Journal of Bioenergetics and Biomembranes, Vol. 15, No. 6, 1983

The Adaptation to Salinity: Protein Synthesis and Some Aspects of Energy Transduction in Fish Gill Mitochondria

N. Suresh,[1,2] K. Shivakumar,[1] and J. Jayaraman[1]

Received December 6, 1982; revised April 20, 1983

Abstract

Exposure of freshwater fish to saline conditions brings about somewhat drastic changes in the mitochondrial energy metabolism. These include abolition of oxidative phosphorylation, ATP-induced contraction of swollen mitochondria and transhydrogenase activity. On the other hand the endogenous calcium levels and protein synthetic capacity are elevated. *In vitro* protein synthesis by mitochondria from freshwater and stressed fish shows qualitative and quantitative variations. Effluxing the excess calcium by treatment with NaCl or inhibiting the protein synthesis by chloramphenicol in stressed mitochondria restores almost all the functions. It is proposed that the energy potential formed by the mitochondrial membrane is channelized to perform different functions and that the ratio of channelization can be altered to suit the needs of the cell.

Key Words: Protein synthesis; energy transduction; salinity adaptation; gill mitochondria.

Introduction

We have demonstrated earlier that when the freshwater fish *Tilapia mossambica* (*Sarotherodon mossambicus*) is exposed to seawater (thereby imposing an ion-osmotic stress), mitochondria isolated from the muscle and gill tissues show drastic changes in both structure and function (Bashyam *et al.*, 1980; Suresh and Jayaraman, 1983). On prolonging the stress conditions, the mitochondria tend to recover to the normal freshwater status. During this recovery period, it was observed that there was a steady increase in the total

[1]Department of Biochemistry, School of Biological Sciences, Madurai Kamraj University, Madurai-625021, India.

[2]Present address: Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, Massachusetts 02254.

0145-479X/83/1200-0379$03.00/0

activities of various mitochondrial electron transport enzymes, whereas their specific activities remained constant (Suresh and Jayaraman, 1983). Analyses of mitochondrial DNA, RNA, and proteins under the above conditions have also shown a steady increase (Meenakshi *et al.*, 1979). To characterize this phenomenon further, we have now studied the protein synthesis in mitochondria isolated from gills of fish under normal and stress conditions.

As a response to stress, the organelles lose their capacity to synthesize ATP from externally added ADP, show a decreased capacity to take up $^{45}Ca^{++}$, and lose their ability to contract in the presence of ATP + Mg^{++}. However, the calcium levels are high and the protein synthetic capacity (as measured by amino acid incorporation) of isolated mitochondria increases severalfold. Further, these changes are transient, i.e., they last only for 2–7 days depending on the magnitude of the stress, and continued exposure of the stress situation restores the various activities to that of the freshwater fish. Interestingly though, the electron transport capacity of the mitochondria (as measured by succinate oxidation) does not show any change throughout the period. A situation thus arises where the conventionally accepted energy production in the form of ATP is knocked out, at least temporarily, but the mitochondria at the same time carry out the highly energy-demanding function of protein synthesis at an enhanced rate.

Based on the analyses of mitochondria isolated from a variety of organisms, we have shown earlier (Honnappa *et al.*, 1975) that a *prima facie* case exists for the differential channelization of energy produced by mitochondria to carry out different functions depending on the needs of the organism. The results mentioned above with fish encouraged us to explore this idea further.

Our logic is that only two parameters namely endogenous calcium levels and amino acid incorporation capacity, are enhanced in the mitochondria isolated from tissues of fish under early stress conditions, whereas many other functions are inhibited. Therefore, would blocking the enhancement of these two parameters by artificial means have any effect on the other functions? Gratifyingly, yes, lending credence to our hypothesis of the differential channelization of energy potential. The results are presented here.

Materials and Methods

Conditions of fish collection, maintenance, exposure to salinity stress, and isolation of gill mitochondria have all been adequately described earlier (Sulochana *et al.*, 1977; Suresh and Jayaraman, 1983). [^{14}C]-Chlorella hydrolysate, specific activity 42 mCi/matom C, was obtained from Bhabha Atomic Research Center, Bombay. The general protocol followed in all

experiments, unless otherwise mentioned, was to transfer the fishes from freshwater to the saline medium and at different time intervals take out samples for analyses. Since it has been shown that the behavioral pattern of mitochondria is identical, except for a shift in the time scale, in both 25 and 50% seawater-exposed fishes, both media were used interchangeably depending on the type of experiment and convenience. All experiments were carried out at least three times, and consistent patterns were found. But since the absolute values showed variation from one batch of fish to another, typical and not statistical results are given.

