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Secondary structure of the promastigote surface protease of *Leishmania*

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By Raman spectroscopic analysis we have determined the secondary structure of the promastigote surface protease, named PSP or gp63, of *Leishmania major*. It consist of nearly 50% antiparallel β-strand, and less than 20% α-helix. These results are contrasted with the predominantly α-helical VSGs of the African trypanosomes and the α-helical metalloproteinase thermolysin. The PSP of *Leishmania* thus represents a novel kind of membrane-anchored protease.

Raman spectroscopy; Membrane-bound protein; Metalloproteinase

1. INTRODUCTION

Promastigotes of all species of *Leishmania* express at their surface an abundant glycophosphatidylinositol (GPI)-anchored 63 kDa glycoprotein, provisionally designated gp63, that can, depending on the species, represent more than one percent of the total cellular protein [1–3]. The function of gp63 has been implicated variously as the major surface protein of the promastigote [1], a major antigen recognized by the infected mammalian host [4,5], as the major complement component 3 (C3) acceptor [6] and directly as the ligand for the macrophage complement receptor 3 (CR3) [7]. Furthermore, gp63 was identified as a neutral-to-alkaline protease able to cleave a variety of polypeptide substrates [8,9] and therefore designated the promastigote surface protease, or PSP. Recently, it has been unambiguously shown to be a zinc metalloproteinase [10]. Promastigotes of 15 species of *Leishmania*, including strains of 13 species isolated from human patients, express the surface protease activity ([11] and R.E., unpublished), indicating that this highly conserved enzyme plays an essential, if as yet undetermined role in the life cycle of the parasitic protozoan. Analysis of the deduced amino acid sequence shows that PSP is synthesized with a 100-amino acid N-terminal preprosequence that is not found on the mature protein, and a short hydrophobic C-terminal domain that is exchanged for the GPI anchor of mature PSP [12]. Secondary structure predictions lead to the conclusion that none of the common secondary structural classes predominate in PSP [13].

In order to obtain experimental information on the structure of PSP, we determined the secondary structure of the purified PSP of *Leishmania major* by Raman spectroscopy. One of the perspectives of our study was to compare PSP with another structurally well-characterized GPI-anchored glycoprotein of protozoan origin, the variant surface glycoprotein (VSG) of *Trypanosoma brucei*, and with a metalloproteinase of bacterial origin, thermolysin. One might expect that the *Leishmania* surface protease would exhibit some structural similarities to *T. brucei* VSG based on the shared mode of membrane anchoring [14], spontaneous homodimer formation, and the taxonomic proximity of the two kinetoplastid protozoans. Crystallographic and Raman spectroscopic analyses have shown that VSGs form elongated dimers composed of 40 to 50% α-helix [15–17].

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Similarly, PSP and thermolysin seem to share a common, highly conserved catalytic site [10,18]. Thermolysin has an α-helix content of 40% and a β-strand content of 30% [19]. Thus, one might expect PSP, like the VSGs and thermolysin, to be predominantly α-helical. Our experimental results indicate the opposite: the PSP of *Leishmania* is predominantly composed of antiparallel β-strands.

2. MATERIALS AND METHODS

2.1. *Organism and culture conditions*

Promastigotes of *L. major* LEM 513 were grown at 26°C in HOSMEM II medium [20], in which fetal calf serum was replaced by 300 mg·l^{-1} bovine serum albumin and 4 mg·l^{-1} Tween 80.

2.2. *Purification of* L. major *LEM 513 PSP*

The detergent-soluble, amphiphilic protease was extracted from whole promastigotes and purified as described in [21] with several modifications. Washed cells were lysed in 10 mM Tris-HCl, 140 mM NaCl, pH 7.4 (TBS), containing 2% Triton X-114, and insoluble cytoskeletal and nuclear material was eliminated by centrifugation. Following a temperature-induced phase separation of the supernatant, a detergent-depleted (<0.6%, w/v) aqueous phase containing molecules soluble in the absence of nonionic detergent, and a detergent-enriched phase (~12%, w/v) containing amphiphilic and hydrophobic molecules, are obtained. The detergent phase was diluted 5-fold with 10 mM Tris-HCl at pH 7.5 containing 2.2 mM lauryldodecylamine *N*-oxide (LDAO, CMC = 2.2 mM) (buffer A) to reduce the viscosity of the detergent solution and then loaded on a 2.5 × 10 cm (40 ml) column of Fractogel TSK DEAE-650 (S) (Merck, Darmstadt, FRG) equilibrated in buffer A. The column was then washed with five bed volumes of the same buffer and the bound proteins were eluted at a flow rate of 5 ml·min^{-1} with a linear gradient of 0 to 500 mM NaCl in buffer A. Fractions containing PSP were identified by their proteolytic activity on casein at pH 8.5. Fractions were pooled, diluted 3 times in buffer B (the same as buffer A, but pH 8.0) and applied to a Mono Q quaternary amine anion-exchange column (5 × 50 mm, 1 ml; Pharmacia AB, Uppsala, Sweden) at 1 ml·min^{-1}, washed with 5 bed volumes of buffer B, and eluted with a linear gradient of 0 to 500 mM NaCl in buffer B. Active fractions were identified as described and assessed for purity by SDS-PAGE [22] on 1 mm-thick, 8–16% polyacrylamide linear gradient minigels (BioRad, München, FRG).

