Preparation and characterization of bovine aortic actin

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(Received 23 November 1984/22 January 1985; accepted 22 February 1985)

A functional vascular smooth-muscle actin from bovine aorta was purified to homogeneity by an original method and was able to polymerize. Aortic actin is composed of two major isoforms and at least two minor ones. This actin was not phosphorylated by either cyclic AMP-dependent protein kinase or C kinase. The physical properties of aortic actin were found to be very similar to those of skeletal-muscle actin, except for amino acid composition (three tryptophan residues instead of four). The aortic actin and skeletal-muscle actin differ in the extent of activation of the Mg-dependent ATPase of skeletal-muscle myosin.

Understanding of muscular-contraction regulatory mechanisms has challenged scientists for more than 20 years. Discovery by Ebashi (1963) and Ebashi & Ebashi (1964) of the troponin complex in skeletal muscle and subsequent studies on the reconstituted regulated actomyosin (actin, myosin, tropomyosin and troponin complex) were key steps in the understanding of the Ca^{2+} regulatory action in skeletal and cardiac muscles. In smooth muscle, the picture is less clear. Indeed, smooth muscle is not an homogeneous tissue and not all the smooth-muscle proteins that are involved in contraction have been purified and fully characterized. As a first step in the study of actin–myosin interaction and of its regulatory mechanisms, we have attempted to purify the different proteins involved in smooth-muscle contraction. The general procedure utilized for the preparation of skeletal-muscle actin from acetone-dried powders (Spudich & Watt, 1971) was generally unsuccessful for smooth muscle.

Specific methods for the preparation of smooth-muscle actin have been reported for uterus (Elce *et al.*, 1981) and gizzard (Sobieszek & Bremel, 1975; Strzelecka-Golaszewska *et al.*, 1980). The first one involves anion-exchange chromatography of the ATP extract followed by gel filtration. The second

procedure starts from an acetone-dried powder and includes an EDTA wash essential for the removal of actinin (Strzelecka-Golaszewska *et al.*, 1980). In contrast with gizzard and uterine actins, mammalian vascular actin is poorly characterized (Ruegg *et al.*, 1965; Gosselin-Rey *et al.*, 1969). None of the above procedures yielded satisfactory results when applied to bovine aorta.

The present paper reports the purification and the characterization of a functional mammalian vascular actin from bovine aorta and outlines its similarities to, and differences from, skeletal-muscle actin.

Materials and methods

Ultrogel AcA 44 was purchased from IBF (Villeneuve-la-Garenne, France); chymostatin, leupeptin and STI were from Sigma. [^{32}P] ATP was purchased from Amersham International.

The catalytic subunit of cyclic AMP-dependent protein kinase was prepared as described by Peters *et al.* (1977). Skeletal-muscle actin was prepared as described by Pardee & Spudich (1982). Skeletal-muscle myosin and its subfragment 1 (SF_1) were prepared as described by Kielly & Harrington (1960) and Weeds & Taylor (1975) respectively, and were generously given by Dr. R. Kassab's group at this Institution.

Protein concentrations

These were determined spectrophotometrically using the following: myosin, $A_{280}^{0.1\%}$ 0.56; SF_1 , $A_{280}^{0.1\%}$ 0.75; skeletal-muscle actin, $A_{290}^{0.1\%}$ 0.637. Alterna-

Abbreviations used: SDS, sodium dodecyl sulphate; DTT, dithiothreitol; STI, soya-bean trypsin inhibitor; SF_1 , myosin subfragment 1; Mg-ATPase, Mg^{2+} -dependent ATPase.

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tively, protein concentrations were determined by the method of Spector (1978), with γ -globulin as the standard.

Gel electrophoresis

SDS/5–20%-(w/v)-polyacrylamide-gel electrophoresis was performed using the discontinuous buffer system described by Laemmli (1970). Two-dimensional polyacrylamide-gel electrophoresis was performed as described by O'Farrell (1975), and the gels were stained with silver (Wray *et al.*, 1981) or with Coomassie Blue.

Sedimentation-velocity analysis

This was performed by using a MSE analytical ultracentrifuge equipped with a scanning absorption optics attachment to monitor the migration of the boundary at 280 nm.