Protein Synthesis

(a) In Isolated Gill Tissue. Gill tissues (about 4 g) were carefully and quickly excised out from the fish and suspended in cold Ringer buffer containing 1% glucose. After equilibration at 30°C in a water bath shaker for a few minutes [^{14}C]-Chlorella hydrolysate (2 μCi/ml) was added. Following 1 h incubation, the tissue pieces were picked out and quickly rinsed in cold Ringer buffer; then the mitochondria isolated. The isolated mitochondria were processed by Bollum's method (1968) with minor modifications. The mitochondrial pellets were treated with cold 10% TCA and after centrifugation, the supernatant was discarded. The precipitates were again suspended in 10% TCA and kept at 70°C for 15 min. The precipitates obtained were successively washed with 10% cold TCA, ethanol, ethanol:ether (1/1 v/v), and finally with ether. The final pellet was dissolved in a minimal volume of 0.1 N NaOH and spotted onto Whatman No. 1 filter paper discs, and the radioactivity was measured in a liquid scintillation counter (efficiency for ^{14}C 80%).

(b) In Isolated Mitochondria. In this series of experiments, gill tissues were excised out and mitochondria isolated by differential centrifugation as before. The mitochondrial pellet was suspended in the normal polarographic medium used for respiration studies. The reaction was initiated by the addition of 2 μCi of [^{14}C]-Chlorella hydrolysate. After 30 min of incubation, the reaction was stopped by the addition of 1 ml 10% TCA, the pellet was processed as described earlier, and the radioactivity was determined. Sterile conditions were maintained throughout to avoid bacterial contamination. Occasionally, aliquots before and after incubation were plated out to verify for the absence of bacteria.

SDS-polyacrylamide gel electrophoresis was carried out using 10% acrylamide gels following the procedure of Weber and Osborn (1969). Staining was done with Coomassie blue. When radioactive samples were analyzed by slab gels, the dried gels were exposed to Kodak X-ray film and then developed. The developed films were scanned using a microdensitometer.

Calcium Efflux from the Mitochondria

Samples containing the required amount of mitochondrial protein were taken in separate tubes. NaCl was added to a final concentration of 20 mM. The mixture was incubated for 3 min at 4°C followed by centrifugation at 10,000 *g* in a Janetzki TH 12 centrifuge for 2 min in the cold. The pellets were washed twice with 0.25 M sucrose and used as such.

Chloramphenicol Treatment of Mitochondria

The mitochondria were incubated with chloramphenicol at a concentration of 200 μg/mg mitochondrial protein for 5 min at 4°C and spun at 10,000 *g* in a Janetzki TH 12 centrifuge for 2 min in the cold. The pellet was referred to as "CAP-treated" or chloramphenicol-treated sample. The treatment with tetracycline was also done in the same way with 200 μg/mg mitochondrial protein.

Analyses of Adenine Nucleotide Content

About 25–30 mg mitochondrial protein was treated with 10 ml cold ethanol, and stirring was maintained overnight at 0°C. The supernatant obtained after centrifugation at 10,000 *g* for 10 min was dried *in vacuo* and the residue dissolved in minimal amount of water. The nucleotides were separated by thin-layer chromatography on polyethylenimine-coated cellulose plates. The solvent used for mono- and dinucleotides was 1 N acetic acid:3 M LiCl, 9:1 (v/v), and for trinucleotides, 2 N acetic acid:2 M LiCl, 1:1 (v/v). After visualising the quenching spots under UV, the spots were eluted with ethanol and estimated spectrophotometrically.

Results

Amino Acid Incorporation into Mitochondria in Isolated Gill Tissue

Figure 1 shows the pattern of amino acid incorporated as proteins in mitochondria. It is seen that even on the first day after imposing the stress, the amount incorporated is almost twice the values before the stress, and is maintained up to about the 6th day. Thereafter it declines steadily to almost the normal levels by about the 15th day.

Amino Acid Incorporation by Isolated Mitochondria

Since our aim was to measure the intrinsic protein synthetic capacity of mitochondria under conditions where other parameters such as ADP/O ratio, etc. are measured, we have used the polarographic medium for these studies.

