CHAPTER 3

RESULTS- I

Activity of pyruvate dehydrogenase A (PDHA) in hamster spermatozoa correlates positively with hyperactivation and is associated with sperm capacitation
3.1 Introduction

Freshly ejaculated mammalian spermatozoa are not competent enough to fertilize the oocyte, but gain competence after a finite period of residence in the female reproductive tract. This time dependent process, during which mammalian spermatozoa become fertilization competent, is termed "capacitation" (Chang, 1951, 1955; Austin, 1951). During capacitation, spermatozoa undergo two distinct physiological changes namely, hyperactivation, during which spermatozoa gain the momentum to proceed towards the oocyte and acrosome reaction, which facilitates the penetration of the oocyte. Concomitant with these changes, at the molecular level, activation of signal transduction cascade leading to increase in tyrosine phosphorylation of proteins is observed in mammalian spermatozoa (Visconti and Kopf, 1998; Lefievre et al., 2002; Jha et al., 2003; Kulanand and Shivaji, 2001; Mitra and Shivaji, 2004; Mitra et al., 2005; Kumar et al., 2006; Mariappa et al., 2006). A few of these capacitation induced tyrosine phosphorylated proteins have been identified and they include the fibrous sheath protein of 95 kDa (FSP 95) (Mandal et al., 1999), calcium binding tyrosine phosphorylation regulator protein (CABYR) (Naaby-Hansen et al., 2002), E1β subunit of pyruvate dehydrogenase (PDHB) (Ficarro et al., 2003) and A Kinase Anchoring Proteins 83 (AKAP's) in human spermatozoa (Carrera et al., 1996); AKAP4 (Jha and Shivaji, 2002), dihydrolipoamide dehydrogenase (DLD) (Mitra and Shivaji, 2004; Mitra et al., 2005), and phospholipid hydroperoxide glutathione peroxidase (GPX4) (NagDas et al., 2005) in hamster spermatozoa and a heat shock protein of 90 kDa (HSP90) (Ecroyd et al., 2003) in mouse spermatozoa. Despite the identification of these tyrosine phosphorylated proteins and attempts to understand their role in capacitation, the molecular basis of capacitation is still a poorly understood phenomenon (Jha et al., 2003; Shivaji et al., 2006).
A simple straightforward method to unravel the molecular basis of capacitation would be to identify as many components, which are essential for capacitation \textit{in vitro} and then work around these components so as to understand the process. It is in this context that it is important to note that the carbon sources glucose, pyruvate and lactate are essential for \textit{in vitro} capacitation of mammalian spermatozoa (Mitra and Shivaji, 2004; Mitra et al., 2005; NagDas et al., 2005; Ecroyd et al., 2003; Bavister and Yanagimachi, 1977). Of these carbon sources, glucose has been shown to be of primary importance for capacitation because of its ability to generate energy through the glycolytic pathway (Urner and Sakkas, 1996; Urner et al., 2001; Mukai and Okuno, 2004), and also due to its requirement for capacitation-associated protein tyrosine phosphorylation (Urner et al., 2001; Travis et al., 2001). In hamster spermatozoa, it was shown that pyruvate-lactate support acrosome reaction (Bavister and Yanagimachi, 1977). Recently, \textit{in vitro} evidences showed the essentiality of glucose in early stages and pyruvate-lactate in later stages of capacitation in hamster spermatozoa (Mitra and Shivaji, 2004). In fact, dihydrolipoamide dehydrogenase, DLD component of pyruvate dehydrogenase complex, which metabolizes pyruvate directly, was identified as the post pyruvate-lactate metabolic enzyme involved in hamster sperm capacitation (Mitra and Shivaji, 2004). In a more recent study, a direct correlation between localization, tyrosine phosphorylation and activity of dihydrolipoamide dehydrogenase with hamster sperm capacitation was established (Mitra et al., 2005). But, since DLD is not the only protein that undergoes capacitation-associated protein tyrosine phosphorylation in hamster spermatozoa (Jha and Shivaji, 2002; Mitra and Shivaji, 2004) identification of the other proteins and characterizing their function would further unravel the molecular basis of sperm capacitation. It is assumed that in addition to DLD other components which constitute the pyruvate dehydrogenase complex (PDHc) may also be involved in sperm capacitation. Pyruvate dehydrogenase (PDHc) is critical in pathways of energy production and is a multienzyme complex consisting of: pyruvate dehydrogenase...
(PDHA), dihydrolipoamide acetyltransferase (DLAT), dihydrolipoamide dehydrogenase (DLD) and E3-binding protein (PDHX). Two additional enzymes are associated with the pyruvate dehydrogenase complex: a specific pyruvate dehydrogenase kinase, which is capable of phosphorylating PDHA at serine residues and a loosely associated specific pyruvate dehydrogenase phosphatase, which reverses the phosphorylation. Activity of PDH in vivo may be regulated by the availability of the substrates pyruvate and lipoamide themselves, as well as by the relative concentrations of NAD/NADH, CoA/acetyl-CoA, adenine diphosphate ADP/ATP, and the proportion of PDHA in its active dephosphorylated state (Young et al., 1998; Jilka et al., 1986; Yeaman et al., 1978).

