letters to nature

Yeast Sm-like proteins function in mRNA decapping and decay

Sundaresan Tharun*†, Weihai He*†, Andrew E. Mayes‡§,
Pascal Lennertz*, Jean D. Beggs‡ & Roy Parker*

* Department of Molecular and Cellular Biology and Howard Hughes Medical
Institute, University of Arizona, Tucson, Arizona 85721, USA
‡ Institute of Cell and Molecular Biology, University of Edinburgh,
King's Buildings, Mayfield Road, Edinburgh EH9 3JR, UK
§ Unilever Research Colworth, Colworth House, Sharnbrook, Bedford,
MK44 1LQ, UK
† These authors contributed equally to this work

One of the main mechanisms of messenger RNA degradation in eukaryotes occurs by deadenylation-dependent decapping which leads to 5′-to-3′ decay[1,2]. A family of Sm-like (Lsm) proteins has been identified, members of which contain the 'Sm' sequence motif, form a complex with U6 small nuclear RNA and are required for pre-mRNA splicing[3–9]. Here we show that mutations in seven yeast Lsm proteins (Lsm1–Lsm7) also lead to inhibition of mRNA decapping. In addition, the Lsm1–Lsm7 proteins co-immunoprecipitate with the mRNA decapping enzyme (Dcp1), a decapping activator (Pat1/Mrt1) and with mRNA. This indicates that the Lsm proteins may promote decapping by interactions with the mRNA and the decapping machinery. In addition, the Lsm complex that functions in mRNA decay appears to be distinct from the U6-associated Lsm complex, indicating that Lsm proteins form specific complexes that affect different aspects of mRNA metabolism.

We proposed that the family of Lsm proteins affects mRNA degradation because lsm1 strains are deficient in mRNA decapping[10] and the Lsm proteins interact with each other[6,7]. Thus, we examined the decay of the MFA2pG and PGK1pG reporter mRNAs[11] in strains with the nonessential LSM1, LSM6 or LSM7 genes (each of which is thermosensitive) deleted, and in strains carrying temperature-sensitive forms of LSM2 or LSM5, or a partial loss-of-function allele of LSM8. The reporter mRNAs are under the control of the GAL1 UAS to allow measurement of their decay rates and contain a poly(G) tract in their 3′ untranslated region (UTR), which traps decay intermediates by blocking the 5′-to-3′ exonuclease in cis[12,13]. Mutations inhibiting mRNA turnover accumulate full-length mRNA and show a decrease in the levels of the decay intermediate[12].

Two observations indicated that loss-of-function alleles of LSM1–LSM7 inhibited mRNA degradation. First, loss-of-function alleles in the LSM1, LSM2, LSM5, LSM6 and LSM7 genes led to increased levels of full-length mRNA and decreased levels of decay intermediate for both the MFA2pG (Fig. 1, in 0 min) and PGK1pG (data not shown) transcripts. Second, the MFA2pG transcript showed a slower decay rate (Fig. 1) in the lsm1, lsm2, lsm5, lsm6 and lsm7 mutant strains as compared with wild-type strains. Although the lsm1, lsm2, lsm5, lsm6 and lsm7 alleles cause thermo-sensitive growth, we observed the effects on mRNA decay at both 24 °C (Fig. 1) and 37 °C (data not shown). The lsm8-1 allele examined did not affect mRNA turnover. The inactivation of the Lsm proteins caused a partial defect in mRNA decay as compared with the complete block seen in dcp1Δ and dcp2Δ strains[14,15], suggesting that Lsm proteins activate mRNA decay. Consistent with this view, an lsm1Δ lsm6Δ double mutant strain did not show an increased defect in mRNA decay (Fig. 1).

To determine which step in mRNA degradation was affected in the lsm mutants, we examined the structure of the MFA2pG mRNA in these strains. Strains deficient in decapping or in 5′-to-3′ exonucleolysis accumulate oligoadenylated, full-length mRNA species, with or without cap, respectively[10,12,14–16]. Thus, we

