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IISc THESES ABSTRACTS

Thesis Abstract (Ph.D.)

A conservation strategy for the birds of the Uttara Kannada district by R. J. Ranjit Daniels.
Research supervisor: Madhav Gadgil.
Department: Centre for Ecological Sciences.

1. Introduction

A conservation strategy for birds involves the consideration of various factors such as the present status of the landscape in concern, characterization of the existing vegetation types, patterns of distribution of vegetation and birds, the biogeographic influences that determined these patterns, history of changes in the bird fauna and the underlying reasons, if any, local factors influencing the bird species diversity and assemblage, habitat preferences of birds and the need to assign conservation values or priorities to birds based on rational attributes and criteria.

2. Present study

The Uttara Kannada district is in the northwestern extreme of Karnataka. Lying between 13° 55′ N–15° 32′ N latitude and 74° 05′ E–75° 05′ E longitude it covers a total area of 10,291 km^2. Located centrally on the Western Ghats it shares most of the climatic, topographic and landscape features of this hill chain, especially a high average annual rainfall of 2500 mm. Birds of Uttara Kannada were studied and listed by early British ornithologists even 50 to 100 years back. From a careful analysis of the changes in land use over the past 100 years and changes in the bird fauna based on a five-year study, it was apparent that the bird fauna of this district has by and large remained stable despite significant changes in the land use. The few species of birds that have possibly disappeared from the district were on the fringes of their geographic ranges. Birds of the drier scrub seem to have suffered the worst due to habitat transformation into irrigated cultivation. The birds which have recently expanded their ranges over the district are those utilizing the newly created irrigation tanks and reservoirs[1].

Though there are still canopied forests covering 60% of the district (LANDSAT imagery for the year 1986), these show various levels of human interference. The present study of vegetation regeneration suggests that most of the forests in the district are recovering from past disturbances. It was also found that the diversity of woody plants increases with higher rainfall and decreases with disturbance (40 species/2400 m^2 and 33 species/2400 m^2 in the least and most disturbed evergreen forests, respectively). This study has also shown that the diversity of woody plants is the highest in evergreen forests and this is positively correlated to the structural complexity of the forests as measured by vertical stratification, free and canopy densities.

The Uttara Kannada district has been divided into five ecological zones based on the distribution of vegetation and birds. They are the coastal, northern (less disturbed) evergreen forest, southern

(more disturbed) evergreen forest, moist deciduous forest and dry deciduous forest/scrub zones. The moist deciduous forest zone has the highest bird species richness of 165 taxa (species and subspecies) of birds whereas the northern evergreen forest has the least diversity of the five zones with 111 taxa. However, this zone has the most distinct bird fauna of the five zones.

Uttara Kannada has an estimated total of 465 ± 20 taxa of birds[1]. Approximately a third of these are migrants visiting the district in winter. Whereas 93% of the migrants are palaearctic birds, 90% of the residents are shared with the southern Western Ghats.

The birds of Uttara Kannada are mostly generalized habitat users utilizing a family of closely related habitat types. A new index for measuring habitat niche breadth that is introduced in this study takes into consideration both the time a taxa of bird spends in each habitat type and also the relatedness between these habitat types. Based on this niche index it is shown that the evergreen forest birds have the least niche breadth of 1.68 whereas the birds utilizing the rocky hill tops have the broadest of 2.27.

Local bird species diversity is the highest (47 taxa/sample) in the moist deciduous forest and not as expected in the evergreen forest (31 taxa/sample). Number of taxa of birds is negatively correlated to the structural complexity of natural forests as measured by canopy and tree density, vertical stratification and plant species diversity. Number of bird taxa per sample is positively correlated to the landscape heterogeneity and heterogeneity of the canopy as measured by the coefficient of variation in the canopy density. Structurally similar vegetation types have similar bird species and this is further exaggerated by proximity of the samples compared.

Assigning numerical values to birds based on four attributes such as geographical restrictedness, narrow habitat preference, taxonomical uniqueness and degree of endangerment suggested that the birds of Uttara Kannada value low on the third and fourth attributes. Geographical restrictedness is apparently the most important attribute to be considered in assigning conservation values to the birds of Uttara Kannada. Birds of the evergreen forest value high under this attribute if considered at the global scale. Habitat specialists are however more in the marshes and hence these habitats take a higher value under this attribute.

3. Conclusion

The findings suggest that birds of the Uttara Kannada district are by and large opportunists doing better in more heterogeneous landscapes. To maintain the existing diversity of the district, it is recommended that a careful mosaic of different habitat types may be maintained as a network. The major habitats identified for this network are the evergreen forests for their endemic bird taxa, the marshes for their habitat specialists and the moist deciduous forests for their high diversity of birds. Most of the secondary habitats such as monocultures of teak, betelnut and eucalyptus can also be incorporated into the conservation plan as they can serve as links between the nodes or centres of diversity which may otherwise get isolated[2].

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Thesis Abstract (Ph.D.)

Antibodies specific to dpG. sequence-dependent binding to dsDNA by Chitra Kannabiran.
Research supervisors: T. M. Jacob and N. Appaji Rao.
Department: Biochemistry.

1. Introduction

Antibodies specific to nucleic acids and their components have several applications[1], and those specific to dpG had been studied earlier in our laboratory[2–5]. It was found that a population of dpG antibodies bind preferentially to dsDNA with dpG-specificity. In the present investigation, dpG antibodies were studied with a view to understand their mode of interaction with dsDNA. The results show that the antibodies bind at dpG residues studied at specific nucleotide sequences in the DNA.

2. Experimental approach

Antibodies specific to dpG were raised in rabbits by immunization with protein conjugates of dpG. IgG was prepared from antisera by sodium sulphate precipitation. Antibody binding to ^{3}H-dpG, ^{3}H-dsDNA and ^{3}H-ssDNA were assayed by nitrocellulose-filter-antibody-binding assay (NFAB). The use of these radioactive ligands enables one to study the antibody population having the highest affinity to any one of these probes in the presence of other antibody populations. Analysis of the specificity of the antibody population binding to a radioactive probe was carried out by competitive inhibition of the binding, using non-radioactive nucleosides, nucleotides, dsDNA and ssDNA as inhibitors. Antibody-DNA binding was also assayed by gel electrophoresis on agarose gels.

3. Results and discussion

Specificity analysis revealed that the populations of antibodies that bind to ^{3}H-dpG, ^{3}H-dsDNA and ^{3}H-ssDNA are different but possess several similar properties. They show higher affinity to dpG compared to other nucleotides and to dsDNA compared to ssDNA. Deoxyribose moeity is highly immunodominant. The role played by phosphate group in antibody binding is different for the three antibody populations. For the ^{3}H-dpg-binding antibodies the negative charges on the phosphate group appear to be inhibitory whereas for the ^{3}H-ssDNA-binding population of antibodies the phosphate group is important for the interaction. However, for the ^{3}H-dsDNA-binding population, the phosphate group is not essential for binding.

The binding of dpG antibodies to sheared *E. coli* ^{3}H-dsDNA increased initially with increasing amounts of antibody but saturation was reached when only 20% of the DNA fragments were bound. This indicated that all fragments of sheared *E. coli* dsDNA do not have dpG-antibody binding sites of sufficient affinity. Experiments using unbound DNA fragments collected from NFAB assays confirmed that they were deficient in antibody binding. These experiments further showed that the binding sites for dpT, dpC, dpA and dpG antibodies are located mostly on different fragments of sheared *E. coli* DNA. Competitive inhibition studies using DNAs of similar composition showed that the occurrence of dpG-antibody binding sites per unit weight differ widely in these DNAs.

Identification of possible binding sites for dpG antibodies was achieved by monitoring the binding to restriction fragments of plasmid and phage DNAs of known sequences, using gel electrophoresis. Computer search revealed two unique sequences—$\frac{GCATC}{CGTAG}$ and $\frac{CAGCTT}{GTCGAA}$—missing from all non-binding DNA fragments but present in all binding fragments. The hexamer contains AGCT which is the

cutting site for the restriction endonuclease AluI. It was observed that digestion of PUC 19 DNA by AluI was inhibited in a time-dependent manner of dpG antibodies. This result proves that CAGCTT is a binding site for the antibodies. It is proposed that sequence-dependent fine structure of DNA and conformation of dpG at the binding site are important for antibody binding.

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Thesis Abstract (Ph.D.)

Antibody probes for DNA fine structure: Studies with dpC antibodies by C. Srikumar.
Research supervisor: T. M. Jacob.
Department: Biochemistry.

1. Introduction

Nucleic-acid-reactive antibodies are of great interest in clinical medicine and are potentially useful for the isolation and estimation of nucleic acids or their constituents as inhibitors of nucleic acid functions, as probes for nucleic-acid structures and as model systems to study protein-nucleic acid interactions[1]. Antibodies have been raised against mono-, di- and tri-nucleotides in our laboratory and their specificities to various ligands studied[2–8]. The interaction of anti-dpG antibodies with dsDNA has been reported previously [9,10]. The present work is a detailed study of the interactions of different populations of anti-dpC antibodies with DNA. It has led to the identification of the most-preferred binding site on dsDNA for a population of anti-dpC antibodies.

2. Materials and methods

Anti-dpC antibodies were raised to rabbits by injecting them with dpC conjugated with BSA or Tg. IgG was prepared from the sera and used for all the studies. ^{3}H-DNA was prepared from the thymine-requiring strain of *E. coli*, W 3110$\bar{T}$ by growing it in M 9 medium supplemented with ^{3}H-thymine.

The binding of anti-dpC antibodies to ^{3}H-DNA was assayed by nitrocellulose filter binding assay as described previously[11]. Gel retardation assays were done on submarine agarose gels in 0.04 M tris-acetate EDTA buffer, pH 7.5, at 4°C.

