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IISc THESES ABSTRACTS

Thesis Abstract (Ph.D.)

Studies on vitamin-carrier proteins: Physicochemical and functional aspects by P.B. Seshagiri.
Research supervisor: P.R. Adiga.
Department: Biochemistry.

1. Introduction

Although the essential requirement of vitamins, like biotin, riboflavin, etc., for embryonic development in the chicken has been known for a long time, the mode of their protein-mediated deposition in the egg was demonstrated only recently by the discovery of specific high-affinity vitamin binding/carrier proteins[1,2]. One such protein discovered was the chicken egg yolk biotin-binding protein (BBP) which was reported to be involved for the yolk deposition of biotin[2]. However, a counterpart of this protein with comparable physicochemical characteristics present in the egg white has not been investigated so far. Therefore, the present study was aimed at the isolation and molecular characterisation of a BBP in the chicken egg white, followed by the extension of similar studies to the rodent model (rat) as well, since there has been no information so far regarding the mode of biotin transport during mammalian embryonic development. Furthermore, studies were also conducted on riboflavin-carrier protein (RCP) of the bonnet monkey in order to understand the mechanism of transport of yet another vitamin *viz.*, riboflavin, during primate reproduction.

2. Experimental procedure

Preparation of the chicken egg white and the serum from pregnant/estrogenised rats was made as described by Murty[3]. From these samples their respective BBP was isolated to homogeneity by employing biotin affinity chromatography[3]. The physicochemical and immunological characterisations of the proteins were performed according to the standardised procedures[3]. Western blot analysis was performed according to the procedure of Towbin *et al*[4]. Two-dimensional tryptic peptide map analysis and *in vitro* translation of poly $(A)^+$ – RNA were carried out by following the standardised procedures of this laboratory[5]. Radioimmunoassay (RIA) for proteins and steroid hormones were performed as described by Murty[3]. Assay of glutathione reductase activity of the monkey erythrocyte hemolysate was according to the procedure of Bamji[6].

3. Main results and conclusions

Attempts were initially made by immunological and biochemical methods, to explore the possibility of a BBP distinct from avidin in the chicken egg white. Ouchterlony immunodouble diffusion and the Western blot techniques revealed that the oviduct cytosols of the estrogenised chicks/laying hens and the crude protein fraction of the egg white contained BBP that exhibited

extensive immunological cross-reactivity with the purified yolk BBP[24]. This protein of the egg white displayed specific [^{14}C]-biotin binding activity and when saturated with the unlabelled biotin, the protein showed thermally induced biotin exchange reaction with [^{14}C]-biotin at 55°C. Analysis of *in vitro* biosynthesised [^{14}C]-labelled egg white proteins in the oviduct tissue explants from estrogenised chicks revealed that *ca.* 2% of the total labelled proteins could be specifically immunoprecipitated with anti-yolk BBP antibodies. These interesting findings led to the isolation of BBP of Mr 67,000 in an homogeneous form from the chicken egg white by employing DEAE-cellulose chromatography followed by biotin affinity chromatography. Several physicochemical and immunological characteristics were very closely similar to that of the yolk BBP and not of avidin. *In vitro* translation and immunoblot experiments indicated that both the liver and oviduct synthesise BBPs of molecular size corresponding to the native protein. Furthermore it appears that the yolk BBP gets deposited in the form of four identical subunits while the white BBP remains unaltered in the egg white.

Preliminary immunological and biochemical studies performed in the rodent model led to the discovery of BBP in the sera of pregnant or estrogen-treated rats. This rat BBP of Mr 66,000 could be isolated to apparent homogeneity by employing biotin-AH Sepharose column. The isolated protein specifically bound [^{14}C]-biotin, exhibited partial immunological homology with the yolk BBP and had a pI of *ca.* 4.1. A heterologous RIA was developed for this protein by using yolk BBP antibodies. By this method, rat BBP showed remarkable immunological cross-reactivity with rat serum albumin (RSA), although other gross immunochemical methods failed to show this cross-reactivity. However, pI values and tryptic peptide maps of these two proteins were different indicating distinct differences in physicochemical properties. Furthermore, it has been demonstrated that unlike RSA, rat BBP was required for the maintenance of pregnancy, presumably to transport biotin to the developing embryo, since active immunisation against yolk BBP of fertile female rats resulted in repeated termination of pregnancy owing to immunoneutralisation of the endogenously circulating maternal BBP.