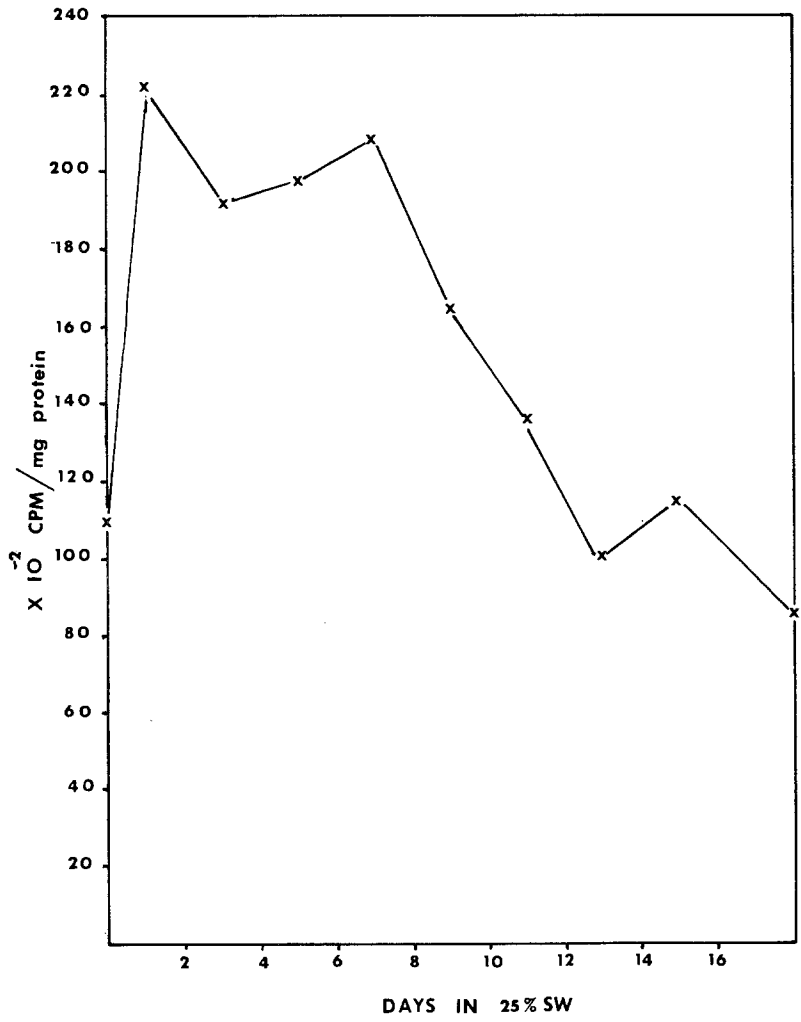

Fig. 1. Amino acid incorporation into mitochondria in isolated gill tissue exposed to 25% seawater. Gill tissues (about 4 g) were carefully and quickly excied out from the fish and suspended in cold Ringer buffer containing 1% glucose. After equilibration at 30°C in a water-bath shaker for 3 min, the reaction was started by the addition of [^{14}C]-Chlorella hydrolysate (2 μCi/ml). Following 1 h incubation, the tissues pieces were picked out, quickly rinsed in cold Ringer buffer, and the mitochondria isolated. The isolated mitochondria were processed as in Materials and Methods.

No additions for ATP regeneration were made. Under these conditions good incorporation was obtained—even better than those reported with all additions (Wheeldon and Lehninger, 1966; Satav *et al.*, 1973). The incorporation by mitochondria from gills of freshwater fish is given in Table I.

Interestingly, addition of substrates such as NADH, glutamate +

Table I. Characteristics of [^{14}C]-Amino Acid Incorporation by Gill Mitochondria of Freshwater Fish (Mitochondria alone, 29,000 cpm/mg protein)[a]

Additions	Stimulation (%)	Inhibition (%)
ATP	59	
ADP		38
NADH		54
NADH + ADP		53
Succinate + rotenone		56
Succinate + ADP		58
Glutamate + Malate		35
TMPD + Ascorbate + antimycin		49
Chloramphenicol		92
Cycloheximide		1
Azide		83
DNP		63
Oligomycin		33
Atractyloside		No effect
Bongkrekic acid		No effect
Shaking		27
Aeration		30