3. Experiments and results

The specificity of anti-dpC antibodies was determined by binding to ^{3}H-DNA and competing with dpC and other non-radioactive ligands using the nitrocellulose filter assay. Three different populations of DNA-binding dpC antibodies were characterized from the antisera: those specific to ssDNA (0.15 M

salt), specific to dsDNA (0.15 M salt) and dsDNA (3.75 M salt). The DNA binding was base-specific in all the three cases.

It is probable that dpC residues in the immunogen assume different conformations by interacting with the lysine side chain through which they are attached to the protein or other parts of the protein. To test this idea, simple model compounds were made by chemically coupling aliphatic alcohols and amines to dpC. When the inhibitory effect of these compounds on ^{3}H-dsDNA binding in 0.15 M salt was studied it was found that all of them are better inhibitors than dpC.

Studies using sheared ^{3}H-DNA (from *E. coli*) fragments recovered from filtrate after dpC-antibody binding revealed that the antibodies do not bind to all the fragments. This gave the clue that there are only limited number of dpC-antibody binding sites on *E. coli* DNA. By competition experiments it was found that the relative abundance of sites is different on different eukaryotic and prokaryotic DNAs.

To identify the actual sites of dpC-antibody binding to dsDNA, agarose gel electrophoresis was resorted to. When antibodies bind to DNA, the DNA either remained at the origin or its movement was retarded. The antibodies definitely bound to two Hinf I restriction fragments of pBR 322 and three of Hind III digest of lambda DNA but not to Hae III-digested ØX174 DNA at all. Computer analysis of the sequence of these fragments revealed the self-complementary sequence GATC as the antibody-binding site. When Bam H1 linkers (GGATCC) were ligated to a non-binding ØX174 DNA fragment, it bound dpC antibodies thus confirming the results of the computer analysis.

4. Discussion

The studies reported here show that there are at least three different populations of DNA-binding anti-dpC antibodies in the antisera elicited. They vary in their specificities. The population which binds to ^{3}H-dsDNA at 0.15 M salt concentration has been shown to be site-specific. The site of binding has been identified as dpC in the sequence GATC, and the antibodies are binding to this sequence probably because this sequence has a specific fine structure with a specific conformation of dpC residues. Thus these antibodies are dpC-specific probes for the fine structure of DNA.

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Thesis Abstract (Ph.D.)

Studies on the biology of thermophilic fungi by A. K. Rajasekaran.
Research supervisor: Ramesh Maheshwari.
Department: Biochemistry.

1. Introduction

Among the eukaryotes, only some fungi possess the ability to grow at temperatures up to 60°C[1]. The thermophilic fungi, as they are called, are an important component of the microflora which develops in self-heating masses of organic matter. They are also widespread in soil. This study was undertaken to obtain some basic information on their growth and physiological characteristics. Such information was expected to prove useful in understanding the ecology and evolution of thermophilic fungi.

2. Experimental

2.1. *Growth*

A thermophilic fungus, *Thermomyces lanuginosus*, commonly found in compost and soil, was selected for most experiments. Its T_{min}, T_{opt} and T_{max} of growth were close to 30, 40 and 60°C, respectively. The values for specific growth rate and the molar growth yield (biomass produced per mole of sugar utilised) of thermophilic fungi studied were comparable to those of mesophilic species, when the measurements were made at the respective temperature optima of growth. These findings discount the belief that the thermophiles grow faster, and have a higher maintenance energy requirement than the mesophiles.

2.2. *Respiration*

Measurements of respiratory rates of mycelia showed that thermophilic fungi do not have a faster rate of metabolism than mesophilic fungi. This suggests that an ability to hold their reaction rates in spite of the increased kinetic energy of the reacting molecules could be the hallmark of thermophilic fungi. Determination of respiratory quotient indicated that at a high temperature (50°C) of growth the main endogenous substrate was carbohydrate, whereas at low temperature (30°C) it was lipid. Determination of C6/61 ratio indicated that the predominant pathway of hexose degradation in *T. lanuginosus* at 50°C was the Embeden-Meyerhof pathway, and at 30°C it was pentose phosphate pathway. This suggested that metabolism in thermophilic fungi readily responds to changes in temperature; the temperature acts as switch in regulating the metabolism.

2.3. *Lipid composition*

Because lipids can exhibit phase transitions as a function of temperature, an inability to adjust the lipid composition of the plasma membrane had been proposed to be a reason for the inability of thermophilic fungi to grow at the low temperatures. The higher content of unsaturated fatty acids in the phospholipid fraction of *T. lanuginosus* grown at 30°C suggested that this is unlikely to be the cause of inhibited growth at the low temperatures.

2.4. *Nutrient uptake system*

Active transport of glucose in *T. lanuginosus* was mediated by a substrate-specific carrier. Such transport seemed to be driven by a proton gradient across the plasma membrane. The possession of a high-affinity sugar transport system (Km $\approx$ 290 μM) would allow the utilisation of substrate when present in low concentrations.

2.5. *Protein breakdown*

An early hypothesis proposed that thermophilic microorganisms can grow and survive at high temperatures because of the ability to rapidly replace heat-labile proteins. The rate of protein breakdown in thermophilic fungi was comparable to that in the mesophilic fungi (3.5% per h). The hypothesis of rapid turn over of protein was therefore discounted as a general mechanism for thermophily.

2.6. *Ecology*

Microbiological examination of soil samples collected from different places in India confirmed the widespread distribution of thermophilic fungi in soil. As the soil temperatures are normally far below the optima for thermophilic fungi, experiments were designed to understand their common occurrence in soil. An immunofluorescent approach was taken to study the autecology of *T. lanuginosus* using the buried-slide technique. The ability of the fungus to grow in soil in the open could not be proved. Experiments were also designed to study the competitive ability of thermophilic fungi in soil plates exposed to diurnally varying thermal regimes. Development of thermophilic fungal colonies in soil plates occurred only when the incubation temperature was varied between 40 and 48°C. The experimental results do not favour the view that thermophilic fungi can normally grow and reproduce in soil. Their widespread presence in soil is considered to be a result of dissemination of propagules from growth in self-heating masses of organic material (compost) wherein high temperatures (up to 55°C) have been recorded.

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Thesis Abstract (Ph.D.)

Colitis-phage lytic enzyme and its gene by Anita Gupta.
Research supervisor: J. D. Padayatty.
Department: Biochemistry.

Colitis bacteriophage is a virulent phage of *Escherichia coli* and has a growth cycle of 35 min with a burst size of 100[1]. It contains 65% protein and 35% DNA. The DNA is double-stranded, is of 33 kilobase (kb) in size and is highly transfective. The phage induces a lysozyme which is involved in the lysis of the infected cells. The lysozyme acts on *Micrococcus lysodeikticus and E. coli* cells. A lysozyme mutant of the colitis phage was not able to produce the lysozyme protein as demonstrated by the inability to act on *M. lysodeikticus* cells and the absence of the immunoreactivity with antibodies

raised against lysozyme. However, the lysozyme-mutant phage was able to lyse the host cell by inducing a lytic enzyme which acts on *E. coli* and not on *M. lysodeikticus* cells. By the expression of the cloned colitis-phage DNA, it was shown that a 1.2-kb *BamHI* DNA fragment codes for the lysozyme while a 2.3-kb *Bam*HI fragment codes for the lytic enzyme [2,3].

The lytic enzyme was partially purified from the lysozyme-mutant-phage-infected cells and to near homogeneity from the *E. coli* cells harbouring cloned lytic enzyme gene by a single-step purification by CM cellulose column chromatography[2]. The lytic enzyme has a molecular weight of 19,000, pH optimum of 7.5 and requires 0.5 mM $MgCl_2$ for optimum activity. It was found to be different from T4, colitis phage and hen egg white lysozymes in its immunoreactivity and substrate specificity.

The DNA form the lysozyme-mutant colitis phage was digested with *Bam*HI and cloned into pBR322 at the *Bam*HI site and the recombinant clones were screened for the lytic enzyme activity. A clone producing lytic enzyme was identified and had an insert DNA of 2.3 kb. This insert was also shown to code for a 20-kd structural protein. The 2.3-kb insert has a single *Cla*I site and produces 1.2- and 1.1-kb *Cla*I-*Bam*HI fragments which were cloned into pBR322. The clone containing the 1.1-kb *Cla*I-*Bam*HI fragment produced the lytic enzyme. The structural gene for lytic enzyme and the promoter were mapped on a 0.8-kb *Bgl*II-*Bam*HI segment of the 1.1-kb DNA fragment by an *in vitro* transcription-translation system derived from *E. coli* MRE 600. The translated products were immunoprecipitated with the antibodies raised against the colitis-phage lytic enzyme, resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and the radioactive protein bands were detected by fluorography.

The 1.1-kb *Cla*I-*Bam*HI DNA fragment was sequenced by the dideoxy chain termination method and a sequence of 1071 nucleotides was obtained. A sequence of 163 amino acids was derived from the nucleotide sequence. The protein containing the 163 amino acids has a molecular weight of 18,050 and contained 13 Arg and 8 Lys residues making the protein basic. The molecular weight, the basicity and the N-terminal amino-acid sequence, Met-Arg-Phe, of the derived protein, are in agreement with those of the lytic enzyme. The derived amino acids at the N-terminus of the colitis-phage lytic enzyme are homologous to the extent of 18–34% with those of T4-, P22- and ϕ29-phage lysozymes[4,5]. The Shine-Dalgarno-like sequence, GGGGT, is 5 nucleotides upstream to the initiator codon, ATG. The primer extention studies showed that the initiation of transcription is at G, 166 nucleotides upstream to the ATG. The sequence TAACGTG and TTATCA, at position −10 and −35 from initiation of transcription may represent the Pribnow box and the RNA polymerase recognition site, respectively.