examined the poly(A) distribution on the MFA2pG mRNA in the lsm mutants with or without fractionation into capped and uncapped fractions by immunoprecipitation with anti-cap antibodies. Strains mutant in LSM1–LSM7 accumulated MFA2pG transcripts with short poly(A) tails (Fig. 2, T lanes), indicating that these strains were deficient in decapping or in 5′-to-3′ exonucleolysis. A substantial amount of the accumulated oligoadenylated species in the lsm1, lsm2, lsm5, lsm6 and lsm7 strains was immunoprecipitated by antisera against the cap structure, similar to the dcp1Δ strain, wherein decapping is blocked (Fig. 2). This indicated that LSM2, LSM5, LSM6 and LSM7 are required, as is LSM1 (ref. 10) for efficient mRNA decapping. Moreover, in strains where the only copy of the essential LSM3 or LSM4 is expressed from a GAL promoter, transfer of the culture to glucose medium (thereby depleting Lsm3 or Lsm4 (ref. 7) led to the accumulation of capped, deadenylated MFA2pG mRNAs (Fig. 2). This observation, together with the co-immunoprecipitation of Lsm3 and Lsm4 with mRNA decay factors (see below), suggests that LSM3 and LSM4 are also required for efficient mRNA decapping.

Two-hybrid analysis has identified interactions between Lsm proteins and the Dcp1 decapping enzyme, the Xrn1 5′-to-3′ exonuclease and Dcp2 (ref. 17). The Lsm proteins also show
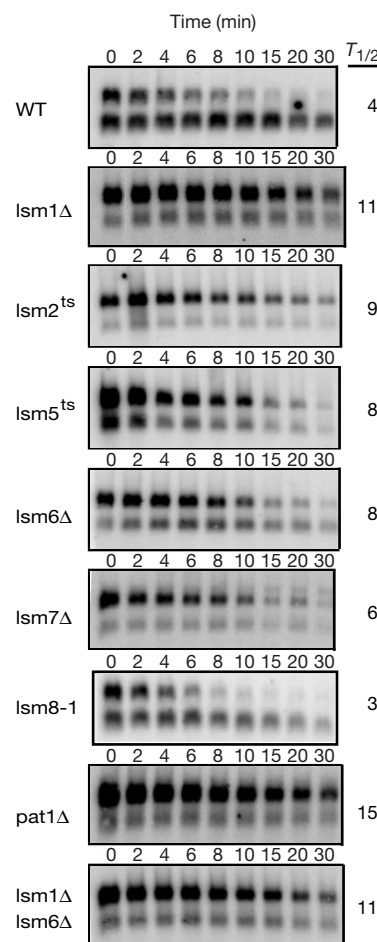

Figure 1 MFA2pG mRNA is stabilized in most lsm mutant strains. The decay of the MFA2pG transcript after repression of transcription by the addition of glucose in lsm mutants and wild-type (WT) strain at 24 °C is shown. The top band is the full-length mRNA and the lower band is the mRNA fragment produced by 5′-to-3′ exonucleolytic digestion to the 5′ side of the poly(G) tract[11]. The strains used were WT (yRP841), lsm1Δ (yRP1365), lsm2^ts (yRP1366), lsm5^ts (yRP1367), lsm6Δ (yRP1369), lsm7Δ (yRP1370), lsm8-1(BP4), pat1/mrt1Δ (yRP1372) and lsm1Δ lsm6Δ (yRP1407).

515

two-hybrid interactions with Pat1 (YCR077c)[17], a protein originally identified through a two-hybrid interaction with topoisomerase II (ref. 18). Pat1Δ strains have multiple phenotypes, including temperature sensitivity, that are similar to mutations in the *MRT1* gene, an uncloned locus known to affect mRNA decapping[12]. There is evidence that *MRT1* and *PAT1* are the same gene. First, the *PAT1* gene complements the temperature sensitivity and mRNA decay defect in *mrt1* strains (data not shown). Second, *pat1Δ* strains show a defect in mRNA decay similar to the *mrt1* alleles (Fig. 1). Third, sequencing of the *PAT1* gene from strains carrying the *mrt1-1*, *mrt1-2* and *mrt1-3* alleles identified mutations creating nonsense codons at amino acids 547, 157 and 308, respectively. Fourth, the *mrt1* alleles and the *pat1Δ* are genetically linked and *pat1Δ* fails to complement the *mrt1* lesions in diploids (data not shown). Thus, *PAT1/MRT1* is required for efficient mRNA decapping.