The above mentioned observations in the rodent model led to the studies on the establishment of functional significance of vitamin-carrier protein, in particular RCP during pregnancy in primates. For this purpose, five female bonnet monkeys of proven fertility were actively immunised against chicken RCP. All the immunised animals elicited good titers of anti-chicken RCP antibodies and certain fraction of these were able to significantly recognise ^{125}I-monkey RCP. The immunisation *per se* did not have any effect on the reproductive cyclicity as monitored by steroid hormonal profiles, as well as the riboflavin status as assessed by erythrocyte glutathione reductase activity. Subsequently, pregnancies of these animals were monitored by estimating, by RIA the levels of progesterone and monkey chorionic gonadotropin. Results obtained over a period of $2\frac{1}{2}$ years indicated that there was immunosuppression of pregnancy in at least four out of five animals at various times of gestation between days 12 and 61 after conception. This effect appeared to depend on circulating antibody titers since animals which escaped pregnancy termination concordantly showed low titers of anti-cRCP antibodies. All the animals towards the end of the study period carried their pregnancy to term and delivered either still born or live babies, presumably due to acquisition of immune tolerance over a period of time upon repeated boosters of the immunogen. These studies clearly demonstrated that the RCP is vitally required to supply riboflavin to the embryo during pregnancy in the primate and this protein-mediated vitamin delivery mechanism seems to be operative in the rodents as well as primates.

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Thesis Abstract (Ph.D.)

Studies on myelin proteins in the developing rat brain by Valli Vissa Akella. Research supervisor: P.S. Sastry.
Department: Biochemistry.

1. Introduction

Understanding the biochemistry and functional basis of the nervous system, in particular the brain, is one of the most challenging aspects of biological research. It is now recognized that the transformation of the embryonic nervous system into the mature adult brain is an extremely complex process where morphological, physiological and biochemical changes occur in an integrated manner[1]. Synaptogenesis, synaptic transmission and myelination are among the most important events of the specific differentiated functions of the nervous system. The myelin sheath is a modified and a highly specialized extension of the Oligodendrocyte plasma membrane. It functions as an insulator and facilitates saltatory conduction. In spite of the many investigations carried out earlier, the phenomenon of oligodendrocyte-axon recognition, the synthesis and assembly of various myelin components, the mechanisms of elaboration of the glial cell membrane sheath are still not clear. Studies on the synthesis and regulation of the myelin-specific components would be helpful in elucidating the molecular mechanisms of myelinogenesis. During the past few decades, abundant information has been obtained on the composition and characteristics of the major myelin components *viz.*, its lipids and proteins. The pathways of lipid biosynthesis and the modulation of the enzymes involved have been elucidated in normal and myelin-deficient mutants. Myelin proteins have been characterized biochemically and immunologically[2]. However, the biosynthesis and modulation of the synthetic machinery of the myelin proteins and the possible synchrony between the myelin lipid and protein biosynthesis are largely unexplored. Many post-translational modifications of myelin proteins have been discovered and characterized but their role in the modulation of myelin function is not known. This investigation envisages studies on myelin protein biosynthesis and one of the post-translational modifications *viz.*, phosphorylation.

2. Experimental

Rats were chosen as the experimental animals since myelination is a postnatal event in this species. Three myelin-specific proteins *viz*, basic protein (BP), proteolipid protein (PLP) and Wolfgram protein (WP) were purified to homogeneity[3] and antisera specific to these proteins were raised in rabbits. Several *in vitro* protein synthesizing systems were tested and compared for their efficiency in synthesizing the myelin-specific proteins using specific antisera for immunoprecipitation. Use of polysomes as the source of mRNA[4] and reticulocyte lysate[5] as the source of protein synthesis gave the best results. In the studies on post-translational modifications, phosphorylation of basic myelin protein and Wolfgram protein was studied using endogenous kinases as enzyme sources and γ-p^{32}-ATP

