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Amino acid consumption by *Chloroflexus aurantiacus* in batch and continuous cultures

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Abstract. Amino acid consumption was studied with batch and continuous chemostat cultures of *Chloroflexus aurantiacus* grown phototrophically in complex medium with casamino acids (Pierson and Castenholz 1974). Amino acids like Arg, Asx, Thr, Ala, Tyr, which were utilized during the early exponential phase by cells grown in batch cultures were consumed in chemostat cultures essentially at any of the dilution rates employed (0.018–0.104 h^{-1}). Those amino acids which were taken up during subsequent phases of growth were consumed in chemostat cultures preferentially at low dilution rates. For example, the consumption of Glx was enhanced during the late exponential phase and at low dilution rates. At high dilution rates Glx was not consumed at all. Since Glx utilization largely paralleled bacteriochlorophyll formation, it is discussed that formation of the photopigment depends on the intracellular availability of Glu as the exclusive precursor for tetrapyrrole synthesis.

Key words: *Chloroflexus aurantiacus* – Continuous culture – Bacteriochlorophyll – Amino acid utilization

The photosynthetic apparatus of green phototrophic bacteria is located in two distinct subcellular structures, the chlorosomes and the cytoplasmic membrane. In the thermophilic, facultatively phototrophic *Chloroflexus aurantiacus*, chlorosomes represent light harvesting units characterized by the presence of bacteriochlorophyll (Bchl) *c*, while the cytoplasmic membrane houses Bchl *a*-protein complexes fulfilling both light harvesting and photochemical reaction center functions. As far as studied, the two functionally different Bchl *a*-protein complexes of the cytoplasmic membrane are present at constant proportion (Feick et al. 1982). However, variations in the proportion of Bchl *c* to Bchl *a*, representing variations in the size of antenna units per photochemical reaction center, have frequently been observed (Pierson and Castenholz 1974; Schmidt et al. 1980; Fuller and Redlinger 1985; Foster et al. 1986).

In a first report it was concluded that these variations resulted from changes in illumination or oxygenation of cultures of *C. aurantiacus* (Pierson and Castenholz 1974). Later, however, it could be shown that, under anaerobiosis and constant illumination, the Bchl *c*/Bchl *a* ratio varied as the growth rate was varied (Oelze and Fuller 1987; Golecki and Oelze 1987). This raises the question as to the nature of nutritional factors controlling the growth rate.

In the experiments reported above, *C. aurantiacus* was grown in a complex medium containing casamino acids as well as yeast extract. Since it is known that *C. aurantiacus* utilizes different amino acids for biomass production with different efficiency (Heda and Madigan 1986), we started the investigations on the control of Bchl protein complexes by analyzing amino acid consumption by *C. aurantiacus* growing in complex medium in either batch or continuous culture systems. It will be shown that the organism exhibited different patterns of amino acid utilization depending on the stage and the rate of growth, respectively.

Materials and methods

Organism and growth conditions

Chloroflexus aurantiacus strain J-10-fl (Pierson and Castenholz 1974) was grown in medium D (Castenholz 1969) supplemented with (w/v) 0.1% yeast extract, 0.25% casamino acids and either 0.05% or 0.02% Gly-Gly at pH 8.3. Cultures used for inoculation were grown with illumination in screw-capped bottles at 56° C. For growth experiments in either batch or in chemostat systems, 1 l jacketed culture vessels (Wheaton-Celstir) were equipped as described previously (Oelze and Fuller 1987).

