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Peroxidase catalysed cross-linking of an intrinsically unstructured protein via dityrosine bonds in the oocyst wall of the apicomplexan parasite, *Eimeria maxima*

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ABSTRACT

Apicomplexan parasites such as *Eimeria maxima* possess a resilient oocyst wall that protects them upon excretion in host faeces and in the outside world, allowing them to survive between hosts. The wall is formed from the contents of specialised organelles – wall-forming bodies – found in macrogametes of the parasites. The presence of dityrosine in the oocyst wall suggests that peroxidase-catalysed dityrosine cross-linking of tyrosine-rich proteins from wall-forming bodies forms a matrix that is a crucial component of oocyst walls. Bioinformatic analyses showed that one of these tyrosine-rich proteins, *Em*GAM56, is an intrinsically unstructured protein, dominated by random coil (52–70%), with some α-helix (28–43%) but a relatively low percentage of β-sheet (1–11%); this was confirmed by nuclear magnetic resonance and circular dichroism. Furthermore, the structural integrity of *Em*GAM56 under extreme temperatures and pH indicated its disordered nature. The intrinsic lack of structure in *Em*GAM56 could facilitate its incorporation into the oocyst wall in two ways: first, intrinsically unstructured proteins are highly susceptible to proteolysis, explaining the several differently-sized oocyst wall proteins derived from *Em*GAM56; and, second, its flexibility could facilitate cross-linking between these tyrosine-rich derivatives. An in vitro cross-linking assay was developed using a recombinant 42 kDa truncation of *Em*GAM56. Peroxides, in combination with plant or fungal peroxidases, catalysed the rapid formation of dityrosine cross-linked polymers of the truncated *Em*GAM56, as determined by western blotting and HPLC, confirming this protein's propensity to form dityrosine bonds.

1. Introduction

The oocyst wall of the apicomplexan parasite, *Eimeria maxima*, is rich in dityrosine (Belli et al., 2003), a molecule involved in the cross-linking of proteins (Belli et al., 2006). The oocyst wall forms in a regulated fashion from the contents of specialised organelles – wall-forming bodies – within the macrogamete stage of the parasite (Ferguson et al., 2003). Two proteins from the wall-forming bodies of *E. maxima*, *Em*GAM56 and *Em*GAM82, have been relatively well characterised and are integral to development of the oocyst wall (Ferguson et al., 2003). Both of these proteins are rich in tyrosine and are processed or degraded into smaller proteins of various sizes (Belli et al., 2006). These smaller proteins are believed to be cross-linked via dityrosine-bonds to help form the oocyst wall and generate its resistance to chemical and environmental insult (Belli et al., 2006).

We explored the structure of *Em*GAM56 in an effort to explain its propensity to be processed into smaller proteins and form dityrosine cross-links, paying particular attention to the possibility that it is an intrinsically unstructured protein (IUP). IUPs have important functions and, indeed, their inherent disorder and flexibility is crucial in some instances. Thus, it has been shown that IUPs are involved in important biological functions such as DNA recognition, molecular assembly, protein modification and modulation of affinity of protein binding (Iakoucheva et al., 2002; Ward et al., 2004; Dunker et al., 2002). Bioinformatic analyses have shown that apicomplexan parasites are rich in disordered proteins (Feng et al., 2006). Moreover, whilst it is known that *Em*GAM56 is processed into smaller polypeptides that are incorporated into the

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oocyst wall (Belli et al., 2003, 2006), these polypeptides are not cleaved from the parent protein at well-established protease cleavage sites. Many IUPs exhibit similar proteolytic sensitivity, a feature that allows for extremely rapid turnover (Tompa, 2005). This is an important characteristic for oocyst wall formation, which is known to occur rapidly using molecules that are stored in the wall-forming bodies of the macrogametocyte (Belli et al., 2003). Therefore, the structure of *Em*GAM56 was analysed by: (i) using a variety of bioinformatic tools; (ii) assessing resistance of the protein to extreme temperatures and pH changes; and (iii) nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopy.

Another feature of IUPs is that the accessibility of tyrosine residues for radical-mediated oxidative cross-linking is increased. Thus, for example, it has been shown that the highly efficient nitration and oxidation of α-synuclein, resulting in the formation of α-synuclein polymers through dityrosine cross-linking, is attributable to the unstructured conformation of the protein, which causes all its tyrosine residues to be exposed in the solvent phase, thereby enhancing the probability for cross-linking reactions (Souza et al., 2000). Cross-linking of tyrosine is often catalysed by peroxidase(s) (Belli et al., 2006). Peroxidase activity has been detected specifically in the wall-forming bodies and the developing oocyst wall of *E. maxima* in situ in the small intestine (Belli et al., 2003, 2006); thus, it is already known that peroxidase activity is located in the appropriate places to catalyse dityrosine cross-linking in this parasite. However, the idea that derivatives of tyrosine-rich proteins such as *Em*GAM56 can actually form suitable dityrosine cross-links has not been tested. Thus, we also aimed to show that a truncated recombinant form of *Em*GAM56 can be cross-linked by peroxides in the presence of peroxidase(s).

