC) during growth till the early mature stage, was obtained by linear regression of N_t over the period of 10 to 14 days. Both N_t and the SLA decreased with increasing age of the primary leaves (Tab. 3).

Assessment of the in vivo action of cycloheximide

In order to determine the energy costs of protein turnover, the in vivo action of CHM was assessed using EFE as a 'reporter' protein. The induction of EFE-activity was maximal at 2 and 10 mM ACC for leaf discs and detached bean leaves, respectively (Fig. 1). CHM effectively inhibited this induction of EFE-activity (Fig. 2), which became evident only after more than 4 h following ACC addition (slopes in Fig. 3). At 80 μM CHM, no increase in
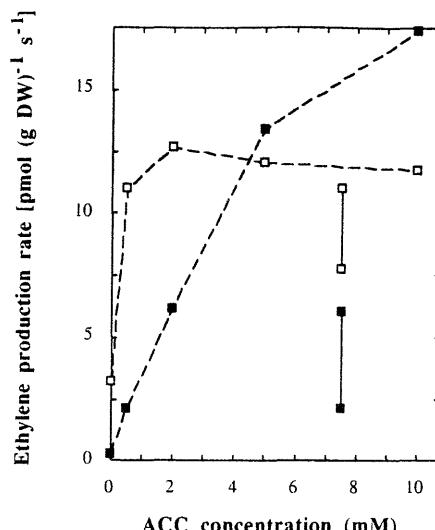
Fig. 1. Ethylene production rate (pmol [g DW]$^{-1}$ s^{-1}) of leaf discs (open symbols) and detached leaves of bean (filled symbols), as dependent on external ACC concentration. LSD values are indicated by the vertical bars (n = 3 for both the leaf discs and detached leaves).
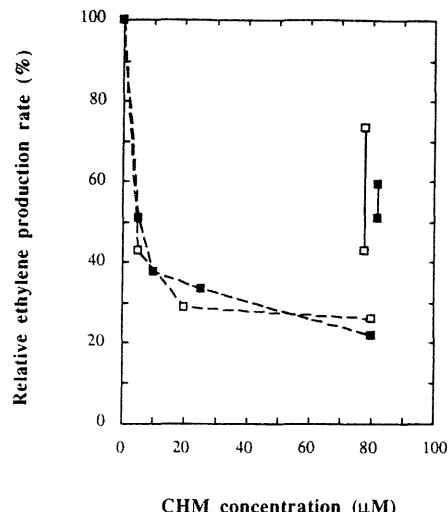
Fig. 2. Relative ethylene production rate of leaf discs (open symbols) and detached leaves (filled symbols) of bean, as dependent on the external CHM concentration. The discs and leaves were incubated in 2 and 10 mM ACC, respectively. LSD values are indicated by the vertical bars (n = 2 and 4 for leaf discs and detached leaves, respectively).

Time (h)

SHAM concentration (mM)
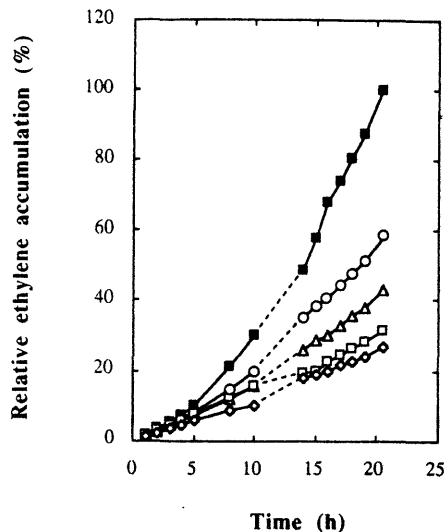
Fig. 3. Ethylene production of primary leaves of bean in the absence (■) and presence of 5 (○), 10 (△), 25 (□) or 80 (◇) μM CHM. In all treatments 10 mM ACC was present and CHM was added at the start of the measurement. The 24 h time course of each treatment was obtained from different leaves for the various periods, separated by the dashed lines. Data from all leaves used in one 24 h time course (i.e. 4 treatments with two leaves per treatment) were expressed as a fraction of the control (0 μM CHM). The slopes of the lines give the relative ethylene production rates (n = 2).

Fig. 5. Response to SHAM of respiration of bean leaf slices. The absence and presence of KCN is represented by ■ and □, respectively. The standard error is given by a vertical bar, unless this value is smaller than the symbol size (n varies for different points from 1 to 10).