1. Pyruvate + TPP $\xrightarrow{\text{PDHA}}$ 2-(alpha-hydroxyethyl)-TPP + CO$_2$

2. 2-(alpha-hydroxyethyl)-TPP + lipoamide $\xrightarrow{\text{DLAT}}$ S-acetyldihydrolipoamide + TPP

3. S-acetyldihydrolipoamide + CoA $\rightarrow$ acetyl-CoA + dihydrolipoamide

4. Dihydrolipoamide + FAD $\xrightarrow{\text{DLD}}$ lipoamide + FADH$_2$

5. FADH$_2$ + NAD$^+$ $\rightarrow$ FADH + NADH + H$^+$

Figure 3.1 Reaction mechanism of pyruvate dehydrogenase complex. PDHc catalyses the oxidative decarboxylation of pyruvate to acetyl CoA and generates the reduced form of nicotinamide adenine dinucleotide (NADH) and CO$_2$, through a series of five reactions involving PDHA, DLAT and DLD.

Mammalian pyruvate dehydrogenase is present both as a somatic form (PDHA1) and as well as a testis-specific form (PDHA2) (Dahl et al., 1990). The somatic form of PDHA1 is located on p22.1 of the human X chromosome, is coded by Pdha1 gene and contains 10 introns and spans approximately 17 kb (Dahl et al., 1990). The testis-specific form of PDHA2 is located on chromosome 4, is coded by Pdha2 gene, completely lacks introns and possesses characteristics of a functional processed gene.
PDHA2 is a homodimer and makes a functional active heterotetramer PDHA, with another homodimer PDHB, reported to be tyrosine phosphorylated in human spermatozoa during capacitation (Ficarro et al., 2003). PDHA (2α+2β) has two active sites and requires thiamine pyrophosphate (TPP) and magnesium as cofactors for its enzymatic activity. PDHA catalyzes the decarboxylation of pyruvate forming a tightly bound enzyme intermediate and liberating carbon dioxide (Figure 3.1). Thus, though much is known about the somatic form (PDHA1) studies related to the function and role of the testis-specific PDHA2 are scanty. PDHA2 is considered one of the most important subunits of PDH complex since perturbations in the activity of this subunit leads to reduced or total loss of function of the entire complex thus resulting in a loss of pyruvate metabolism and ATP production. PDHA2 perturbations are known to cause neurological abnormalities and lactic acidosis (Young et al., 1998). In case of plants, antisense inhibition of mitochondrial PDHA2 in anther tapetum causes male sterility (Yui et al., 2003). Moreover, PDHA in gram-negative bacteria is a homodimer, whereas in gram-positive bacteria and eukaryotes, it is a heterotetramer (Fries et al., 2003; Johnston et al., 1997; Neveling et al., 1998). The known exceptions from this rule are Zymomonas mobilis and Thiobacillus ferrooxidans, both of which are gram-negative and have been shown to possess heterotetrameric enzymes (Neveling et al., 1998).

In this chapter PDHA2 is identified as a tyrosine-phosphorylated protein from the proteome of capacitated spermatozoa of golden hamster (Mesocricetus auratus). N-terminal sequencing of the protein and the translated cDNA sequence indicated that the protein was similar to the mammalian testis-specific PDHA2. PDHA exhibited a non-canonical extra mitochondrial localization in the principal piece of hamster spermatozoa. Further, the time course of phosphorylation of PDHA2 and the activity of PDHA correlate with the capacitation status of hamster spermatozoa.
3.2 Results

3.2.1 Identification of a basic 41 kDa capacitation dependent tyrosine-phosphorylated protein in capacitated hamster spermatozoa

