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Biochemical and Biophysical Research Communications 309 (2003) 749–754
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Structural and evolutionary consequences of unpaired cysteines in trypsinogen

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Received 13 August 2003

Abstract

Vertebrate trypsins usually contain six disulfide bonds but human trypsin 1 (PRSS1) contains only five and human trypsin 2 (PRSS2) contains only four. To elucidate possible evolutionary pathways leading to the loss of disulfide bonds, we have constructed mutants lacking one or two cysteines of four disulfide bonds (C22–C157, C127–C232, C136–C201, and C191–C220) in rat anionic trypsinogen and followed their expression in the periplasm of *Escherichia coli*. When both cysteines of any of the above-mentioned disulfide bonds were replaced by alanines we found, as expected, proteolytically active enzymes. In the case of C127–C232 (missing from both human trypsins) and C191–C220 both single mutants gave active enzymes although their yield was significantly reduced. In contrast, only one of the single mutants of disulfide bonds C22–C157 and C136–C201 (missing from human trypsin 2) was expressed in *E. coli*. In the case of these disulfide bonds, we obtained no expression when the solvent accessible molecular surface of the free cysteine residue was the smaller one, indicating that a buried unpaired cysteine was more deleterious than one on the surface of the molecule.

Keywords: Trypsinogen; Human; Cysteine; Disulfide; Evolution; Heterologous protein expression; Gel electroforesis

The history of trypsinogen evolution is an interesting example both of gain and of loss of disulfide bridges. Trypsins isolated from the higher vertebrates contain six disulfide bonds. Of these three (C42–C58, C168–C182, and C191–C220)[3] are present in bacterial, fungal, and insect trypsins as well. The same disulfide bridges are also conserved in most serine proteinases belonging to clan SA (for nomenclature see [1]). These disulfide bonds form short loops in the vicinity of the His 57 (C42–C58), as well as of the substrate binding pocket (C168–C182 and C191–C220). During the evolution of the vertebrate lineage tunicata (*Boltenia* and *Bortyllus*) gained two cysteines forming disulfide bridge C136–C201, while the final disulfide set was reached already in lamprey by recruiting disulfide bridge C22–C157 and C127–C232 [2]. These "new" disulfide bonds form large loops; two of them connect the two domains of trypsin (C22–C157 and C127–C232).

Interestingly human trypsins, as revealed by the cloning of two human trypsin cDNAs, as well as by genome sequencing, do not fit into this pattern. Large-scale sequencing of the human T cell receptor (TCR) locus provided us with two exciting discoveries [3]. First, it was found that there were two groups of trypsinogen genes, one towards the 3′ end of TCR β locus and another towards the 5′ end of TCR β locus. On the basis of this spatial separation a new nomenclature for trypsinogens was suggested: group I trypsinogens are those found 3′ in the TCR β locus, group II trypsinogens are those found 5′ in the TCR β locus. There are no functional group II trypsinogens in the human genome, while the three apparently functional genes T4, T6, and T8 belong to group I. By comparing with the cDNA sequences, T4 was identified as the cationic form,

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[3] The chymotrypsin numbering is used throughout.

0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.08.064

i.e., human trypsinogen 1, while T8 was found to be identical to anionic human trypsinogen 2 [4]. Interestingly, the synthenic relationship of the trypsinogen and TCR genes was found in the mouse and the chicken genome as well.

The second interesting finding, more relevant to the topic of our present work, is that in eight out of nine trypsinogen genes, including all the expressed ones, the disulfide bridge between C127 and C232 is lost. Moreover T8, which is identical to human trypsinogen 2, contains only four disulfide bonds as a consequence of loosing C136 and C201 as well. Therefore, these trypsins can be regarded as natural disulfide mutants.