To investigate the significance of the two-hybrid interactions between the Lsm proteins and the proteins required for mRNA decapping, we immunoprecipitated epitope-tagged proteins Lsm1–Lsm8 and determined whether Dcp1 or Pat1/Mrt1 co-immunoprecipitated by using western analysis. Dcp1 co-immunoprecipitated with Lsm1–Lsm7, although the efficiency of co-immunoprecipitation varied (Fig. 3a). No co-immunoprecipitation of Dcp1 with Lsm8 could be detected, however, even after long exposures. Similarly, Pat1/Mrt1 also co-immunoprecipitates with Lsm1–Lsm7 proteins, but not with Lsm8 (Fig. 3b; and data not shown).

The Lsm and Sm proteins are RNA-binding complexes and function in splicing by assembling with snRNAs[3,4]. To investigate whether Lsm proteins affect decapping through interactions with mRNA, we determined whether mRNAs co-immunoprecipitated with Lsm proteins (Fig. 4). Lsm1, Lsm5 and Dcp1 co-immunoprecipitated with a small (1−3% of the total) but clearly detectable amount of *CYH2* mRNA. In contrast, Lsm8 did not co-immunoprecipitate the *CYH2* mRNA in amounts significantly above background levels. Moreover, we did not detect U6 snRNA in immunoprecipitates of Lsm1 and Dcp1, which indicates that
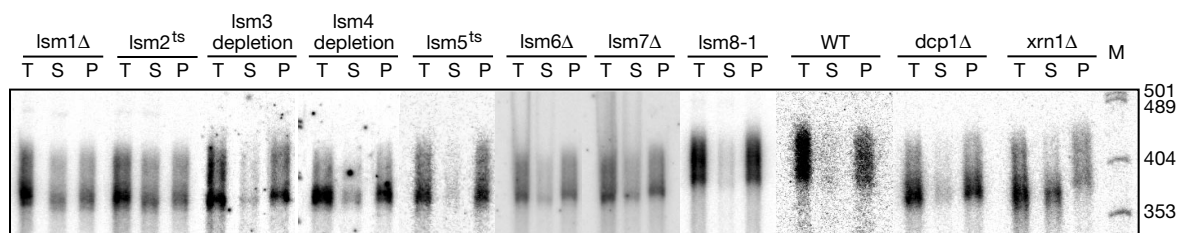

Figure 2 Lsm mutants accumulate oligoadenylated, full-length capped mRNAs. Steady-state mRNA from various *lsm* mutants, *dcp1Δ*, *xrn1Δ* and wild-type strains were analysed on a polyacrylamide northern blot with or without immunoprecipitation with anti-cap antibodies. Strains expressing *LSM3* and *LSM4* under *GAL* control were shifted to glucose for 12 h before analysis, which has been shown to deplete these proteins[7]. T, total RNA; P, RNA extracted from the immunoprecipitate; S, supernatant. Radiolabelled *Hpa*II-digested pUC18 DNA was used as marker (M).
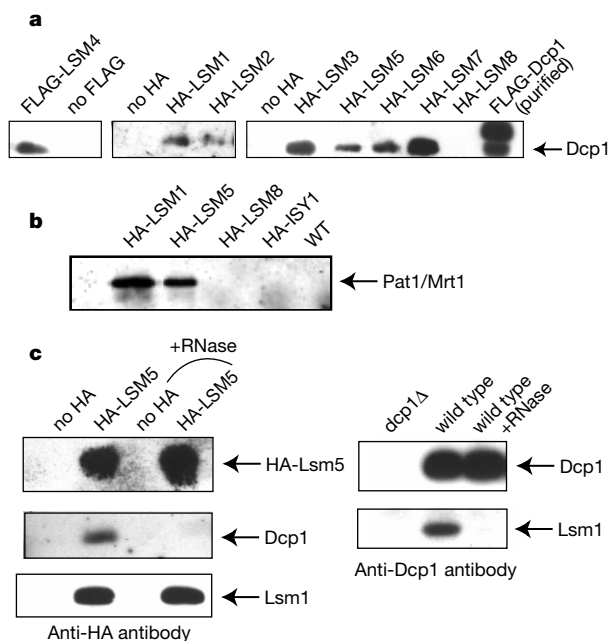

