Preprotein binding by ATP-binding site mutants of the Bacillus subtilis SecA

van der Wolk, J.P W; Klose, M; Freudl, R; Driessen, A.J.M.

Published in:
Biological membranes

Publication date:
1994

Pryer, N.K., Wuestehube, L.J., and Schekman, R. (1992) Vesicle-mediated protein sorting. Annu. Rev. Biochem. **61**: 471-516.

Roberts, C.J., Nothwehr, S.F & Stevens, T.H. (1992) Membrane protein sorting in the yeast secretory pathway: evidence that the vacuole may be the default compartment. J. Cell Biol. **119**: 69-83.

Romanos, M.A., Scorer, C.A., and Clare, J.J. (1992) Foreign gene expression in yeast: a review. Yeast **8**: 423-488.

Schreuder, M.P., Brekelmans, S., Ende, H. v.d., and Klis, F.M. (1993) Targeting of a heterologous protein to the cell wall of *Saccharomyces cerevisiae*. Yeast **9**: 399-409.

Sleep, D., Belfield, G.P., and Goodey, A.R. (1990) The secretion of human serum albumine from the yeast *Saccharomyces cerevisiae* using five different leader sequences. Bio/technology **8**: 42-46.

Takeshige, K., Baba, M., Tsuboi, S., Noda, T. & Ohsumi, Y. (1992) Autophagy in yeast demonstrated with proteinase deficient mutants and conditions for its induction. J. Cell Biol. **119**: 301-311.

Verbakel, J.M.A. (1991) Heterologous gene expression in the yeast *Saccharomyces cerevisiae*. PhD Thesis, University of Utrecht, Utrecht, The Netherlands.

Voorhout, W., Leunissen-Bijvelt, J., Leunissen, J., Tommassen, J. & Verkleij, A.J. (1989) Immuno-gold labelling of *Escherichia coli* cell envelope components. In: Immuno-gold labelling in Cell Biology (ed. by A.J. Verkleij & J.L.M. Leunissen), pp. 292-304. CRC Press, Boca Raton, Florida.

Ward, E.S., Güssow, D., Griffiths, A.D., Jones, P.T., and Winter, G. (1989) Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*. Nature **341**: 544-546.

Ward, M., Wilson, L.J., Kodama, K.H., Rey, M.W., and Berka, R.M. (1990) Improved production of chymosin in *Aspergillus* by expression as a glucoamylase-chymosin fusion. Bio/technology **8**: 435-440.

Woolford, C.A., Noble, J.A., Garman, J.D., Tam, M.F., Innis, M.A., and Jones, E.W. (1993) Phenotypic analysis of proteinase A mutants. J. Biol. Chem. **268**: 8990-8998

PREPROTEIN BINDING BY ATP-BINDING SITE MUTANTS OF THE *BACILLUS SUBTILIS* SecA

J. van der Wolk, M. Klose,* R. Freudl* and A.J.M. Driessen

Department of Microbiology

University of Groningen

Kerklaan 30

9751 NN Haren

The Netherlands

The preprotein *translocase* of *Escherichia coli* (Wickner *et al.*, 1991) guards preproteins from the site of synthesis at the ribosome in the cytosol to the processed form to be released into the periplasm. SecA (Schmidt *et al.*, 1988) is the ATP-hydrolysing, peripheral subunit of the *translocase* (Brundage *et al.*, 1990; Wickner *et al.*, 1991), and plays an essential role in preprotein translocation (Lill *et al.*, 1989). The low endogenous ATPase activity of SecA is stimulated by interactions with acidic phospholipids, preproteins and the SecY/E protein (Lill *et al.*, 1989, 1990; Brundage *et al.*, 1990). This stimulated ATPase activity initiates translocation which is further driven by ATP hydrolysis and Δp (Schiebel *et al.*, 1991; Driessen, 1992). Biochemical studies suggest that SecA possess three ATP binding sites (Lill *et al.*, 1989; Oliver, 1993). Only one domain shows a significant level of sequence similarity to the Walker A- and B-motifs for a NTP-binding site (Walker *et al.*, 1982) (Fig. 1). Both regions are highly conserved among different bacterial and algal SecA homologues. To analyze the function of this putative ATP binding site, we started a site-directed mutagenesis approach and changed critical residues of the A-domain (Klose *et al.*, 1983; van der Wolk *et al.*, 1993) of the *Bacillus subtilis* SecA homolog (Overhoff *et al.*, 1991). Now we report on the localization of the B-domain and further characterized ATP- and preprotein-binding activities of the mutants.