First studies on the disulfide bonds of bovine cationic trypsin showed that sodium borohydride reduces selectively two disulfide bonds in trypsinogen and in trypsin which are nonessential for activity [5]. However, when the reduction was followed by S-alkylation of C191 and C220 there was a significant loss of esterase activity and no amidase activity was detected [6]. In light of recent mutagenesis studies the inactivity of the chemically modified trypsin does not per se prove the essential nature of this disulfide bond. It is more likely that the bulky groups used for the chemical modification induce severe distortion of the substrate binding pocket. Elimination of disulfide bond C191/C220 by site directed mutagenesis resulted in a mutant which folded correctly in heterologous expression systems and retained significant activity both on ester and amide substrates [7,8]. Replacement of the cysteines C191 and C220 by alanines did not decrease the stability of the mutant in urea induced denaturation experiments [7]. At the same time the pH profile of the mutant enzyme became narrower as a consequence of a drop in the high pK_a value, indicating some loss of stability of the Ile16–Asp194 salt bridge essential for the stabilization of the active trypsin conformation [8].

In this study, we present data on the expression of disulfide mutants of rat trypsinogen II in *Escherichia coli* periplasmic expression system. The disulfide bonds involved are C22–C157, C127–C232, C136–C201, and C191–C220. The cysteines were always replaced by alanines. Besides the double mutants where both cysteines of a disulfide bond were mutated, all single mutants, containing an unpaired cysteine, were also constructed. An analogue of human trypsinogen T8, i.e., mutant C127A/C136A/C201A/C232A was also prepared.

Materials and methods

Construction of mutant rat trypsinogens. Mutations were introduced by site-directed mutagenesis performed with the megaprimer method [9]. As template for the preparation of the single mutants with one unpaired cysteine, the native rat trypsinogen cDNA was used. Double mutants and the mutant lacking disulfide bridges C127–C232 and C136–C201 were obtained with the help of the previously prepared single mutants used as templates. Mutations were confirmed by sequencing and the mutant cDNAs were cloned into the pTRAP vector [10].

Expression and isolation of native and mutant proteins. The native and mutant zymogens were expressed constitutively into the periplasmic space of *E. coli*. Transformed *E. coli* cells were incubated overnight in 3 ml LB medium. Optical density of the overnight culture was measured at 590 nm. Based on these data the same number of cells was used for isolation of the expressed proteins. Isolation was performed as described below. Cells were centrifuged (4000 rpm) at 4 °C, resuspended in 25% of the initial volume in a solution containing 20% sucrose, 30 mM Tris–HCl (pH 8), and 1 mM EDTA, and kept on ice for 10 min. After another centrifugation (4000 rpm) at 4 °C, cells were resuspended in 15% of initial volume in ice-cold distilled water and kept on ice for 10 min. The periplasmic fraction was obtained as the supernatant of the third centrifugation (12,000 rpm) at 4 °C. The fractions were lyophilized and kept at −20 °C.

Analysis of the trypsinogen expression. The presence of the trypsinogens in the periplasmic fractions was confirmed by SDS–PAGE in reducing and nonreducing conditions,, followed by Western blotting. Just before electrophoresis lyophilized samples prepared from 3 ml cultures were dissolved in 100 μl of 1 mM HCl and mixed with equal volume of 2× SDS sample buffer with or without mercaptoethanol. After 5 min incubation in boiling water 10 μl were loaded to 10 well minigels (BioRad Mini-Protean II). Acrylamide concentration was 15% and Dalton Mark VII-L (Sigma) standard was used for molecular mass calibration. After electrophoresis proteins were transferred to nitrocellulose membrane (Hybond-C extra, Amersham) in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) by 200 mA at 4 °C for 3 h. For immunological detection, as first antibody we used a polyclonal antibody raised in rabbits against rat trypsinogen, the second antibody was a horseradish peroxidase-labelled anti-rat IgG. Enzymatic activity was detected by Ni-dimethyamido benzidine reaction.

Detection of trypsin activity by zymograms. Zymogens were activated by incubation with enterokinase solution. Lyophilized periplasm obtained from 3 ml culture was incubated in 100 μl of 50 mM NaCl, 5 mM $CaCl_2$, and 20 mM MES, pH 6 containing 30 ng enterokinase (Sigma E 0885, purified on an immobilized ecotin column [11]) at 37 °C for 1 h. Samples were then mixed with equal volume of 2× SDS sample buffer without mercaptoethanol. After 10 min incubation at 65 °C, 10 μl was loaded on a 15% polyacrylamide gel containing 1% (w/v) gelatine. After electrophoresis the gel was washed for 20 min in a 1% Triton X-100 solution, incubated for 1 h at 37 °C in a buffer containing 50 mM Tricin (pH 8.0), 100 mM NaCl, 10 mM $CaCl_2$, and 0.05% Triton X-100, and finally stained with a Coomassie brilliant blue solution. The sensitivity of this method is comparable to that of Western blotting.