Chemical analyses

In order to determine Bchl *a* and Bchl *c* contents, culture samples were removed from the culture vessel, sedimented by centrifugation and extracted with acetone/methanol (7:2, v/v) at room temperature in the dark for 20 min. Pigment concentrations were calculated on

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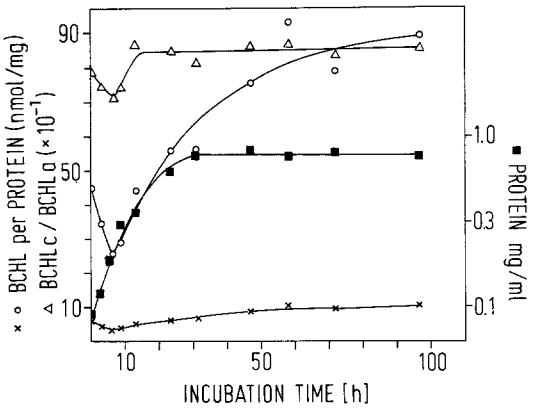

Fig. 1. Phototrophic growth of *Chloroflexus aurantiacus* in batch culture. The cells were grown at 57° C in complex medium containing casamino acids (0.25%), yeast extract (0.1%), and Gly-Gly (0.05%) as described in the Materials and methods section. The culture was illuminated with 50 klx of white light. Cell protein (■), nmol bacteriochlorophyll *a* per mg of cell protein (x), nmol bacteriochlorophyll *c* per mg cell protein (○); bacteriochlorophyll *c* per bacteriochlorophyll *a* (△) × 10

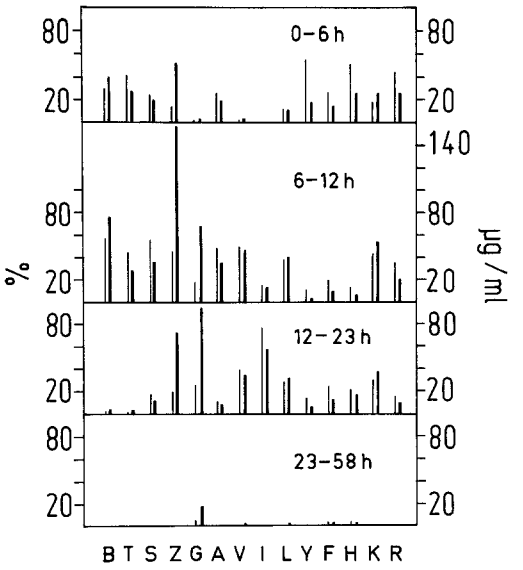

Fig. 2. Amino acid consumption by *Chloroflexus aurantiacus* growing phototrophically in batch culture as described in the legend to Fig. 1. Amino acid analyses were performed with culture fluids isolated after culture intervals as indicated. Left thin lines: percent consumed per total amount of an individual amino acid present in fresh medium; right bold lines: amount (µg/ml) of amino acid consumed. Asx (B), Thr (T), Ser (S), Glx (Z), Gly (G), Ala (A), Val (V), Ile (I), Leu (L), Tyr (Y), Phe (F), His (H), Lys (K), Arg (R)

the basis of molar absorption coefficients of $E_{767} = 76\ mM^{-1}\ cm^{-1}$ (Clayton 1966) and $E_{666} = 74\ mM^{-1}\ cm^{-1}$ (Feick et al. 1982) for Bchl *a* and Bchl *c*, respectively.

Protein contents of washed cell suspensions were estimated colorimetrically (Lowry et al. 1951).

Amino acid analysis

Culture samples were centrifuged (48,000 × *g*, 4° C, 30 min). The cell-free supernatants (200 µl each) were dried by a stream of nitrogen gas at 40° C and subjected to total acid hydrolysis (4 M HCl, 105° C, 18 h) in sealed tubes. After removal of HCl by a stream of nitrogen gas at 40° C the dry residue was dissolved in 600 µl sodium citrate (0.1 M) buffer, pH 2.2, containing 2,2′-thiodiethanol and 50 µl hydrolysate was analyzed in an automatic amino acid analyzer, model LC 6001 (Biotronik, München, F.R.G.) equipped with an autosampler (sample injector BT 7040). Elution of the cation exchange column (resin type BTC 2710) was performed sequentially with five sodium citrate buffers (0.1 M, pH 3.31, 13 min; 0.1 M, pH 3.87, 22 min; 0.08 M, pH 4.3, 15 min; 0.06 M, pH 5.20, 25 min; 0.03 M, pH 9.95, 30 min) at a flow rate of 20 ml/min. The initial column temperature of 50° C was increased to 58° C after an elution time of 23 min followed by an increase to 63° C after 70 min. Amino acids were detected after their elution from the column by ninhydrin reaction at 100° C and were quantitatively determined by absorbance measurements at 570 nm and 440 nm (for Pro), respectively.