2. Materials and methods

2.1. Bioinformatic analysis of protein sequence of EmGAM56

Structural analysis of *Em*GAM56 was performed using the bioinformatics programs PSIPRED (McGuffin et al., 2000), APSSP2 (Raghava, 2002), SSpro (Cheng et al., 2005), GOR4 (Garnier et al., 1996) and SOPMA (Geourjon and Deléage, 1995) for secondary structure predictions and FoldIndex© (Prilusky et al., 2005), IUPred (Dosztanyi et al., 2005), RONN (Yang et al., 2005) and DISPROT (Sickmeier et al., 2007) for disorder predictions. The protein sequence of *Em*GAM56 was imported into a window of each web server and then submitted for secondary structure predictions or disorder/intrinsically unfolded predictions. The secondary structure predictions for *Em*GAM56 were assigned to each amino residue, whereas for disorder predictions the results were presented in a graph indicating disorder probability or tendency for each amino residue.

2.2. Construction of a truncated recombinant version of EmGAM56

The recombinant construct containing DNA fragments encoding amino acids 102–225 (corresponding to base pair numbers 469–840 of *emgam56*) was generated according to methods described previously (Sambrook and Russell, 2001). DNA was amplified by PCR using specific oligonucleotide primers (CACCATGTCCAACAGAATGAACGCTGCCATG as the forward primer and GCTCCTGCCCTTTCTGCCCATATTT as the reverse primer), and *remgam56* (Belli et al., 2004, 2009) as the DNA template. The reaction was carried out as follows: initial denaturation at 95 °C for 5 min; denaturation at 95 °C for 30 s; primer annealing at 55 °C for 1 min (annealing temperature varied slightly from each reaction for optimal amplification); extension at 72 °C for 90 s; repetition of the whole cycle 35 times; and a final extension at 72 °C for 10 min. The DNA fragment was then cloned into a pET101/D-TOPO® vector according to the manufacturer's instructions (Champion™ pET101 directional TOPO® expression kit, Invitrogen™, Australia), followed by transformation into *Escherichia coli* TOP10 competent cells (Invitrogen™, Australia). DNA sequencing was carried out to verify that the recombinant constructs contained the correct sequence of DNA insert. The recombinant construct, designated *emgam56.469–840*, was sent to the Australian Genome Research Facility, Qld, Australia, for automatic double-stranded sequencing of plasmids using dideoxy dye-terminator chemistry and ABI automated sequencers. DNA was sequenced in both forward and reverse directions. The consensus sequences were obtained by aligning sequenced DNA fragments to *emgam56* using AssemblyLIGN™ (Oxford Molecular Ltd., UK) software to confirm identity.

2.3. Generation of recombinant proteins for structural analyses

The recombinant protein arising from the construct *emgam56.469–840* was over-expressed in *E. coli* strain BL21 Star™ cells (supplied by Champion™ pET101 directional TOPO® expression kit, Invitrogen™, Australia), followed by protein purification as described previously (Belli et al., 2004) with a minor modification. Briefly, *E. coli* strain BL21 Star™ cells containing *emgam56.469–840* were induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside for 3 h at 30 °C. Bacterial pellets, harvested by centrifugation at 12,000*g* for 5 min, were subsequently used for protein purification. The recombinant protein, designated *Em*GAM56.469–840, was purified using a Hitrap chelating column (GE Healthcare, Australia) and the protein concentration was measured using a Bio-Rad protein assay based on the Bradford dye-binding method. The purified protein was desalted and buffer-exchanged in 50 mM sodium phosphate buffer pH 6.8 using a PD-10 desalting column (GE Healthcare, Australia), resulting in a single band on silver-stained SDS–PAGE. The protein was stored at −20 °C until needed.

2.4. Effect of boiling and pH change on the structure of rEmGAM56.469–840

To examine the effect of extreme temperature on the structural integrity of recombinant *Em*GAM56 (r*Em*GAM56) and r*Em*GAM56.469–840, the proteins were boiled for 10, 20, 30 and 40 min and then analysed by SDS–PAGE and immunoblotting as described previously (Belli et al., 2002a) using mouse anti-recombinant 56 kDa antibodies (mouse anti-r*Em*GAM56 antibodies) (Belli et al., 2003). Additionally, the effect of pH was tested by addition of hydrochloric acid (HCl), citric acid or sodium hydroxide: (i) HCl was added to produce incubation media of pH 5, pH 3.99, pH 3.01, pH 2 and pH 1.03; (ii) citric acid was added to produce an incubation medium of pH 4; and (iii) sodium hydroxide was added to produce an incubation medium of pH 11. The samples were analysed by SDS–PAGE, followed by immunoblotting as described previously (Belli et al., 2002a) using mouse anti-recombinant 56 kDa antibodies (mouse anti-r*Em*GAM56 antibodies) (Belli et al., 2003).

2.5. Structural analysis of rEmGAM56.469–840 by NMR and CD spectroscopy

The recombinant protein, r*Em*GAM56.469–840 (in 50 mM sodium phosphate buffer pH 6.8; Sigma), was concentrated using a centrifugation concentrator (Vivaspin500 with a 3,000 Da molecular mass cut-off), followed by centrifugation at 15,000*g* for 10 min; the final protein concentration was ~1.5 mg/ml. Before the NMR spectrum was recorded, 5% $^{2}H_2O$ was added and then the sample