Calculation of solvent accessible molecular surfaces of cysteine residues. The solvent accessible molecular surfaces of cysteine residues were calculated with the dms program of the MidasPlus 2.1 (UCSF). The contact, the reentrant, and the total molecular surface areas were used as defined by Richards [12]. The calculation was performed on wild type rat trypsin (PDB [13] accession code: 1ANE [14]) with the solvent molecules removed prior to the calculation. For each cysteine residue the contact and reentrant surface areas were calculated with a probe size of 1.4 Å. Solvent accessible molecular surface was calculated as the sum of contact and reentrant surface areas.

Results

The analysis of the expression of different trypsinogen mutants relies on SDS–PAGE of the periplasmic extracts. The low level of expression and the presence of several bacterial proteins did not allow the direct observation of

the expressed trypsinogens in the periplasmic extracts. Moreover β-lactamase, a 25 kDa protein produced by the expression vector also, migrates identically to rat trypsinogen in reducing SDS–PAGE. Therefore, we detected trypsinogen by means of Western blotting using a polyclonal antibody against rat trypsinogen.

Fig. 1A displays a Western blot of different rat trypsinogen mutants containing even number of cysteines, as well as human trypsinogen 2. The intensity of the major band of about 25 kDa corresponds to the level of expression, the polyclonal antibody used for detection fully cross-reacts with human trypsinogens and with the mutants as well. Fig. 1A shows nearly uniform level of expression. In some cases, we observed even higher expression of mutant than that of the wild type enzyme.

There is a considerable difference in the yield, however, if mutants containing odd number of cysteines were expressed (Fig. 1B). The intensity of the 25 kDa bands differs widely. Mutants C157A, C232A, C136A, and C191A, containing unpaired cysteine residues C22, C127, C201, and C220, respectively, are expressed at a relatively high level, at about 25–65% of the wild type. Mutant C127A (free cysteine 232) is produced with a significantly lower yield. In this blot, proteolysed forms are also seen in wild type and in most of the mutant samples. In case of mutant C22A (free cysteine 157, Fig. 1B, lane 3) only the proteolysed forms are found, while the expressed amount of mutant C201A (free cysteine 136, Fig. 1B, lane 6) is below the detection limit under these experimental conditions. It is striking that in the case of each disulfide bond there is one single cysteine-containing derivative that can be expressed at a significantly higher level than the other.

SDS–PAGE in the absence of reducing agent reveals further differences in the electrophoretic pattern of the disulfide and single cysteine mutants (Fig. 2A). Wild type rat trypsinogen forms a single major band whose electrophoretic mobility is significantly higher than the mobility of reduced trypsinogen. This increase in the electrophoretic mobility is due to the more compact shape of the oxidized trypsin molecule cross-linked by six disulfide bonds. In case of disulfide mutants the majority of the protein also forms a single band. These bands however have always lower mobility compared to the wild type trypsinogen. The decrease in the mobility is roughly proportional to the size of the loop formed by the eliminated disulfide bond. Around the major bands