The leader sequence of the lytic enzyme messenger RNA is 166 nucleotides long and can form stem-and-loop structure which may protect the messenger RNA from the nuclease digestion. The transcription-termination signal, the invert repeat, and the termination site, the oligo Ts, were at 7 and 28 nucleotides, respectively, downstream to the translation termination site, TAA. It was followed by another invert repeat at 85 nucleotides, which also may act as a signal for rho-dependent transcription termination[6]. The RNAs isolated from colitis-phage-infected cells hybridized with the cloned gene for the lytic enzyme and they were resolved into four species of RNAs of sizes 1100, 800, 700 and 560 nucleotides. This indicated that the initiation and/or termination of transcription of lytic enzyme messenger RNA take place at more than one site.

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Thesis Abstract (Ph.D.)

Quantitative studies on the expression of rinderpest virus genes in acute and persistent infections by Deepa A. Bhavsar.
Research supervisor: M. S. Shaila.
Department: Microbiology and Cell Biology.

1. Introduction

A large number of publications have appeared regarding measles virus (MV) persistence *in vitro*[1,2] but hardly any information is available on the persistence of rinderpest virus (RV). An *in-vitro* persistent infection of RV in Vero cells has been established earlier in this laboratory. This cell line is referred to as Pi-2 and the basic mechanisms of persistent infection of RV have been investigated in detail[3,4]. Characterisation of biochemical and molecular biological parameters leading to the maintenance of persistent RV infection has led to some interesting findings, which, in turn, have helped us in providing a basis for understanding the molecular mechanisms underlying RV persistence and viral pathogenicity.

The present investigation aims at analysing the quantitative differences in the transcription of individual virus genes leading to mRNA synthesis in Pi-2 cells with the help of gene-specific probes. An attempt has also been made to quantify the amounts of antigenome and genome RNA in Pi-2 cells and compare with those made during lytic infection. It is hoped that by comparing the synthesis of viral mRNA and genomic RNA during acute and persistent infections of the virus, it should be possible to determine the changes occurring in the genome structure and/or its expression, which lead to the establishment of the persistent state.

2. Experimental programme

To obtain information on the amount of virus-specific transcripts, genomic RNA and antigenomic RNA made in Pi-2 cells and in acutely infected cells it was essential to have all the viral genes molecularly cloned and to prepare strand-specific RNA probes. Therefore, the major part of the present work describes the identification of individual gene-specific recombinant clones from different cDNA libraries and their characterisation and subcloning in *in-vitro* transcription vector. To use the transcripts synthesised *in vitro* as a strand-specific probes, their polarity was determined by hybridisation with poly $(A)^+$ RNA from infected cells as a source of (+) sense RNA. Using the gene-specific cDNA probes, the amounts of transcripts for each gene made in acutely infected cells and Pi-2 cells have been quantified in a population of poly $(A)^+$ RNA. Similarly, the amounts of

genomic [(−) sense] RNA and antigenomic [(+) sense] RNA in total RNA of Pi-2 cells and acutely infected cells were quantified with the help of strand-specific RNA probes derived from the cloned DNA.

Several bi- and tri-cistronic cDNA clones were identified; characterisation of these clones has enabled us to establish the gene order. As one of the bicistronic clones was derived from an expression library, the proteins expressed by it in *E. coli* have been analysed by immunoscreening with different types of antibodies and in western blots.

Finally, an attempt has been made to obtain information on the possibility of RV persistently remaining in the body of the animal (cattle or sheep), either experimentally infected or in field.

3. Results and conclusion

The screening of RV RBOK cDNA library in pUC13 with radiolabelled nucleic-acid probes has yielded N, P/C, M, N-P/C, P/C-M and N-P/C-M gene-specific clones and screening of the cDNA library in λ gt11 with antibody probes has yielded N, M and F-H gene-specific clones. The screening of Pi-2 cDNA library in λ gt10 with nucleic-acid probes has helped us to obtain N, M and F gene-specific clones.

In the case of bicistronic clone F-H5 in λ gt11, the reactivity of the expressed antigens with three monoclonal antibodies to RV H protein indicates the possibility of complete H sequence being represented. Further, immunoreactivity of the expressed protein with the antipeptide antibodies against $-NH_2$ and $-COOH$ termini of F1 indicates that the clone F-H5 may contain the entire coding regions corresponding to H and F proteins. Western-blot analysis demonstrates that the F protein is expressed as a fused product with β-galactosidase ($M_r \sim 165000$), while the M_r of the H protein is ~ 65000. Similar analysis of a clone H14 demonstrates a protein with M_r 40,000, suggesting that complete H-gene sequence is not represented in the clone.

Quantification of individual mRNA species made in RV-infected cells with the help of gene-specific radiolabelled cDNA probes reveals the characteristic attenuation of transcription at each gene junction. The levels of transcripts decrease progressively with the distance of the 6 cognate genes from the 3′ end of the genome, thus giving rise to a gradient of polarity in transcription. The gradient gets steeper at the junction of M and F genes. The transcription polarity in Pi-2 cells also follows the same pattern, but with lesser amounts of each mRNA species. Since the quantities of transcripts for the genes near the 3′ end of the viral genome are relatively high, whereas those for the distal genes progressively and sharply diminish, it appears that the altered ratios of mRNAs in Pi-2 cells may most likely lead to reduced expression of the viral-envelope proteins. This could account for the impediment of viral budding and thereby favouring its persistence. Although the M gene is transcribed in Pi-2 cells, the mRNA is not translatable in heterologous cell-free translation system. The presence of non-translatable M transcripts may be due to either point mutations in the coding region of the M gene resulting in premature termination of the protein synthesis or occurrence of point mutations in the 5′ non-coding region which may reduce the ribosome binding or initiation of translation.

The identification of RV RBOK N-P/C, P/C-M and N-P/C-M cDNA clones indicates that the gene order in this region is N-P/C-M. Further, characterisation of a bicistronic clone F-H and a monocistronic L clone establishes the RV gene order as 3′-N-P/C-M-F-H-L-5′.

Most of the clones have been characterised for the size of the insert and subcloning of the genes has been carried out in the *in-vitro* transcription vector pGem 1 to obtain strand-specific RNA probes. Quantification of the genomic and antigenomic RNA with strand-specific radiolabelled RNA probes in total RNA isolated from RV acutely and persistently infected cells suggests the presence of increased

amounts of antigenome [(+)-sense] RNA in Pi-2 cells with a corresponding decrease in the negative polarity genome. The ratio of genomic RNA to antigenomic RNA is remarkably lower (0.6) in case of Pi-2 cells total RNA, compared to that of the acutely infected cells (1.6). The increase in the amount of anti-genomic RNA indicates that in Pi-2 cells, in addition to the lowered production of transcripts, there is an aberrant form of replication which ignores the regulatory signals involved in the synthesis of negative polarity genomic RNA from positive polarity antigenome template. This altered synthesis of RNA may be occurring as a consequence of accumulated mutations.

Finally, an attempt has been made to obtain information on the possibility of RV persistently remaining in the body of the animal (cattle or sheep), either experimentally infected or in the field. It is significant to note that in the absence of clinical symptoms, the virus persists in the animal for up to 4 weeks and possibly longer. Further, this virus remains in the form which is unable to cause the disease in its host, either cattle or sheep.

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Thesis Abstract (Ph.D.)

Purification, kinetic mechanism and regulation of nitrate reductase from *Candida utilis* by T. Satyabhama.
Research supervisors: G. Ramananda Rao and M. S. Shaila.
Department: Microbiology and Cell Biology.

1. Introduction

Assimilatory nitrate reductase (NAR), the first enzyme in the pathway of nitrate assimilation catalyzes the reduction of nitrate to nitrite[1]. As NAR is a highly regulated enzyme and plays a critical role in the regulation of nitrate assimilation, its properties and regulation have been extensively investigated in plants, algae, fungi and bacteria[2–5]. In fungi such as *Aspergillus* and *Neurospora* high levels of NAR are found in cultures only when they are grown with nitrate or nitrite in the absence of ammonium. This has led to the general consensus that NAR is induced by nitrate and repressed by ammonium[6,7]. In *Neurospora* the increased NAR appears to result from *de novo* synthesis of the

enzyme. In contrast, in the green alga *Chlorella*, NAR activity is absent in nitrogen-starved or ammonium-grown cultures but the cells do contain NAR precursors. Investigation in plant system has revealed that though NAR is substrate inducible, *de novo* synthesis of enzyme can take place even in the absence of nitrate in response to other factors. With the help of recombinant DNA clones, it has been shown in barley and squash that induction is brought about by an increase in the steady-state level of NAR mRNA. Thus, there seems to be marked differences in the regulation of NAR. The present study was aimed at gaining some insight into the regulation of this enzyme in eukaryotes like yeasts which are somewhat higher in phylogenetic ladder compared to prokaryotes.

Candida utilis was used since it provides an ideal system, by virtue of its rapid growth, non-pathogenicity and adaptability to grow on nitrate as sole source of nitrogen. The aspects which were investigated in detail include: 1. Purification and characterization of the catalytic properties of assimilatory NAR. 2. Kinetics of the homogeneous enzyme to provide insight into the catalytic mechanism of the enzyme. 3. Regulation of the enzyme by studying the levels of NAR protein, activity and mRNA under conditions of induction and representation.