3. Results and discussion

The developmental profile for BP synthesis was compared in two systems *viz*, a homologous polysome system and a reticulocyte lysate system where brain polysomes were used as the source of mRNA without further deproteinization. In both systems, a comparable profile of BP synthesis was obtained. It was shown that the brain acquires the capacity to synthesize BP before the fifth postnatal day. Maximal rates of synthesis were observed during the period of active myelination. This profile agrees well with the observations of Carson *et al*[6] and Zeller *et al*[7] on BP synthesis in the developing mouse brain.

WP synthesis *in vitro* was shown for the first time. The developmental profiles for WP and PLP synthesis showed that these two proteins are also synthesized before the fifth postnatal day. The synthesis of the three proteins BP, PLP and WP proceeds at different rates which is not commensurate with their quantity in myelin. Synthesis of WP reached its maximum levels first and this observation supports the hypothesis of Roussel *et al*[8] that WP may be a prerequisite for myelination.

The *in vitro* phosphorylation of BP by protein kinases endogenous to myelin was characterized. The effect of age on BP phosphorylation was tested. It was shown that the BP phosphorylating capacity is present in purified myelin throughout development. In an attempt to explain the existence of 2′, 3′–cyclic nucleotide 3′–phosphohydrolase, a myelin marker enzyme, the effect of 2′, 3′–cAMP (the *in vitro* substrate) and 2′–AMP (the product) of this enzyme on BP phosphorylation was tested. 2′, 3′–cAMP but not 2′–AMP stimulated the BP phosphorylation significantly. This effect was observed in myelinating and adult animals and was absent in aging animals. Polyamines, spermine and spermidine did not affect BP phosphorylation by kinases endogenous to purified myelin. No evidence was obtained for the phosphorylation of the major myelin protein–PLP at any age.

The phosphorylation of the minor myelin protein WP has been characterized for the first time. A basal kinase and a 3′, 5′–cAMP-dependent protein kinase (kinase A) were identified as the kinases responsible for WP phosphorylation. These enzymes exhibited a difference in their ontogeny; while basal kinase phosphorylated WP throughout development, kinase A-mediated WP phosphorylation appeared only after maturation and increased with age. WP phosphorylation by both the kinases was stimulated by 2′, 3′–cAMP. Polyamines, spermine and spermidine inhibited WP phosphorylation. The regulation by these two small molecules *viz.*, 2′, 3′–cAMP and polyamines was found to be age dependent. The phosphoaminoacid in WP was identified for the first time as phosphoserine.

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Thesis Abstract (Ph.D.)

Regulation of oxidative phosphorylation in heat-exposed rats by R.S. Puranam. Research supervisors: T. Ramasarma and C.K. Ramakrishna Kurup. Department: Biochemistry.

1. Introduction

Exposure of endotherms to heat stress triggers physiological changes to counter the adverse effects of elevated environmental temperature. Under these conditions the requirement for energy of the body decreases Since the mitochondrion is the powerhouse of the cell, changes in respiratory activity are to be anticipated in heat stress. Thyroxine has been implicated in the regulation of mitochondrial oxidative function. However, the precise mechanism of mitochondrial response to heat stress and the manner in which it is modulated by thyroxine are little understood. Experiments designed to throw light on both these aspects are presented in this thesis.

2. Materials and methods

Male albino rats (110–120 g) of the Wistar strain were exposed to heat stress (36–37°C) in a specially designed ventilated chamber for different periods of time Animals maintained at 23–24°C served as control. Hypothyroidism was produced by thyroidectomy or by dietary administration of 6-propyl-2-thiouracil (0.16%) Diet and water were given *ad lib.*

Table I
Effect of heat exposure on mitochondrial oxidative activity

Source of mitochondria	Oxygen uptake (% of control)	
	– cytochrome *c*	+ cytochrome *c*
Kidney	69 ± 17	99 ± 21
Liver	69 ± 10	71 ± 5

The rate of succinate oxidation by mitochondria isolated from animals exposed to 36–37°C for 20 days is given taking the corresponding rate of control animals (kept at 23°C) as 100. The values are the mean ± SD of 10–16 animals

Kidney and liver mitochondria were prepared by differential centrifugation[1]. Oxidative phosphorylation was determined by polarography. The rate of generation of H_2O_2 was measured by the decrease in scopoletin fluorescence. The activity of cytochrome oxidase was determined using ferrocytochrome *c* as electron donor. The cytochrome content was determined by difference spectra[2].