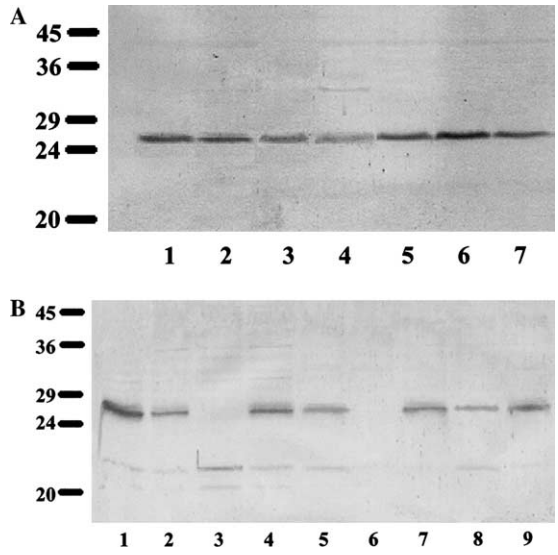

Fig. 1. (A) Electrophoretic mobility of trypsinogens with an even number of cysteines under reducing conditions. Lane 1: wild type trypsinogen, lane 2: C22A/C157A mutant, lane 3: C127A/C232A mutant, lane 4: C136A/C201A mutant, lane 5: C191A/C220A mutant, lane 6: C127A/C232A/C136A/C201A mutant, and lane 7: human trypsinogen 2. (B) Electrophoretic mobility of trypsinogens with an odd number of cysteines under nonreducing conditions. Lane 1: wild type trypsinogen, lane 2: C157A (C22 free cysteine), lane 3: C22A (C157 free cysteine), lane 4: C232A (C127 free cysteine), lane 5: C127A (C232 free cysteine), lane 6: C201A (C136 free cysteine), lane 7: C136A (C201 free cysteine), lane 8: C220A (C191 free cysteine), and lane 9: C191A (C220 free cysteine).

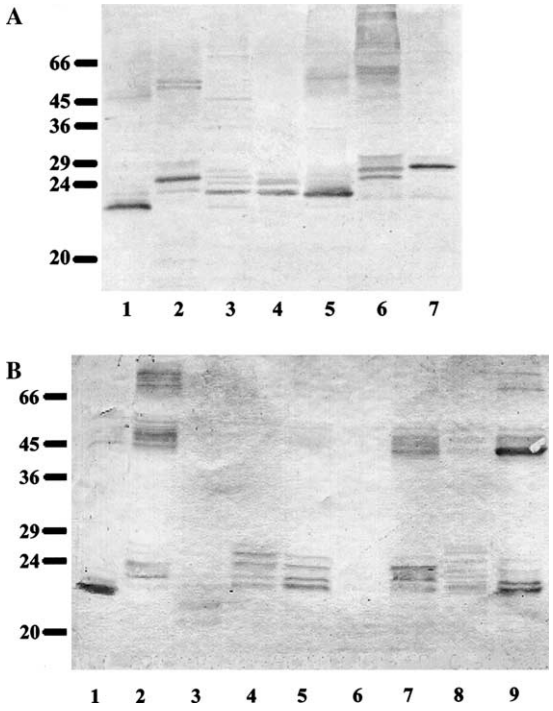

Fig. 2. (A) Electrophoretic mobility of trypsinogens with an even number of cysteines under nonreducing conditions. Lane 1: wild type trypsinogen, lane 2: C22A/C157A mutant, lane 3: C127A/C232A mutant, lane 4: C136A/C201A mutant, lane 5: C191A/C220A mutant, lane 6: C127A/C232A/C136A/C201A mutant, and lane 7: human trypsinogen 2. (B) Electrophoretic mobility of trypsinogens with an odd number of cysteines under nonreducing conditions. Lane 1: wild type trypsinogen, lane 2: C157A (C22 free cysteine), lane 3: C22A (C157 free cysteine), lane 4: C232A (C127 free cysteine), lane 5: C127A (C232 free cysteine), lane 6: C201A (C136 free cysteine), lane 7: C136A (C201 free cysteine), lane 8: C220A (C191 free cysteine), and lane 9: C191A (C220 free cysteine).

there are faint bands indicating the presence of scrambled disulfide derivatives too. Besides these presumably monomeric forms, bands in the 45 kDa region indicate the presence of disulfide linked dimers too. This pattern became dominant in the double disulfide mutant C127A/C232A/C136A/C201A where there is no major band, but numerous bands of nearly equal intensity populate the region of monomeric and dimeric forms. Regarding the disulfide content this mutant corresponds to human trypsinogen 2, but the latter molecule does not show any tendency to form incorrect disulfide bonds; there is a single well defined band in human trypsinogen 2 (Fig. 2A, lane 7)

All single cysteine derivatives (except C201A, Fig. 2B, lane 6) of rat anionic trypsinogen give multiple bands in nonreducing SDS–PAGE (Fig. 2B). In mutant C22A (Fig. 2B, lane 3) high mobility bands indicate proteolytic degradation, while there is no detectable band in C201A (Fig. 2B, lane 6). The most homogeneous sample is mutant C191A (Fig. 2B, lane 9). The most intense band in this case is at about 45 kDa which corresponds to the range of disulfide linked dimers. The predominant cysteine forming the disulfide bond is C220 in both monomers (Likó et al, unpublished results). The multimeric forms were also highly populated in mutants C157A, C136A, and C220A. C157A and to lesser a extent C191A contain several bands in the 80 kDa region too. These are probably disulfide linked tetramers.

