Journal of Bioenergetics and Biomembranes (1977) **9**, 363–372

Modification of Amino Acid and Sugar Transport in Uncoupler-Adapted *Euglena gracilis**

Joseph S. Kahn and Randy T. McConnell†

Department of Biochemistry, North Carolina State University, Raleigh, North Carolina 27607

Received 22 August 1977

Abstract

Uncoupler-adapted *Euglena gracilis* have a greatly impaired capacity to take up and incorporate some exogenously supplied amino acids and sugars. The degree of inhibition varies widely from >90% in the case of valine or glucose to none in the case of histidine. The inhibition is due to a decreased activity of the transport mechanism itself and is not due to either a lesion in the control mechanism for endogenous amino acid or sugar synthesis nor to a direct inhibition of the transport mechanism by uncouplers.

No preferential labeling of mitochondrial membranes by [^{14}C]amino acids occurs during the process of adaptation, a time when no cell division occurs. Apparently, during the long time required for adaptation, there occurs no major modification of mitochondrial proteins which could explain the subsequent resistance to uncouplers.

Introduction

Euglena gracilis has been shown [1, 2] to undergo a reversible physiological adaptation, which does not involve a mutation, to effectors of oxidative phosphorylation. The effectors tested include the uncouplers 2,4-

* Contribution from the Department of Biochemistry, School of Agriculture and Life Sciences and School of Physical and Mathematical Sciences. Paper No. 5179 of the Journal Series of the North Carolina Agricultural Experiment Station, Raleigh, North Carolina 27607.

† Present address: Molecular Biology Department, Burroughs-Wellcome Company, Research Triangle Park, North Carolina 27707.

This journal is copyrighted by Plenum. Each article is available for $7.50 from Plenum Publishing Corporation, 227 West 17th Street, New York, N.Y. 10011.

dinitrophenol (DNP),‡ carbonylcyanide, *m*-chlorophenylhydrazone, and 1799, as well as the inhibitors oligomycin and tri-*n*-butylchlorotin. The effectors were not excluded from the cells, and cells adapted to any one of the effectors showed resistance to all of the others. When adapted, growth and metabolism of the cells were hardly distinguishable from those of normal cells, and doubling time was only slightly extended. Efficiency of utilization of exogenous carbon source (ethanol, glutamate) was only slightly reduced in adapted cells. The adaptation was lost after three to four generations in effector-free medium. It appeared that lowering of "energy charge" rather than the presence of the effectors *per se* induced the adaptability. Mitochondria isolated from the DNP adapted cells did not show any appreciable difference when compared to normal mitochondria, with regard to their respiration rate, ATPase activity, P:O ratios, or the concentration of the effectors required for 50% inhibition of phosphorylation. Substrate consumption for growth was only slightly higher in adapted cells than in normal ones. There was no evidence of the presence of any protein that could bind uncouplers tightly.

We report here that uncoupler adaptation in *Euglena* does not entail a major modification of mitochondrial proteins during the process of adaptation. We will also report on major changes in amino acid and sugar transport in adapted cells as compared to normal ones.

Materials and Methods

Preparation of UV-Bleached Mutants

Wild-type cells of *Euglena gracilis*, Z strain, were plated out on nutrient agar and illuminated for 2 min with a GE G30T8 germicidal lamp at a distance of 18 in. The plates were than kept under incandescent light at room temperature for 1 week. The few green colonies that appeared were removed with a wire loop and the plate flooded with growth medium and incubated for 4 hr with occasional gentle shaking. Samples of that medium were then plated on fresh agar plates and incubated in the light for a week, after which the flooding and replating were repeated. Finally, the fastest growing colony was removed and grown in liquid culture. The repeated flooding did not only ensure the selection of a rapidly growing mutant, but tended also to eliminate mutants with significant flagellar damage.

Cell growth in heterotrophic medium and harvesting were done as

‡ Abbreviations used: DNP, 2,4-dinitrophenol; 1799, 1,7-hexafluoro-2,6-dihydroxyd2,6-*bis* (trifluoromethyl)-4-heptanone; SDS, sodium dodecyl sulfate.

previously described [3], as was the isolation and purification of mitochondria [4].