Viscosity

This was determined by using a Cannon microviscosimeter at 25°C. Densities were measured with a digital density meter (DMA 40, Parr, Graz, Austria).

Nucleotide and ion binding

The amount of bound bivalent cation was determined, after treatment of the sample with AG 50 resin (X8; 0.5 ml of resin/ml of actin solution), by atomic-absorption spectrophotometry (Varian model 1150). The actin-bound ATP was measured after removal of free nucleotide by treatment with AG 1 resin (X2; 0.5 ml of resin/ml of actin solution) as described by Strzelecka-Golaszewska *et al.* (1980).

$[^{32}\text{P}]\text{P}_i$

$[^{32}\text{P}]\text{P}_i$ liberated during actin polymerization was determined by the method of Reimann & Umfleet (1978).

Proteolytic cleavage of G-actin

All enzymic cleavages of skeletal-muscle or aortic G-actins were performed at 25°C in Hepes buffer (pH 7.6)/0.2 mM-CaCl₂/0.1 mM-ATP/0.5 mM-DTT, except for thrombin and *Staphylococcus aureus* proteinase, where 1 mM-EDTA and 1% SDS were added respectively. The time course of the cleavage was monitored by 0.1% SDS/15%-(w/v)-polyacrylamide-slab-gel electrophoresis, samples being taken at suitable times. The enzyme/actin molar ratio was 1:5 for trypsin, chymotrypsin, thrombin, *Staph. aureus* proteinase and 1:10 for thermolysin. The enzymic reactions were terminated by adding 1 vol. of the reaction mixture to 5 vol. of sample buffer (containing 9 M-urea, 1% SDS and 1% DTT). The sample was loaded on a 0.1% SDS/

15%-polyacrylamide slab gel and subjected to electrophoresis.

Amino acid analyses

These were performed on a Beckman model 120 B analyser. Cysteine was determined as cysteic acid (Moore, 1963). Phospho-amino acid analyses were carried out by the method of Capony & Demaille (1983). Tryptophan was determined after hydrolysis with methanesulphonic acid in the presence of tryptamine (Simpson *et al.*, 1976).

Electron microscopy

Actin (1 mg/ml) was polymerized with 100 mM-KCl/1 mM-MgCl₂, diluted to 0.1 mg/ml with polymerization buffer, and pipetted on to a 200-mesh Formvar carbon-coated grid. After 1 min, the grid was washed with 10 drops of polymerization buffer and negatively stained with 10 drops of 1% (w/v) uranyl acetate. The grids were air-dried and examined with a Jeol 200 CX electron microscope operating at 80 kV with a 20 μm aperture.

Activation of the Mg-ATPase of skeletal myosin and SF₁ by aortic or skeletal-muscle actin

Comparative activation of skeletal-muscle myosin ATPase with aortic or skeletal-muscle actin was carried out at 25°C in 60 mM-KCl/10 mM-Hepes (pH 7.0)/2 mM-ATP/3 mM-MgCl₂, in the presence of 0.1 mM-CaCl₂ or 1 mM-EGTA. The reaction was initiated by the addition of ATP and terminated by addition of 5% (w/v) trichloroacetic acid. Identical experiments were performed with skeletal-muscle myosin SF₁, except that the KCl concentration was lowered to 10 mM. Phosphate liberated was automatically determined as described by Terasaki & Brooker (1976).

Purification of bovine aortic actin

All steps were performed at 4°C. Fresh aortas were dissected from fat and connective tissue, ground in a meat grinder for 3 $\times$ 10 s and homogenized for 30 s in a Waring Blender with 3 vol. of 50 mM-KCl/15 mM-MgCl₂/0.2 mM-DTT/2 mM-EGTA/10 mM-Tris/HCl (pH 7.6)/3% (v/v) Triton X-100. The homogenate was centrifuged at 10000g for 10 min and the supernatant was discarded. This step was repeated three times. Three additional washes with the same buffer without Triton X-100 were then performed. The resultant pellet was homogenized (2 $\times$ 30 s bursts in a Waring Blender) with 3 vol. of 40 mM-imidazole/HCl (pH 7.5)/10 mM-ATP/2 mM-EGTA/4 mM-EDTA/1 mM-DTT/leupeptin (1 mg/l)/chymostatin (1 mg/l)/STI (10 mg/l). This homogenate was centrifuged for 30 min at 10000g and the supernatant filtered through glass wool. The actomyosin contained in