EFE activity was found in detached leaves. In leaf discs 20 μM had a similar effect.

Respiration and inhibition of protein synthesis

In the presence of CHM, dark respiration of growing leaves was inhibited by approximately 20 to 34% of the control ($P < 0.05$; Fig. 4A). In full-grown leaves, CHM inhibition was at most 11% of the control ($P < 0.05$; Fig.

4B) and only present after 6.9 and 11 h. SHAM blocked the alternative path (Fig. 5), but did not affect the respiration of 12 to 16 days old bean leaves in the presence or absence of 80 μM CHM (data not shown). Thus, the P/O_2-ratio was 6 and CHM did not cause a decrease in respiration by an effect on the efficiency of oxidative phosphorylation, assuming that the external NADH oxidation and the rotenone insensitive bypass are not operating (cf. Lambers 1985). The respiration rate of the cell suspension was inhibited by up to 37%, after addition of CHM ($P < 0.05$; Fig. 6). This shows, similar to the data of discs of growing leaves, that the present method has

Time (h)
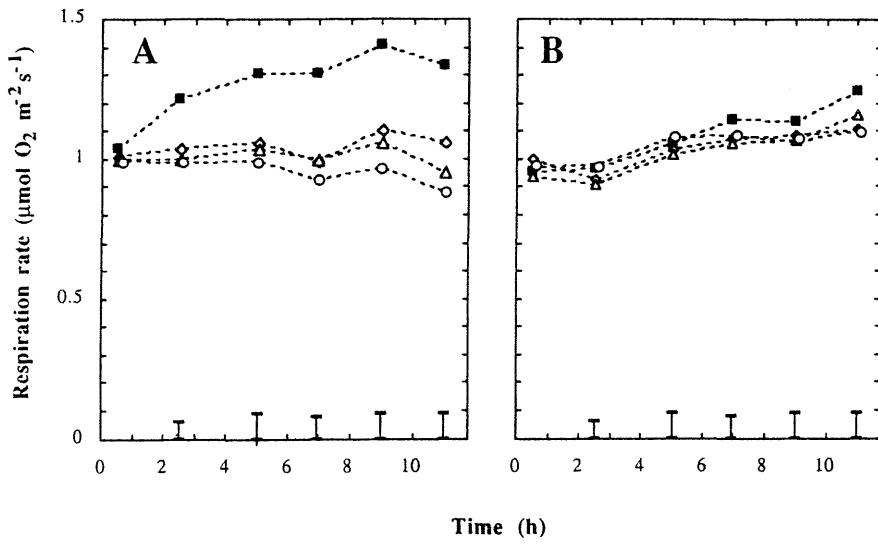
Fig. 4. Effect of the absence (■) and presence of 5 (○), 20 (△) or 80 (◇) μM CHM on the respiration rate of leaf discs of growing (A) and full-grown (B) primary bean leaves. CHM was added at the start of the measurement. LSD values are given by the vertical bars at the bottom of the figure (n = 9 for both ages).

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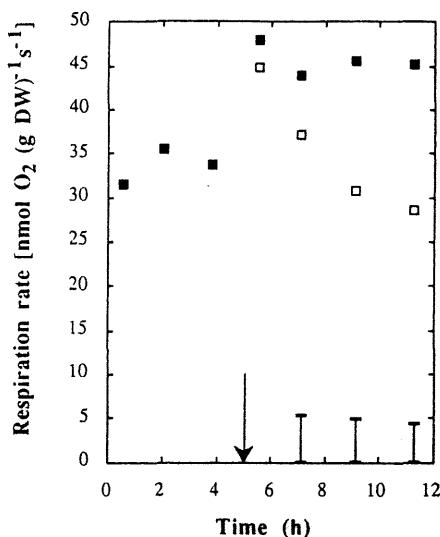
Fig. 6. The effect of 20 μM CHM (□) on the respiration rate of cell suspensions compared to that of untreated cells (■). The time of CHM addition (5 h) is indicated by an arrow. LSD values are indicated by the vertical bars at the bottom of the chart (n = 16).

adequate sensitivity to detect the respiratory costs of protein synthesis (involved in turnover), when the rate of synthesis is quantitatively important.