Protein concentration of purified PSP was determined by the method of Bradford [23] using ovalbumin as standard.

2.3. *Preparation of PSP for Raman spectroscopy*

Fifteen mg of purified amphiphilic PSP was dialysed extensively against 5 × 1 l of 50 mM ammonium hydrogen carbonate, pH 8.0 (no detergent), then lyophilized. The lyophilized, salt-free protein was redissolved in a minimum volume of Milli-Q (Millipore) ultrapure water (>18 MΩ). Samples of 10 μl, containing 1 mg of PSP, were transferred to glass capillaries for spectroscopic analysis. Aliquots of 100, 50, 10, and 5 μg of the concentrated PSP were evaluated by SDS-PAGE for eventual degradation. Several bands of lower molecular size were detected with the most-heavily overloaded samples; this material amounts to less than 5% of the intact 63 kDa protease.

2.4. *Raman spectroscopic measurements*

The Raman measurements and data analysis were performed as described previously [24]. The sample was irradiated with the 514.5 nm line of an Ag^+ laser, the light intensity at the sample being 50 mW. The slit width of the double monochromator was set at 5 to 6 cm^{-1}. For one measurement, typically 100 scans across the region of the amide I band were recorded in order to obtain a satisfactory signal-to-noise ratio. The scan speed was 2 steps per s with a step size of 0.2 Å corresponding to 0.6 to 0.7 cm^{-1}. The fluorescence background was about 10 times

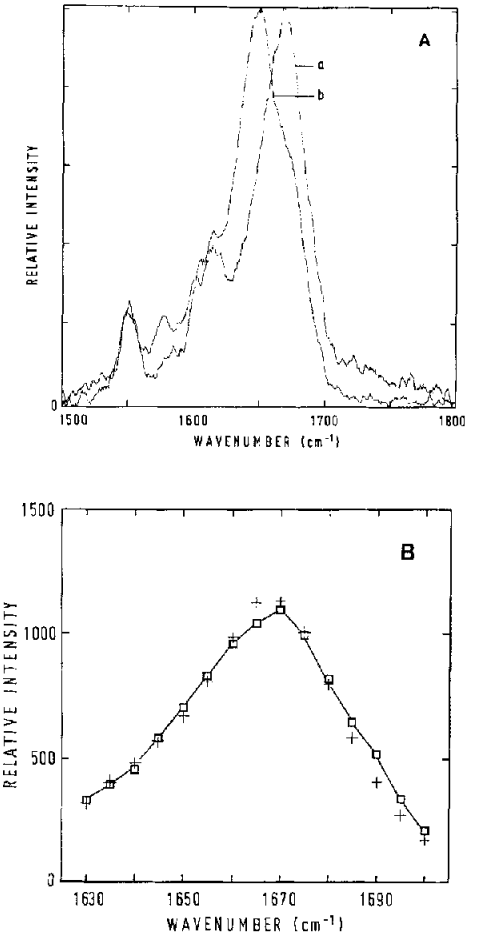

Fig.1. (A) The Raman amide I band of *Leishmania major* PSP is shown as a. For comparison, the amide I band of *Trypanosoma brucei* VSG MIT at 1.2 [16] is shown as b. (B) 15-point representation of the amide I band of PSP (□) together with the fit by a superposition of the amide I bands of 15 reference proteins (+).

higher than the amide I band, but relatively smooth, so that it could be subtracted as a flat baseline.

For the data analysis in terms of protein secondary structure, we used the second method of Williams [25] with 15 reference proteins and $k_{rank} = 3$ (in his notation). This implies that for one measurement, the percentages of the secondary structure classes are obtained with an error of ± 3%.