the supernatant was dissociated by a dropwise addition of ATP and Mg^{2+} to a final concentration of 20mM-ATP and 150mM- $MgCl_2$ and the cloudy solution was centrifuged at 60000g for 1h. The precipitate was dehydrated by repeated washes with acetone precooled at $-20^{\circ}C$ and air-dried overnight at $4^{\circ}C$. This procedure yielded 6–10g of acetone-dried powder per kg of fresh aorta. The powder can be stored at $-20^{\circ}C$ for several months. Crude actin preparations were obtained by extraction of the acetone-dried powder with 50 vol. of 1M-KCl/1mM-DTT/leupeptin (1mg/l)/chymostatin (1mg/l)/STI (10mg/l) at $4^{\circ}C$ for 10min. The suspension was centrifuged for 10min at 20000g. The pellet was washed with water, centrifuged again and extracted for 3h at $4^{\circ}C$ with 50 vol. of ATP containing buffer [1mM-ATP/1mM-DTT/0.2mM- $CaCl_2$ /10mM-imidazole/HCl (pH 7.5)/leupeptin (1mg/l)/chymostatin (1mg/l)/STI 10mg/l]. The suspension was centrifuged for 10min at 20000g and actin contained in the supernatant was polymerized for 1h at $20^{\circ}C$ by addition of 100mM-KCl and 1mM- $MgCl_2$. Polymerizable actin was pelleted by centrifugation for 3h at 100000g, the pellet suspended in the extraction buffer and depolymerized by 24h dialysis against the same buffer. Final purification was performed by gel-filtration chromatography on Ultrogel AcA 44 equilibrated with 1mM-ATP/0.2mM- $CaCl_2$ /1mM-DTT/2mM imidazole/HCl, pH 8. Actin-containing fractions were determined by gel electrophoresis, pooled, and concentrated by vacuum dialysis against the ATP/ Ca^{2+} buffer.

Results

Skeletal actin is generally isolated from acetone-dried powder. When this procedure was attempted with fresh aorta by the method of Spudich & Watt (1971), the actin contained in the ATP/ Ca^{2+} extract was found to be non-polymerizable. The procedure described in the Material and methods section yields polymerizable actin. The protein patterns obtained in the various steps of the purification procedure are shown in Fig. 1. Selective removal of actin from skeletal-muscle acetone-dried powders containing tropomyosin is generally obtained by extraction at $0^{\circ}C$ and polymerization with high concentrations of KCl. When applied to the aortic powder, such a procedure resulted in a considerable contamination of actin with tropomyosin.

In order to minimize the tropomyosin contamination, we pre-extracted the acetone-dried powder with 1M-KCl, which mainly removes tropomyosin along with some actin (Fig. 1, lane 2). The following ATP/ Ca^{2+} extract therefore contained a

Fig. 1. SDS/5–20%-polyacrylamide-gel electrophoresis of samples taken at the various steps of actin purification. Lanes 1 and 6, M_r standards [from top to bottom: phosphorylase *b* (94000), albumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20100) and lactalbumin (14400)]. Lane 2, KCl extract of acetone-dried powder (80 μ g); lane 3, ATP/ Ca^{2+} extract (80 μ g); lane 4, actin after one cycle of polymerization–depolymerization (50 μ g); lane 5, actin pool after chromatography of 80 μ g on Ultrogel AcA 44.

low amount of tropomyosin (Fig. 1, lane 3). This extract was polymerized by the addition of KCl and $MgCl_2$, but still contained trace amounts of tropomyosin (Fig. 1, lane 4), which were removed by gel-filtration chromatography. The first peak contained tropomyosin and actin associated under native conditions with some higher- M_r contaminants, whereas actin eluted in the second peak was close to 95% pure (Fig. 1, lane 5). The purity of bovine aortic actin was checked by gel electrophoresis under various conditions; 0.1% SDS/5–20%-(w/v)-polyacrylamide-gradient-gel electrophoresis showed a single band of M_r 42000. Two-dimensional gel electrophoresis showed the presence of four spots (pI 5.62, 5.56, 5.51, 5.46) with the same M_r of 42000 (Fig. 2). The purification procedure is summarized in Table 1.

