Proc. Indian Acad. Sci., Vol. 88 B, Part I, Number 2, April 1979, pp. 137–144,
© printed in India.

Some aspects of protein metabolism in the skeletal muscles of frog (*Rana cyanophlictis*) during cold acclimation

SHAKUNTHALA SRIDHARA
Department of Zoology, Central College, Bangalore University, Bangalore 560 001
Present address : Department of Vertebrate Biology, University of Agricultural Sciences, GKVK Campus, Bangalore 560 065

MS received 21 June 1978; revised 8 January 1979

Abstract. In spite of great evidence of increased protein synthesis during cold acclimation in many poikilotherms, little is known about protein turnover rates during thermal stress. Studies on protein levels and its catabolism in the different skeletal muscles of *Rana cyanophlictis* revealed muscle specific compensation to temperature. The variability in the adaptation of different muscles is attributed to trophic influences of the nervous system primarily and secondarily to acclimation.

Keywords. Protein metabolism; skeletal muscles; thermal acclimation; muscle specificity; trophic influence.

1. Introduction

Compensatory increase in protein synthesis was demonstrated during cold acclimation in several poikilotherms based on levels of protein, RNA and protein bound amino acids (Rao 1967; Prosser 1967). Translational compensation of protein synthesis was observed in the skeletal muscle and rotational compensation occurred in the gills and liver of goldfish (Das and Prosser 1967). Subsequent studies by Das and Krishnamoorthy (1969) revealed increased rate of radioactive amino acid incorporation into muscle proteins of goldfish. However for greater turnover, the rates of synthesis and degradation must be balanced. Although, lysosomal activity was shown to increase during warm adaptation (Hazel and Prosser 1970), protein metabolism in the skeletal muscles of frog is little understood in terms of synthesis and degradation during thermal acclimation.

Materials and methods

Rana cyanophlictis in the weight range of 20–25 g were cold acclimated by keeping them in bread boxes (30 cm × 15 cm × 15 cm) containing water to a depth of 6·25 cm and maintaining them in a refrigerator whose temperature was adjusted to 12±1° C. Each box accommodated three frogs and the duration of acclimation

was 15 days. Locomotor activity of the animals was unaffected by acclimation. Animals kept at room temperature (25±3° C) constituted controls. Both categories of frogs were forcefed daily on 5 g of either frog muscle or foot of pond snail. After pithing the frogs, the skeletal muscles, viz., sartorius, gracilis major, triceps femoris, adductor longus and brevis, semimembranosus, gastrocnemius, tibialis anticus, peroneus, deltoid, pectoralis, rectus abdominus and whole heart were removed, weighed and used for biochemical assays.

2·1. *Total protein*

Weighed amount of tissue was homogenised in 5 ml of cold distilled water, to which an equal amount of 10% TCA was added. The precipitated protein was collected after centrifugation and quantitatively estimated by biuret method (Layne 1957).

2·2. *Sarcoplasmic proteins*

As no enzyme is reported to be capable of degrading myosin and other contractile proteins (Kohn 1965) sarcoplasmic proteins of the muscle homogenate are suggested to serve as endogenous substrate for the lysosomal activity and hence their levels were also estimated according to the basic principle described by Barany *et al* (1965) for contractile proteins..

RNA was extracted and purified according to Smillie and Krotokov (1960). The RNA content of the extract was estimated by orcinol colour reaction. Bovine liver RNA was used for the preparation of calibration curves.

Total free amino acids in the TCA precipitated (5%) supernatant of the muscles were colorimetrically estimated according to Oser (1965). For autolytic, cathepsin and proteolytic activities, the muscles were homogenised in ice cold 0·25 M sucrose in an all glass homogeniser. The muscle concentration in the homogenate was uniformly maintained at 5% to which a few drops of 0·1% Triton X-100 were added. The clear supernatant was collected after standing the homogenate in the cold for an hour and then centrifuging at 3000 g at 0° C in an international PR-6 centrifuge.

2·3. *Autolytic activity*

One ml of the supernatant was incubated at 37° C in a water bath for 1 hr. An unincubated, TCA denatured homogenate served as the control. At the end of the incubation period the autolytic activity was stopped and proteins precipitated by the addition of equal volume of 10% TCA. After centrifugation the supernatant was analysed for total amino acids by the method of Moore and Stein (1954).

2·4. *Cathepsin activity*

5% muscle homogenate was mixed with equal volume of 0·2 M phosphate buffer at 7·2 pH and incubated at 37° C in a water bath for 3 hr. Incubation was followed by protein denaturation and amino acid estimation as described earlier (Moore and Stein 1954).