[a] Mitochondrial protein corresponding to about 1 mg was used per assay. The incubation medium was the same used for oxygen uptake. It contained 0.25 M sucrose, 0.005 M Tris-HCl, pH 7.4, 0.001 M EDTA, 0.005 M potassium phosphate buffer, pH 7.4, and 0.005 M $MgSO_4$. Wherever indicated, glutamate (10 mM), malate (5 mM), succinate (10 mM) NADH (5 mM), TMPD (1 mM), ascorbate (10 mM), ATP (1 μM), ADP (5 μM), oligomycin (5 μM) rotenone (2 μM), chloramphenicol (200 μg/ml), cycloheximide (250 μg/ml), atractyloside (1 μM), bongkrekic acid (1 μM), antimycin (1 μM), azide (10 mM), and DNP (1 mM) were used at these concentrations. The reaction was initiated by the addition of 2 μCi of [^{14}C]-Chlorella hydrolysate. After 30 min of incubation the reaction was stopped by the addition of 1 ml of 10% TCA and the pellet was processed as described in Materials and Methods.

malate, succinate + rotenone, and ascorbate + TMPD decreased the extent of incorporation. Inhibition of incorporation by succinate has been reported by Wheeldon and Lehninger (1966) and by Lamb *et al.* (1968). The former authors suggested that the addition of respiratory subtrates might divert some of the amino acids to other metabolic pathways such as transamination, thereby reducing the amino acid pool size. Kroon (1964) has shown that maintenance of the pyridine nucleotides in a reduced form is unfavorable for protein synthesis. Our results fit in with these observations.

Externally added ATP showed about 60% stimulation, whereas addition of ADP, with or without an oxidizable substrate, decreased the incorporation. It is possible that the phosphorylation of ADP drains the available energy potential away from protein synthesis (Suresh and Jayaraman, unpublished).

Almost 90% inhibition was achieved by chloramphenicol, and there was practically no inhibition by cycloheximide, showing that the incorporation was mitochondrial (microbial contamination being ruled out). The high inhibition by azide (83%) and by DNP (63%) shows the process to be linked to the electron transport system and generation of membrane potential. The low

inhibition with oligomycin and the absence of an effect by either atractyloside or bongkrekic acid is somewhat surprising, and this is not understood. Shaking and aeration also inhibited the process by 30%. Oxidative phosphorylation could be favored by shaking or aeration, resulting in the channelization of energy available for phosphorylation.

Amino Acid Incorporation by Mitochondria Isolated from Tissues of Fish Under Stress

Figure 2 shows the results of an experiment where fish were subjected to stress, and, at different stages, the capacity of isolated gill mitochondria to incorporate amino acids was studied. There was a very sharp increase in the extent of incorporation within 2 days after exposure to stress (in this case 50% seawater), and the levels were restored to normal soon after. Some of the characteristics of this incorporation are given in Table II. Here also, the results are somewhat similar to the freshwater mitochondria.

Characterization of Mitochondrial Proteins

Figure 3 shows the densitometric tracings of slab gels of mitochondrial proteins from gills of freshwater, stressed, and adapted fish. As is seen, five new proteins with molecular weights of 11, 13, 14.5, 18, and 25 kdaltons are seen only in the mitochondria from stressed fish. The identities of these are at present unknown.

In a different experiment, the acid-precipitable material obtained after incubating isolated mitochondria with [^{14}C]-amino acids was separated by slab gel electrophoresis and then radioautography carried out. Figure 4 shows the densitometric tracings of one such radioautogram. Although it is well established that mitochondria can synthesize about 10 proteins, several bands are seen, although many of them are faint. Jeffreys and Craig (1976) also obtained similar results. But the striking observation is that two new proteins of molecular weight 49 and 61 kdaltons are labeled significantly under the stress conditions.

Appearance of new proteins under stress conditions is not a new phenomenon, since recently Lin and Klingenberg (1980) reported the appearance of an uncoupler protein in bovine adipose tissue mitochondria during cold exposure. Identity of the proteins synthesized under our conditions and correlation between the results of staining and radioautograms are being worked out.

Study of Nucleotide Levels

In an earlier communication (Suresh and Jayaraman, 1983) we have shown that the mitochondria from stressed fish have lost the ability to