3. Results and conclusions

Both kidney and liver mitochondria showed a 20–30% decrease in the rate of active oxidation of succinate on exposure of rats to heat stress. In the case of kidney the decrease was corrected on the addition of cytochrome *c*. Liver mitochondria did not respond to cytochrome *c* addition (Table I).

Kidney mitochondria of heat-exposed animals showed decreased rate of H_2O_2 generation when α-glycerophosphate was used as electron donor. The activity of the dehydrogenase was also lowered[3].

Table II
Effect of heat exposure on the content of cytochromes in renal and hepatic mitochondria of the rat

Source of mitochondria	Cytochrome	P moles/mg protein	
		Control	Heat-exposed
Kidney	C	588 ± 24	389 ± 22
	aa_3	243 ± 28	238 ±44
Liver	C	227 ± 16	173 ± 31
	aa_3	213 ± 17	116 ± 29

Animals were exposed to heat stress for 20 days. The cytochrome content was determined from difference spectra.

In heat-exposed animals, the content of cytochrome *c* in kidney mitochondria decreased by about 50%. In liver mitochondria cytochrome oxidase content decreased significantly (Table II). These findings explain the differential response of oxidation by kidney and liver mitochondria to exogenous cytochrome *c*.

In order to see whether the above effects of heat exposure are mediated by thyroxine, the effect of thyroidectomy and administration of the antithyroid agent propylthiouracil, on mitochondrial oxidations was examined. Both kidney and liver mitochondrial oxidations decreased under hypothyroid conditions. But the decrease was not corrected fully by the addition of cytochrome *c* to the reaction system. The content of cytochrome *c* showed a partial decrease in mitochondria. Other cytochromes were not affected. These results reveal that the deleterious effect of heat stress on mitochondrial oxidation may not be entirely mediated by thyroxine. The fact that the levels of thyroxine in circulation do not decrease on continuous exposure to heat supports the above conclusion.

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Thesis Abstract (Ph.D.)

Studies on polymerization of acrylonitrile and acrylamide by Bhabatosh Shaha.
Research supervisor: V.R. Pai Verneker.
Department: Inorganic and Physical Chemistry.

1. Introduction

The present investigation is aimed at studying the polymerization of acrylonitrile (AN) and acrylamide (AM) with different initiators — lithium and sodium metals in particular by varying polymerization conditions like temperature, solvent and concentration. The elucidation of the initiation mechanism and correlation of X-ray crystallinity with NMR stereoregularity have also been envisaged.

2. Studies on polymerization of acrylonitrile

Acrylonitrile was polymerized by free radical as well as ionic initiators under varying conditions of temperature, solvent and concentration. The various PANs obtained were characterized by a number of techniques like XRD, UV, IR, ^{1}H NMR, ^{13}C NMR spectroscopy and viscometry.

It is known[1] that a typical PAN gives two peaks in its XRD pattern — one intense, around 2 θ value of 17° and the other quite diffuse around 29°. It has been made possible in the present

investigation to bring about appreciable changes in the intensity as well as in the number of peaks in PAN *e.g.* AIBN-initiated PANs have been found to give additional XRD peaks around 22.4° and 12° under ambient conditions (0.4% initiator, bulk polymerized) and at 60°C (solution polymerized with low monomer concentration and *in vacuo*) respectively. The intensity of the peak around 17° could be significantly enhanced in lithium-initiated PAN (Li-PAN) obtained at −33°C compared to that obtained at 65°C in spite of about 25-fold increase in molecular weight.

X-ray crystallinity of various free radical-initiated PAN remained fairly constant with a crystallinity index (X) of 30–40% in spite of wide variations in polymerization conditions. However, X-ray crystallinity of anionic PAN have been found to be low and could be varied over a relatively wider range *viz*., 0–26%.