The present study confirms our earlier observations (Jha and Shivaji, 2002; Mitra and Shivaji, 2004) that hamster spermatozoa following incubation in a medium that is conducive for capacitation for 5 h at 37°C in a CO₂ incubator, exhibit time-dependent and capacitation-associated increase in tyrosine phosphorylation of proteins in the range of 35–95 kDa and pl 4-9 (Figure 3.2 A-D) following immunoblot analysis using monoclonal antiphosphotyrosine antibody (clone 4G10). However, when the antibody to phosphotyrosine was pre-incubated with O-phospho-L-tyrosine before use, the antibody did not cross-react with any of the proteins on the immunoblot (data not shown). In non-capacitated spermatozoa phosphorylation of proteins on the tyrosine residue was not visible (Figure 3.2 C). The prominent tyrosine phosphorylated proteins so far identified in capacitated hamster spermatozoa include precursor of hamster pro-AKAP4 (97 kDa), AKAP4 (previously known as AKAP83; 83 kDa), DLD component of pyruvate dehydrogenase (56 kDa) (Jha and Shivaji, 2002; Mitra and Shivaji, 2004; Mitra et al., 2005) and GPX4 (NagDas et al., 2005). In the present study, attempts have been made to identify a basic tyrosine phosphorylated protein of molecular mass of 41 kDa and pl ~ 8.4, which was detected only in capacitated hamster spermatozoa (Figure 3.2 D). This particular phosphorylated protein (41 kDa and pl 8.4) following N-terminal sequencing yielded 15 residues, which on BLAST analysis (Altschul et al., 1990) (Figure 3.2 E) showed that the candidate protein was PDHA2 (EC 1.2.4.1), a constituent of PDHA (Figure 3.2 E). The first residue of the candidate protein aligned with the 31st residue of PDHA2 (EC 1.2.4.1), thus indicating that the 41 kDa tyrosine-phosphorylated protein is PDHA2, the mature testis-specific form of pyruvate dehydrogenase from hamster spermatozoa (Dahl et al., 1987).
Figure 3.2 Tyrosine phosphorylation of PDHA2 in capacitated hamster spermatozoa. (A-D) 2D-PAGE and immunoblot analysis of the total proteins of non-capacitated (A and C) and capacitated (B and D) spermatozoa of hamster. In A and B the gels were stained with Coomassie Brilliant Blue R250 whereas in C and D the gels were probed with monoclonal anti-phosphotyrosine antibody. The mol. wt. standards and pl are indicated. Known capacitation dependent tyrosine phosphorylated proteins of hamster spermatozoa namely AKAP4, DLD and PDHA2 are circled and labeled 1, 2 and 3 respectively in figure 3.2 A, B and D. Note that PDHA2 with a mol. wt. of 41 kDa separates into three different protein spots with pl of 8.2, 8.4 and 8.6 respectively (figure 3.2 A, B, D and F). (E) Comparison of the N-terminal sequence of the 41 kDa and pl 8.4 protein of capacitated hamster spermatozoa with PDHA2 of Rattus norvegicus. (F) Immunoblot analysis of the total proteins of capacitated spermatozoa of hamster with monoclonal anti-PDHA2 antibody. (G) Immunoblot analysis of PDHA2 of hamster spermatozoa at different time points (0 to 7 h) during capacitation using anti-PDHA2 antibody. Anti-tubulin antibody was used as a control for equal loading of protein. (H) Solubilization experiments indicate that PDHA2 of hamster spermatozoa is associated with the detergent-soluble supernatant (S) fraction and not the detergent insoluble pellet (P) fraction following immunoblot analysis using anti-PDHA2 antibody. Solubilization of hamster spermatozoa using 0.05% Triton-X 100, followed by immunoblot analysis using anti-PDHA antibody indicated that both PDHA2 (41 kDa) and PDHB (36 kDa) are associated with pellet fraction (TP) and not with soluble fraction (TS).
Further more, using monoclonal anti-PDHA2 antibody (clone 9H9AF5, Molecular probes, Eugene, USA) it was further confirmed by Western blot analysis that the candidate protein is indeed PDHA2 (Figure 3.2 F). In addition to this protein, two other proteins of identical molecular weights but with pIs of 8.2 and 8.6 also cross-reacted with the monoclonal anti-PDHA2 antibody (Figure 3.2 F). Thus, it appears that PDHA2 exists as three different protein spots in hamster spermatozoa (Figure 3.2 F). The levels of these three proteins remained unchanged during the process of capacitation (Figure 3.2 G). The same blot was also stripped and probed with alpha tubulin antibody to confirm equal loading. Densitometric evaluation of the protein levels of PDHA2 did not show significant differences during the process of capacitation. The reason we studied the levels of PDHA2 during capacitation was to demonstrate that the levels of the protein was unaltered during capacitation, but the phosphorylation level changed (compare Figures 3.2 D and G).

Immunoblot analysis of the detergent-soluble (supernatant) and detergent-insoluble (pellet) fractions of hamster spermatozoa using monoclonal anti-PDHA2 clearly indicated a single protein band of 41 kDa only in the soluble fraction of hamster spermatozoa treated with 5 mM DTT and 0.05% Triton X-100 indicating that PDHA2 is associated with the detergent soluble fraction of sperm (Figure 3.2 H). PDHA2 was not detectable in the detergent-insoluble fraction (Figure 3.2 H). Treatment with Triton X-100 alone failed to solubilize PDHA2 (Figure 3.2 H) and it was detected only in the pellet fraction.