To determine amino acid or sugar incorporation into cell carbohydrate or proteins, 0.2–1μCi of uniformly labeled [^{14}C]amino acids or sugars was added to 50 ml of growth medium either carrier free (~250 mCi/mmole, $<10^{-7}$ M) or with 2×10^{-3} M carrier. After incubation, cells were harvested by centrifugation (1000 × *g*, 5 min) and washed twice with 10^{-3} M NaCl + 2×10^{-3} M of the amino acid or sugar used. The cells were finally extracted with 10 ml of 4.5% cold trichloroacetic acid, followed with 5 ml of 1% of the same. The pellet was solubilized with 1 N NaOH and the radioactivity of both the pellet and the TCA extract was determined by scintillation counting using an Omnifluor (New England Nuclear)/Triton X-100 cocktail (2 : 1). In every case the activity in the TCA extract was negligible compared to that in the pellet.

For short-term rates of uptake cells were harvested in mid-log phase, washed, and resuspended in fresh medium. After shaking for 1 hr, 2–4 μCi [^{14}C]amino acid or sugar was added with 2×10^{-3} M carrier. Samples containing 2.5 mg protein were removed at zero time and at 10-min intervals, mixed with excess unlabeled amino acid or sugar, centrifuged rapidly at 1200 × *g* for 5 sec, the pellet resuspended in the unlabeled additive, and recentrifuged. The pellet was finally suspended in 2.5 ml of 10^{-3} M NaOH, and 1 ml taken for counting. Total time elapsed between removal of sample and addition of the NaOH was <2 min. In preliminary experiments we found that the initial rate of uptake was linear for at least 1 hr, and for as long as 4 hr in the case of some amino acids.

Paramylum from cells grown in ^{14}C-labeled amino acids or sugars was purified by precipitating the washed cells with 1 N $HClO_4$, then extracting the pellet twice with 95% ethanol. The pellet was then suspended in 1 N $HClO_4$ and heated in a boiling water bath for 2 hr. The precipitate was collected by centrifugation (12,000 × *g*, 10 min), washed twice with distilled water, dissolved in 1 N NaOH, and held at room temperature for 2 hr. Perchloric acid was then added to pH 3, and the precipitate collected by centrifugation (37,000 × *g*, 20 min) and washed twice with distilled water. Samples for dry weight, carbohydrate, protein, and radioactivity assays were collected from the washed cells, $HClO_4$-digested precipitate, and NaOH-digested precipitate. The final pellet contained about 15% protein by weight. A plot of protein content versus radioactivity extrapolated to 0 protein corrected for any residual activity due to the protein.

Protein was determined by the method of Lowry et al. [5], using bovine serum albumin as standard.

Labeled amino acids were purchased from New England Nuclear and from ICN.

Results

Cells of *Euglena gracilis* that were induced to adapt by growth in the presence of arsenate [2], then transferred to medium containing DNP, and labeled with [^{3}H]phenylalanine, showed no significant enrichment of the label in the mitochondrial membrane fraction (Table 1). In addition, when cells in the process of adaptation were labeled for short periods of time (3 hr) with [^{14}C]phenylalanine, the amount of label in the mitochondria relative to the whole cell actually decreased (Table II). The same cells, when treated with 100μg/ml of chloramphenicol—an inhibitor of mitochondrial protein synthesis—in addition of the DNP, required the same length of time (150–160 hr) to adapt. Taken together, these data indicated no significant protein synthesis in the mitochondria during adaptation.

One striking feature in Table II was the fact that even the adapted cells (after 150 hr), which were growing vigorously, incorporated only a fraction of the label as compared to normal cells (time 0). Consequently, we investigated further the utilization of exogenous amino acids by normal and adapted cells. Preliminary experiments had indicated that within 12–16 hr the bulk of the protein of the cells introduced into the labeled medium turned over so that between those times a constant specific activity of the protein was reached. The data (Table III) show that the adapted cells have a drastically reduced capacity to utilize most amino acids in the medium for protein synthesis. The reduction was essentially the same whether the concentration of amino acids in the medium was 2×10^{-3} or $< 10^{-7}$ M. The higher concentration was potentially more than sufficient to