3. RESULTS

The Raman spectrum in the range of 1500–1800 cm^{-1} of *L. major* PSP is shown in fig.1A, together with the previously obtained spectrum of a VSG of *T. brucei*. It is evident that the amide I bands around 1650 cm^{-1} of the two proteins differ, while their tryptophan bands around 1550 cm^{-1} coincide. The maxima of the amide I bands lie at 1668 cm^{-1} for PSP and at 1650 cm^{-1} for VSG. These two numbers indicate that the secondary structure of PSP is predominantly β-stranded, whereas VSG is predominantly α-helical. For a detailed analysis, the amide I band of PSP was fitted by a superposition of the spectra of 15 reference proteins as shown in fig.1B. From this fit, the percentages of secondary structure classes were obtained as listed in table 1. The total β-strand content of PSP is 52%, of which nearly all is antiparallel.

In table 1, the results of independent Raman spectroscopic analyses of two different preparations of *L. major* PSP are listed. They differ by only 1%, which is less than the error of ± 3% arising from the data analysis. Hence, the overall error in the result for the percentage of secondary structure classes is about ± 4%.

For comparison, the percentages of the secondary structure classes of a VSG of *T. brucei* and of *Bacillus thermoproteolyticus* thermolysin are included in table 1.

Table 1

Secondary structure of the *Leishmania* promastigote surface protease (PSP) determined from the Raman amide I bands of two different samples

	$^{+}H_o$ (%)	H_d (%)	S_a (%)	S_p (%)	T (%)	U (%)	H_{tot} (%)	S_{tot} (%)
PSP sample 1	7	9	47	4	21	12	16	51
PSP sample 2	6	9	48	4	21	12	15	52
VSG MITat 1.2	33	16	23	3	15	10	49	26
Thermolysin	26	14	25	5	19	12	40	30

For comparison, the secondary structures of *Trypanosoma brucei* variant surface glycoprotein (VSG) MITat 1.2 [16] and of *Bacillus thermoproteolyticus* thermolysin [19] are included. $^{+}H_o$, ordered α-helix; H_d, disordered α-helix; S_a, antiparallel β-strand; S_p, parallel β-strand; T, turn; U, undefined; H_{tot}, total α-helix; S_{tot}, total β-strand

4. DISCUSSION

Raman spectroscopic analysis of the promastigote surface metalloproteinase of *L. major* shows that the structure of this protein is more than 50% β-stranded. Previous structural predictions, based on the analysis of the deduced amino acid sequence of *L. major* PSP, failed to identify the predominant β-structure. In these studies, the entire, unprocessed translation product was analysed, not the sequence of the mature glycoprotein lacking the extensive N-terminal signal and prosequences and the hydrophobic C-terminal peptide, which were predicted to be extensively α-helical [12,13]. When only the amino acid sequence of the mature protein is considered as the basis for structural prediction, a slight, but not striking predominance of β-structure over α-helix is found.

PSP is clearly distinct from the variant surface glycoproteins of African trypanosomes, which are predominantly α-helical. Although both PSP and VSG are major GPI-anchored surface glycoproteins of taxonomically related protozoan parasites, and both show a tendency to form noncovalent dimers in detergent solution, the two are clearly structurally unrelated. PSP differs also from the well-characterized metalloproteinase thermolysin, which is approximately 40% α-helical. It should be noted, however, that serine proteases like trypsin and chymotrypsin, the cysteine protease papain, and the zinc metalloenzyme carbonic anhydrase resemble PSP in being predominantly β-stranded [26,27].

These differences between PSP and the VSGs and thermolysin should not be surprising, given the lack of similarities among these proteins at the amino acid sequence level. The high β-sheet content of PSP, compared to the enriched α-helical content of the VSGs [16] and thermolysin [19], indicates that, in spite of catalytic, topologic, or taxonomic similarities, the promastigote surface proteinase PSP represents a novel category of protein, thus far unique to the genus *Leishmania*. The

peculiar secondary structure of PSP may confer upon it the marked stability to heat and detergent-mediated denaturation observed in vitro [9] and its resistance to the activities of a variety of proteases, including its resistance to self-degradation at both the surface of living promastigotes and in detergent solution (R.E., unpublished). The striking degree of conservation of this novel enzyme within the genus ([11], R.E., unpublished), as well as the presence of highly homologous genes in the different species of *Leishmania* examined by Button and McMaster [12], indicate that PSP plays an essential role in the life cycle of the parasite.

Further structural studies on *Leishmania* PSP are clearly justified. Crystallization of the enzyme and subsequent X-ray diffraction analysis may help to identify the natural substrates of PSP, and thus clarify the role of this abundant protease in the life cycle of *Leishmania*. Further, the determination of a high resolution three-dimensional structure of PSP could permit the rational design of specific inhibitors of therapeutic value in the treatment of the human leishmaniases.

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