### 3.2.2 Sequencing of hamster Pdha2 gene and testis specific expression

To confirm that the tyrosine phosphorylated PDHA2 is a testis specific isoform, hamster testis cDNA was PCR amplified and sequenced using two overlapping primer sets namely FPE1-RPL and RP4-RP2 which amplified two overlapping fragments of
cDNA of 850 bp and 500 bp respectively when hamster testis cDNA was used as the template (Figure 3.3 A, B). These fragments when sequenced using the above four and two other nested primers (NE3 and FP1) yielded a cDNA sequence of ~1.12Kb for Pdha2 (EMBL Acc. No. AM279660). In silico translation of the cDNA sequence, yielded a sequence of hamster PDHA2 protein, which matched with the testis-specific form of rodent PDHA2 and showed >80% similarity with rat (AAH 78757.1; 82%) and mouse (P35487; 81%) testis-specific form of PDHA2 respectively (Figure 3.4). The obtained sequence did not cover the 16 amino acid residues at the C-terminal end (Hawes et al., 1995). The in silico translated sequence included functionally important domains such as the thiamine pyrophosphate (TPP) binding domain (GDGAAQGQVAEYNLSALWKLPCVF), the catalytic domain (TYRYHGHSMSDPGISYRREEVQSMRS) and interestingly the mitochondrial signal sequence (MRKMLASLVHSVGSVPAMRGGLASLH) (Figure 3.4). The TPP binding domain and the catalytic domain have been shown to be conserved in PDHA2 of human, yeasts and bacteria (Hawes et al., 1995). The testis specific form of PDHA2 of hamster differed from the somatic form of PDHA2 of rat (CAA78146.1) and mouse (AAH07142.1) to the same extent (23%) but in both the forms TPP binding domain, the catalytic domain and the signal sequence were conserved. The testis and somatic isoforms of PDHA2 of mouse also differed by 11% (lanello and Dahl, 1992).

The primer set FPE1 and RPL which specifically amplified a fragment of 850 bp of Pdha2 when cDNA from hamster testis was used as the template did not yield any amplicon when cDNA from somatic tissues such as liver, brain, kidney, spleen, heart, muscle, ovary and oviduct (Figure 3.3 C) were used further confirming that the hamster sperm associated PDHA2 is a testis-specific form. The amplified product of 850 bp after sequencing and BLAST analysis was found to be 76%, 84% and 85% similar to the testis-specific form of Pdha2 of human, rat and mouse respectively. Gapdh primers were
used as a positive control to amplify the cDNA of Gapdh from all the above tissues (Figure 3.3 C).

**Figure 3.3 Nucleotide sequence of hamster testis Pdha2.** Hamster testis cDNA was used as a template to amplify two overlapping fragments of Pdha2 of 500 bp (A and lane 1 of figure B) and 850 bp (A and lane 2 of figure B) respectively using the primer sets RP4 and RP2 and FPE1 and RPL (A). DNA size markers of 1kb were used in lane 3 of figure B. The amplified products were sequenced using four sets of primers (FPE1 and RPL, RP4 and RP2, FPE1 and RPE1 and NE3 and FP1 as indicated in A). (C) Tissue specific expression of Pdha2 was confirmed by RT-PCR using total RNA from testis (T), liver (L), brain (B), kidney (K), heart (H), muscle (M), spleen (S), ovary (OV) and oviduct (O1) of hamster using the testis-specific primers FPE1 and RPL. Amplification of Gapdh was used as a positive control for the amplification of the cDNA from all the above tissues (C). In both gels of C, 100 bp DNA ladder was used as a size marker in the first lane.
Figure 3.4 *In silico* translated sequence of hamster PDHa2. The deduced amino acid sequence of PDHA2 of hamster spermatozoa (EMBL Acc. No. AM279660) is compared with that of mouse (P35487), rat (AAH78757.1) and human (P29803) testis-specific PDHA2. The alignment was constructed by using ClustalW (http://www.ebi.ac.uk). The mitochondrial localization sequence is underlined and the arrow indicates the cleavage site between the mature and the precursor protein. The conserved TPP binding domain and the proposed catalytic domain are boxed. Identical amino acids in the four proteins are indicated by (*), gaps by (-) and conserved residues by (.).
3.2.3 Validation of rabbit polyclonal antibody against bovine pyruvate dehydrogenase (PDHA)