Physical properties of bovine aortic actin

Table 2 summarizes the physical properties of aortic actin. The amino acid composition of bovine aortic actin is very similar to that of skeletal-muscle actin (Table 3). The presence of one residue of methylhistidine per molecule and the absence of trimethyl-lysine (which is present in cytoplasmic actin; Vandekerckhove *et al.*, 1984) were noted.

Fig. 2. Two-dimensional gel electrophoresis of column-purified aortic actin

The isoelectric-focusing run was performed in the presence of a 2% Ampholine mixture (pH 4–6/pH 3–10, 4:1, v/v), 9.5M-urea, 2% Nonidet P40 and 3.5% acrylamide. The second-dimensional running gel was 0.1% SDS/15% polyacrylamide. (a) 10 μ g of aortic actin was loaded and Coomassie Blue-stained; (b) 3 μ g of aortic actin was loaded and silver-stained.

Table 1. Purification of bovine aortic actin from 500 mg of acetone-dried powder

	Total protein (mg)	Actin (mg)	Purification (fold)	Yield
KCl extract	392	225	57.4	
ATP/Ca ²⁺ extract	252	219	87.1	1
Polymerization and depolymerization	111	90	81.2	0.44
Gel filtration on Ultrogel AcA 44	94.2	89.5	95.0	0.37

Table 2. Physical properties of bovine aortic actin

Parameter	Value
M_r	
By gel filtration	41 600
By gel electrophoresis	41 000
By ultracentrifugation	41 994
R (Stokes radius)	2.74 nm (27.4 Å)
$s_{20,w}$ (sedimentation coefficient)	3.0S
$D_{20,w}$ (diffusion coefficient)	$6.38 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$
$\bar{v}$ (partial specific volume)*	0.729 ml $\cdot$ g ⁻¹
$A_{274}^{0.1\%}$, $A_{274}^{0.1\%}$, $A_{290}^{0.1\%}$ (absorption coefficients)	$\left\{ \begin{array}{l} 0.787 \\ 0.365 \end{array} \right.$

* From amino acid composition.

Also, bovine aortic actin has a lower content of aromatic amino acids than has the homologous protein from skeletal muscle.

The *N*-terminal residue was found to be blocked when the molecule was submitted to Edman degradation (Edman & Begg, 1963). Phospho-amino acids or sugars were not detected.

The u.v.-absorption spectrum exhibits a maximal absorbance at 274 nm; the absorption coefficient ($A_{274}^{0.1\%}$) was calculated by assuming 27 leucine and 29 alanine residues per molecule of M_r 42 000 and was found to be 0.787. The amount of bound Ca²⁺ after removal of free Ca²⁺ was determined to be $1 \pm 0.02 \text{ mol/mol}$ ($\pm$ S.D., $n = 3$).

The amount of ATP bound determined after removal of free nucleotide was found to be $0.95 \pm 0.07 \text{ mol/mol}$ ($\pm$ S.D., $n = 3$).

Polymerization of bovine aortic actin

The bovine aortic actin polymerizes in the presence of 100 mM-KCl and 1 mM-Mg²⁺ at pH 8.0, and its specific viscosity is linearly related to actin concentration. The extrapolation to zero specific viscosity gives the critical concentration for actin polymerization. The value of 0.92 μ M is comparable with that obtained for skeletal-muscle actin. The polymerization of G-actin is accompanied by ATP splitting at a rate of 1.4 mol of P_i/h per mol of actin.

Table 3. *Amino acid composition of rat uterus, chicken gizzard, rabbit skeletal-muscle and bovine aortic actin*
 Rabbit skeletal-muscle actin values are reproduced from the known amino acid sequence (Collins & Elzinga, 1975),
 rat uterus values from Elce *et al.* (1981) and chicken gizzard values from Vandekerckhove & Weber (1979b).