Figure 3 Lsm proteins co-immunoprecipitate with proteins involved in mRNA decay. **a**, Dcp1 co-immunoprecipitates with proteins Lsm1–Lsm7. Epitope-tagged Lsm proteins were immunoprecipitated and the immunoprecipitates were analysed by western blot for Dcp1. **b**, Co-immunoprecipitation of Pat1 with HA-tagged Lsm proteins. Epitope-tagged Lsm proteins were immunoprecipitated and the immunoprecipitates were analysed by western blot for Pat1. Isy1, a protein involved in pre-mRNA splicing[22], was used as a control. **c**, Interaction between Dcp1 and Lsm proteins is RNA dependent. Immunoprecipitation of Dcp1 (right panel) and HA−LSM5 (left panel) from lysates of different strains (indicated above the lanes) was done with ('+RNase') or without prior RNase treatment and presence of co-immunoprecipitating proteins (Dcp1 and Lsm1) was analysed by western blot using polyclonal Dcp1 or Lsm1 antisera.

these interactions are RNA specific (Fig. 4b; and data not shown). These results suggest that Lsm proteins interact with mRNA molecules and thereby modulate decapping. The co-immunoprecipitation of Lsm proteins with Dcp1, but not with each other or Pat1, is sensitive to RNase treatment (Fig. 3c and data not shown). This suggests that either the Lsm complex and Dcp1 interact directly in a manner dependent on one or both of these proteins binding mRNA, or that these proteins interact simultaneously with the same mRNA molecules. Thus, the co-immunoprecipitation of Lsm proteins, Dcp1 and mRNA may reflect the detection of a transient mRNP (ribonuclear protein particle) organization that exists before mRNA decapping.

These physical interactions suggest that the Lsm proteins have a role in cytoplasmic mRNA degradation. Consistent with this view, Lsm1, Lsm7, Dcp1 and Pat1 can be detected in the cytoplasm by immunofluorescence (Fig. 5). Lsm7 was also found in both compartments, consistent with it affecting both mRNA decay and splicing[6,7]. Some nuclear staining was observed for both Dcp1 and Lsm1, suggesting that these proteins may also have a nuclear function.

Several observations indicate that a complex, consisting of Lsm1–Lsm7, is involved in mRNA decay and that a different complex, consisting of Lsm2–Lsm8, is associated with U6 and involved in pre-mRNA splicing. First, although mutations in *LSM2–LSM7* affect both mRNA decay and pre-mRNA splicing, mutations in *LSM1* only affect mRNA decay, and lesions in *LSM8* affect splicing but not mRNA decay (Fig. 1)[7,8,10]. Second, proteins Lsm2–Lsm7 co-immunoprecipitate with both U6 snRNA[6,7,9] and mRNA decay factors (Fig. 3), whereas Lsm1 does not immunoprecipitate U6 snRNA[6,7] but does pull down Dcp1 and Pat1/Mrt1 (Fig. 3). In contrast, Lsm8 does co-immunoprecipitate U6 snRNA[6–9] but does not pull down Dcp1 or Pat1/Mrt1 (Fig. 3). Third, immunoprecipitation of Lsm8 co-immunoprecipitates Lsm2–Lsm7, but not Lsm1 (ref. 6). Fourth, a pair of distinct complexes, each consisting of seven members, would be consistent with the known organization of Sm and Lsm proteins[19]. This suggests that the Lsm proteins form at least two distinct complexes that are involved in different aspects of RNA metabolism.

The function of the Lsm proteins in decapping appears to be to interact with the mRNA substrate and accelerate decapping, perhaps by promoting rearrangements in mRNP organization that
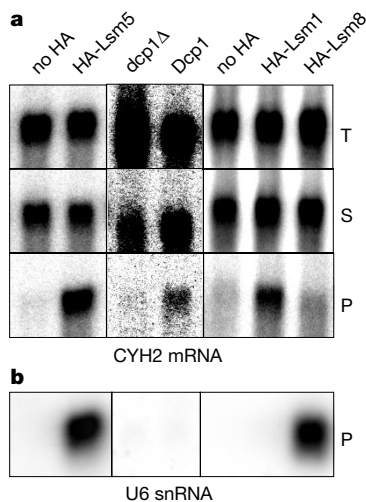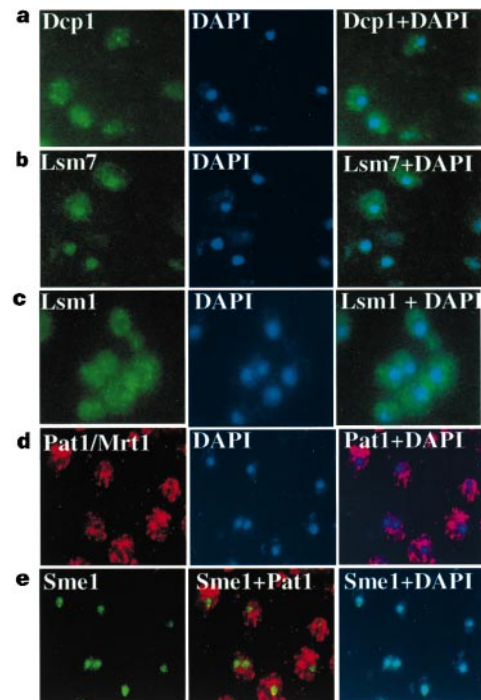