The commercially procured monoclonal PDHA2 antibody which successfully recognized PDHA2 as a 41 kDa protein in capacitated hamster spermatozoa by 2D-PAGE immunoblot analysis was not suitable to localize PDHA2 in hamster spermatozoa in immunofluorescence studies. Various dilutions of the antibody up to 1:1 did not yield any positive signal implying that probably the monoclonal antibody was unable to recognize the epitope associated both with intact and demembranated spermatozoa (data not shown). It is known that a homodimer of PDHA2 and an homodimer of PDHB together form a functionally active PDHA. Therefore, by localizing PDHA one could indirectly infer the localization of both PDHA2 and PDHB provided both these subunits are present. In fact, when a polyclonal anti-PDHA antibody (a gift from Prof J G Lindsay, University of Glasgow, UK) was used it was observed that anti-PDHA antibody cross-reacted with two proteins of mol. wt. 36 and 41 kDa (Figure 3.5 A). The 41 kDa protein appeared as three distinct spots and was earlier identified to be PDHA2 by N-terminal sequencing (Figure 3.5 A). The 36 kDa protein was identified to be PDHB based on its mol. wt. (36 kDa), pi (4.5-5.5) and also by MALDI analysis as determined earlier (Fujinoki et al., 2004). The assignment of the 41 and 36 kDa proteins as PDHA2 and PDHB was also confirmed using an anti-PDHc antibody, which was made against whole PDH complex (PDH) minus PDH DLD component (a generous gift from Dr R A Harris, Indiana University School of Medicine, USA) and known to cross-react with DLAT, PDHA2 and PDHB (Figure 3.5 B). Thus the results validate the specificity of polyclonal anti-PDHA to PDHA2 and PDHB. This polyclonal anti-PDHA antibody has been used in all future experiments to detect PDHA2 and PDHB in immunoblot and immunofluorescence studies. The homology between testis and somatic isoforms of PDHA are very high so it is possible that polyclonal antibody against PDHA will recognize both the isoforms of PDHA.
Figure 3.5 Immunoblot analysis of the proteins of hamster spermatozoa using polyclonal anti-PDHA antibody and anti-PDHc antibody. Hamster sperm proteins following 2D-PAGE and Western blot analysis with polyclonal anti-PDHA antibody detected all the three protein spots of PDHA2 (41 kDa) and PDHB (36 kDa) (A); anti-PDHc antibody detected DLAT (72 kDa), PDHA2 and PDHB (B). The molecular weights and isoelectric points (pl) are indicated.

3.2.4 Extra mitochondrial localization of PDHA in mammalian spermatozoa

The localization of PDHA in hamster caudal spermatozoa was studied using anti-PDHA antibody using indirect immunofluorescence microscopy. Intense staining was observed only in the principal piece of cauda epididymidal spermatozoa (PP; Cy3: red) (Figure 3.6 A). No staining was observed in the head (H), midpiece (MP) or other regions of the tail (Figure 3.6 A and 3.6 B). PDHA is a mitochondrial enzyme and therefore it was anticipated that PDHA would be present in the midpiece (mitochondrial compartment) rather than the principal piece. The absence of staining in the midpiece could be due to the inability of the antibody to penetrate the mitochondria. To rule out this possibility, immunofluorescence staining was studied following permeabilization of spermatozoa with Triton X-100, which is known to expose mitochondrial proteins. In these permeabilized spermatozoa also staining was observed only in the principal piece and no staining was observed in the mid piece (Figure 3.6 C). Further, when monoclonal antibody to sperm mitochondrial protein phospholipid glutathione peroxidase (GPX4)
(donated by Prof. Kuhn, Humboldt University Medical School Charite, Germany) was used as a marker for sperm mitochondria (midpiece), as anticipated, localization was observed only in the midpiece (MP) of the spermatozoa [FITC: green] (Figure 3.6 E). Immunofluorescence studies using the secondary antibody alone did not yield any staining on the spermatozoa (Figure 3.6 G).

Further, to investigate the fate of PDHA during hamster sperm capacitation, immunofluorescent studies were performed with hamster spermatozoa samples prepared at different time points during capacitation. Staining was observed only in the principal piece and the intensity of staining remained unaltered in this region of the spermatozoa both in non-capacitated (0 to 1.5 h) and capacitated spermatozoa (3-7 h) (Figure 3.6 H).

The generality of the non-canonical extra mitochondrial localization of PDHA observed in hamster spermatozoa was also investigated in ejaculated human and cat spermatozoa using anti-PDHA antibody. In both cat and human spermatozoa staining was not observed in the midpiece. The staining in cat spermatozoa differed from that of hamster spermatozoa in that PDHA appeared to be present in the principal piece and in the posterior region of the head (Figure 3.6 I). In human spermatozoa staining was seen only in the posterior region of the head (Figure 3.6 K). Immunoblot analysis of cat and human spermatozoal proteins confirmed that the PDHA antibody was cross reacting with both the subunits namely PDHA2 (41 kDa) and PDHB (36 kDa) (Figure 3.6 M). Thus the results indicate that PDHA exhibits non canonical extra mitochondrial localization in mammalian spermatozoa.
Figure 3.6 Immunofluorescent localization of PDHA and GPX4 in hamster spermatozoa. Immunofluorescent localization of PDHA and the sperm mitochondrial marker GPX4 (Phospholipid glutathione peroxidase) in hamster spermatozoa using anti-PDHA antibody and anti-GPX4 antibody respectively. PDHA is present only in the principal piece (PP) of the spermatozoa both in non-capacitated (A and C) and capacitated spermatozoa (H). The intensity of staining remained unaltered during the process of capacitation (H). Staining was not observed in the end piece (EP), mid-piece (MP) or the head (H) region (blue due to DAPI staining). Triton-X 100 treated spermatozoa also showed PDHA in the principal piece (C). PHGPx localized to the midpiece of the spermatozoa (E). Figure B, D, F, J and L are phase contrast micrographs of figure A, C, E, I and K. Spermatozoa treated only with the secondary antibody did not show any localization on the spermatozoa (G). (I-L) Immunofluorescent localization of PDHA in cat and human spermatozoa using anti-PDHA antibody. In cat spermatozoa PDHA localizes in the posterior region of the head (H) and the principal piece (PP) of the tail (I) whereas in human spermatozoa PDHA was present only in the posterior region of the head (H) (K). The bar in figure (A-H) represents 10 µm and (I-L) represents 20 µm. Immunoblot analysis was also performed on cat and human spermatozoal proteins using anti-PDHA antibody, which detected PDHA2 (41 kDa) and PDHB (36 kDa) (M).
3.2.5 Localisation of PDHA in fibrous sheath (FS) of hamster spermatozoa by confocal microscopy