2.5 *Proteolytic activity*

10 ml of clear 5% homogenate of each muscle was dialysed at 4° C for 2 hr against 500 ml of 0·25 M sucrose. The assay mixture containing 1 ml of dialysate, 1 ml of 1% casein (w/v) in 0·0005 M sodium bicarbonate and 1 ml of 0·1 M $CaCl_2$ was incubated at 37° C for 3 hr. The assay method was adapted from Krishnamoorthy (1971). The reaction at the end of incubation period was stopped by adding 2 ml of ice cold 10% TCA, the resultant mixture was centrifuged and the amino acid content of the supernatant was determined as usual (Moore and Stein 1954).

The total initial protein content of the homogenate prior to incubation was estimated by micro-biuret method (Itzhaki and Gill 1964) and considered for calculating the rate of enzyme activity. The autolytic, cathepsin and proteolytic activities were expressed as μ mol amino acids released/h/mg initial muscle homogenate protein.

3. Results

Total proteins did not change in most muscles except in semimembranosus and cardiac. In the former the levels were elevated while a decrease characterised the latter (table 1). The sarcoplasmic protein content did not alter on cold acclimation

Table 1. Changes in the levels of proteins, amino acids and RNA in the muscles of cold acclimated frogs.

Muscle	Total Proteins*		Sarcoplasmic Proteins*		Amino acids*		RNA*	
	Cont.	Accli.	Cont.	Accli.	Cont.	Accli.	Cont.	Accli.
Sartorius	148±18	158±47	42±19	66±34	11±2	11±4	1·27±0·8	1·22±0·4
Gracilis major	175±47	173±41	47± 5	52±18	14±3	10±6	0·89±0·3	0·93±0·2
Triceps femoris	170±21	172±32	43± 6	41± 7	8±4	9±2	0·81±0·4	1·62±0·5
Adductor longus and brevis	161±36	167±37	44±12	53±20	8±3	16±6**	7·71±0·5	1·37±0·2**
Semi-membrano-sus	171±31	246±75**	65±10	74±25	12±3	11±3	0·73±0·3	1·82±0·6**
Gastrocne-mius	171±32	188±24	50±17	47±27	12±4	10±2	0·79±0·5	1·14±0·5
Tibialis-anticus	166±24	181±42	62± 9	56±23	24±6	20±8	1·1 ±0·7	1·87±0·1
Peroneus	180±39	170±18	65± 7	48±33	21±6	25±5	0·56±0·2	1·57±0·7
Deltoid	158±35	156±29	62± 9	51±37	25±5	24±4	0·91±0·3	1·76±0·4
Pectoralis	163±37	174±52	37±17	59±27	19±4	28±6**	1·36±0·7	1·92±0·4
Rectus abdo-minus	150±32	159±25	44± 8	34±19	14±4	14±3	1·00±0·4	1·63±0·3**
Cardiac	225±33	178±31**	73± 8	42±29	28±4	41±14**	1·78±0·2	2·96±1·0**

* mg/g muscle.

** Statistically significant change from control values.

(table 1). The total free amino acid levels were not affected except in adductors, pectoralis and cardiac which exhibited increased concentrations. Similarly the RNA levels increased only in adductors, rectus, semimembranosus and cardiac but remained unaltered in other muscles (table 1).

In vitro autolytic degradation decreased in sartorius and gracilis on cold adaptation (table 2). It increased in adductors, gastrocnemius, tibialis, peroneus, deltoid, pectoralis, rectus and cardiac while in others it did not change (table 2). Cathepsin activity of many muscles was not affected except in sartorius and gracilis where an increase was noticed (table 2).

Significant decrease in the proteolytic activity was seen in sartorius, gracilis, triceps, gastrocnemius and deltoid due to low temperature acclimation (table 2). An increased activity was noticed in tibialis, peroneus, rectus and cardiac while the remaining muscles were not affected (table 2).

4. Discussion

The muscle mass forms 45% of total adult body weight and two-thirds of the muscle proteins are made up of myofibrillar proteins and hence these proteins form a major proportion of body weight. In addition the protein turnover rate of normal muscles is two to three times more than that of liver proteins suggesting their dominant role in the overall protein metabolism of body (Millward 1970). Hence changes induced by thermal acclimation in protein metabolism signify the extent and nature of physiological compensation undergone by the organism during thermal acclimation.