2. Purification and properties

Procedure was standardized to purify NAD(P)H-nitrate oxidoreductase (EC 1.6.6.2) to homogeneity. Initial attempts to use fractionation by ammonium sulphate which is used for purification of NAR from other sources led to rapid inactivation of NAR. Addition of oxidizing agent like ferricyanide to the crude extract prevented inactivation of the enzyme. Further, use of PMSF throughout purification helped in stabilizing the enzyme. Thus employing protamine sulphate precipitation, ammonium sulphate fractionation, DEAE-cellulose chromatography and affinity chromatography on Blue Sepharose®, NAR from *C. utilis* was purified (600 fold) to electrophoretic homogeneity with an yield of 60%. The purified enzyme resembled NAR from other systems in possessing the associated activities like cytochome *c* reductase and reduced viologen nitrate reductase. It has a sedimentation coefficient of 8.7 S and molecular weight of 200,000 ($\pm$ 2,000) as determined by source-density-gradient centrifugation and gel filtration. Analysis on SDS-PAGE showed that the enzyme is a homodimer consisting of two subunits of molecular weight 100,000. The isoelectric point of the enzyme is 6.5. The difference spectra of the pure enzyme indicated absorption maxima for the reduced enzyme at 423 nm, 557 nm and a broad shoulder centred around 527 nm. The oxidized enzyme showed an oxidized Soret peak at 413 nm. The results indicate the presence of cytochrome b_{557} in the enzyme molecule. Inhibition of the enzyme by sulphydryl agents indicated that sulphydryl groups are required for enzyme activity. Metal-binding agents like cyanide and azide strongly inhibited the enzyme. *C. utilis* NAR is subject to a reversible redox inactivation in the presence of NADH and cyanide. Preincubation with cyanide and NADH reduces enzyme activity by 80%, ferricyanide prevents such inhibition. Addition of nitrate of preincubation mixtures protects the enzyme against inhibition. Detailed analysis of the effect of thiourea and cyanide further revealed that inhibition by these agents is dependent on the oxidation-reduction status of the enzyme.

3. Kinetic mechanism

NAR from *C. utilis* is bispecific with respect to its specificity for reduced pyridine nucleotide. It can use both NADH and NADPH. However, NADH is a more efficient substrate and gives a higher rate, though the affinity is similar to NADPH. In the reaction catalyzed by NAR, the reduction of nitrate to nitrite involves two substrates and two products. Hence, it is a bi bi reaction. The intersecting initial velocity patterns obtained with respect to nitrate and NADH indicated that the kinetic mechanism is sequential, wherein both the substrates should bind to the enzyme before any products

Table I
Product inhibition patterns obtained for *Candida utilis* nitrate reductase

Inhibitory product	Variable substrate			
	NADH		Nitrate	
	Subsaturating nitrate	Saturating nitrate	Subsaturating NADH	Saturating NADH
Nitrite	NC	UN	NC	NC
NAD^+	C	C	NC	NI

C = competitive; NC = non-competitive; UC = uncompetitive; NI = no inhibition.

are released. Further, product inhibition studies revealed that the mechanism is ordered sequential (Table I). Analysis of the results indicates that NAD(P)H binds to the enzyme prior to the binding of nitrate. There is formation of ternary complex, and reduction of nitrate occurs. The order of release of products is nitrate followed by NAD^+. Studies with dead-end inhibitor like thiocynate also supported this mechanism.

4. Regulation

Using *in-situ* assay technique, *in-vivo* regulation of levels of NAR in *C. utilis* was examined in the presence of nitrate and ammonium. Nitrate ions were absolutely essential for induction and maintenance of high levels of NAR activity. Cells grown on ammonium show no enzyme activity. Kinetics of NAR induction in the presence of nitrate and fall in activity in ammonium medium were followed as a function of time. On nitrogen starvation the induced cells exhibit a reduction in *in-vivo* activity of the enzyme and the rate of loss is accelerated by the presence of ammonium. NAR-specific antibodies were used to check the levels of NAR protein. Ouchterlony double-diffusion tests and SDS-PAGE of immunoprecipitates of cell-free extracts from cells pulse-labelled with [^{35}S]-methionine clearly demonstrate the absence of NAR protein in ammonium-grown cells. The cell-free extracts from cells grown in ammonium medium failed to relieve the inhibition of NAR activity by its specific antibody further confirmed the above result. Quantitation of the NAR protein by ELISA indicated that *de novo* synthesis of enzyme occurs on induction in the presence of nitrate which parallels the appearance of enzyme activity. Also attempts to elucidate the level at which nitrate serves as an inducer, by using inhibitors like glutamine, tungsten and cycloheximide, indicated that fresh synthesis of NAR occurs on the addition of nitrate.

To understand whether the regulation of the NAR is at the transcriptional or post-transcriptional level, use of recombinant DNA technology was employed. Using NAR-specific antibodies, polysomes enriched with NAR message were selected. cDNA prepared from NAR message was used to screen the cDNA library (prepared from total polyA$^+$RNA isolated from nitrate-induced cells of *C. utilis*). An NAR-specific clone was identified. The insert from the plasmid pNAR6 obtained from NAR-specific-cDNA clone was used to measure NAR mRNA levels under various conditions. The results showed that there is an increase in the steady-state level of the NAR-specific RNA in the presence of nitrate. Therefore, nitrate regulates the NAR at transcriptional level while ammonium regulates both at transcriptional and post-transcriptional level.

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Thesis Abstract (Ph.D.)

Spectroscopic studies on two biological amphiphiles: melittin and bilirubin by Kasturi Lahiri Puranam.

Research supervisor: P. Balaram.
Department: Molecular Biophysics Unit.

1. Introduction

Molecules which possess both hydrophobic and hydrophilic parts are said to be amphiphilic or amphipathic[1]. Many important processes in biological systems involve molecules which are amphiphilic, *e.g.*, endocytosis, immunorecognition, motility, energy transduction and nerve conduction. This work deals with studies on two interesting biological amphiphiles, melittin and bilirubin. The former which is a 26-residue polypeptide is the main cytotoxic component of the bee venom[2]. Being an amphiphilic peptide, melittin is extremely membrane active. Bilirubin, a tetrapyrrole, is the end product of heme degradation in humans and the disorder in its metabolism has been implicated in neonatal hyperbilirubinemia[3]. The conformational variability and aggregation state of melittin has been investigated in the present study in aqueous, non-aqueous and lipid environments. The ionophoretic property of melittin and its voltage-dependent insertion into membranes has also been studied. Studies with bilirubin include attempts at mimicking its serum-albumin binding site and its structure and conformation in bile-salt micelles.

2. Experimental methods

A wide variety of spectroscopic techniques have been employed to realize the goals of this study. These include difference ultraviolet, circular dichroism (CD), fluorescence and nuclear magnetic resonance (NMR). One-dimensional nuclear Overhauser effect (NOE) measurements have been extensively used to delineate the conformation of micelle-packaged bilirubin. Chromatographic techniques of gel filtration and high-performance liquid chromatography (HPLC) have been used to

determine micelle size and purity, respectively. Standard procedures were used to synthesize tetraacetyl melittin and the bilirubin-amino acid adducts.

3. Results and conclusions

The conformation of melittin has been studied in various organic solvents and found to be remarkably solvent dependent. An extended 'β-sheet-like' structure has been proposed for melittin in the apolar solvent, dioxane. Transition from one conformation to another has been observed in different solvents. This behaviour is similar to that of membrane-active signal peptides[4]. Fluorescence-polarization measurements using the red-edge excitation of the tryptophanyl residue of melittin have been shown to be a useful parameter to probe the aggregation state of melittin in aqueous and particularly lipid environments. When the red-edge polarization data are considered in conjunction with solvent isotope effects the difference between the monomeric and aggregated melittin becomes very clear (Fig. 1). The interaction of melittin with synthetic lipids was monitored by this method and also by determination of the extent of acrylamide quenching of the Trp residue. The depth of insertion of melittin into various lipid bilayers has been found to be dependent on the gel-to-liquid crystalline phase-transition temperature of the lipid. Also, a salt-induced aggregation of lipid-bound melittin has been observed.

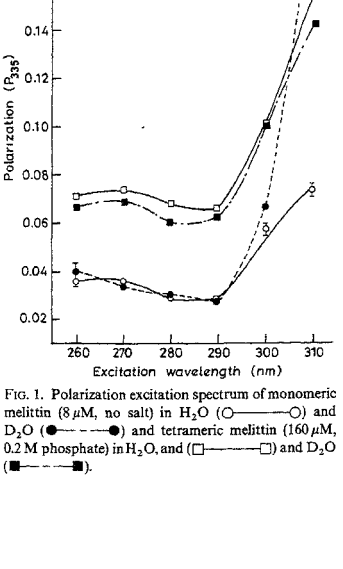

FIG. 1. Polarization excitation spectrum of monomeric melittin (8 μM, no salt) in H_2O (○———○) and D_2O (●- - - -●) and tetrameric melittin (160 μM, 0.2 M phosphate) in H_2O, and (□———□) and D_2O (■- - - -■).

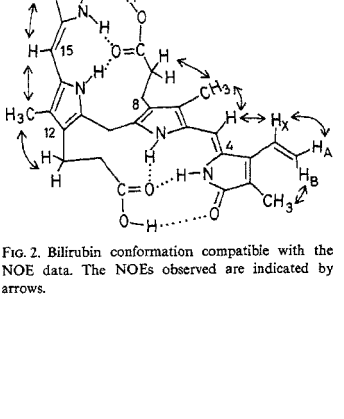

FIG. 2. Bilirubin conformation compatible with the NOE data. The NOEs observed are indicated by arrows.

The ion transporting ability of melittin has been investigated by difference UV spectroscopy using a liposome-bound metallochromic dye, arsenazo III. Melittin has been found to affect transport of Ca^{2+} ions. The voltage-dependent insertion of melittin into bilayers as proposed earlier[5] has also been demonstrated by this assay.

Attempts to model the bilirubin binding on human-serum albumin involved studying the interaction of bilirubin with symmetrical alkyl diamines, chiral mono- and diamines and basic polypeptides[6]. The induction of optical activity in the otherwise inactive bilirubin and enhancement of the bilirubin fluorescence have been found to be excellent probes to monitor such interactions. Binding of bilirubin to the alkyl diamines has been found to be dependent on the length of the diamine as well as solvent. Theoretical calculations based on such an observation have led to the proposal of a novel 'quasicyclic' conformation of bilirubin which is solvent dependent[7]. In addition, chirality has been introduced in bilirubin itself by coupling it to chiral amino acids. The CD spectrum of such adducts has been found to be solvent dependent, once again indicating a difference in the bilirubin conformation.