Solution-polymerized Li-PAN and sodium-initiated PAN (Na-PAN) are colored and coloration in these PANs has been found to run parallel to dielectric constant (DEC) of the polymerization medium. UV absorption maximum with Li-PAN or Na-PAN has been found to undergo a blue shift with decrease in polymerization temperature.

IR spectrum of anionic PAN shows a shoulder/peak at 2190 cm^{-1}, to the nitrile band at 2240 cm^{-1}, which could be attributed to metallation[2] of the nitrile group.

In contrast to typical free radical-initiated PAN typical colored anionic PAN obtained at relatively higher temperature has been found to give one extra group of peaks around 2.7δ in ^1H NMR spectrum and one or more peaks in the region 10–16δ in ^{13}C NMR spectrum. The intensity of these peaks has been found to decrease with decrease in polymerization temperature. Li-PAN obtained at −33°C does not show any of these peaks; incidentally it is colorless. Quite logically, these extra peaks, found in ^1H and ^{13}C NMR spectrum of colored anionic PAN has been attributed to some colored structures.

Whereas free radical-initiated PANs are known[3] to be predominantly heterotactic (~50%), anionic PANs have been found to have a pronounced isotactic contribution (40% or more) from ^{13}C NMR stereoregularity point of view.

Metallic sodium has been found to be more effective as an initiator compared to lithium towards bulk polymerization and solution polymerization of acrylonitrile (AN) from low dielectric solvents. However, both have been found to be equally reactive when polymerization is carried out from high dielectric constant solvents.

Li-PAN obtained at 65°C and Na-PANs obtained at 65°, RT (~27°) and −33°C show much resemblances amongst themselves in respect of their UV, IR, ^1H NMR, ^{13}C NMR spectra, XRD pattern as well as molecular weight and color. These polymers show quite resemblances to typical anionic PAN obtained with phenylmagnesium bromide at 45°/RT, n-butyllithium at 65°C in respect of characteristics mentioned above. However, Li-PAN obtained at −33°C resembles more closely the typical free radical-initiated PAN in respect of its spectral and other behaviour. Further, both lithium- and sodium-initiated AN systems have been found to give ESR signals; though the magnitude of 'g' differed marginally in the two cases, they differed significantly in respect of line shape as a function of temperature. Moreover, while lithium-initiated polymerization of AN could be totally quenched with 1% hydroquinone that with sodium initiation continued uninterruptedly under similar conditions. It has also been found in the present study that while both lithium and sodium could polymerize acrylamide (AM), in bulk, to poly- -alanine *via* 1,4-addition at 100°C, lithium-initiated solution polymerization of acrylamide from dioxan at 19°C resulted, unlike sodium, in the formation of both PBAL and PAM. The formation of PAM is regarded as polymerization through 1,2-addition *via* free radical mechanism.

All the above studies strongly point to the fact that metallic lithium behaves as a free radical initiator at low polymerization temperature (−33°C, for AN, +19°C for AM) while it behaves as an ionic initiator at higher polymerization temperature (+65°C for AN, +100°C for AM) Based on these, a tentative reaction mechanism has been postulated

3. Studies on polymerization of acrylamide

Polyacrylamide (PAM) was obtained both by free radical and ionic initiators and characterized by similar techniques used for characterization of PANs Polyacrylamide (PAM) has been found to be essentially an amorphous polymer with its X-ray crystallinity seldom exceeding 15% However, X-ray crystallinity of poly-β-alanine (PBAL) which is known to be crystalline, could be varied, in the present study, over a wide range *e g* 40–100% depending on polymerization conditions

Earlier workers[4,5] have reported three XRD peaks for PBAL obtained with a number of initiators. In the present study it has been possible to have PBAL having two or more peaks depending on polymerization conditions

PAM has been found to be essentially atactic (~50%) as has also been observed by other workers[6,7]. However, in PBAL isotactic and syndiotatic contributions have been found to far outweigh atactic contribution, the two together contributing up to 80% or more

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