Figure 5 Cellular localization of Dcp1, Pat1/Mrt1, Lsm7 and Lsm1 by immunofluorescence analysis. **a–c**, Strains expressing HA-tagged Dcp1 (AEMY121 (**a**)), Lsm7 (AEMY51 (**b**)) or Lsm1 (AEMY28 (**c**)) were stained with anti-HA antibodies and anti-rat (fluorescein isothiocyanate) secondary antibody (green fluorescence, left panel), or subjected to 4,6-diamidino-2-phenylindole (DAPI) nuclear staining (blue fluorescence, middle panel). A merger of the two panels is shown in the right panel in each case. **d**, AEMY54 cells were stained with anti-Pat1 antibodies and anti-rabbit (cy3) secondary antibody (red fluorescence, left panel), or DAPI nuclear stain (blue fluorescence, middle panel). **e**, As a control, cells expressing the HA-tagged Sme1 nuclear protein (AEMY54) were stained with anti-HA antibodies and anti-rat (fluorescein isothiocyanate) secondary antibody (green fluorescence, left panel). A merge of HA–Sme1 staining and Pat1 staining (middle panel), and a merge of HA–Sme1 staining and DAPI nuclear staining (right panel).

lead to dissociation of the cytoplasmic cap-binding complex, which is an inhibitor of decapping[20]. A general role for Lsm proteins in modulating RNP structure in a variety of contexts by affecting RNA–RNA and/or protein–RNA interactions would be consistent with the proposal that their function in pre-mRNA splicing is to facilitate RNP rearrangements during splicing[7,21]. □

Methods

Yeast strains and plasmids

Figure 4 Lsm proteins and Dcp1 co-immunoprecipitate mRNA. RNA was extracted from the immunoprecipitate (P) of Dcp1, HA–Lsm5, HA–Lsm1 or HA-Lsm8 and analysed by northern blot for *CYH2* mRNA (**a**) or U6 snRNA (**b**). Similar analysis of aliquots of the lysate (T), and supernate (S) for the *CYH2* mRNA is also shown (**a**).

LSM7–HA–TRP1); IDY2 (ref. 22) (*MATa trp1-1 his3Δ200 isy1Δ::HIS3 pCA23* (*HA–ISY1, CEN URA3*)). For Lsm4, the MCY4 (ref. 5) strain expressing *GPD* promoter-controlled FLAG–Lsm4 protein from a plasmid (pRP961) was used. This strain has a chromosomal *LSM4* gene under *GAL* UAS control and was grown in glucose medium for the experiment. MCY4 (ref. 5) (*MATa LEU2–GAL–LSM4*) was compared with its isogenic wild-type strain w303 (*MATα ade1-101 his3-Δ1 trp1-289 ura33-52*) without epitope-tagged proteins.

RNA analysis

Transcriptional shut-off experiments, northern analysis and immunoprecipitation of RNA were performed as described[12,13,15]. Oligo oRP140 (5′-ATATTGATTAGATCAGG-AATTCC-3′) was used to probe for *MFA2pG* mRNA.