Interaction of bilirubin with bile salts like cholate, deoxycholate and dehydrocholate has been monitored using various spectroscopic methods, mainly ^1H NMR. Fluorescence and CD results indicate the possibility of such interactions even below the critical micelle concentration of bile salts. Gel filtration has been used to examine the size of the bilirubin-cholate complex. The elution profile was found to be critically dependent on pH of the eluant.

The bilirubin-cholate system has also been studied extensively by ^1H NMR methods[8]. The spectrum of bilirubin in the 1:4 bilirubincholate complex has been assigned by spin decoupling and NOE measurements. The low-field spectra of hydrated and micelle-solubilized bilirubin are found to be distinctly different. A detailed NOE study of this complex has led to observation of significantly large negative NOEs, which have been ascribed to intra- and inter-cholate and also to inter-bilirubin-cholate interactions. The observed NOEs have helped in the delineation of the conformation of the micellized bilirubin molecule (Fig. 2).

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Thesis Abstract (Ph.D.)

A constraint algorithm for molecular dynamics simulation and an analysis of lipid conformation

by Indira Chandrasekhar.
Research supervisor: V. Sasisekharan.
Department: Molecular Biophysics Unit.

1. Introduction

Two programs to examine the structure and dynamics of lipid systems are described. An increasing awareness of the flexibility and polymorphism of biological macromolecular systems[1-3] and the role of thermal fluctuations and dynamic transitions on biomolecular function makes it imperative to study motion in these systems. Of particular interest is the lipid bilayer membrane which is known to be rich in a variety of motions, both fast and slow.

Well-perfected conformational analysis techniques were used to examine the conformation of lipids, both natural and synthetic, as sound conformational data are not available on these molecules. An unambiguous and systematic nomenclature scheme for amphipathic lipids was proposed to provide a framework for these studies. Further, the interaction of a disaccharide trehalose with a phosphoglycerolipid bilayer was studied using molecular modelling methods[4,5], as trehalose is believed to stabilise the biomembrane during water depletion in anhydrobiotic organisms. The other program involved the development of a molecular dynamics (MD) program for examining motion in a system subject to geometric constraints.

2. Nomenclature and conformational analysis

The widely used method of lipid nomenclature due to Sundaralingam[6] was found unsatisfactory as (i) it is incompatible with the norms set out by the IUPAC-IUB Commission on Biochemical Nomenclature[7] as regards the assignment of the absolute configuration of the glycerolipids, and (ii) it is specific to the glycerolipids and cannot easily be extended to include the different natural and synthetic lipid classes of interest. A general, rationally extendable method of nomenclature developed by the author is shown in Figs 1 and 2 for the glycerolipids and a class of synthetic compounds, the glutamic-acid amphiphiles. The method is independent of the molecular backbone of the compound and focusses instead on the central asymmetric carbon of the molecule. The structural features of the system such as separation of the first heteroatom along the chain and the separation of any of the chain atoms from the heteroatom can be easily distinguished (see, for example, Fig. 2).

The conformational flexibility of the glycerolipids was evaluated using a novel three-parameter approach and a complex semi-empirical potential energy function[1]. The glutamic acid lipid, on the other hand, was examined using a simple non-bonded and torsional expression. These lipids form a class of lipid analogues which mimic natural lipids, but on prolonged incubation below the phase-transition temperature undergo a morphological transition to helical tubular forms[8]. The two systems reveal a marked similarity and adopt variations on the familiar 'arm-chair' structure of long-chain amphipaths.

3. The molecular constraint force algorithm

An MD program was developed for examining motions in systems with a bond constraint of the type

$$(r_i - r_j)^2 - d_{ij}^2 = 0 \tag{1}$$

where r_i and r_j are the position vectors of the ith and jth atoms and d_{ij} is the separation between

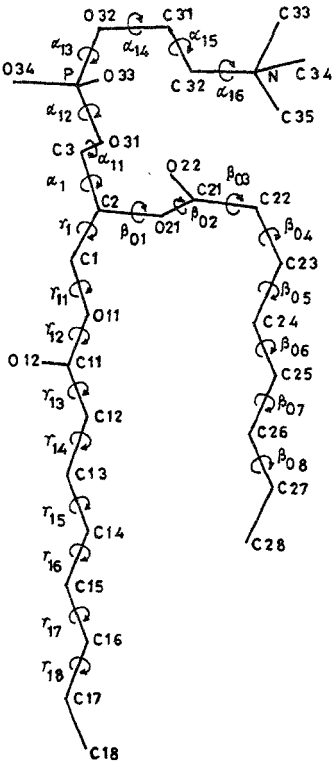

FIG. 1. Atomic and torsional nomenclature for the glycerolipid.

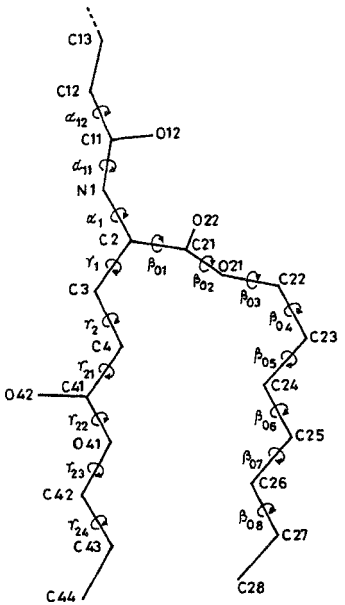

FIG. 2. The atomic and torsional nomenclature for the glumatic acid amphiphile.

them. The Newton's equation of motion

$$\Sigma m_i d^2 r_i / dt^2 = F \tag{2}$$

is modified because of the constraint to

$$\Sigma m_i d^2 r_i / dt^2 = F + G \tag{3}$$

where F is the external force and G the force due to the constraints. The Molecular Constraint Force

method developed by the author is an iterative technique where a restoring force is applied successively to linked atomic particles to maintain fixed distances. The magnitude of the restoring force is evaluated using a finite difference estimate, which is very efficient.

The algorithm was tested on a 12×12 array of tetraatomic molecules with a Lennard–Jones potential and time steps of $10^{-16}, 10^{-15}$ and 10^{-14} seconds. The program was found to be stable, efficient and allowed an application of constraints in any order.

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Thesis Abstract (Ph.D.)

Structure of Belladonna mottle virus at 5.5Å resolution by Sanjeev Kumar Munshi.
Research supervisor: Mathur R. N. Murthy.
Department: Molecular Biophysics Unit.

1. Introduction

Belladonna mottle virus (BDMV) is a highly infectious plant virus reported from several geographical locations in Europe and the United States. It belongs to the tymogroup of spherical plant viruses. The virus particles have a diameter of approximately 300 Å. One hundred and eighty subunits of coat protein of M_r 20 kd encapsidate a genomic RNA of M_r 2.0×10^6. Purified preparations of tymoviruses contain several species of particles including those devoid of nucleic acid, reflecting the ability of protein subunits to assemble into virus-like particles without the aid of nucleic acid. Many of the currently accepted concepts on viral architecture owe their initial development to research on tymoviruses. However, high-resolution three-dimensional structure of any tymovirus is not known to date. This work reports the first attempt of structure determination of a tymovirus at 5.5 Å resolution[1,2].

2. Purification and crystallization

BDMV was propagated on *Nicotiana glutinosa* and purified from the infected leaves by differential centrifugation. Purified virus particles, characterized by analytical ultracentrifugation and electron

microscopy, were found to be a mixture of intact particles (115 S) and empty shells (54 S). The intact particles were separated from the empty capsids by sucrose density-gradient centrifugation. Virus particles were crystallized from 0.1 M Na citrate, pH 5.6 by vapour diffusion technique using 2.5–3.0% polyethylene glycol 8000 as the precipitant. Crystals were characterized by precession and oscillation photography. They belong to rhombohedral R3 space group with $a = 300$ Å and $\alpha = 60°$. Only one molecule of BDMV is compatible with the unit cell, indicating that the molecular 3-fold is aligned with the crystal 3-fold[3].

3. Data collection and processing

Three-dimensional X-ray diffraction data were collected by screenless oscillation photography. Crystals were mounted with their 3-fold aligned with the camera spindle. A total of 70 photographs with an oscillation range of 1° and overlap between consecutive pictures of 20% were recorded to yield a complete asymmetric unit of the reflection data. Films were digitized on a Joyce Lobel film scanner at 50 μ intervals and the resulting images were used to obtain integrated intensities of reflections to a resolution of 5.5 Å. The data on different films were scaled and subjected to the post-refinement procedure resulting in improved crystal orientation, estimates of partiality and cell parameters. The post-refined cell parameters were $a = 295.38$ Å and $\alpha = 59.86°$. The final merging R-factor for the entire data set was 12.7% for reflections with $I/(I) > 2.0$[3].

4. Structure solution

Self-rotation function studies were carried out using data between 11 and 15 Å resolution. These studies unambiguously established the icosahedral symmetry of BDMV particles and their orientation in the crystal cell. *Ab-initio* phase determination was attempted by assuming BDMV particles as hollow spheres. The computed phases for low-order reflections (30 Å) were refined by molecular replacement technique utilizing the known 20-fold noncrystallographic symmetry. However, the refinement was unsuccessful as ripples of negative density persisted in the electron density maps.

Further attempts of phase determination were based on the observed similarity in the tertiary structure of the coat proteins of spherical viruses. Cross-rotation function studies between BDMV and southern bean mosaic virus (SBMV) clearly demonstrated that the structure of BDMV was similar to that of SBMV[4]. Hence, a molecule of SBMV was suitably placed in BDMV unit cell and used as a starting model. Phases were computed up to 6.0 Å resolution and refined by density averaging. Phase extension and refinement were carried out in three parts, 300–9.5 Å, 300–7.5 Å, 300–5.5 Å. At the end of refinement, the crystallographic R-factor and correlation coefficient were 18.2% and 0.87, respectively.