Results

Growth in batch culture

In order to study the course of casamino acid consumption on the basis of single amino acids, *Chloroflexus aurantiacus* was grown phototrophically in batch culture. Typical growth kinetics observed under these conditions are demonstrated in Fig. 1. Since exponentially growing cells were used for inoculation, the cultures started to grow without significant lag phase. Specific contents of Bchl *c* and, to a lesser extent, of Bchl *a* decreased during the first phase of growth and increased again after about 6 h of growth. This decrease was due to a lag in Bchl formation while cell protein formation started directly at the beginning of the experiment. Overall, this initial decrease in specific Bchl contents resulted in a relatively slight decrease in the Bchl *c* to Bchl *a* ratio from 8–7, followed by an increase until, after about 15 h of growth, the ratio reached a constant value of 8.5.

At that time the culture approached about half of its biomass level finally attained after 30 h of cultivation. This infers that, in the second half of growth, increased self-shading because of one doubling of cell mass did not affect the ratio of Bchl *a* to Bchl *c*.

Amino acid levels in the culture fluids were analyzed after the following periods of cultivation: period 1 (0–6 h), period 2 (6–12 h), period 3 (12–23 h) and period 4 (23–58 h). These periods correspond to the early (1) and the intermediate (2) phases of exponential growth, the phase of decreasing growth rate (3), and the stationary (4) phase of growth. From the levels of amino acids present in fresh and in spent medium, respectively, amino acid utilization during the four periods was calculated. The results are depicted together with the percentages utilized of individual amino acids present in fresh medium (Fig. 2). Contamination by cellular material of *C. aurantiacus* could be excluded by the absence of peptidoglycan-specific components (Jürgens et al. 1987) such as amino sugars (muramic acid, glucosamine).

Obviously, the patterns of amino acid consumption changed when the cultures passed different phases of growth. For example, during the exponential phase (0–12 h) by far most of Asx, Thr, Ala, Tyr, and Arg were used up. On the other hand, in the course of period 3 cells preferentially utilized Gly, Glx as well as Ile. In the stationary phase of growth amino acid utilization was largely restricted to Gly. With respect to the latter amino

Table 1. Steady state cell protein levels as well as specific bacteriochlorophyll (Bchl) *a* and *c* contents of chemostat cultures of *Chloroflexus aurantiacus* grown at constant illumination (50 klx) and different dilution rates (D) as indicated

D	Protein	Bchl *a*	Bchl *c*
(h^{-1})	(mg/ml)	(nmol/mg protein)	
0.018	0.74	5.4	53
0.031	0.56	7.0	34
0.049	0.43	7.5	26
0.075	0.37	5.0	14
0.104	0.21	2.8	4

acid, it should be noted that most of Gly was derived by hydrolysis from Gly-Gly, i.e. the buffer substance. Analyses performed with non-hydrolyzed samples revealed that by the end of period 4 up to 65% of the buffer substance had been utilized.

When estimating the rate of amino acid utilization, it should be considered that the cell protein level reached by the culture at the end of period 1 was doubled in the course of period 2. This indicates that, within periods 1 and 2, *C. aurantiacus* consumed comparable amounts of Asx, Ser, Ala, and Lys. However, utilization of Glx was significantly increased and utilization of Gly and Val was only started in the second period of exponential growth.