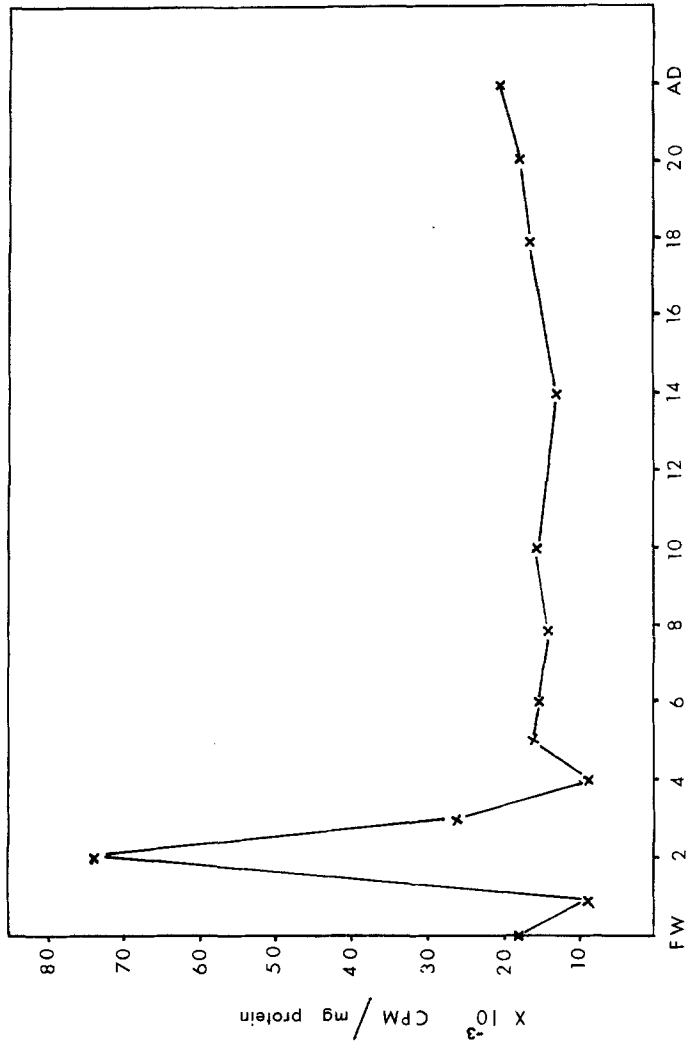

Fig. 2. Amino acid incorporation in mitochondria isolated from fish exposed to 50% seawater. The protocol for amino acid incorporation is the same as described in Table I.

Table II. ^{14}C-Amino Acid Incorporation in Stressed Mitochondria (50% Seawater, 2nd Day; Mitochrondria alone, 95,248 cpm/milligram Protein)

Condition	Inhibition (%)
Succinate + rotenone	24
Succinate + rotenone + ADP	20
Succinate + rotenone + ADP + oligomycin	38
Succinate + rotenone + ADP + azide	66
Succinate + rotenone + ADP + CAP	80
Succinate + rotenone + CAP	81
Succinate + rotenone + CHI	No inhibition
Succinate + rotenone + DNP	62

[a]The protocol for amino acid incorporation was the same as described in Table I.

synthesize ATP from externally added ADP. The increased amino acid incorporation, a highly energy-requiring reaction, takes place under such conditions. The results in Table I show that this protein synthesis is inhibited under phosphorylating conditions (substrate + ADP); its response to oligomycin is sluggish and, further, atractyloside and bongkrekic acid, the inhibitors of the ATP/ADP translocase system, do not have any effect. Thus, it was of interest to analyze the intramitochondrial nucleotide levels of these different mitochondria (Table III). Under stress conditions, AMP levels were not

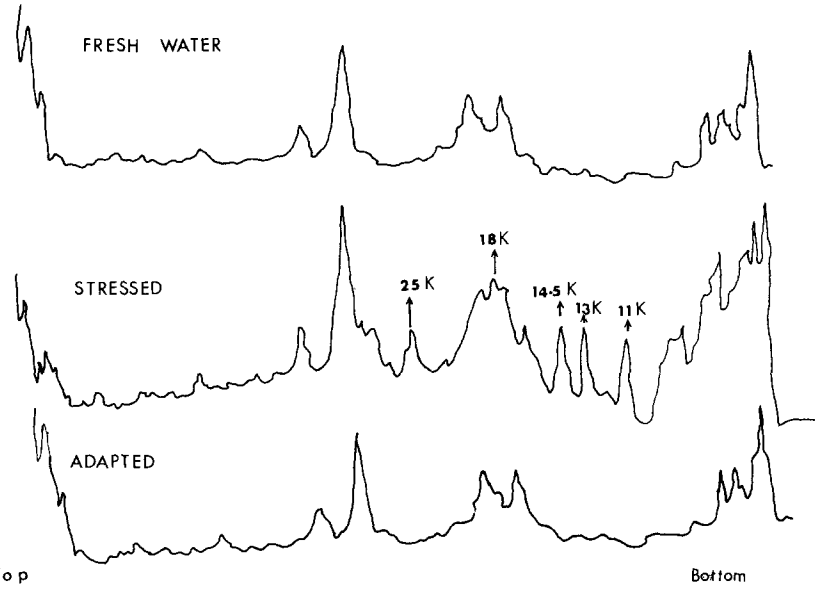

Fig. 3. Densitometric tracings of slab gels of mitochondrial proteins from gills of freshwater, stressed, and adapted fish after SDS-polyacrylamide gel electrophoresis of whole solubilized mitochondria.