CHM supplied during the respiration measurements, was sufficient to block the induction of EFE-activity determined the first 6 h afterwards, for discs of both growing (10 and 12 days old) and full-grown (19 days old) leaves (data show a similar pattern as in Fig. 2). Thus, the absence of a CHM effect on respiration was not due to poor penetration during the respiration measurements.

Calculations of respiratory costs of protein turnover

For full-grown leaves, the theoretical decrease of the respiration rate after CHM treatment was calculated following Equation 1 (input data in Tab. 3; in the absence of growth s_{CHM} equals zero and thus r_{CHM} equals m_{CHM}). This

theoretical CHM effect varied by an order of magnitude, depending on the literature values of K_d and E_{CHM} (Tab. 4; derivation E_{CHM} in Tab. 1). Despite this variation, the CHM effect should be detectable as shown by a comparison of the theoretical values of m_{CHM} (Tab. 4) to both the lowest (0.06 μmol O_2 m^{-2} s^{-1}) and highest (0.09 μmol O_2 m^{-2} s^{-1}) just detectable difference between respiration rates (i.e. the LSD values in Fig. 4B). Furthermore, with the highest detection limit of respiration observed as input in Equation 1 (s_{CHM} equals zero), K_d-values higher than 0.7×10^{-6} s^{-1} should cause a significant decrease of respiration (Tab. 5). The highest CHM inhibition of respiration observed yields a K_d of 1.1×10^{-6} s^{-1} (Tab. 5). These K_d-values are both within the range of literature values (Tab. 4). The present calculations show that non-significant CHM effects on the respiration rate of full-grown leaves cannot be ascribed to insensitivity of the method, but are due to relatively low turnover rates.

For growing leaves, the significance of the costs of protein turnover was examined by calculating m_{CHM} (Eqn 3; Tab. 5), as the difference between the observed CHM effect (r_{CHM}; Fig. 4A) and the calculated s_{CHM} (Eqn 2; Tab. 5). The K_d-values corresponding to this m_{CHM}, as derived from Equation 1, are $\leq 2.5 \times 10^{-6}$ s^{-1} (Tab. 5). These estimates are within the range of literature values (values and references in Tab. 4; average $K_d = 2.6 \times 10^{-6}$ s^{-1}), also indicating that the sensitivity of the present method is adequate for detecting differences in respiration rate due to CHM inhibition of protein (re)-synthesis.

Discussion

Assessment of the in vivo action of cycloheximide

The optimal ACC (2 mM) and CHM (20 μM) concentrations for leaf discs, agreed with those used by Philosoph-Hadas et al. (1986; 3 mM ACC and 20 μM CHM for discs of tobacco leaves). The induction of EFE-activity by its substrate ACC became only evident after a lag-time of 4 h (cf. the slopes of the curves in Fig. 3). A period of 4 h is sufficient to enable induction of de novo protein synthesis (Lewin 1990). From the results presented in Figs 2 and 3 and from the observations following the respiration

Tab. 4. Theoretical values of m_{CHM} (CHM-affected respiration for protein turnover), as dependent on protein degradation constants (K_d) and specific costs of protein synthesis as affected by CHM (E_{CHM}). Calculation is according to Eqn 1, for full-grown primary leaves of bean. *, m_{CHM} Significant in all measurements (i.e. values higher than largest LSD in Fig. 4B).

$K_d \times 10^6$ (s^{-1})	m_{CHM} (nmol O_2 m^{-2} s^{-1})		References
	$E_{CHM} = 10.8$	$E_{CHM} = 15.3$	De Visser et al. (1992)
0.306	38	54	Dungley and Davies 1982
0.389	49	69*	Ibid.
1.113	139*	197*	Ibid.
2.265	284*	402*	Ibid.
2.377	298*	422*	Eising and Gerhardt 1987
3.241	406*	575*	Barneix et al. 1988
4.861	609*	863*	Davies 1982
5.961	748*	1 060*	Eising and Gerhardt 1987

Tab. 5. Derivation of the degradation constants (K_d; using Eqn 1) corresponding with the various maintenance respiration rates for protein turnover (m_{CHM}). For growing leaves, s_{CHM} was calculated according to Eqn 2. In full-grown leaves no net synthesis occurs ($s_{CHM} = 0$). m_{CHM} is the difference of r_{CHM} and s_{CHM} (Eqn 3). The values of s_{CHM}, m_{CHM} and K_d are based on an E_{CHM} value of 10.8 or 15.3 mol ATP (mol peptide bond)$^{-1}$ (cf. Tab. 1 and De Visser et al. 1992) as indicated by the superscript a or b, respectively.