*Institut für Biotechnologie 1, Forschungscentrum Jülich GmbH, Postfach 1913, 5170 Jülich, Germany

NATO ASI Series, Vol. H 82
Biological Membranes:
Structure, Biogenesis and Dynamics
Edited by Jos A. F. Op den Kamp

		A-region		B-region
Bacillus subtilis	96	NIAEMKTGEGKTLTSTLP	196	RPLHFAVIDEVDSILIDEARTPLII
Escherichia coli	98	CIAEMRTGEGKTLTATLP	198	RKLHYALVDEVDSILIDEARTPLII
Pavlova lutherii	94	KIAEMKTGEGKTLVAILP	194	NGFEFAIIDEVDSVLIDEARTPLII
Antithamnion sp	90	KIAEMKTGEGKTLVAMLT	190	RDFFFAIIDEIDSILIDEARTPLII
		****.*******.. *.		. . .*..**.**.**********
		GxTGxGKT		RxxxhhhhDEADxhh
				RxxxhhhhDEADxhh

Fig 1. Sequence alignment of the putative ATP-binding domains of SecA homologs.

EXPERIMENTAL PROCEDURES:

Bacteria, plasmids and materials. Wild-type and mutant *B. subtilis secA* genes were cloned into the expression vector pTRC99A under regulatory control of the *trc* promoter/*lac* operator (Klose et al., 1993). *E. coli* strain JM109 (*recA1, endA1, hyrA96, thi⁻, hsdR17, relA1, supE44,* λ⁻, *Δ(lac-proAB), (F', traD36, proAB, lacIq2ΔM15*) harbouring the plasmids pMKL40, pMKL20, and pMKL21 (Table 1) was used for purification of SecA proteins as described (van der Wolk *et al.*, 1993). pMKL18 contained the *E. coli* wild-type *secA* gene (Klose *et al.*, 1983). SecA growth complementation experiments were performed with *E. coli* strains MM52 (MC4100, *secA51*^{ts}), BA13 (MC4100, *secA13*^{am}, *supF*^{ts}, *zch::TN10*), MM54 (*geneX*^{am}, *supF*^{ts}) and JM105.1 (*sec51*^{ts}, *leu::Tn10, thi, rpsL, endA, sbcB15, hspR4, Δ(lac-proAB)*, [F', *traD36, proAB, lacIqΔM15*]) (*See* Klose *et al.*, 1993). ProOmpA and OmpA were isolated as described (*See* Driessen, 1992).

Proteolytic digestion. The conformation of SecA was probed by the sensitivity to Staphylococcal V8 protease (Shinkai *et al.*, 1991). Reaction mixtures (50 μℓ) contained: 60 μg of SecA, 50 mM TrisCl (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$ and 1 mM DTT. ATP (4 mM), (pro-)OmpA (640 μg mℓ$^{-1}$), and/or *E. coli* phospholipid vesicles (800 μg ml^{-1}) were added as indicated. Controls received 0.48 M urea. After 10 min preincubation at 37°C, 450 ng of V8 protease was added and aliquots (10 μℓ) were withdrawn at different time intervals. Reactions were terminated with 5 μℓ of blocking buffer (10 mM PMSF, 6 % SDS, 150 mM TrisCl [pH 6.8], 30% [v/v] glycerol, 15 mM DTT, and 0.03

% bromophenol blue) and heating for 5 min at 95°C. Samples were analyzed by SDS-PAGE.