5. Analysis of the structure

Analysis of the final electron density map revealed that the tertiary structure of BDMV is similar to that of SBMV. However, several stretches of the polypeptide chain appeared to be in altered conformation in BDMV when compared to SBMV. These stretches are located at the intersubunit corners and at the periphery of the virus coat. The map also revealed the presence of large holes along the icosahedral three-fold axes. These channels are large enough for the transport of ligands such as polyamines.

6. Stability studies

Biochemical studies on BDMV revealed the presence of polyamines such as spermidine, putrescine and spermine. The replacement of these polyamines by monovalent cations resulted in the loss of

stability of the particles at alkaline pH. The amino-acid sequence of BDMV coat protein does not contain a segment equivalent to the basic amino-terminal arm of SBMV found interacting with RNA. Hence, it is possible that polyamines confer stability to BDMV particle by interacting with RNA and their loss through the channels observed in the electron density map leads to loss of particle stability[5].

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Thesis Abstract (Ph.D.)

Studies on a chito-oligosaccharide-specific lectin from *Coccinia indica* by Ashok R. Sanadi.
Research supervisor: A. Surolia.
Department: Molecular Biophysics Unit.

1. Introduction

Lectins are cell-agglutinating and carbohydrate-binding proteins of plant and animal origin[1]. Though first discovered in the late nineteenth century, research on lectins gained momentum only in the 1940s when it was shown that certain lectins agglutinate human blood groups in a selective manner. Two observations made in the 1960s that (i) certain lectins induce mitogenicity in lymphocytes, and (ii) certain lectins preferentially agglutinate transformed cells as compared to their normal counterparts led to the widespread use of lectins as tools in biology and medicine. In addition, the use of lectins in the purification and characterisation of glycoconjugates and carbohydrates is being widely exploited. These applications provide the impetus to the purification and characterisation of lectins from a variety of sources.

Lectins occur in various tissues of many organisms, a notable case being the phloem exudate of Cucurbits, where lectins constitute up to 25% of total protein. Since most lectins studied have been from seeds, and the only phloem exudate lectin studied in any detail is *Luffa* lectin[2], it is necessary to study these lectins in detail. This is all the more essential in view of the fact that the function and role of phloem exudate lectins is still a matter of dispute. The first step to achieve this end would be to thoroughly understand the structure-function relationship of these lectins.

2. Results and discussion

Coccinia indica agglutinin (CIA) was isolated from the exudate of *Coccinia indica* fruits, and purified to homogeneity using an affinity matrix of denatured soybean agglutinin coupled to Sepharose 4B.

Macromolecular characterisation showed that the protein is a dimer of identical subunits held together by non-covalent forces. The molecular weight of each subunit is 16,000. The protein has a large number of cysteines; carboxyamindylation was thus carried out to increase protein stability and achieve higher protein concentrations. Purity of the protein (which is not glycosylated) was demonstrated by electrophoresis, gel filtration, analytical ultracentrifugation and N-terminal analysis (glycine).

Hemagglutination experiments indicated that CIA is not blood-group specific. Inhibition experiments indicated that the lectin is specific only to $\beta(1\rightarrow4)$-linked oligomers of GlcNAc. Inhibitory potency increases with oligomer size, the monosaccharide failing to inhibit activity even at very high concentrations.

Hemagglutination inhibition is a useful technique but can be used to obtain only semi-quantitative information relative to a particular saccharide used as a reference. In order to obtain precise quantitative information, fluorescence spectroscopic methods were utilized.

The enhancement of fluorescence and the concomitant blue shift that occur upon ligand binding are strongly indicative of the involvement of tryptophan residues in binding activity. Since the sugars themselves are not intrinsically hydrophobic, this increased hydrophobicity as sensed by the tryptophans could arise out of one or a combination of the following factors. Firstly, there could be a conformational change on binding—subtle or otherwise, resulting in a more hydrophobic environment around the tryptophanyl residue(s). It has been established in the case of lysozyme by X-ray techniques that the position of the tryptophan changes by 0.75 Å on ligand binding. Secondly, ligand binding could result in an exclusion of water molecules from around the tryptophan(s), leading to increased hydrophobicity. Lastly, the orientation of the sugar ring(s) on binding could result in the clustering of the CH_2 group and/or protons at certain topological distributions in the sugar-binding site close to the tryptophan(s), with the same effect on the tryptophan side chain(s).

Ligand-size-dependent enhancement has also been observed in other cases—WGA[3], rice germ lectin[4] and *Luffa* lectin[2]. This implies a unique orientation of the tryptophan(s) with respect to different saccharide units of the ligand, which in turn suggests that the tryptophan(s) involved in the binding process is (are) placed to one side of the centre of the binding site. Moreover, since the maximum perturbation of the emission maximum occurs only on binding to the tetrasaccharide and higher oligomers, the position of the tryptophan involved in binding activity can be ascribed to the subsite where the fourth sugar resides, or in close proximity of it.

The association constants (K_a) of binding to various ligands were studied, and from the temperature dependence of these values, the thermodynamic parameters of binding were obtained. The K_a values increase with ligand size implying that the binding site is a highly extended one consisting of subsites, each accommodating one sugar residue.

The enthalpy change upon binding is much higher than the corresponding values for any other $(GlcNAC)_n$ binding lectin, suggesting better complementarity of the ligands and the binding site. From the entropy, free energy and enthalpy values all indicate that the ligand most complementary to the binding site is the tetrasaccharide, tetraose.

In order to understand the structure-activity dependence of a lectin, it is necessary to identify the amino-acid residues involved in binding activity. It has been observed that tryptophan residues are involved in many cases, and this residue has received well-documented attention. A simple method is fluorescence quenching, where one studies the quenching of tryptophanyl fluorescence by low molecular weight agents. Quenching by iodide, acrylamide and succinimide was studied. It is seen that iodide quenching is not affected by ligand binding, whereas both the neutral quenchers are. This shows that the trytophans involved in binding activity are deeply buried, and are thus not accessible to the charged iodide ion with its large sphere of hydration.

Using extrinsic fluorescence of a reporter group linked to the sugar, information can be obtained about the binding of unlabelled sugars as well. This is done by monitoring the change in fluorescence upon displacement of the labelled sugars from the protein by the inhibitory-free sugars. Moreover, since the parameter being followed is the fluorescence of the ligand itself, a more reliable picture of the binding process can be obtained.

The fluorescence intensites of all the Meumbelliferyl glycosides were substantially quenched upon binding to CIA, except in the case of Umb.GlcNAc, where there was no change at all. The extent of quenching was observed to be ligand-length dependent – 62.5% for Umb.biose and 100% for both Umb.triose and Umb.tetraose. Among the possible factors that can cause this quenching of Me-Umb. fluorescence anchored at or near the binding region as a result of carbohydrate-specific binding is the polarity of the environment which seems to be very important. This is evident from the decrease in relative fluorescence of the three ligands. This differential interaction of the indicating group in the three ligands upon being bound by the protein is also evident from the shifts in the emission maxima that occur with binding. There is a blue shift of 2 nm in the case of Umb.biose, and 5 nm in both Umb.triose and Umb.tetraose.

The data obtained by titration of a fixed concentration of CIA with varying concentrations of Umb.tetraose at 26°C, analysed according to Scatchard[5], gave a straight line. The association constant for the binding was $3.5 \times 10^5\ M^{-1}$ and the number of binding sites per protein molecule (dimer, M, 32000) was 2.

The thermodynamic parameters for the binding of all the three ligands were obtained by doing a temperature-dependent study. From the data obtained, it was concluded that the reporter group occupies an independent subsite in each case, with the terminal, non-reducing sugar residue protruding from the binding pocket in the case of Umb.tetraose. Moreover, it was possible to unambiguously assign the tryptophan to the fourth subsite or adjacent to it. A model based on our conclusions is shown in Fig. 1.

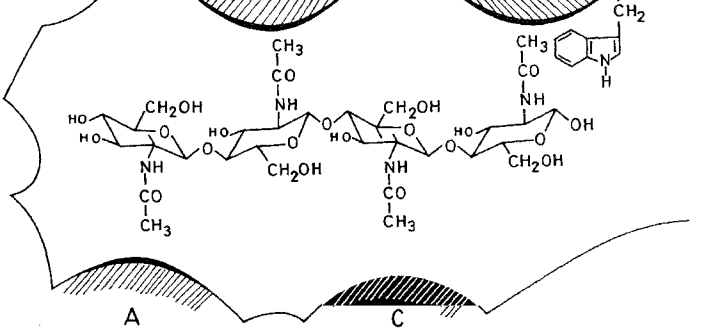

FIG. 1.

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Thesis Abstract (Ph.D.)

Structural transition in DNA and its effect on protein–DNA interactions by N. Ramesh.
Research supervisor: Samir K. Brahmachari.
Department: Molecular Biophysics Unit.

1. Introduction

The demonstration of sequence-dependent conformational flexibility of DNA[1] and the discovery of left-handed Z-DNA[2] paved the way for detailed study on the possible role for such structure of DNA. In order to further our understanding on the factors controlling the structural transitions in DNA and the effect of DNA sequence/structure-specific control events during gene expression, studies were carried out on certain model systems which form the basis of this work.

The basic aim of the present study has been to understand the factors controlling the structural transition in DNA from B to Z form, its stabilization and its effect on protein–DNA interactions. The various aspects that have been investigated are: a) importance of critical cation balance in the control of B to Z transition in synthetic polynucleotide such as poly(dg-dC), b) effect of B to Z transition in DNA on the protein–DNA interactions, c) stabilization of altered structures (like Z-form) in supercoiled plasmid and their preservation in the presence of Z-stabilizing factors, d) dynamic nature of B to Z transition, e) probable role for DNA structural transition in the origin of DNase I hypersensitivity and gene expression.