In batch culture, the cells pass different stages of limitation. The degree of amino acid limitation may be assumed as important reason for the observed sequence of amino acid utilization. Further informations on this aspect should be derived from experiments with continuous chemostat cultures, which can be arrested at different stages of limitation.

Growth in continuous culture

It has been reported that increasing the dilution rate ($D = \mu$, i.e. the growth rate) of a constantly illuminated chemostat culture of *C. aurantiacus* resulted in decreases in the steady state biomass level and in the Bchl *c*/Bchl a ratio (Oelze and Fuller 1987). Comparable experiments were performed in the present investigation and, for reference, cell protein levels together with specific Bchl contents attained by *C. aurantiacus* at representative steady states are compiled in Table 1.

Amino acid analyses performed with hydrolyzed culture supernatants showed that steady state concentrations of practically all of the amino acids increased with increasing D. From the concentrations of individual amino acids present in fresh and spent medium, respectively, amino acid consumption was calculated (Table 2). Again, contaminating amino acids from cellular material of *C. aurantiacus* were absent as improved by the lack of peptidoglycan-specific amino sugars (Jürgens et al. 1987) such as muramic acid or glucosamine. Evidently, consumption of amino acids decreased with increasing D. It should be noted, however, that, with individual amino acids, the magnitude of these decreases showed considerable differences. For example, Glx was the amino acid

Table 2. Amounts of amino acids (μg/ml) consumed by *Chloroflexus aurantiacus* grown phototrophically (50 klx) in continuous chemostat culture at different dilution rates (D) on complex medium (Pierson and Castenholz 1974)

Amino acid	D (h^{-1})				
	0.018	0.031	0.049	0.075	0.104
Asx	98.9	91.2	84.9	80.1	51.4
Thr	51.5	50.0	47.7	46.2	20.2
Ser	44.0	41.5	31.4	25.6	5.3
Glx	132.2	104.4	49.5	5.4	0
Ala	38.8	30.8	26.5	25.5	10.0
Val	71.4	39.5	25.5	14.6	1.0
Ile	21.0	27.9	20.0	16.0	4.2
Leu	51.4	45.6	37.4	30.7	15.1
Tyr	12.4	21.9	11.3	10.0	0
Phe	16.9	14.2	13.7	9.6	5.0
His	25.5	31.8	17.1	23.1	20.4
Lys	105.0	50.0	41.0	40.0	28.0
Arg	33.7	28.4	25.3	25.0	21.5
Total	740	577	425	330	155

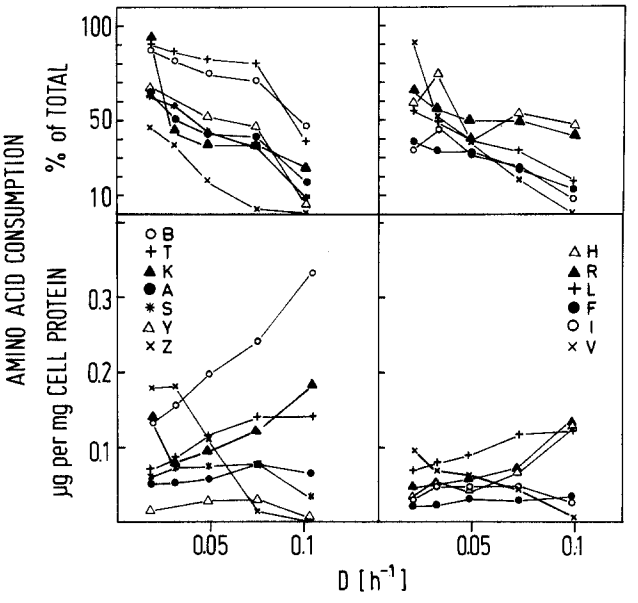

Fig. 3. Amino acid consumption by *Chloroflexus aurantiacus* growing phototrophically in chemostat culture at different dilution rates (D [h^{-1}]). For other growth conditions and symbols, see the legends to Figs. 1, 2. Upper part: percent consumed per total amount of an amino acid present in fresh medium; lower part: amount of an amino acid consumed per cell protein (μg/mg)