Table 1. Plasmids

Plasmid	Amino acid substitution	*B. subtilis secA* allele	Reference
pMKL04	none	Wild-type	Klose *et al.*, 1993
pMKL20/pMKL200	$Lys^{101}\rightarrow$ Asn	K101N	Klose *et al.*, 1993
pMKL21/pMKL210	$Lys^{106}\rightarrow$ Asn	K106N	Klose *et al.*, 1993
pMKL4041	$Asp^{207}\rightarrow$ Asn	D207N	M.Klose, unpublished
pMKL4040	$Asp^{215}\rightarrow$ Asn	D215N	M.Klose, unpublished

ADP binding. Binding of ADP to SecA was assayed using the fluorescent analog 1,N^6-ethenoadenosine-5'-diphosphate (ε-ADP) (Molecular Probes, Eugene, OR). Solutions contained: 200 μg mℓ$^{-1}$ of SecA, 50 mM TrisCl (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and 10 μM ε-ADP. Reactions received 5 mM of indicated nucleotides, 400 μg mℓ$^{-1}$ proOmpA and/or 0.3 M urea. Fluorescence polarization measurements at 20°C were performed with an SLM 4800C (Aminco, Urbana, IL) fluorimeter equipped with Glan-thompson polarizers. Excitation and emission was at 300 and 410 nm, respectively. The polarization (P) of ε-ADP fluorescence was calculated according to: $P = [(R_{verr}/R_{horz})-1] / [(R_{verr}/R_{horz})+1]$ where R_{verr} and R_{horz} are the ratios of parallel and perpendicular signals with the excitation polarizer placed in the vertical and horizontal orientation, respectively.

RESULTS:

Expression of B. subtilis secA A- and B-domain mutant genes in E. coli. Site-directed mutations were introduced in critical residues of the A- and B-domain of the putative catalytic ATP-binding site of the *B. subtilis* SecA (*See* table 1). Mutant proteins were expressed in various *E. coli* strains to test their ability to complement the growth-defects of conditional lethal *secA* mutations. After a shift to the nonpermissive temperature (42°C), full length, but non-functional SecA protein is synthesized in MM52 (Schmidt *et al.*, 1988), a truncated amino-terminal SecA fragment is synthesized in BA13 (Cabelli *et al.*, 1988), while due to a strong polar effect in the stop codon of *geneX*, no

SecA is synthesized in MM66 (Schmidt *et al.*, 1988). *E. coli* JM105.1 contains a single copy of the *lacI*^{q} gene on the F' episome (Klose *et al.*, 1993). When the temperature sensitive strains MM52, BA13 or MM66 were transformed with plasmids pMKL40, wild -type *B. subtilis* SecA protein was found to complement the growth defects of these strains at the non-permissive temperature, provided that the protein is not brought to high expression level (Table 2) (Klose *et al.*, 1993). With pMKL210 and pMKL440 only poor growth was observed, even at the permissive growth temperature (Table 2). Lys^{106} and Asp^{215} therefore seem to be crucial for complementation of the *E. coli secA* mutant strains by the *B. subtilis* (this paper) and *E. coli* (M. Klose, unpublished) SecA.

Table 2. Growth complementation of *E. coli secA* mutants

secA Allele	Plasmid	*E. coli* strain							
		MM52		JM105.2		BA13		MM66	
		30°C	42°C	30°C	42°C	30°C	42°C	30°C	42°C
		G I	G I	G I	G I	G I	G I	G I	G I
	pTRC99A	+ +	- -	+ +	- -	+ +	- -	+ +	- -
B. subtilis									
Wild type	pMKL40	+ +	+ -	+ +	+ -	+ +	+ -	+ +	+ -
K101N	pMKL200	+ +	+ -	+ +	+ -	+ +	+ -	+ +	+ -
K106N	pMKL210	(+) -	- -	(+) -	- -	(+) -	- -	(+) -	- -
D207N	pMKL441	+ +	+ -	+ +	+ -	+ +	+ -	+ +	+ -
D215N	pMKL440	(+) -	- -	(+) -	- -	(+) -	- -	(+) -	- -
E. coli									
Wild type	pMKL18	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +

E. coli strains were grown on LB plates supplemented with 0.5 % glucose (G) or 1 mM IPTG (I). Growth temperatures were 30 or 42°C; growth; (+), poor growth; -, no growth.

ATP enhances the V8 protease resistance of wild-type SecA, but not that of K106N SecA. Soluble *E. coli* SecA is sensitive to digestion by protease V8. ATP and the non-hydrolyzable analog AMP-PNP enhance the resistance of SecA against V8 digestion (Shinkai *et al.*, 1991), suggesting a conformational change upon binding of ATP. *B. subtilis* SecA proteins were purified and incubated with V8 protease. Wild-type SecA was rapidly digested by V8 protease, and was more resistant in the presence of ATP (Fig. 2A). K106N SecA was not protected against V8 protease digestion in the presence of ATP (Fig. 2B). These data suggest that Lys^{106} is involved in an ATP-dependent conformational change of SecA.