Localisation of PDHA in the fibrous sheath (FS) of the flagellum of hamster spermatozoa was performed by confocal microscopy using polyclonal anti-PDHA antibody and anti-AKAP4 antibody of hamster, which was earlier demonstrated by us to be localized in the FS of hamster spermatozoa. Hamster spermatozoa showed localization of both AKAP4 and PDHA in the principal piece and merger of the images indicated that AKAP4 and PDHA are colocalised (Figure 3.7 A) thus implying that PDHA is present in the FS in hamster spermatozoa. In the confocal studies, optical sections (0.2 μm each) of the spermatozoa were obtained and the innermost sections in which the localization was observed were merged to ascertain colocalization (Mitra et al., 2005).

3.2.6 Subcellular localisation of PDHA in fibrous sheath (FS) of hamster spermatozoa

Localisation of PDHA in hamster spermatozoa was also studied by fractionating the spermatozoa into heads, tails, FS and outer dense fibres (ODF) (Figure 3.7 B) which were then subjected to immunoblot analysis using anti-PDHA antibody. Proteins of the head did not yield any band following western blot analysis (data not shown) but in the tail two bands of mol. wt. 41 and 36 KDa were detected corresponding to PDHA2 and PDHB respectively associated with FS and not ODF. Using anti-AKAP4 antibody as a marker for FS and anti-outer dense fiber 2 (ODF2) antibody as a marker for ODF (Gift from Dr Sigrid Hoyer-Fender, Georg-August-Universitat, Gottingen, Germany) it was demonstrated that FS and ODF prepared cross-reacted with the respective antibodies (Figure 3.7 B). Anti-AKAP4 antibody cross-reacted with AKAP4 [83 kDa protein in FS] (Jha and Shivaji, 2002) and anti-ODF2 antibody cross-reacted with two proteins of mol. wt. 45 kDa and ~ 70 to 80 kDa, characteristic of the ODF2 protein (Cristoph et al., 1999)
(Figure 3.7 B). These studies confirm the association of PDHA with FS of hamster spermatozoa.

3.2.7 Tyrosine phosphorylation of PDHA in the principal piece of hamster spermatozoa

Since PDHA2 exhibits capacitation dependent tyrosine phosphorylation it was interesting to check colocalization of PDHA2 using anti-PDHA antibody and tyrosine phosphorylation using anti-phosphotyrosine antibody in capacitated hamster spermatozoa and was monitored by confocal microscopy (Figure 3.7 C). The results indicated co-localization of PDHA and tyrosine phosphorylation in the tail of capacitated spermatozoa but not in the head region (Figure 3.7 C). In other mammalian spermatozoa it was also observed that most of the capacitation dependent tyrosine phosphorylated protein localize in flagella of capacitated hamster spermatozoa (Jha and Shivaji, 2002; Leclerc et al., 1997; Si and Okuno, 1999; Uner et al., 2001; Mitra et al., 2005).
Figure 3.7 Association of PDHA2 with fibrous sheath of hamster spermatozoa. Localisation of PDHA in the fibrous sheath of hamster spermatozoa by confocal microscopy (A). Confocal microscopy studies show colocalisation of PDHA (Cy3: red) with fibrous sheath (FS) marker AKAP4 (FITC: green), in the principal piece of hamster caudal spermatozoa. Immunoblot analysis (B) using anti-AKAP4 antibody (panel 1), anti-ODF2 antibody (panel 2) and anti-PDHA antibody (panel 3) confirm that PDHA2 and PDHB are present only in the fibrous sheath (FS) and it is absent in outer dense fiber (ODF). ODF markers were not present in FS but were present in ODF (panel 2). The FS marker AKAP4 was detected only in the FS fraction (panel 1). Colocalisation of PDHA and phosphotyrosine proteins in the (PP) of capacitated hamster spermatozoa determined by confocal microscopy (C). The spermatozoon was first labeled with anti-phosphotyrosine antibody (green) followed by anti-PDHA antibody (red).
3.2.8 Pyruvate dehydrogenase activity during hamster spermatozoal capacitation