TABLE I. Labeling of Cell Fractions of *Euglena gracilis* Transferred to Dinitrophenol-Containing Medium After Introduction in Arsenate-Containing Medium [a]

Cell fraction	L-(^{3}H)Phenylalanine (cpm/mg protein)	Activity relative to whole cell
Whole cells	53,330	1.00
1000 × *g* fraction	12,400	0.23
Mitochondria	42,540	0.80
Mitochondrial matrix	18,700	0.35
Mitochondrial membranes	72,970	1.37
20,000 × *g* supernatant	83,100	1.56

[a] Cells were incubated 48 hr in medium containing 2×10^{-3} M sodium arsenate in addition to the 2×10^{-3} M sodium phosphate. Cells were harvested and resuspended in 30 ml of growth medium containing 10^{-5} M dinitrophenol and carrier-free L-(^{3}H)phenylalanine. Incubation was terminated after 30 hr when microscopic examination revealed signs of active, adapted cells.

TABLE II. Incorporation of [^{14}C]Phenylalanine into Whole Cell and Mitochondrial Protein of *Euglena gracilis* during the Adaptation Period [a]

Time sample Removed (hr)	L-[^{14}C]Phanylalanine (cpm/mg protein)		Ratio Mitochondria/ whole cell
	Mitochondria	Whole cell	
0	7347	8270	1.00
5	358	378	1.07
25	233	319	0.82
50	156	284	0.62
75	164	446	0.41
100	163	332	0.55
125	134	325	0.46
→150	243	523	0.52
190	207	365	0.64

[a] At 0 time, dinitrophenol (1×10^{-5} M) was added to a culture of exponentially growing *Euglena*. At the times indicated, samples of cells were removed, harvested, resuspended in a small volume of fresh medium containing carrier-free L-[^{14}C]phenylalanine, and incubated for 3 hr. Arrow indicates the time cells became adapted, i.e., commenced growing.

supply the needs of the cells without endogenous synthesis of that particular amino acid, the maximal depletion of the medium being 35% (serine) [6].

In all these experiments, the label in the TCA-soluble pool represented only a fraction of that in the protein. This pool size, however, was subject to the same decrease in adapted cells as was the protein pellet.

During 18–22 hr of incubation in the absence of DNP, adapted cells deadapt only partially [1]. In the absence of DNP, the adapted cells also show a decreased incorporation of [^{14}C]amino acids (Table III). However, since adapted cells accumulate high concentrations of DNP which will not leak out, the inhibition could be due to the remaining DNP in the cells which would have to be diluted out by cell division before increased amino acid uptake could commence. We consequently made use of the fact that the uncoupler 1799, which induces adaptation interchangeably with DNP, is not accumulated by the cells but leaks out readily due to its higher p*K*, the cells being permeable only to the un-ionized uncouplers [2]. We inoculated cells adapted to 1799 as in the experiments described in Table III and found that valine and phenylalanine incorporation in these cells was still partially inhibited as in DNP-adapted cells (Table IV). We concluded from this that at least part of the reduce incorporation of amino acids was not due to an inhibition of the preexisting transport mechanism by the uncoupler.

TABLE III. Incorporation of [^{14}C]Amino Acids into the Protein of Normal and DNP-Adapted Cells of *Euglena gracilis*[a]

Cell type and addition	Val	His	Phe	Ser	Leu	Pro	Arg
A. Medium containing 2×10^{-3} M of [^{14}C]amino acid indicated (μmole/mg protein)							
Normal	0.212	0.009	0.128	0.287	0.114	0.036	0.014
Adapted	0.057 (27)	0.012 (133)	0.053 (41)	0.226 (77)	0.116 (102)	0.008 (21)	0.006 (41)
Adapted + 10^{-5} M DNP	0.023 (11)	0.013 (136)	0.039 (30)	0.141 (49)	0.075 (65)	0.003 (9)	0.005 (34)
B. Medium containing carrier-free (<10^{-7} M) of [^{14}C]amino acid indicated (cpm/mg protein)							
Normal	7568	372	1663	12252	3742	2628	129
Adapted	1473 (19)	308 (83)	512 (31)	7794 (64)	3130 (84)	232 (9)	89 (69)
Adapted + 10^{-5} M DNP	450 (6)	290 (79)	306 (18)	7905 (65)	1153 (31)	183 (7)	84 (65)

[a] Cells were grown in medium with L-amino acid additions as indicated, 18 hrs for normal, 22 hr for adapted cells (about two doubling times), and then harvested and treated as described in Methods. Numbers in parentheses are percent of normal. Average of three experiments.