In sharp contrast to the highly heterogeneous pattern seen in Western blots of the trypsinogen samples, the zymograms of enterokinase activated periplasmic extracts show only one major proteolytically active band. In wild type trypsin the band above 24 kDa represents the autolytically cleaved so-called two-chain trypsin form. All double mutants contained active forms irrespective of the heterogeneity seen in Western blots. The active band in the zymogram usually corresponds to the band with the highest migration in the Western blot (Fig. 3A). In accordance with the western blots we found no enzymatic activity in mutants C22A and C201A (Fig. 3B).

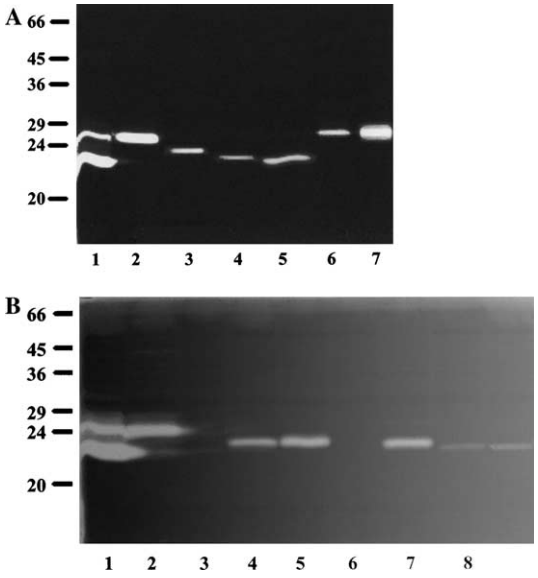

Fig. 3. (A) Zymograms of trypsinogens with an even number of cysteines. Lane 1: wild type trypsinogen, lane 2: C22A/C157A mutant, lane 3: C127A/C232 mutant, lane 4: C136A/C201A mutant, lane 5: C191A/C220A mutant, lane 6: C127A/C232A–C136A/C201A mutant, and lane 7: human trypsinogen 2. (B) Zymograms of trypsinogens with an odd number of cysteines. Lane 1: wild type trypsinogen, lane 2: C157A (C22 free cysteine), lane 3: C22A (C157 free cysteine), lane 4: C232A (C127 free cysteine), lane 5: C127A (C232 free cysteine), lane 6: C201A (C136 free cysteine), lane 7: C136A (C201 free cysteine), lane 8: C220A (C191 free cysteine), and lane 9: C191A (C220 free cysteine).

Discussion

The major goal of our study is to investigate the consequences of the presence of unpaired cysteine in a protein containing multiple disulfide bonds. Such situation is inevitable in the evolution of vertebrate trypsinogen where both gain and loss of disulfide bonds can be observed. In order to model the intermediate single cysteine containing forms we replaced cysteines by alanines. The rationale behind this choice is that we wanted to add a neutral residue which would not establish any specific interactions. Such interactions in different environments of the eliminated cysteines might obscure the effects of unpaired cysteines.

We are fully aware of the fact that that our prokaryotic model expression system is different from the eukaryotic one. Previous studies showed that the *E. coli* periplasmic expression system is capable to produce correctly folded wild type trypsinogen and several mutants of it [8,9,14,15]. Moreover, active trypsin can also be expressed [16]. On the other hand, it is also known that the expression level of proteins in *E. coli* is correlated to their stability and incorrectly folded proteins are removed by the proteolytic system of *E. coli* [17,18]. These data indicate that this economical and fast expression system might be suitable to analyze the correctness of folding in different disulfide mutants of trypsin.

Our results show that all double mutants were able to form enzymes with proteolytic activity although the expression level as well as the efficiency of correct disulfide bond formation is different in mutants missing various disulfide bridges. This finding is not surprising because there are several trypsins with only three, four or five disulfide bonds.