2. Material and methods

All the reagents were of Analar grade. Poly(dG-dC) and poly(dG-dm^{5}C) were form PL Biochemicals. Wheat germ was obtained from Krishna Flour Mills, Bangalore. Biorex 70 was from Biorad, Blue Sepharose was prepared as reported earlier[3].

E. coli DNA polymerase I assays were performed in the presence of labelled nucleotide and the extent of incorporation was determined after removal of free nucleotides, as reported earlier[4]. S1 nuclease digestion, end labelling, alkaline agarose gel analysis of poly(dG-dC) and poly(dG-dm^{5}C) were performed according to Maniatis *et al*[5].

The UV, CD and sedimentation analysis were performed in Beckman DU 8B spectrophotometer, Jasco J-20 spectropolarimeter and Beckman analytical ultracentrifuge, respectively.

Plasmids were purified by CsCl-EtBr density-gradient centrifugation. Topoisomers of the plasmids were prepared by relaxing the plasmids in the presence of different concentrations of EtBR. Z-DNA

antibody was a gift from Dr. B. D. Stollar, Z-DNA binding protein and DNA topoisomerase I were isolated from wheat germ.

Footprinting on supercoiled and linear plasmids were done using DNAse I and the labelled fragments were analysed on an 8% denaturing polyacrylamide gel.

3. Results and discussion

3.1. *Critical cation balance in B to Z transition*

Monovalent ions like Li^+ with high charge density and large hydration shell are known to stabilize DNA in the B form[6]. Ethanol-induced B to Z transition was absent in the Li salt of poly(dG-dC). In addition, the B to Z transition of Na salt of poly(dG-dC) induced by 60% ethanol was destabilized by Li^+. Mg^{++} and hexamine cobalt chloride could compete out Li^+ to induce B to Z transition in Li salt of poly(dG-dC). Cobalt complexes with fewer than six amine functions were found to induce a B to Z transition at a higher concentration when compared to the hexamine complex. Model building studies showed that two amine functions in one plane of the hexamine complex hydrogen bonded to N7 and O6 of G and another amine function which is out of plane, interacts with the phosphate of the next residue in the 5′ direction. Later, crystal structure studies by Rich and coworkers[7] showed that this was the mode of binding of hexamine cobalt complex to Z-DNA.

3.2. *Recognition of B and Z forms of DNA by E. coli DNA polymerase I*

The effect of B to Z transition in synthetic polymers such as poly(dG-dC) and poly(dG-dm^{5}C) on their ability to act as template for the polymerase function of *E. coli* DNA polymerase I was studied. Poly(dG-dC) in the Z form was found to be less effective as a template for *E. coli* DNA polymerase I in comparison to the B form. Sedimentation and 3′ OH end analysis confirmed the presence of near-identical number of initiation sites on the polymers. Further studies under near physiological conditions using the polymers in the B and Z forms showed a one-to-one correlation with respect to B to Z transition and the decrease in the polymerase activity conforming the above observations. This inhibitory effect of the Z structure in DNA synthesis provides a possible mechanism by which small stretches of Z-DNA may act as modulators of DNA synthesis.

3.3. *DNA supercoiling and B to Z transition*

Supercoiling has been postulated to play an important role in the modulation of gene expression[8]. Circular DNA was used as a model system to understand the effect of supercoiling on structural transition and the maintenance of altered structure in relaxed plasmid in the presence of Z-stabilizing factors. To create circular DNA with different levels of supercoiling, purification of topoisomerase I from wheat germ was carried out. Factors such as hexamine cobalt chloride, Z-DNA antibody, and Z-DNA binding protein could stabilize Z structure in relaxed plasmid provided they were initially bound to supercoiled plasmids. Restriction enzyme digestion with BssHII, of a family of topoisomers, and analysis on a two-dimensional agarose gel showed that B to Z transition was dynamic under the influence of supercoiling. The supercoiling may modulate many of its actions through changes in the DNA structure or by promoting binding of transacting factors.

3.4. *Recognition of B and Z forms of DNA by DNase I*

The structural requirements for DNase I action, a hypersensitivity of which is correlated with activation of the gene[9], was probed using model systems. Z form of poly(dG-dm^{5}C) was completely resistant to DNase I in comparison to the digestion of the B-form. Comparison of DNase I footprinting

of supercoiled and linearised plasmids under single-hit conditions, indicate a marked cleavage of unaltered B form conformation by DNase I as opposed to the altered region. The B–Z junction was also found to be highly sensitive to DNase I action. Even in the absence of a protein factor an altered structure like Z-DNA going over to B conformation could give rise to DNase I hypersensitivity. Thus the occurrence of hypersensitivity need not necessarily involve alteration of the structure to a form other than the B polymorph, where structural transition is a causative factor for DNase I hypersensitivity.

4. Conclusions

In conclusion, it has been possible to show that i) Li^+ inhibits B to Z transition in poly(dG-dC), ii) hexamine cobalt complex induces a structural transition to the Z form by stabilizing the structure through hydrogen bonding with its amine functions, iii) Z form of the DNA is less effective as a template for *E. coli* DNA polymerase I in comparison to the B form, iv) B to Z transition is dynamic under superhelical tension, v) Z structure can be stabilized even in relaxed plasmids in the presence of trans factors, vi) in the absence of a protein factor an altered structure like Z-DNA going over to B conformation could give rise to DNase I hypersensitivity.

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Thesis Abstract (Ph.D.)

Synthesis and structural studies of self-complementary oligodeoxyribonucleotides by Kumud Majumder.
Research supervisor: Samir K. Brahmachari.
Department: Molecular Biophysics Unit.

1. Introduction

The right-handed double-helical structure of DNA[1], which paved the way for an upsurge in the development of molecular biology, did not emphasize the importance of the sequence dependence of DNA conformation. As a result, the DNA molecule was considered to be relatively monotonous in structural terms. However, with the availability of the structural details of a large number of oligonucleotides during the last decade, the sequence-dependent structure of DNA has been recognised to be a rule rather than an exception. In fact, the inherent flexibility of the polynucleotide backbone enables different polymeric forms of DNA to exist in equilibrium[2,3]. Nevertheless, the rules governing the formation of a sequence-specific secondary structure are yet to be understood.

Thus, the basic aim of the present study has been to understand the role of sequence-specific backbone conformation of DNA-oligomers in the process of protein-DNA interaction and/or in the conformational interconversions of the DNA itself. Towards this goal, studies were carried out on the following aspects: (i) solution conformation of some CG-oligomers containing restriction endonuclease (RE) sites; (ii) solution conformation of the AT-tetramers and its correlation with the lack of RE specificity in these tetranucleotides; (iii) the role of 5'-phosphate and sequenece in B→Z transition of alternating CG-hexamers in solution; (iv) conformation of $d(ACGT)_n$ and $d(AGCT)_n$ sequences with special reference to B→Z interconversion; and (v) significance of flanking bases in the sequence-specific recognition and cleavage by restriction enzymes.

Since a large quantity of highly pure oligonucleotides were necessary for the above structural studies we have also carried out the (vi) chemical synthesis of oligodeoxyribonucleotides using an optimal amount of monomer; and (vii) development of convenient and easy methods of HPLC purification of the synthetic oligonucleotides.

2. Materials and methods

All the reagents were of the best grade available commercially. The reagents were distilled/dried wherever necessary. Triethylammonium acetate (TEAA) and triethylammonium bicarbonate (TEAB) were prepared as per published methods[4,5].

The deoxyoligonucleotides – d(AATT), d(TTAA), d(TATA), d(CCGG), d(GGCC) and d(CCGGCCGG) were synthesised by the solid-phase method on silica gel matrix using a column fitted with sintered disc. Both phosphotriester and phosphoramidite routes were employed. The reaction intermediates such as the mononucleoside phosphodiesters and phosphoramidites were synthesised by suitable modifications of the existing methods[6,7]. ^{32} P-labelling was carried out according to Maniatis *et al.*[8]

The HPLC analysis was carried out on a Beckman 342 gradient HPLC system. The anion exchange (AE) HPLC purification was carried out using Ultrasphere AX-10 or Partisil SAX columns (25 cm long, 4.6 mm i.d., 10 μm particle size). The reversed-phase (RP) Ultrasphere ODS column (25 cm long, 4.6 mm i.d., 5 μm particle size) was used for the RP-HPLC analysis.

The UV, CD and NMR spectra were recorded, respectively, on Beckman DU 8B, JASCO J-20 and Bruker 270 MHz (or 500 MHz) NMR spectrometers.

3. Results and discussion

3.1. *Synthesis and purification of oligonucleotides*

The synthesis of the oligonucleotides was carried out by following the repeat-coupling approach[4,5]. This method, based on the principles of repetitive solvent extraction, provided a 60–65% saving of the monomer block per base addition.

The HPLC purification was carried out on RP- or AE- columns or a combination of both. In lieu of using an elevated temperature and non-volatile buffers we have analysed the samples at room temperature using volatile buffers. This was possible because the samples were injected in an already denatured (by heating as a formamide solution) state[4,5]. This, thus, provides a better alternative to the existing HPLC methods. These techniques were utilised in the purification of the oligomers used in the present study. The oligomers were characterised by the enzymatic methods[5].