TABLE IV. Incorporation of [^{14}C] Amino Acids into the Protein of Normal and 1799-Adapted Cells of *Euglena gracilis*[a]

	Valine (μmole/mg protein)	Phenylalanine
Normal	0.233	0.141
Adapted	0.150	0.106
Adapted + 10^{-5} M DNP	0.042	0.078
Adapted + 2×10^{-4} M 1799	0.126	0.087

[a] Experimental procedure as in Table III, except that cells were adapted using 2×10^{-4} M 1799 instead of DNP. [^{14}C]amino acids were 2×10^{-3} M. Average of three experiments

We tested the incorporation of glucose, fructose, sucrose, and glycerol under the same conditions as for the amino acids. Sucrose was not incorporated at all. The results (Table V) show that the uptake of sugars was also drastically inhibited in the adapted cells.

Short-term experiments showed that the initial rates of uptake of amino acids and glucose follow a pattern similar to that of the long-range incorporation (Table VI). In the case of valine and phenylalanine, the rate increased somewhat after about 2 hr of incubation, but the ratio of rates between normal and adapted cells did not change.

With the exception of serine, all the amino acids we tested are not readily metabolized to other amino acids by *Euglena gracilis* [7]. Howevern they could be metabolized to other cell components, particularly paramylum and lipids. In the long-range experiments, however, we found only traces of radioactivity in the lipid fraction when serine or leucine was supplied to the cells and essentially none in the case of valine and phenylalanine. In the case of paramylum 5–10% of the label was found in that fraction regardless of treatment.

TABLE V. Incorporation of [^{14}C] Glucose, [^{14}C]Fructose, or [^{14}C]Glycerol into Pramylum of Normal and DNP-Adapted *Euglena gracilis*[a]

Cell type and addition	Glucose (nmole incorporated/μmole subunit of paramylum)	Fructose	Glycerol
Normal	6.15	11.80	1.51
Adapted − DNP	0.50	0.90	0.73
Adapted + DNP	0.08	0.22	0.45

[a] Conditions as in Table III, with 2×10^{-3} M of indicated ^{14}C-labeled sugar added to medium.

TABLE VI. Initial Rate of Uptake of Amino Acids and Glucose by Normal and DNP-Adapted Cells of *Euglena gracilis*[a]

Addition	Normal	Adapted (nmole/mg protein/min)	
		−DNP	+DNP
Val	0.136	0.064 (47)	0.022 (16)
Phe	0.074	0.051 (69)	0.026 (35)
Leu	0.213	0.192 (90)	0.132 (62)
Ser	0.216	0.178 (82)	0.117 (54)
His	0.014	0.016 (111)	0.012 (86)
Pro	0.013	0.011 (85)	0.004 (33)
Glucose	0.0468	0.0080 (17)	0.0024 (5)

[a]Experimental design described in Methods. Numbers in parentheses are percent of normal. Average of two experiments. All additions at 2×10^{-3} M.

Discussion

The absence of any perferential incorporation of [^{14}C]amino acids into mitochondrial membranes during the process of adaptation (Tables I and II) indicates that the adaptation does not involve the preferential synthesis of major new mitochondrial proteins. Moreover, in experiments separating the mitochondrial membranes of [^{3}H]phenylalanine-labeled cells on SDS-acrylamide disc gel electrophoresis, no specific peptides that were preferentially labeled were found [8].

The extent to which exogenously supplied amino acids supplant the endogenously synthesized ones varies greatly from one amino acid to the other. Using the data of Miller and Kempner on the amino acid composition of whole *Euglena* Cells [6] for comparison, we found that normal cells would use exogenous valine, phenyalanine, and serine preferentially (>67%), whereas histidine, proline, and arginine were used only to a limited extent (<11%). Leucine was intermediate (26%). However, there was no correlation between utilization of exogenous amino acids and the inhibition of their uptake due to the adaptation process: The most affected amino acids were valine, which was readily utilized, and proline, which was utilized only poorly.