Cold acclimation resulted in an increase in the protein content of liver, gill and muscle of goldfish (Murphy 1961; Das and Prosser 1967), body wall of earthworms (Rao 1967), abdominal fat body and haemolymph of *Rhodinus prolixus* (Okasha 1964) and in rat liver (Vaughan *et al* 1958). The albumin increased and α-1-globulin decreased in the blood of *Salmo gairdneri* (Meisner and Hickman 1962) during adaptation to cold. Concurrently warm acclimation induced an increase in the levels of free amino acids in the body wall of earthworms, blood of freshwater mussel (Rao 1967), haemolymph of *Rhodinus* sp. (Okasha 1964), liver, gill and muscle of goldfish (Das and Prosser 1967). A significant increase was also noticed in the RNA content of freshwater mussel tissues and gills of goldfish (Rao 1967; Das and Prosser 1967). Attempts to correlate changes in protein synthesis during thermal adaptation with changes in RNA synthesis led these authors to conclude that the net synthesis of proteins in the goldfish during low temperature acclimation may be due to altered synthesis of RNA from microsomes rather than from nuclear fractions. Perfused liver of cold adapted toadfish exhibited higher rates of protein synthesis compared to warm adapted ones. A similar increase up to 60% was noticed in the aminoacyl transferase activity of toadfish during acclimation to cold (Prosser 1973). Jankowsky (1960) reported 70% increase in the rate of ^{14}-glycine incorporation into total protein in the thigh muscles of cold acclimated *Rana temporaria* compared to frogs acclimated to high temperature. Earlier Mews (1957) obtained similar results of increased ^{14}C-glycerol incorporation in the leg muscles of *Rana esculenta*. The ^{14}C-glycerol incorporation was more in the liver than in the muscle. Further liver protein of female frogs contained more radioactive

Table 2. Changes in the autolytic, cathepsin and proteolytic activity of frog muscles during cold acclimation.

Muscle	Autolytic activity*		Cathepsin activity*		Proteolytic activity*	
	Cont.	Accli.	Cont.	Accli.	Cont.	Accli.
Sartorius	0·01 ± 0·002	0·005 ± 0·001**	0·01 ± 0·005	0·04 ± 0·028**	0·032 ± 0·01	0·02 ± 0·005
Gracilis major	0·06 ± 0·02	0·005 ± 0·001**	0·005 ± 0·001	0·03 ± 0·01**	0·015 ± 0·005	0·005 ± 0·001**
Triceps femoris	0·01 ± 0·005	0·015 ± 0·001	0·01 ± 0·005	0·015 ± 0·005	0·03 ± 0·01	0·005 ± 0·001**
Adductor longus and brevis	0·01 ± 0·004	0·03 ± 0·01**	0·015 ± 0·006	0·01 ± 0·005	0·015 ± 0·01	0·015 ± 0·008
Semimembranosus	0·07 ± 0·02	0·08 ± 0·03	0·01 ± 0·005	0·015 ± 0·004	0·01 ± 0·001	0·015 ± 0·005
Gastrocnemius	0·01 ± 0·022	0·03 ± 0·002**	0·01 ± 0·004	0·01 ± 0·002	0·01 ± 0·004	0·006 ± 0·001**
Tibialis anticus	0·01 ± 0·002	0·06 ± 0·03**	0·30 ± 0·01	0·03 ± 0·015	0·01 ± 0·002	0·015 ± 0·002**
Peroneus	0·01 ± 0·002	0·03 ± 0·005**	0·035 ± 0·01	0·03 ± 0·02	0·01 ± 0·002	0·02 ± 0·002**
Deltoid	0·01 ± 0·005	0·07 ± 0·03**	0·03 ± 0·01	0·035 ± 0·02	0·03 ± 0·01	0·01 ± 0·002**
Pectoralis	0·01 ± 0·004	0·03 ± 0·01**	0·01 ± 0·002	0·03 ± 0·015	0·001 ± 0·001	0·015 ± 0·005
Rectus abdominus	0·01 ± 0·005	0·03 ± 0·01**	0·03 ± 0·01	0·025 ± 0·015	0·01 ± 0·008	0·06 ± 0·02**
Cardiac	0·01 ± 0·004	0·09 ± 0·02	0·03 ± 0·01	0·032 ± 0·015	0·01 ± 0·005	0·09 ± 0·02**

* μ moles amino acids/mg protein hr

** Statistically significant from control values.

carbon than males while the muscle protein incorporation rates were more dependent on adaptation temperature. He also demonstrated greater proteolytic activity of cell homogenates in cold acclimated frogs. In contrast Bishop and Gordon (1967) did not observe any change in muscle protein levels of *Bufo boreas* consequent on thermal acclimation.