Leaf tissue	r_{CHM}	s_{CHM} (nmol O_2 m^{-2} s^{-1})	m_{CHM}	$K_d \times 10^6$ (s^{-1})	Remark on the value of r_{CHM}
Growing leaves	260	221[a]–313[b]	–[b]– 39[a]	–[b]–0.43[a]	Lowest observed effect (Fig. 4A)
	442	221[a]–313[b]	129[b]–221[a]	0.99[b]–2.42[a]	Highest observed effect (Fig. 4A)
Full-grown leaves	91	0	91	0.51[b]–0.73[a]	Highest detection limit (Fig. 4B)
	140	0	140	0.79[b]–1.12[a]	Highest observed effect (Fig. 4B)

measurements (similar pattern as shown in Fig. 2), it is concluded that induction of EFE-activity is a useful probe for evaluating the in vivo action of CHM.

The effect of CHM on respiration

The respiration rate of leaf discs after approximately 5 h in the absence of CHM follows the trend we observed on primary leaves of intact bean plants: respiration (μmol O_2 m^{-2} s^{-1}) = – 0.051 × age (days) + 2.11 (n = 31) for ages between 10 and 20 days. Therefore, leaf discs are considered representative of intact leaves.

CHM inhibits 80% of the energy costs involved in protein turnover (Tab. 1, cf. De Visser et al. 1992) of all nucleus-encoded proteins, irrespective of their individual rates and pathways of turnover and synthesis. The fraction of protein synthesis in the organelles (F_{org}) was assumed to be 30%, because (1) less than 10% of all the mitochondrial proteins are encoded by and synthesized in the mitochondria (Leaver and Gray 1982), and (2) ribulose bisphosphate carboxylase/oxygenase, which constitutes approximately 1/3 of total organic nitrogen in mesophyll cells (Dalling 1987), is for approximately 75% of its subunits encoded by DNA of the chloroplast (Ellis 1981, Mullet 1988) and (3) most of the other chloroplast proteins are encoded by the nucleus (Ellis 1981).

After correcting the *measured* CHM effect on respiration of growing leaves (r_{CHM}; Tab. 5) for all processes involved in protein synthesis and for the synthesis in the organelles by using Eqn 5, total costs of overall protein synthesis (including protein turnover when present) were up to 59% (i.e. $r_p = 0.77$ μmol O_2 m^{-2} s^{-1}) of total dark respiration (approximately 1.3 μmol O_2 m^{-2} s^{-1}; Fig. 4A). Similarly, overall theoretical costs of *net* protein synthesis of a growing leaf, without regarding turnover, were 24 to 42% (i.e. $s_p = 0.32$ to 0.55 μmol O_2 m^{-2} s^{-1}; using Eqn 5 and s_{CHM} given in Tab. 5) of the respiration measured in the absence of CHM. Thus, using Eqn 6, m_p is found to be 17 to 35% of total respiration.

In full-grown leaves, the inhibition of respiration in the presence of CHM (6.9 and 11 h; Fig. 4B) occurred later than expected, as both ethylene production and respiration of growing leaves showed a CHM response after 4 and 2.5 h, respectively. From the largest CHM effect observed in full-grown leaves (r_{CHM} equal to m_{CHM} as s_{CHM} is zero; 11%; Fig. 4B), the fraction of respiration associated with protein turnover in all cell organelles (m_p) was calculated to be up to 17–21% of leaf dark respiration rate (cf. Eqn 4). These values represent an upper limit, as (1) the largest CHM effect was used in the calculation and (2) secondary effects cannot be excluded completely, despite the short-term measurements (see next paragraph on non-specific effects of CHM). From the low values for the upper limit, we conclude that protein turnover does not require a major amount of respiratory energy in full-grown primary bean leaves. These low costs must be due to a relatively low turnover rate in this tissue, as it is unlikely that the specific costs of protein turnover (values in Tab. 1; after De Visser et al. 1992) have been overestimated.