Effect of proOmpA on V8 protease sensitivity of wild-type and K106N SecA. B. subtilis SecA ATPase activity is stimulated by proOmpA and the protein binds to translocation intermediates of proOmpA (van der Wolk *et al.*, 1993). On the other hand, K106N SecA blocks the Δp-dependent chase of such intermediates possibly through tight binding. Addition of proOmpA to the *B. subtilis* SecA, preincubated with ATP, restored the sensitivity to V8 digestion to the level when no ATP was present (Fig. 2A). A strong effect was observed with the K106N SecA, suggesting that this mutant binds proOmpA with greater affinity than the wild-type. OmpA had only little effect on the V8 protease sensitivity (not shown). These results demonstrate that K106N SecA interacts with preproteins.

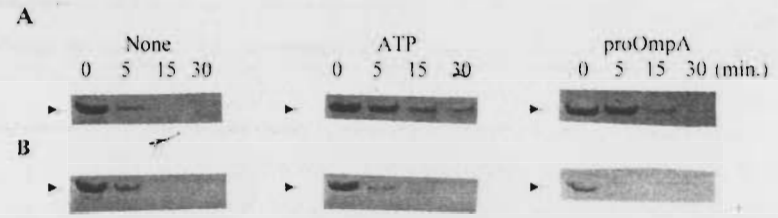

Fig 2. V8 protease sensitivity of wild-type (A) and K106N (B) SecA.

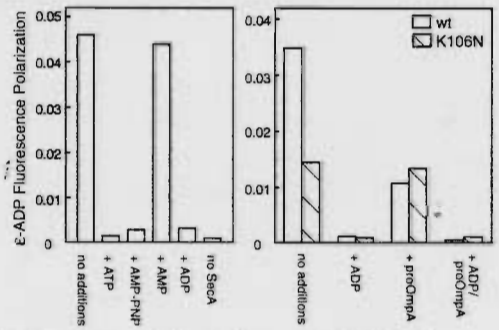

Fig 3. Binding of ε-ADP to wild-type and K106N SecA.

Binding of ε-ADP to wild-type and K106N SecA. ε-ADP was used to assay ADP binding to SecA through fluorescence polarization measurements (Fig. 3A). Bound ε-ADP was chased by an excess ATP, ADP or AMP-PNP, while AMP was ineffective. K106N SecA still binds ADP (Fig. 3B, hatched bars), although with lower specificity than the wild-type (open bars). ProOmpA elicits release of bound ε-ADP from the wild-type to the level found for the K106N SecA. ProOmpA had no effect on the binding of ε-ADP to K106N SecA, while further release required an excess ADP. These results suggest that binding of proOmpA to SecA lowers its affinity for ADP, while K106N SecA appears to be frozen in this low-affinity ADP-binding state.

DISCUSSION:

In a previous communication (van der Wolk *et al.*, 1983), we have shown that Lys^{106} is an essential residue of the A-domain of the catalytic ATP-binding site. The *in vivo* complementation assays now identify Asp^{215} as part of the B-domain. This site differs from the one suggested by Koonin and Gorbalenya (1992). Further work will involve the purification of this mutant in order to analyze its activities *in vitro*.

A conceptional model for the active site of SecA based on the structure of RecA which has been solved at 2.7 Å with bound ADP (Story and Steitz, 1992) is presented in Fig. 4. In RecA, the enzymatic mechanism of ATP hydrolysis involves activation of a water molecule by a glutamate residue for an in-line attack of the γ-phosphatepresented by the invariable lysine (Lys^{106} in SecA) of the A-domain. The position of the glutamate residue in SecA is unknown. The γ-phosphate is positioned such that Mg^{2+}, stabilized by the invariable aspartate (Asp^{215}) of the B-domain, bridges the oxygen atoms of the β- and γ-phosphates such that the phosphate oxygens, the Mg^{2+} ion and the Oγ of threonine of the A-domain (Thr^{107}) all lie in the same plane. In addition, the γ-phosphate is in contact with the so-called C-domain, not yet identified in SecA. The site for preprotein binding may be formed upon interaction between the β- and γ-phosphates and residues of the C-region that lie adjacent to the phosphate-binding loop. In analogy with the RecA model, ATP hydrolysis is expected to destroy interactions between the nucleotide and residues of the C-region, resulting in a change in conformation of the