Composition (residues per molecule of M_r 42000)

Amino acid	Source of actin . . .	Rat uterus	Chicken gizzard	Rabbit skeletal muscle	Bovine aorta
Asx		33.2	33	34	32
Thr*		25.1	26	28	22
Ser*		23.1	24	22	23
Glx		41.3	40	39	35
Pro		18.7	19	19	21
Gly		35.5	28	28	30
Ala		30	29	29	29
Cys		6.5	6	5	6
Val†		19.7	21	21	16
Met		15.2	15	16	17
Ile		25.3	29	29	21
Leu		28.5	27	26	26
Tyr		13.4	16	16	15
Phe		11.9	12	12	13
Lys		19.3	19	19	18
Arg		16.8	18	18	18
His		8.2	8	8	8
MeHis		N.D.	1	1	1
Trp‡		N.D.	4	4	3

* After extrapolation to zero-time hydrolysis.

† From the 72h-hydrolysis value.

‡ After hydrolysis with methanesulphonic acid.

Proteolytic cleavage of skeletal-muscle and bovine aortic actin

Fig. 3 shows that aortic actin, like skeletal-muscle actin, is organized in domains that can be separated after enzymic cleavage. The comparative cleavage of skeletal-muscle and aortic actins, at the same enzyme/substrate ratio, by trypsin, chymotrypsin and thermolysin, yield identical fragmentations, the formation of a 35000- M_r peptide and a rapid conversion into a 33000- M_r form.

However, the cleavage with *Staph. aureus* proteinase and thrombin indicate some differences. The former, both in the case of skeletal-muscle and aortic actin, yields three major peptides (M_r 29000, 22000 and 16000). The kinetics of the cleavage and the relative proportion of each peptide show that skeletal-muscle actin is less susceptible than aortic actin. More evident are the kinetic differences in the thrombin cleavage; for aortic actin, in addition to the 39000- and 29000- M_r peptides common to the two actins, we observed the presence of a doublet of M_r 14000 and 12000.

Phosphorylation of aortic actin

Attempts to phosphorylate bovine aortic actin

by the catalytic subunit of cyclic AMP-dependent protein kinase or by phospholipid- and Ca^{2+} -dependent kinase (C kinase; enzyme/substrate ratio 1:100) were unsuccessful; less than 1% of actin was phosphorylated after 2h of incubation.

Electron microscopy

Electron micrographs of negatively stained preparations of actin polymerized with 100mM-KCl/1 mM-MgCl₂ show the presence of predominantly long filaments and some shorter ones (Fig. 4). The measure of the filament length before and after gel filtration is very difficult, since the length of the filaments is dependent on the technique of washing and staining. However, the filaments are longer and straighter after gel filtration (Fig. 4b) than before (Fig. 4a). The fact that a higher quantity of shorter filaments was found in actin preparations that had not been purified by gel filtration could be due to the presence of contaminants eliminated on gel filtration.

These contaminants could contain some capping or severing factors, as suggested by the presence of a 93000- M_r protein. Addition of skeletal-muscle heavy meromyosin to aortic actin filaments gives a pattern identical with that of skeletal-muscle actin (Fig. 4c).

Fig. 3. Comparative kinetics of the cleavage of rabbit skeletal-muscle (i) and bovine aortic (ii) actin by different proteolytic enzymes
(a) chymotrypsin; (b) thermolysin; (c) *Staph. aureus* V8 proteinase; (d) thrombin. Abbreviation used: st, standards (identical with those in Fig. 1.).

Activation by actin of the Mg-ATPase of skeletal myosin

The time course of ATP hydrolysis by actomyosin reconstituted from skeletal-muscle or aortic actin and skeletal-muscle is shown in Fig. 5. The experiment was performed with a myosin/actin ratio of 1:12. The comparison revealed that the extent of activation of the skeletal-muscle myosin Mg-ATPase of the reconstituted actomyosins, aortic actin+skeletal-muscle myosin is 1.6 times lower than the skeletal-muscle actin+skeletal-muscle myosin system. This difference persisted at higher ATP concentrations (results not shown). The experiment with skeletal-muscle myosin SF₁ confirms the difference in the extent of activation of skeletal-muscle and aortic actin. The activation of the Mg-ATPase of skeletal SF₁ by aortic actin is 3.75 times lower than that obtained with skeletal actin.

Discussion

It is now accepted that actin in smooth muscles has a dual role: participation in the contractile

process as well as a cytoskeletal contribution. In spite of the importance of actin, only gizzard actin preparations have been well described (Strzelecka-Golaszewicka *et al.*, 1980) and no good method for the preparation of vascular smooth-muscle actin has been reported as yet. The aim of this present work was to purify and characterize a functional mammalian vascular smooth-muscle actin, in order to study further the actin-myosin interaction and its regulatory mechanisms.