Immunology

The immunoprecipitation and immunofluorescence experiments followed standard protocols. The HA–Lsm protein immunoprecipitations shown in Fig. 3a and c were done using anti-HA antibody sepharose. Lysis and washing buffer was IPD1 buffer (50 mM Tris pH7.5, 50 mM NaCl, 2 mM MgCl$_2$, 0.1% Nonidet P-40) containing 1 mM β-mercaptoethanol and 1× 'COMPLETE' protease inhibitor mix (Boeringer Manheim). Bound proteins were eluted with 50 mM Tris pH7.5, 100 mM NaCl, 2 mM MgCl$_2$, 1% SDS, 1 mg ml^{-1} HA peptide and 1× COMPLETE protease inhibitor mix, and analysed for presence of Dcp1 by western blotting[23]. The Dcp1 immunoprecipitation shown in Fig. 3c was done similarly using anti-Dcp1 polyclonal antibodies and protein A sepharose, and the co-immunoprecipitating Lsm1 was revealed in western blots using anti-Lsm1 polyclonal antibodies. Elution buffer was as above but without HA peptide. The immunoprecipitation of FLAG–Lsm4 shown in Fig. 3a was done similarly using anti-FLAG antibody agarose (Sigma). Lysis was in IPD2 buffer (50 mM Tris pH7.5, 25 mM NaCl, 2 mM MgCl$_2$, 0.2% Nonidet P-40). IPD2 buffer without Nonidet P-40 was used to wash the beads. Elution buffer was as above but with 200 μg ml^{-1} FLAG peptide. HA–Lsm immunoprecipitation in Fig. 3b was done using anti-HA antibodies and protein A sepharose. Lysis and washing buffer was 6 mM HEPES pH7.9, 150 mM NaCl, 2.5 mM MgCl$_2$, 0.05% Nonidet P-40. Immunoprecipitated proteins were extracted by boiling pellets in SDS sample buffer and western analysed using anti-Pat1 polyclonal antibodies. To study the RNA dependence of co-immunoprecipitations, lysates were treated with 0.5 μg μl^{-1} RNase A for 10 min at room temperature before preclearing. To study the co-immunoprecipitation of RNA, total RNA extracted from the immunoprecipitates was analysed by northern blotting using *CYH2* complementary DNA probe or U6 oligo probe (oRP 759: 5′-GACCAAATGTCCACGAAGGG-3′).

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Structural basis for the anticoagulant activity of the thrombin–thrombomodulin complex

Pablo Fuentes-Prior*, Yoriko Iwanaga*, Robert Huber*, Rene Pagila†, Galina Rumennik†, Marian Seto†, John Morser†, David R. Light† & Wolfram Bode*

* *Max-Planck-Institut für Biochemie, Abteilung Strukturforschung Am Klopferspitz 18a, D-82152 Martinsried, Germany*
† *Berlex Biosciences, Richmond, California 94804, USA*

The serine proteinase α-thrombin causes blood clotting through proteolytic cleavage of fibrinogen and protease-activated receptors and amplifies its own generation by activating the essential clotting factors V and VIII[1]. Thrombomodulin[2], a transmembrane thrombin receptor with six contiguous epidermal growth factor-like domains (TME1–6), profoundly alters the substrate specificity of thrombin from pro- to anticoagulant by activating protein C (see, for example, reference 2). Activated protein C then deactivates the coagulation cascade by degrading activated factors V and VIII[2]. The thrombin–thrombomodulin complex inhibits fibrinolysis by activating the procarboxypeptidase thrombin-activatable fibrinolysis inhibitor[3]. Here we present the 2.3 Å crystal structure of human α-thrombin bound to the smallest thrombomodulin fragment required for full protein-C co-factor activity, TME456. The Y-shaped thrombomodulin fragment binds to thrombin's anion-binding exosite-I, preventing binding of procoagulant substrates. Thrombomodulin binding does not seem to induce marked allosteric structural rearrangements at the thrombin active site. Rather, docking of a protein C model to thrombin–TME456 indicates that TME45 may bind substrates in such a manner that their zymogen-activation cleavage sites are presented optimally to the unaltered thrombin active site.

Thrombomodulin consists of a lectin-like amino-terminal domain, followed by a hydrophobic segment, six contiguous epidermal growth factor (EGF)-like domains, an *O*-glycosylated serine/threonine-rich domain, a transmembrane segment and a short cytoplasmic tail (Fig. 1a). Upon thrombomodulin binding, thrombin activation of both protein C and thrombin-activatable fibrinolysis inhibitor (TAFI) is enhanced by at least three orders of magnitude[2,3]. Different hypotheses have been proposed to explain the thrombomodulin-induced switch in thrombin specificity. Mutagenesis studies (see, for example, ref. 4) and experiments with fluorescence[5] or spin label[6] active-site probes indicate that thrombomodulin binding may cause allosteric changes in the