The results of the present study are not uniform in the skeletal and cardiac muscle of *Rana cyanophlictis*. Based on the current observations the muscles can be tentatively categorised into five types :

4.1. *Those showing greater protein synthesis*

Although protein synthesis rates were not studied as such, increased levels of total protein and RNA in the muscles can be assumed to represent accumulation of proteins as a result of greater synthesis. Such a state is seen in semi-membranosus.

4.2. *Those indicating greater turnover*

Decreased protein and increased RNA coupled with greater degradation activities could be due to greater turnover of proteins. The increased degradation is also supported by higher levels of free amino acids on cold acclimation (tables 1 and 2). The one muscle which exhibited such compensation was cardiac.

4.3. *Muscles exhibiting inverse or no acclimation*

Enzymes concerned with degradation of metabolites are known to show no compensation or their activities change in reverse direction (Prosser 1973). Adductors, tibialis, peroneus, pectoralis and rectus fit into this category.

4.4. *Muscles exhibiting partial compensation*

Some of the muscles, showing inverse or no compensation for autolytic activity, show at the same time compensation for proteolytic activity. Gastrocnemius and deltoid belong to this type. Similarly autolysis and proteolysis exhibit compensation in the sartorius and gracilis muscle but not their cathepsin activity.

4.5. *Muscles exhibiting compensatory proteolytic activity*

Only one muscle, viz., triceps shows decreased proteloysis on cold acclimation.

Das (1967) demonstrated tissue specificity of compensation to acclimation in goldfish with greater stabilisation of protein molecules in gills as a consequence of cold acclimation. Dean (1969) observed that the response of muscle to temperature acclimation was different from that of other tissues. In the present study even among skeletal muscles the pattern of protein metabolism differs from muscle to muscle. The muscle specificity of protein metabolism is the result of altered functional demands during thermal stress (Sridhara and Krishnamoorthy 1979). The functions of the studied muscles include flexing and pulling the limb ventrally forward (sartorius), bending and pulling the thigh anteriorly and posteriorly (adductors), extending the crus and drawing the leg forward (triceps), pulling the femur backward and flexing/extending the crus (gracilis), adducting and pulling the thigh, flexing and extending

the leg (semimembranosus, gastrocnemius), pulling the ankle (peroneus), extending and pronating the foot (tibialis), forelimb movement (pectoralis and deltoid) and contraction of body cavity is effected by rectus (Holmes 1922 ; Noble 1954). These different activities are achieved by the trophic influences of the innervating nerves. The fact that lysosomes change/increase during neuro-muscular pathological conditions like hereditary muscular dystrophy (Tappel *et al* 1962), denervation atrophy (Pollack and Bird 1968; Krishnamoorthy 1971, 1972) and also during vitamin E deficiency (Bond and Bird 1966) demonstrate that they are under the control of trophic influences of the nerve. Many muscle enzymes like actomyosin ATPase respond to functional demands in muscles (Gutmann *et al* 1971; Guth and Wells 1972). Krishnamoorthy and Shakunthala (1974) have shown that these demands are varied and result due to altered respiratory behavior during cold acclimation. The trophic influences of the nerve act as the fulcrum for functional demands of the muscles regulating the muscle enzyme activities (Guth 1969). Thus the primary adaptation during thermal acclimation must be in the nervous system and the varied response of different muscles in their protein metabolism is initially due to trophic influences of the nerves followed by secondary changes resulting from cold acclimation.

Acknowledgements

The author is grateful to Dr R V Krishnamoorthy for suggesting the problem and to late Dr K Pampapathi Rao for providing facilities and encouragement. Financial assistance of CSIR, New Delhi, is acknowledged.

References

Barany M K, Barany K, Reckard T and Volpe A 1965 Myosin of fast and slow muscles of rabbit; *Arch. Biochem. Biophys.* **109** 185–191

Bishop L G and Gordon M S 1967 Thermal adaptation of metabolism in anuran amphibians; in *Molecular mechanism of temperature adaptation* ed. C. L Prosser (Washington DC): American Association for Advancement of Science)

Bond J S and Bird J W C 1966 Cathepsin activity of liver and gastrocnemius muscle fractions in early vitamin E deficiency; *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **25** 242

Das A B 1967 Biochemical changes in tissues of goldfish acclimated to high and low temperatures—II. Synthesis of proteins and RNA of subcellular fractions and tissue composition; *Comp. Biochem. Physiol.* **21** 469–485

Das A B and Prosser C L 1967 Biochemical changes in tissues of goldfish acclimated to high and low temperature—I. Protein synthesis; *Comp. Biochem. Physiol.* **20** 449–467

Das A B and Krishnamoorthy R V 1969 Biochemical changes of muscle proteins in goldfish (*Carassius auratus*) during thermal acclimation; *Experientia* **25** 594–595