The activity of PDHA in hamster spermatozoa capacitated in TALP was assayed using an established method (David et al., 1985; Marina et al., 2005) (for details see Materials and Methods). PDHA was enzymatically active both in non-capacitated and capacitated spermatozoa (Figure 3.8 A). The activity increased progressively with capacitation and attained a peak between 3 to 5 h and declined marginally thereafter. The activity at 3 h was significantly different when compared to the activity at 0 and 7 h during capacitation (P < 0.05). Further, the activity peak at 3 h coincided with maximum number of spermatozoa that get hyperactivated at this time point. Correlation studies indicated a positive correlation between enzyme activity and percentage of hyperactivated spermatozoa (Figure 3.8 A) with a correlation coefficient (r_s) of 0.863 and the correlation was significant at the level of 0.01. However, when the activity of PDHA was compared with the number of spermatozoa undergoing the acrosome reaction the correlation coefficient (r_s) was 0.274 and it was not significant at 0.01 level (Figure 3.8 A). Increase in PDHA activity also matched the temporal sequence of increase in capacitation dependent tyrosine phosphorylation of PDHA2 during capacitation (Compare figures 3.8 A and 3.8 B). The motility of the hamster spermatozoa during the course of capacitation was around 80%.
Figure 3.8 Activity of PDHA during capacitation of hamster spermatozoa. Correlation of PDHA activity with hamster sperm hyperactivation and acrosome reaction. The PDHA activity (■) and the percentage hyperactivated spermatozoa (○) were found to be positively correlated (Spearman’s correlation coefficient = 0.863) and were significant at P < 0.01 (A). Correlation coefficient of 0.274 was found between PDHA activity and percentage acrosome reaction (○). Asterisks indicate significant difference (P < 0.05 as determined by Student’s t test) in PDHA activity at 3 h compared to activity at 0 and 7 h time points. The percentage motility during the period of incubation was around 80%. Figure 3.8 B shows capacitation dependent increase in tyrosine phosphorylation of PDHA2 in hamster spermatozoa. The histogram represents the intensity of PDHA2 tyrosine phosphorylation as determined using a densitometer.
3.3 Discussion

Mammalian sperm capacitation, a process which confers fertilization competence to spermatozoa, was discovered about half a century ago (Austin, 1952; Visconti and Kopf, 1998; Jha et al., 2003), but as yet the molecular basis of capacitation is still not completely understood (Kulanand and Shivaji, 2001; Jha and Shivaji, 2002; Visconti et al., 1995; Visconti et al., 1999; Cohen Dayag and Eisenbach, 1994; Shivaji et al., 2006). In this chapter, we demonstrate that PDHA2, a testis-specific form which metabolizes pyruvate directly, is tyrosine phosphorylated in capacitated hamster spermatozoa. PDHA2 is a housekeeping enzyme with a significant role in metabolism. The molecular weight of hamster sperm PDHA2 is ~41 kDa and it exists as three different tyrosine phosphorylated proteins with identical molecular weights and the pIs were 8.2, 8.4 and 8.6 respectively (Figure 3.2 A, B, D and F). Further, all the three proteins cross-reacted with PDHA2 monoclonal antibody. PDHA2 in maize also exists as a multiple protein consisting of four to five proteins (Jay et al., 1998). PDHA2 is not associated with the soluble fraction of hamster spermatozoa. Using combination of DTT as a reducing agent and Triton X 100 as a detergent, PDHA2 was completely solubilized. The cDNA derived amino acid sequence of hamster sperm PDHA2 exhibited >80% similarity with the testis-specific PDHA2 of rat and mouse. Further, the TPP binding motif and the catalytic site are conserved as in all the PDHA2 sequences. The tissue specificity was also confirmed using a set of primers specific for the testis form (Figure 3.3 C). A total of 18 residues of tyrosine were found in PDHA2. Fourteen of the tyrosine residues were conserved in mouse, rat, hamster and human and some of these conserved residues were located at sites important for the activity of the enzyme such as the residue at position 195 which is adjacent to the TPP binding domain and three residues within the proposed catalytic domain at positions 292, 294 and 306 respectively. Further, three of the tyrosine residues at positions 157, 247 and 277 were predicted to be potential sites for tyrosine phosphorylation [NetPhos 2.0 Server] (Blom et al., 2004). Two of these residues at
positions 157 and 247 are conserved in mouse, rat, hamster and human. Fujinoki et al., (2004) observed that PDHB is phosphorylated at the serine residue and associated with the regulation of motility activation in hamster spermatozoa. In human spermatozoa, PDHB was identified to be tyrosine phosphorylated during capacitation, using MALDI approach (Ficarro et al., 2003). This chapter demonstrates for the first time capacitation-dependent tyrosine phosphorylation of PDHA2 in a spermatozoon.