preprotein-binding regions causing release of the preprotein by SecA. ATP and ADP both stabilise the conformation of SecA; the conformation stabilized by the binding of ATP has a higher affinity for the preprotein as evident from cross-linking (Akita *et al.*, 1990) and dynamic light-scattering experiments (unpublished). Close proximity of the ATPase active site and the region likely to be involved in preprotein binding is suggestive of an allosteric mechanism by which the binding of the nucleotide affects the binding of the preprotein. Both domains may be highly interacting as signal peptides act as competitive inhibitors of SecA-translocation ATPase, while ATP antagonizes this inhibition (Cunningham and Wickner. 1989). Binding of the preprotein to SecA discharges the bound nucleotide possibly through lowering of the binding affinity (Shinkai *et al.*, 1991; this paper). This phenomenon is lost with the K106N mutant which binds ATP only with low affinity, maybe at an alternative low affinity binding site. We propose that the K106N SecA is prone to high-affinity preprotein binding. The inability to hydrolyse ATP (van der Wolk *et al.*, 1993) prevents the release of bound preproteins, thereby blocking preprotein translocation at the membrane translocation sites.

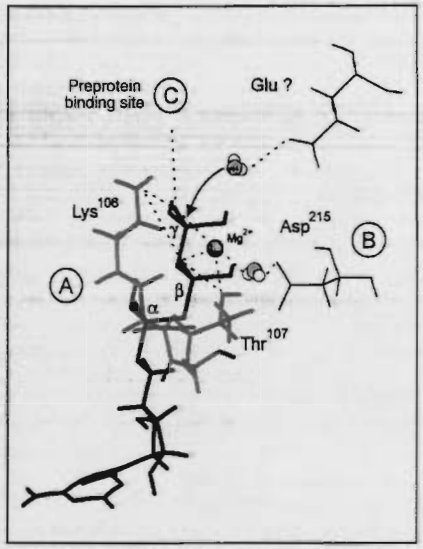

Fig 4. Model for the SecA ATP-binding site.

ACKNOWLEDGEMENTS:

This work was supported by the Netherlands Organization for Scientific Research (N.W.O.) and the Netherlands Foundation for Chemical Research (S.O.N.).

LITERATURE:

Akita M, Sasaki S, Matsuyama S-I, Mizushima S (1990) SecA interacts with secretory proteins by recognizing the positive charge at the amino terminus of the signal peptide in *Escherichia coli*. J Biol Chem 265:8164-8169

Brundage L, Hendrick JP, Schiebel E, Driessen AJM, Wickner W (1990) The purified

Escherichia coli integral membrane Protein SecY/E is sufficient for reconstitution of SecA-dependent precursor protein translocation. Cell 62:649-657

Cabelli RJ, Chen L-L, Tai PC, Oliver DB (1988) SecA protein is required for secretory protein translocation into *E. coli* membrane vesicles. Cell 55:683-692

Cunningham K, Wickner W (1989) Specific recognition of the leader region of precursor proteins is required for the activation of translocation ATPase of *Escherichia coli*. Proc Natl Acad Sci USA 86:8630-8634

Driessen AJM (1992) Precursor protein translocation by the *Escherichia coli* translocase is directed by the protonmotive-force. EMBO J 11:847-853

Klose M, Schimz K-L, van der Wolk J, Driessen AJM, Freudl R (1993) Lysine^{106} of the putative catalytic ATP-binding site of the *Bacillus subtilis* SecA protein is required for functional complementation in *E. coli secA* mutants *in vivo*. J Biol Chem 268:4504-4516

Koonin EV, Gorbalenya AE (1992) Autogenous translation regulation by *E. coli* ATPase SecA may be mediated by an intrinsic RNA helicase activity of this protein. FEBS Lett 298:6-8