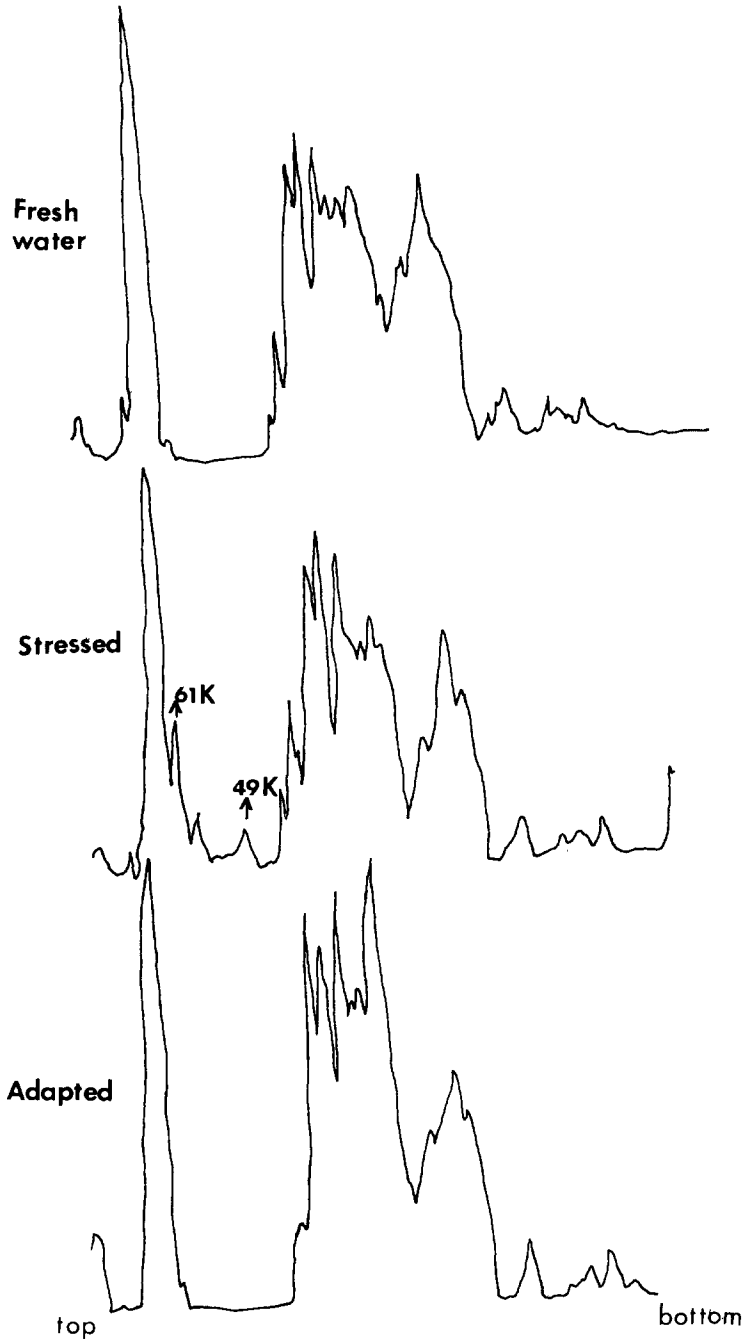

Fig. 4. Densitometric scanning of the autoradiogram of mitochondrially synthesized proteins from freshwater, stressed, and adapted mitochondria after SDS-polyacrylamide gel electrophoresis of whole solubilized mitochondria.

Table III. Free Adenine Nucleotide Pool in Mitochondria

Mitochondria	AMP	ADP	ATP	ATP/ADP
	(nmol/mg)			
Freshwater	195	125	194	1.55
Stressed (50% seawater, 2nd day)	206	81	218	2.69
Adapted (50% seawater, 22nd day)	168	137	144	1.05

decreased, but the ATP/ADP ratio increased about 75%. On adaptation, the ratios reverted almost back to normal.

Effect of Altering the Endogenous Calcium Levels

For this series of experiments, mitochondria were isolated from the gill tissues of freshwater fish (FW mitochondria) and from fish under stress (2nd day in 50% seawater—referred to as SW mitochondria). The following pretreatments were given to these preparations before assaying for the various parameters:

(a) FW mitochondria—control.