Studies in hamster spermatozoa have shown extra mitochondrial localization of enzymes such as ATP synthase F1 beta subunit (ATP5B) in principal piece (Fujinoki et al., 2003), dihydrolipoamide dehydrogenase (DLD) in principal piece and head (Mitra et al., 2005) and pyruvate dehydrogenase E1β (PDHB) in flagellum of hamster spermatozoa (Ficarro et al., 2003). Similarly, voltage dependent anion channels (VDAC2 and VDAC3), which are mitochondrial porins in somatic cells, are found to be associated with outer dense fibres in bovine sperm flagella (Hinsch et al., 2004) and phospholipid glutathione peroxidase (GPX4) another cytosolic protein in somatic cells, is also found to be associated with mitochondria in mature rat spermatozoa (Roveri et al., 1992). The results of this chapter also demonstrates that PDHA exhibits extra mitochondrial non-canonical localization in the principal piece of hamster spermatozoa (Figure 3.6 A, C, H) in the principal piece and in the posterior region of the head in cat spermatozoa (Figure 3.6 I) and in the posterior region of the head in human spermatozoa (Figure 3.6 K) in comparison to its canonical, mitochondrial localization in somatic cells. Reorganization of proteins during sperm capacitation is an important phenomenon (Grace et al., 2002) but PDHA does not show any change in localization during the time course of capacitation (Figure 3.6 H). It is noteworthy to mention that the homology between testis and somatic isoforms of PDHA is high, so it is possible that the polyclonal anti-PDHA antibody used in this study is likely to recognize both the isoforms.
Spermatozoal fibrous sheath is known to act as a scaffold for protein kinase A (PKA) anchoring proteins and glycolytic enzymes (Fawcett, 1975; Eddy et al., 2003; Cao et al., 2006; Storey and Kayne, 1975). In hamster spermatozoa PDHA2 is associated with FS (Figure 3.7 A, B) and can be solubilised using 0.05 % Triton-X 100 and DTT as a reducing agent (Figure 3.2 H). Confocal studies also confirmed that PDHA in hamster spermatozoa is associated with FS (Figure 3.7 A) based on the colocalization observed between AKAP4, a marker of FS, and PDHA (Figure 3.7 A, C). This result is interesting since it is already known that the glycolytic apparatus is localized in the principal piece of mammalian spermatozoa and lends support to the idea of compartmentalized metabolic pathways in spermatozoa (Fawcett, 1975). The association of PDHA with FS highlights the importance of PDHA in providing byproducts like NADH (Tajima et al., 1987; Tajima et al., 1990), which has been implicated in aiding pyruvate metabolism in the mitochondria of pancreatic islets (Eto et al., 1999). Presence of the mitochondrial localizing signal sequence in PDHA is intriguing considering that the enzyme is localized at an extra mitochondrial site, the principal piece of the spermatozoon.

Capacitation-associated increase in protein tyrosine phosphorylation has been observed in spermatozoa of a number of mammalian species (Mitra et al., 2005). As yet, it is not clear whether phosphorylation is the cause or effect of capacitation. In eukaryotes, phosphorylation-dephosphorylation cycle of PDHA2 is a strong regulator of PDH activity (Linn et al., 1969a, 1969b) and the entire pyruvate dehydrogenase complex when phosphorylated is enzymatically-inactive (Dahl et al., 1987; Patel and Korotchkina, 2001). In hamster spermatozoa, the activity of the PDHA (Figure 3.8 A) followed the temporal sequence of increase in protein tyrosine phosphorylation of PDHA2 during capacitation, which peaks around the third to the fifth hour of capacitation and stays so till seventh hour (Jha and Shivaji, 2002) (Figure 3.8 B). It is also interesting to note that PDHA activity profile and the temporal increase in the percentage of hyperactivated
spermatozoa are positively correlated with a correlation coefficient ($r_s$) of 0.863, implying that PDHA activity and hyperactivation are positively correlated (Figure 3.8 A). If this contention is right it should indeed be possible to demonstrate that inhibition of PDHA should inhibit hyperactivation. It was also observed that PDHA activity is not correlated with the acrosome reaction.

This chapter on phosphorylation, localization and enzymatic activity of PDHA in mammalian spermatozoa clearly indicate the involvement of PDHA in sperm capacitation. A question that arises is what is the role of PDHA in sperm capacitation? There are definitive evidences presented in the next chapter (Chapter 4) indicating that pyruvate dehydrogenase complex is crucial for the generation of reactive oxygen species (ROS) (Starkov et al., 2004), which has been implicated in capacitation (Aitken et al., 1997; de Lamirande et al., 1997). Further, increase in activity of pyruvate dehydrogenase complex could lead to increase in utilization of pyruvate-lactate thus leading to generation of more NADH. NADH thus generated could be used by NADH oxidase for generating ROS required for capacitation. NADH oxidase activity and ROS generation have also been linked to motility of human spermatozoa (Zini et al., 1998). Bennelli et al., (2002), reported that a novel Y243S mutation in the PDHA2 subunit leads to decrease in interaction with thiamine pyrophosphate (TPP), a cofactor necessary for activity of PDH. Next chapter focuses on studies to identify the role of extra mitochondrially localized PDHA in sperm capacitation.

3.4 References

All references are listed at the end of the thesis.