3.2. *Structural studies on CG-oligomers*

The solution studies of d(CCGG), d(CCGGCCGG) and d(GGCC) were carried out to obtain a comparison of their solution conformation with that of the reported crystal structures of the relevant oligomers[9,10]. The spectroscopic (UV, CD, ^{31}P-NMR, 1- and 2-D ^{1}H-NMR) and chromatographic (gel filtration HPLC) studies on these sequences showed that the conformation of d(CCGG) and d(CCGGCCGG) in low-salt solution at low temperature is unique and differs considerably from that of d(GGCC). The possibility of aggregation, triple-helical structure and Z-conformation of d(CCGG) has been analysed and ruled out. The available results suggest that the sequences d(CCGG) and d(CCGGCCGG) form alternating B-DNA, while d(GGCC) adopts an A-DNA-like structure. Further, studies carried out under simulated crystallisation conditions suggest that the observed similarity in the crystal structures of d(^{t}CCGG) and d(GGCC)$_2$ is due to the specific conditions used for crystallisation.

3.3. *Structural studies on AT-tetramers*

The solution conformation of the three AT-tetramers d(AATT), d(TTAA) and d(TATA), their correlation with the reported crystal structure of the related tetramer-pd(ATAT)[11] and the lack of RE specificity in these sequences have been investigated. The results of the UV, CD and ^{31}P-NMR and ^{1}H-NMR analyses show that these tetramers do not form proper duplex in low-salt (<0.1 M NaCl) solution at low temperature ($<5°$C). Thus, it is conjectured that the inability to form suitable duplices in solution will result in these sequences adopting an average local conformation in a DNA polymer in solution. This, in turn, is probably responsible for the apparent lack of RE specificity of AT-tetrameric sequences.

3.4. *Effect of 5′-phosphate on B→Z transition*

The observation of Z-DNA conformation in crystals and solution state for different synthetic and natural sequences [12,13] of DNA has prompted us to take a close look at the various factors that control the biologically important B→Z transition of DNA. Here, the influence of 5′-phosphate on the B→Z transition has been reported. The results indicate that the two model CG hexamers, *viz.*, pd(CG)$_3$ and pd(GC)$_3$ exist in B-DNA conformation at low temperature in low-salt solution. The

CD and difference-UV studies showed that pd$(CG)_3$, unlike d$(CG)_3$[14], does not undergo a B→Z transition in 5M NaCl. This clearly suggests that the presence of a 5'-phosphate inhibits the salt-induced B→Z transitions in small CG-oligomers. The oligomer pd(GC) was used as the control since d$(GC)_3$ itself does not form Z-DNA[13,14]. Molecular model-building studies on pd$(CG)_3$ were also carried out to study the inhibitory role of the 5'-phosphate group.

3.5. *Structural studies on d$(ACGT)_n$ and d$(AGCT)_n$ sequence*

Poly (dA-dC).poly(dG-dT), as opposed to poly(dG-dC), requires stringent conditions to undergo B→Z transition in solution[12,13]. On the other hand, d(CGTACGTACG) has been shown to form Z-DNA in the crystalline state[15]. Since 5'-CpG-3' stacks facilitate the formation of Z-DNA, the question as to whether an oligomer of the type d$(ACGT)_n$ will also undergo B→Z transition in solution remains open. We have found that both d$(ACGT)_n$ and d$(AGCT)_n$ when $n=2$ exist in B-conformation in low-salt solution at low temperature and does not undergo salt-induced B→Z transition in solution. Anti-Z-DNA antibody (Ab) did not bind to the d$(ACGT)_n$ polymer, synthesised by enzymatic ligation of d$(ACGT)_2$, even at high concentration of NaCl or hexamine-cobalt chloride. Also, under these conditions the presence of Ab could not prevent the poly d(ACGT) from being cleaved by the RE-RsaI ($GT^{\downarrow}AC$). This suggests that d(ACGT) sequences, in spite of having the same base composition as poly(dA-dC).poly(dG-dT), fail to undergo B→Z transition under the experimental conditions. As an interesting offshoot of the above study it was found that unless the recognition sequence of the REs RsaI and AluI were flanked by two extra base-pairs, cleavage did not occur. This finding was verified with yet another RE, *viz.*, HaeIII digestion of d(CCGGCCGG).

4. Conclusions

It has been possible to show that: (i) depending on the base sequences, tetranucleotide sequence could exhibit distinctly different conformation in solution; (ii) a subtle change in base sequence in conjunction with environmental factors can bring about structural transition which might be the key factor for the specific recognition by enzymes like restriction endonucleases; (iii) specific recognition and cleavage of DNA by some of the REs requires flanking sequences on either side; (iv) the critical charge neutralisation and specific polarity of the sequence can affect structural transitions (like B→Z) in DNA; and (v) in the alternating sequences a minimum stretch of CG-sequences are required for the formation of the Z-conformation.

These studies point to the importance of base sequence as well as the environmental factors in deciding the specific structures of DNA. Although the understanding of a few aspects of the recognition of small well-defined sequences by REs has been attempted here, a proper picture as to how sequence-specific recognition of nucleic acids by proteins takes place is yet to emerge.

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Thesis Abstract (Ph.D.)

Theoretical studies on some aspects of cation transport in membranes by N. Sreerama.
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Department: Molecular Biophysics Unit.

1. Introduction

Transport of ions across membranes is a crucial process in all living organisms due to its coupling with bioenergetics. This ion transport is brought about by molecules such as valinomycin and other ionophores, and transmembrane proteins such as bacterio-rhodopsin. Both the transport process and the agents of transport are studied by various methods and these have resulted[1] in two basic mechanisms of transport (carrier and channel), and two basic processes (facilitated and active). In

this work, facilitated transport in ionophores valinomycin and enniatin B[2] are studied by considering electrostatic potential as the index of ion binding, and the active transport process in bacterio-rhodopsin[3] is studied, using model compounds, by *ab-initio* methods. Also, ionic hydrogen bonds with carbon as the donor were studied by *ab-initio* methods and their importance in biological molecules is examined.

2. Methods

Theoretical methods were chosen depending on the size of the system and the nature of the parameter studied. Small molecules which modelled liganding groups, amino-acid side chains and retinal-Schiff base (SB), and characterised hydrogen-bonded systems were studied by *ab-initio* methods. Both STO-3G and 4-31G basis sets were used and calculations were done using GAUSSIAN 74 and GAUSSIAN 80 series of programs. Electrostatic potential of small molecules was calculated by fitting *ab-initio* density matrix to a gaussian-density basis and solving the Poisson equation analytically, while that of large molecules was calculated using monopole approximation. Interaction energy between two ionophores was calculated by empirical methods.

3. Facilitated transport

The binding of the ion to the ionophore is investigated by considering the electrostatic potential as the index. The liganding capacities of amide, ester and ether oxygens are compared by considering model compounds. The liganding capacities are in the order amide, ether, ester. The liganding capacities are relatively uninfluenced by the extension of the chain. Electrostatic potential maps were found to depict the spatial extension of the liganding capacities.

Different conformations of ionophores, valinomycin and enniatin B were considered to study the facilitated transport. They were first brought to a common reference frame with the centre of the molecule as the origin and the path of the ion as the Z-axis. This was done by constructing the least-squares plane through the C_α atoms and transforming the coordinate system. STO-3G atomic charges were obtained by suitable fragmentation and calculations on model compounds. The electrostatic potential maps were generated in the planes both parallel and perpendicular to the Z-axis and compared with the crystal structures. The maps predicted the position of the ion in the crystal structure property and a potential minimum existed along the path of the ion. The negative potential falls off at the edge of the molecule in valinomycin, but extends beyond the edge in enniatin B. This might have a consequence in determining their relative stability in the hydrophobic environment. Also, the dimerisation in enniatin B is influenced by electrostatic interaction and in valinomycin by van der Waals' interaction.

Different factors influencing the motion of the ion were investigated by considering their influence on the potential profile along the path of the ion (Z-axis). Conformational change influences the potential as the positions of the liganding groups with respect to the path of ion are changed. Ion gradient, which is a crucial factor in facilitated transport, is simulated by considering the induction effects of an ion complex on the potential profile. This introduces asymmetrical elevation of the profile and a reversal of stability was observed in profiles with double minima. Transfer of ion from one ionophore to another was studied by dimerisation and induction effect on the dimers. While in enniatin B dimerisation was sufficient enough to transfer the ion, in valinomycin both dimerisation in proper conformation and induction were needed. Also, it was found that relay mechanism was more favoured by enniatin B than valinomycin.

4. Active transport

On absorbing light at 568 nm bacterio-rhodopsin (bR) undergoes photocycle transporting 1–2 protons across the membrane. Chromophore retinal linked to lysine *via* a Schiff base linkage plays a crucial role in the proton transport[3]. It undergoes all *trans* to 13-*cis* isomerisation and de-/reprotonates during the photocycle. A hydrogen-bonded chain formed by the side chains of amino-acid residues is suggested to explain the transport[4]. *Ab-initio* studies on compounds that model retinal-SB and amino-acid side chains were carried out. The π-energy levels, π-MO diagrams and charges on these compounds were analysed, and proton affinites were compared. It was found that *trans-cis* isomerisation does not bring about the deprotonation. The process of deprotonation of retinal-SB was examined. Factors like geometry distortion and environment change the proton affinity and they might play an important role in the proton transport in bR.

These results were supplemented by studies on proton transfer between Schiff base and related nitrogen compounds. Proton-transfer profiles were constructed and characterised. The effect of a neighbouring hydrogen bond on the proton-transfer profile was investigated and its consequences on the proton transfer in bR were examined.

5. $(C\text{–}H..X)^+$ Hydrogen bonds

The ionic hydrogen bonds with carbon as the donor were studied by *ab-initio* methods. The hydrogen bond strength and the C..X distances were characterised as functions of the changes on carbon atom such as changes in hybridisation and substitution and lengthening of the chain. They were found to be comparable to the neutral hydrogen bonds with oxygen and nitrogen as the donors. Electrostatic potential near the donor group along the hydrogen bond was correlated to the bond strength. Importance of these in biological molecules such as protonated histidine, acetylcholine and zwitterionic glycine were examined. In these molecules, the hydrogen bond strength was found to be quite substantial (5–15 kcal).

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