preferentially utilized at low D while at high D it was not consumed at all. On the other hand, the D-dependent decreases in the consumption of Asx, Thr, Arg, His, Phe were relatively low. Gly, largely derived from Gly-Gly in the course of sample hydrolysis, was essentially not utilized by chemostat cultures of *C. aurantiacus*. At the most, 15% of the original amount of Gly was consumed at the lowest D of 0.018 h^{-1}.

Calculation of the percentage of amino acids consumed revealed that only about 50% of Glx was utilized at the lowest D-value and none at the highest (Fig. 3).

Asx and Thr were the amino acids which were consumed at highest proportions in the range of $0.018-0.075\ h^{-1}$. At $D = 0.104\ h^{-1}$ about 50% of these amino acids remained detectable in the growth medium. His and Arg exhibited the least pronounced D-dependent variation and were consumed up to about 50%. The relative consumption of most of the other amino acids analyzed decreased from about 60% at low to 10–20% at high D.

The amounts of individual amino acids consumed per cell protein, i.e. specific consumption of amino acids is depicted in Fig. 3B. Interestingly, specific utilization of Asx and, to a minor extent, of Thr and Lys increased with decreasing D. The opposite was true with Glx, which showed the strongest decrease under the same conditions, while utilization of Ala, Ser, Thr, Phe and Ile per cell protein was largely independent of variations in D.

Discussion

Comparison of the patterns of amino acid consumption by batch and chemostat cultures of *C. aurantiacus* reveals that utilization of most of those amino acids, which were taken up during early phases of growth in batch culture, decreased relatively slightly upon increasing D. This holds true for Asx, Arg, and, except for the highest D-value, also for Thr, Ser, Ala, and His. Steady state concentrations of those amino acids, which were utilized at later stages of batch growth, preferentially decreased at low D-values. For example, Glx uptake was largely restricted to the lower D-values, while at higher D-values hardly any Glx disappeared from the medium. A comparable dependency was observed with respect to Val. The most pronounced dependence on the growth stage was observed with Gly, respectively Gly-Gly. This compound, hardly used by chemostat cultures, became the preferred substrate in the latest phases of growth in a batch culture. This indicates that about two thirds of Gly-Gly i.e. the buffer substance may be consumed by batch cultures of *C. aurantiacus*.

Overall, the sequential as well as the D-dependent utilizations are reminiscent of diauxic growth with combinations of a more and a less preferred substrate. In any case, it is important to note that cultures of *C. aurantiacus* are subject to significant changes in nutritional conditions, when growing with a mixture of amino acids in batch culture or in chemostats at different D-values. This in turn should create physiologically different populations.

Utilization of Glx is of particular importance with respect to Bchl synthesis and, thus, the formation of the photosynthetic apparatus. *C. aurantiacus* is known to synthesize tetrapyrroles via the C_5-pathway, which utilizes Glu as the only precursor (Avissar et al. 1989; Kern and Klemme 1989). As a matter of fact, either with batch or with chemostat systems, the highest rate of synthesis particularly of Bchl *c* was observed when the cells consumed the highest amounts of Glx. The importance of this nutritional effect on the control of Bchl *c* synthesis is demonstrated by the behaviour of cells in the chemostat system. Through previous investigations it is known that raising D in a constantly irradiated continuous culture decreases the irradiance effective within the culture (Pipes and Koutsoyannis 1962). This should lead to an increase in the formation of Bchl *c* as well as in the Bchl *c*/Bchl *a* ratio. The opposite, however, was observed when D was increased under conditions of the present investigation. This suggests that the decreased availability of Glx within the cells rather than light controlled Bchl formation.

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