The similarity of the results between the initial rates of uptake and the long-range incorporation indicates that the differences in utilization between normal and adapted cells are the result of a lesion in the transport system and not in the control system for endogenous amino acid or sugar synthesis. In addition, the fact that the reduction in [^{14}C]amino acid utilization in adapted cells was essentially the same whether or not carrier

was added to the labeled amino acid (2×10^{-3} versus $< 10^{-7}$M) also suggests that the lesion had to be in the transport system. Otherwise growth of normal cells in the carrier-free experiments would have been drastically inhibited, which it was not. The similarity of inhibition of incorporation at both concentrations of amino acids also indicated that the defect in the transport system was one of amount and not of a modification which would make it less efficient. This similarity of the inhibition is really unusual, and would indicate that the transport mechanism was inefficient at low amino acid concentrations, otherwise the cells would have depleted the medium rapidly in the carrier-free system. The fact that 1799-adapted cells, which do not retain the uncoupler when transferred to 1799-free medium, remained inhibited even in the absence of DNP or 1799 corroborates this conclusion (Table IV). If the inhibition of incorporation in these cells occurred because of a direct inhibition of the transport system by the uncoupler, the removal of uncoupler should have reversed the inhibition completely. Since it did not, the inhibition had to result from a decrease in the transport system *per se.*

In a recent publication [9], the uptake and incorporation of a number of [^{14}C]amino acids, including glutamate, by *Euglena gracilis* was shown to be inducible by preincubation for 3 hr. The cells in our study were grown in the presence of glutamate and, thus, should have had a fully induced transport system. In our study, amino acid and sugar uptake by adapted cells was inhibited even after the uncoupler was removed, and showed no further induction. Probably the induction system was not affected by the uncouplers. In another study, pretreatment of *Euglena gracilis* with nigericin, an ionophore, caused an inhibition of short-term uptake of DNP and glucose [10] probably due to a change in membrant permeability. Neither the effect of amino acids on their transport [9] nor the effect of nigericin on DNP and glucose uptake was dependent on protein synthesis. Both these studies were carried out, however, with normal cells, and involved only short-term changes. They are thus not comparable to uncoupler-adapted cells.

According to Kempner and Miller [7], serine alone of the labeled amino acids we used was converted to other amino acids in any significant amount. The fact that neither the lipids nor the paramylum showed any significant labeling by amino acids was not surprising, considering that the cells were growing with ethanol as carbon source, and growth on amino acids as sole carbon source is much slower.

The data presented here provide the first major difference between normal and uncoupler-adapted *Euglena gracilis.* The drastic reduction in the activity of the transport system in adapted cells could represent an abortive attempt at energy conservation by minimizing energy expenditure

for uptake. Since this would necessitate an increased endogenous synthesis, the net result of such an attempt would be a waste of energy. We have as yet no clue to the physiological significance of the reduced amino acid incorporation in the adapted cells.

Acknowledgments

We wish to thank Mrs. Thea Schostag and Mrs. Karen Klein for their excellent technical assistance throughout this work. The uncoupler 1799 was the gift of the E. I. DuPont Co. through the courtesy of Dr. P. Heytler.

References

1. J. S. Kahn, *Arch. Biochem. Biophys.,* **159** (1973) 649–650.
2. J. S. Kahn, *Arch. Biochem. Biophys.,* **164** (1974) 266–274.
3. I. C. Chang and J. S. Kahn, *J. Protozool.,* **17**(4) (1970) 556–564.
4. D. B. Datta and J. S. Kahn, *J. Protozool.,* **24**(1) (1977) 187–192.
5. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.* **193** (1951) 265–275.
6. J. H. Miller and E. S. Kempner, *J. Protozool.,* **23**(3) (1976) 444–446.
7. E. S. Kempner and J. H. Miller, *Biochemistry,* **4** (1965) 2735–2739.
8. R. T. McConnell, M. S. Thesis, North Carolina State University (1977).
9. B. Parthier, *Biochem. Physiol. Pflanzen,* **166** (1974) 555–560.
10. W. R. Evans, *J. Biol. Chem.,* **246** (1971) 6144–6151.