(b) FW mitochondria loaded with cold calcium by preincubation under optimal conditions of calcium uptake.

(c) Calcium-loaded mitochondria as (b) but treated with NaCl to efflux

Table IV. Effect of Calcium on Different Mitochondrial Functions[a]

Mitochondrial parameters/treatment	(1)	(2)	(3)	(4)	(5)
Succinate oxidation (natoms O_2/min/mg protein)	21	22	21	24	22
Respiratory control index	2.2	1.0	2.0	1.0	2.0
ADP/O ratio	1.9	0	1.7	0	1.6
Transhydrogenase (units/mg protein)	0.0178	0.0054	0.0106	0.0105	0.0156
[^{3}H]-ADP binding[b] (nmol ADP bound/mg protein)	11.7	0	9.2	4.4	11.2
[^{14}C]-amino acid incorporation (cpm/mg protein)	29000	37300	Not tried	95248	Not tried

[a](1) FW mitochondria—control; (2) FW mitochondria loaded with cold Ca^{++} by preincubation under optimal conditions of calcium uptake; (3) Ca^{++}-loaded mitochondria as (2) but treated with NaCl to efflux Ca^{++}; (4) SW mitochondria under stress; (5) SW mitochondria exposed to NaCl to efflux Ca^{++}.

[b]The procedure of Henderson and Lardy (1970) was followed. The assay mixture contained, in 1 ml: 250 mM sucrose, 5 mM Tris-HCl (pH 7.4) buffer, 5 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 1 mg mitochondrial protein, and [^{3}H]-ADP, 0.2 μCi (specific activity 760 mCi/mmol). After 20 min incubation at 30°C, mitochondria were pelleted down at 10,000 *g* in a TH 12 centrifuge and washed with cold buffer once. The pellets were dissolved in minimal volume of 0.1 N NaOH, spotted on filter paper discs, and the radioactivity determined.

Table V. Effect of Inhibiting Mitochondrial Protein Synthesis on Various Functions[a]

Mitochondrial status/parameters	(1)	(2)	(3)	(4)
Succinate oxidation (natoms O_2/min/mg protein)	21	22	24	24
Respiratory control index	2.2	2.0	1.0	2.0
ADP/O ratio	1.9	1.9	0	1.8
Transhydrogenase[b] (units/mg protein)	0.0178	0.0160	0.0105	0.0157
[^{3}H]-ADP binding (nmol ADP bound/mg protein)	11.7	10.8	4.4	9.2
Swelling and contraction (Δ OD/min)				
Endogenous	−0.010	Not tried	−0.010	Not tried
+ Succinate	−0.010	0	−0.010	0
+ ATP + Mg^{++}	+0.015	−0.0075	−0.015	+0.01
Calcium levels (nmol/mg protein)	580[c]	600[c]	950	1000
Calcium uptake capacity (nmol/mg protein)	32	36.9	3.3	5.6

[a](1) Freshwater mitochondria; (2) freshwater mitochondria pretreated with chloramphenicol; (3) stressed mitochondria; (4) stressed mitochondria pretreated with chloramphenicol.
[b]In all cases 1 mM DNP abolished the activity.
[c]After preloading with calcium.

calcium. The presence of the Na^+–Ca^{++} antiport in rat liver mitochondria has been shown by Crompton *et al.* (1976), and such an antiport is present in our system also (unpublished results).

(d) SW mitochondria—under stress.

(e) SW mitochondria exposed to NaCl to efflux calcium.

Several parameters have been measured with these mitochondria, and typical results are summarized in Table IV. It can be seen clearly that (a) the succinate oxidation rate is not affected in any of the preparations; (b) the respiratory control index, ADP/O ratio, and [^{3}H]-ADP binding are decreased by high endogenous calcium levels [preparations (b) and (d)]; and (c) protein synthesis is enhanced by elevated endogenous calcium levels [preparations (b) and (d)].

Effect of Inhibiting Mitochondrial Protein Synthesis

In a second series of experiments, we inhibited mitochondrial protein synthesis with chloramphenicol or tetracycline and then assayed various parameters. Tetracycline results are not shown as they were very similar to the results with chloramphenicol. The results are summarized in Table V. It is seen from Table V, that inhibition of protein synthesis by chloramphenicol restores at least four deranged functions to normal levels:

1. ADP/O ratio
2. $^{45}Ca^{++}$ uptake

3. Transhydrogenase
4. [^{3}H]-ADP binding

It is significant that the endogenous calcium is not affected by treatment with antibiotics.