Purification procedure

In our hands, polymerizable actin could not be prepared from acetone-dried powders of bovine aorta, whether or not extensive EDTA washes were carried out. Another method that does not involve acetone-dried powder (Elce *et al.*, 1981) was also found to be unsatisfactory. On the basis of the above findings we developed a method which was adapted for use in the aorta.

The major difficulty encountered in aorta was the presence of a high content of connective tissue and consequently a low proportion of muscle cells. To overcome this histological problem without tedious dissection, we purified aortic actin from an

Fig. 4. *Electron micrographs of bovine actin preparations polymerized with 100mM-KCl/1mM-MgCl₂, pH8.0, before (a) and after (b) gel filtration, and in the presence of skeletal heavy meromyosin (c)*

Fig. 5. Activation of skeletal-muscle myosin ATPase by skeletal-muscle actin (■) and aortic actin (●). The conditions are given in the Materials and methods section.

actomyosin preparation. A mixture of proteinase inhibitors is used during the preparation of the actomyosin extract to inhibit the proteinases that are known to be very active in smooth muscle (Uyemura *et al.*, 1978).

Phosphorylation of actin

Unlike chicken gizzard G-actin, which was reported to be phosphorylated by the cyclic AMP-dependent protein kinase (Walsh *et al.*, 1981), purified aortic actin is poorly phosphorylated. This discrepancy could be due to a difference between bird and mammalian actins or due to the presence of a contaminant protein in gizzard actin preparations. The second hypothesis appears more probable, since the method of preparation used (Persechini *et al.*, 1981) did not utilize the gel-filtration or ion-exchange chromatography steps known to be necessary in order to obtain purified actin (Pardee & Spudich, 1982). Contradictory results have also been reported for skeletal-muscle actin. However, the positive reports for G- or F-skeletal-muscle actin (Pratje & Heilmeyer, 1972; Grazi & Magri, 1979; Grazi *et al.*, 1980), always show a very low stoichiometry of ³²P incorporation. The phosphorylation by C kinase also showed a very low incorporation (less than 1%), but this poor incorporation could be significant if a specific isoform is involved.

Actin isoforms

The presence of three tryptophan residues instead of four in the published sequence (Vanderkerckhove & Weber, 1979a) could be due to the different methods of actin preparation. These different methods may favour the purification of a given isoform(s) and therefore explain differences

in the tryptophan content of these preparations. Analysis of the actin isoforms by isoelectric focusing showed that bovine aortic actin contained four forms and a low amount of an acidic spot that could be a phosphorylated form of actin; the number of isoforms was dependent on the quantity loaded and the staining procedure. Four distinct spots were detected when a low quantity of actin was loaded and silver staining used, instead of two with Coomassie Blue staining. Identical patterns (four spots) were also obtained when isolated smooth-muscle aortic rat cells were analysed (results not shown). However, the minor isoforms revealed by silver staining (Fig. 2b) are not representative of their relative amounts. Indeed, we obtain a saturation staining of the major isoforms, and their amount is minimal in comparison with the minor isoforms.

In conclusion, the bovine aortic actin has been isolated in a functional state by a specific method and characterized. The results show that this *functional* mammalian vascular actin is somewhat different from skeletal-muscle or gizzard actin. These differences could be important enough to explain the differential activation of the Mg-ATPase of skeletal-muscle, uterine or gizzard myosin by smooth-muscle or skeletal-muscle actin (Sheetz *et al.*, 1976; Cooper & Pollard, 1982; Suzuki *et al.*, 1978; Strzelecka-Golaszewska & Sobieszek, 1981; Sobieszek & Small, 1977). The purification, characterization and the use of specific antibodies against each isoform appear to be necessary steps in assigning a precise role to the isoforms.

This investigation was supported by a research grant from INSERM (CRL 823025). We acknowledge Dr. C. Le Peuch and Dr. R. Kassab's group for their gift of proteins and Dr. J. P. Capony for amino acid analysis. We are especially grateful to Dr. J. Demaille and Dr. S. Katz for stimulating discussion and advice. We thank Ms. B. Pechere and Ms. C. Brenié for expert assistance in the preparation of the manuscript.

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