Lill R, Cunningham K, Brundage L, Ito K, Oliver DB, Wickner W (1989) SecA protein hydrolyzes ATP and is an essential component of protein translocation ATPase of *Escherichia coli*. EMBO J 8:961-966

Lill R, Dowhan W, Wickner W (1990) The ATPase activity of SecA is regulated by acidic phospholipids, SecY, and the leader and mature domains of precursor proteins. Cell 60:271-280

Oliver DB (1993) SecA protein: autoregulated ATPase catalysing preprotein insertion and translocation across the *Escherichia coli* inner membrane. Mol Microbiol 7:159-165

Overhoff B, Klein M, Spies M, Freudl R (1991) Identification of a gene fragment which codes for the 364 amino-terminal amino acid residues of a SecA homologue of *Bacillus subtilis*: further evidence for the conservation of the protein export apparatus in gram-positive and gram-negative bacteria. Mol Gen Genet 228:417-423

Schiebel E, Driessen AJM, Hartl F-U, Wickner W. (1991) $\Delta\tilde{\mu}_{H^+}$ and ATP function at dif ferent steps of the catalytic cycle of preprotein translocase. Cell 64:927-939

Schmidt MG, Rollo EE, Grodberg J, Oliver DB (1988) Nucleotide sequence of the *secA* gene and *secA*(ts) mutations preventing protein export in *Escherichia coli*. J Bacteriol 170:3404-3414

Shinkai A, Mei LH, Tokuda H, Mizushima S. (1991) The conformation of SecA, as re vealed by its protease sensitivity, is altered upon interaction with ATP, presecretory proteins, everted membrane vesicles, and phospholipids. J Biol Chem 266:5827-5833

Story RM, Steitz TA (1992) Structure of the recA protein-ADP complex. Nature 355: 374-376

van der Wolk J, Klose M, Breukink E, Demel RA, de Kruijff B, Freudl R, Driessen AJM (1993) Characterization of a *Bacillus subtilis* SecA mutant protein deficient in translocation ATPase and release from the membrane. Mol Microbiol 8:31-42

Walker JE, Saraste M, Runswick MJ, Gay NJ (1982) Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes have a common nucleotide binding fold. EMBO J 1:945-951

Wickner W, Driessen AJM, Hartl F-U (1991) The enzymology of protein translocation across the *Escherichia coli* plasma membrane. Annu Rev Biochem 60:101-124

Internal Disulfides in the Diphteria Toxin A-Fragment Block Its Translocation to the Cytosol

Pål Ø. Falnes, Seunghyon Choe*, Inger H. Madshus, R. John Collier†, and Sjur Olsnes.
Department of Biochemistry
Institute for Cancer Research
Montebello
0310 Oslo
Norway

INTRODUCTION

A number of protein toxins from plants and bacteria efficiently kill eukaryotic cells by inhibition of protein synthesis. The toxin binds to receptors on the plasma membrane and is endocytosed, before the enzymatically active part of the toxin is translocated to the cytosol. Here it exerts its action by inactivating a crucial component of the cellular protein-synthesizing machinery.

The molecular mechanisms involved in the translocation process have been studied in most detail for diphtheria toxin, the main pathogenicity factor in clinical diphtheria (For reviews, see London, 1992, Sandvig and Olsnes, 1991). Diphtheria toxin is secreted as a polypeptide chain of 58 kD from *Corynebacterium diphtheriae*, and can easily be cleaved by trypsin into two fragments A (21 kD) and B (37 kD), joined by a disulfide bond. Diphtheria toxin entry into cells is initiated by binding of its B-fragment to specific surface receptors. Subsequently, the exposure to low pH in endosomes induces the translocation of the A-fragment to the cytosol. Translocation can also be induced at the level of the plasma membrane by exposing cells with surface-bound toxin to low pH. Low pH has been shown to induce unfolding and the exposure of hydrophobic regions in the toxin molecule, and this has lead to the speculation that unfolding of the toxin may be necessary for the translocation to occur.

---

*Molecular Biology Institute and Department of Chemistry and Biochemistry, University of California, Los Angeles, USA
†Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, USA

NATO ASI Series, Vol. H 82
Biological Membranes:
Structure, Biogenesis and Dynamics
Edited by Jos A. F. Op den Kamp