The fraction of respiration associated with protein turnover obtained by the present approach, is in the lower part of the range of experimental values derived from relationships between respiration rate and protein-N (5 to 76%; after Tab. 2 in De Visser et al. 1992). This supports the contention that such a relationship between respiration rate and protein-N may be determined by more than the process of protein turnover alone. For example respiratory costs involved in nocturnal carbohydrate export might be higher in leaves with higher protein content (cf. De Visser et al. 1992). Also the theoretical estimates of the fraction of leaf respiration associated with protein turnover (De Visser et al. 1992) are somewhat higher than the present experimental values. For full-grown leaves, this is due to the higher turnover rate as assumed by these authors ($K_d = 1.39 \times 10^{-6}$ s^{-1}), than calculated from our measured CHM inhibition of dark respiration rate (Tab. 5). For growing leaves, the differences might be attributed to the relatively high overall dark respiration rates in our growing leaves. Clarkson et al. (1992) observed 20 and 31% inhibition of the respiration of apical and basal parts of barley roots, respectively, after a 2-h incubation with CHM. A direct comparison of this relative CHM effect to the present results is hampered by the functional differences of leaves and roots. Assuming E_{CHM} to be 10.8 and 15.3, this respiratory inhibition would be explained by K_d values of 2.7 to 1.9×10^{-6} s^{-1}, respectively (i.e. assuming 10% dry matter, $P/O_2 = 5$ and $N_t = 3$). These values are within the range of experimental estimates in the literature (see Tab. 4), but relatively high compared to the K_d value of our tissue, especially for full-grown leaves (Tab. 5). In conclusion the present values for

relative costs and rates of protein turnover are relatively low compared to literature data.

The relatively low values derived for the turnover rates (Tab. 5; especially for full-grown leaves) compared to literature values (e.g. Tab. 4, Clarkson et al. 1992), might be due to a discrepancy in the turnover rate between the light and dark period. Unfortunately, the present method does not allow the determination of the respiratory costs of protein turnover in the light. Therefore, the present values are based on the assumption that turnover rates are constant during the diurnal cycle. The differences between the results mentioned above, do not necessarily indicate that this assumption is incorrect. For example, the K_d-values that can be derived from the regression data in Tab. 2 in De Visser et al. (1992) when using an E_{sp} of 11.8 to 20.8 (i.e. 1.5 to 7.5×10^{-6} s^{-1}), are also based on measurements in the dark. There is clearly a need for more information concerning the diurnal pattern of protein turnover.

Evaluation of the action of cycloheximide

The induction of EFE activity proved to be a useful probe for evaluating the in vivo action of CHM. To justify the present experimental approach, primary effects of CHM on the energy transfer should also be excluded (Ellis and MacDonnald 1970). Primary effects of CHM on energy metabolism have been posed (Dheidah and Black 1976), be it on indirect evidence like the reaction time. On the other hand, several studies support the hypothesis that the effects of CHM on respiration are indirect. Renosto and Ferrari (1975) found that the mechanism of sulfate transport inhibition by CHM in plant tissue is a blocking of the synthesis of new proteins, not a disruption of energy metabolism. A primary effect of CHM on energy supply was also excluded in studies with heterotrophic tobacco cells (Rennenberg et al. 1989). Clarkson et al. (1992) concluded from the absence of an effect of CHM on the uptake of P_i that the general effect of CHM on energy metabolism is minimal over a period of at least 4 to 8 h. Summarizing, CHM does not affect respiration by a primary effect in the present short-term experiments. This conclusion is supported by the absence of an effect of CHM on the respiration rate of full-grown leaves (Fig. 4B). Inhibition of metabolic processes by CHM due to the disappearance of the proteins involved, is unlikely in the present short-term measurements. Even so, the implication of such indirect effects would be that the upper limits of energy costs for protein turnover are overestimated, and thus does not affect the present conclusions.

Conclusions

The ACC-induction of EFE activity is a useful probe to calibrate and verify the in situ action of inhibitors of protein synthesis like CHM. Protein turnover in a *growing primary leaf* of bean requires maximally 17–35% of total dark respiration. Turnover costs in *full-grown pri-*

mary leaves are relatively low (i.e. at most 17–21% of total dark respiration), but in the range of theoretical values (De Visser et al. 1992).

The present method offers perspectives to determine respiratory costs associated with protein synthesis and turnover for different species in various environments. Other inhibitors of cytosolic protein synthesis (e.g. an inhibitor of transcription like cordycepin; Galling 1982), might also be applied as long as they do not have primary effects on respiration. It should be noted that a disadvantage of transcription inhibitors is a continuing translation from existing mRNA's, which will result in a delayed effect on protein synthesis.

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