Dean J M 1969 The metabolism of thermally acclimated trout (*Salmo gairdneri*); *Comp. Biochem. Physiol.* **29** 185–196

Guth L 1969 in *Neurosciences Research Progress Bulletin* (Cambridge, Mass: MIT Press) Vol 7

Guth L and Wells J B 1972 Physiological and histochemical properties of the soleus muscle after denervation of its antagonists; *Exp. Neurol.* **36** 463–472

Gutmann E, Schiaffino S and Hanzlikova V 1971 Mechanism of compensatory hypertrophy in skeletal muscle of the rat; *Exp. Neurol.* **31** 451–464

Hazel J and Prosser C L 1970 Interpretation of inverse acclimation to temperature; *Z. Vgl. Physiol.* **67** 217–228

Holmes S J 1922 *The Biology of the frog*; (New York: The Macmillan Company)

Itzhaki R F and Gill D M 1964 A microbiuret method for estimating proteins; *Anal. Biochem.* **9** 401–410

Jankowsky H 1960 Uber die Hormonale Beeinflussung der temperatur-adaptation beim Grassfrosch (*Rana temporaria* L.); *Z. Vgl. Physiol.* **43** 392–410

Kohn R R 1965 Denervation muscle atrophy: An autolytic system in vitro; *Am. J. Pathol.* **47** 315–323

Krishnamoorthy R V 1971 Increased proteolytic activity in muscular atrophy of denervated frogs; *Enzymologia* **41** 249–259

Krishnamoorthy R V 1972 Increased lysosome activity in muscular atrophy of denervated frogs; *Enzymologia* **43** 353–358

Krishnamoorthy R V and Shakunthala N 1974 Increased RBC count and pulmonary respiration in cold adapted frogs; *J. Exp. Biol.* **61** 285–290

Layne D 1957 Spectrophotometric and turbidimetric methods for measuring proteins; in *Methods in enzymology*, eds S P Colowick and N O Kaplan (New York: Academic Press)

Mews H H 1957 Uber die Temperatur-adaptation der eiweisspaltenden und synthetsierenden Zelfermente Von Forschen; *Z. Vgl. Physiol.* **40** 356–362

Millward D J 1970 in *Lysosomes in biology and pathology* eds D J Dingle and R T Dean (New York: North-Holland Publishing Co.)

Moore S and Stein W H 1954 A modified ninhydrin reagent for the photometric determination of amino acids and related compounds; *J. Biol. Chem.* **211** 907–913

Murphy M B 1961 Tissue metabolism of goldfish (*Carrassius auratus*) acclimated to warm and cold temperatures; Ph.D. Thesis, University of Illinois

Meisner H and Hickman C P 1962 Effect of temperature and photoperiod on the serum proteins of the rainbow trout, *Salmo gairdneri*; *Can. J. Zool.* **40** 127–130

Noble G K 1954 in *The biology of amphibia* (New York: Dover Publications Inc.)

Okasha A V K 1964 Effects of high temperature in *Rhodinus prolixus* (Stahl); *Nature* (*London*) **204** 1221–1222

Osser B L 1965 *Hawk's physiological chemistry* (New York: McGraw-Hill Book Co.)

Pollack M S and Bird J W C 1968 Distribution and particle properties of acid hydrolases in denervated muscle; *Am. J. Physiol.* **215** 716–722

Prosser C L 1967 *Molecular mechanism of temperature adaptation* (Washington DC: American Association for Advancement of Science)

Prosser C L 1973 *Comparative animal physiology* Vol 1 *Environmental physiology* (Philadelphia: W B Saunders Co.)

Rao K P 1967 Biochemical correlates of temperature acclimation in *Molecular mechanism of temperature adaptation* ed C L Prosser (Washington D.C.: American Association for Advancement of Science)

Smillie R M and Krotokov G 1960 General extraction procedure for the estimation of nucleic acids in plants; *Can. J. Bot.* **58** 43–45

Sridhara S and Krishnamoorthy R V 1979 Muscle specific compensatory changes in the myosin ATPase activity of the frog, *Rana cyanophlictis* during cold acclimation; *Comp. Physiol. Ecol.* **4** 19–21

Tappel A L, Zalkin H, Caldwell K A, Desai I D and Shibko S S 1962 Increased lysosomal enzyme in genetic muscular dystrophy; *Arch. Biochem. Biophys.* **96** 340–346

Vaughan D A, Hannon J P and Vaughan L N 1958 Associated effects of diet, environmental temperature and duration of exposure on major constituents of the liver of rats; *Am. J. Physiol.* **194** 441–445