The data presented in this work indicate that in the folding milieu provided by the *E. coli* periplasm a buried unpaired cysteine is more harmful then one on the surface of the molecule. This is evident from our results on the elimination of disulfide bonds C22–C157 and

Table 1
Solvent accessible molecular surface of free cysteines and the expression level of the mutants

Mutant	Free Cys	Solvent accessible molecular surface (Å^3)	Expression[a]
C157A	C22	17.4	++
C22A	C157	8.6	—
C232A	C128	60.7	++
C128A	C232	27.8	++
C201A	C136	10.7	—
C136A	C201	15.1	++
C220A	C191	20.5	+
C191A	C220	21.8	+

[a] Expression level was determined by semiquantitative evaluation of zymograms.

Table 2
Partial nucleotide sequence alignment of trypsinogen genes

Gene or pseudogene	Sequence around C136 Exon 3	Sequence around C201 Exon 5
T1	*aatccaGTTttaaat*	*GcaatcTGCaatggg*
T2	*cctatgGATttgata*	*GccatcTGCaatggg*
T3	*actcagGGAtttggt*	*GtggtcTGCaacgga*
T4	**acgaagTGCctcatc**	**GtggtcTGCaatgga**
T5	*accaagTTCctcatc*	*GtggtcTGCaacgga*
T6	*accgagTGCcttatc*	*GtggtcTGCaatgga*
T7	*actgagTGCctcatc*	*GtggtcTGCaataga*
T8	**accgagTCCctcatc**	**GtggtcTCCaatgga**
T9	**actgagTGCctcatc**	**GtggtcTGCaacgga**

Sequence alignment was made by ClustalW [21]. Sequences are from GeneBank Accession Nos. NG 001333 and X72781.

C136–C201. In these cases only one of the single cysteine containing mutants is expressed, those which have larger accessible molecular surface areas (Table 1). The cysteine to alanine mutation evidently creates a cavity in the molecule, but it is clear that its effect would be opposite, i.e., a mutant containing the unpaired cysteine on the surface would be less stable. The observed detrimental effect of the buried unpaired cysteines might be explained by their potency to initiate the formation of incorrect disulfide bonds. These aberrant proteins are evidently degraded by the proteolytic enzymes of *E. coli*. When both cysteines in a disulfide bond have large accessible molecular surface areas both single cysteine containing trypsinogens can be expressed, and these forms result in enzymatically active trypsins as well. We found two such disulfide bonds in trypsinogen C127–C232 and C191–C220. The former one is missing from human trypsins, while both are missing from chymases [19].

Regarding the evolution of the human trypsins we found that the two eliminated disulfide bonds show different patterns. Both single mutants of C127–C232 proved to be able to form active enzyme. In all three expressed human trypsins C127 is replaced by a proline (CCT) and C232 is replaced by a tyrosine (TAC). For both positions however there is a pseudogene which still contains a cysteine codon (TGC), in pseudogene T2 at position corresponding to C127 and in pseudogene T3 at position corresponding to C232. These remnants indicate that the human trypsins have evolved from a six disulfide bond containing precursor. The mutations leading to the loss of cysteines spread through the expressed genes probably by gene conversion as it is suggested for missense mutations linked to hereditary pancreatitis [20].

Disulfide bond C136–C201 is missing only from human trypsin 2 (gene T8). In this case the TGC codon of both cysteines was changed to TCC resulting in a serine. Our data show that mutation of C136 leads to a proteolytically active form, while the other mutant can not be expressed. If we compare the expression pattern of mutants with the aligned nucleotide sequences of trypsinogen genes around the C136 and C201, we find an interesting correlation. In the pseudogenes the sequence around C136 is highly variable, while the sequence around C201 is surprisingly conserved (Table 2). Since we found that mutation of C136 results in a "viable" enzyme while the other mutation does not, it is reasonable to suppose that in the process of the elimination of disulfide bond C136–C201 the first step was the elimination of C136 which is buried in the interior of the molecule.

Acknowledgments

This work was supported by grants from the Hungarian Scientific Research Fund (OTKA T037568) and by the Hungarian Ministry of Education (NKFP 1/10). We thank Professor L. Gráf and Professor R. Naudé (University of Port Elizabeth) for critical reading of the manuscript and Ms. K. Béres for excellent technical assistance.

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