Discussion

The biogenesis of mitochondria as a response to stress conditions such as hypoxia, hyperfunction of organs, cold acclimation, endurance exercise, etc. (Holloszy, 1967, 1975; Meerson and Pomoinitsky, 1972; Davies *et al.*, 1981) has been well documented. In fishes transferred from freshwater to seawater, there is an increase in the chloride cells (Roelofson and Van Deenen, 1973). The increased activity of glutamate dehydrogenase activity along with an increase in [^{3}H]-thymidine incorporation into DNA in the epithelial cells of the gill lamella of young salmon in seawater suggests the formation of cells rich in mitochondria (Conte and Lin, 1967). We have observed increased protein synthesis in our studies.

Granting, in a physiological sense, that increased protein synthesis under stress conditions is a defense mechanism of the organism from the bioenergetic point of view, this is an enigmatic situation. Here is an organelle, deprived of its supposedly main function of energy production, namely ATP synthesis (as measured by the conventional method of ADP/O ratio), carrying out, in an enhanced manner, the highly energy-requiring process of protein synthesis.

There are some plausible explanations: (a) As we have shown earlier (Suresh and Jayaraman, 1983), under stress conditions mitochondria are loaded with calcium. This ion is known to stimulate translation. Further, calcium also keeps the mitochondria in a swollen state, thereby enhancing the permeability of amino acids. (b) There is rapid recycling of the nucleotide pool within the mitochondria itself, so that dependence on external resources is minimized. Klingenberg (1976) has demonstrated the presence of enzymes such as AMP kinase and GTP-AMP P_i transferase in the intramembranal space of mitochondria. It is pertinent to mention that our earlier report of loss of ATP synthesis was based not on the measurement of the quantity of ATP but on the ADP/O ratio. Changes in the nucleotide pool have also been shown to be influenced by calcium (Gomez-Puyou, *et al.*, 1979).

Two significant, perhaps preemptive, conclusions emerge from the results presented here and from our earlier publications: (i) As an immediate response to stress, the mitochondria show decreased capacity to synthesize ATP from externally added ADP and also several energy-dependent functions such as swelling and contraction and transhydrogenase and ion uptake. However, protein synthesis, an energy-requiring process, is enhanced.

Another parameter is the enhanced level of endogenous calcium. (ii) More importantly, artificial expulsion of calcium from stressed mitochondria or blocking of protein synthesis by chloramphenicol restores all the lost functions. The last result would rule out the possibility of any permanent damage caused to the mitochondria.

The following working hypothesis may be suggested. The electron-transport system generates the same energy potential even during the stress period. However, the priorities of the cell in energy utilization to combat the stress have changed. More mitochondrial proteins need to be synthesized to cope with the situation. Evidence that increased mitochondrial protein synthesis takes place under these situations has been presented earlier. Therefore the energy potential generated across the mitochondrial membrane is channeled toward protein synthesis at the expense of oxidative phosphorylation. It is known, for example, that calcium uptake takes precedence over oxidative phosphorylation (Rossi and Lehninger, 1964). However, since protein synthesis requires ATP, this ATP must be derived from internal resources. Gratifyingly, we have found a significant increase in the ATP levels in stressed mitochondria. This derives support from the work of Klingenberg (1976), who has demonstrated the presence of enzymes such as AMP kinase and GTP-AMP P_i transferase in the intermembranal space. We strongly favor this nucleotide recycling process.

The role of calcium is somewhat intriguing. The data show that stress causes influx of large amounts of calcium. This influx may block oxidative phosphorylation and calcium uptake rates. Since calcium can also enhance translation, the calcium accumulation may, at least in part, account for many of the effects observed in stressed mitochondria. One exception is the observation that inhibition of protein synthesis by chloramphenicol or tetracycline reverses the effects of the stress observed without affecting the endogenous levels of calcium.

In conclusion, we propose that when a system is challenged with an adverse environmental milieu, the energy transduction pattern is altered to annul the deleterious effects of the stress. Where one function needs to be stimulated to meet the new demands, the energy required for it is derived at the expense of other functions, the total energy potential remaining constant. Verification of this hypothesis by measuring the potentials generated under different situations of stress is under way.

Acknowledgments

N.S. is grateful to the University Grants Commission and the Council of Scientific and Industrial Research, New Delhi, and S.K. is grateful to the

Indian Council of Medical Research, New Delhi, for financial support. The authors are grateful to Mrs. Archana Suresh for typing the manuscript.

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