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Conformational analysis of the GTP-binding protein MxA using limited proteolysis

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Abstract Guanosine triphosphate (GTP)-binding proteins are known to function as molecular switches that cycle between GTP-bound and guanosine diphosphate (GDP)-bound states. Switching is achieved by the fact that G-proteins in the GTP-bound conformation can interact with a certain set of effector molecules while they interact with a different set of partners in their GDP-bound conformation. The antiviral properties of the interferon-induced MxA protein are critically dependent on the ability of MxA to bind GTP. Using limited proteolysis we analyzed the conformations of the MxA protein under nucleotide-free, GDP-bound, and GTP-bound conditions. We find that whereas the conformations of nucleotide-free MxA and GDP-bound MxA are essentially similar, GTP-binding causes a dramatic change in the conformation of MxA.

Key words: G-protein; MxA; Proteolysis; Nucleotide exchange

1. Introduction

Guanine nucleotide-binding proteins (G-proteins) assume different conformations depending upon the identity of the nucleotide occupying the nucleotide-binding pocket on the protein. This also leads to change in the functional activity of the protein. These proteins show significant conformational changes upon hydrolysis of the bound guanosine triphosphate (GTP) to guanosine diphosphate (GDP) [1]. This aspect of the architecture of G-proteins provides the basis for these proteins acting as molecular switches. These molecular switches represent an 'ON' state in their GTP-bound conformation and an 'OFF' state in the GDP-bound conformation.

The conformational differences resulting from the binding of different nucleotides can be studied by limited proteolysis of the G-protein. The interferon α/β-induced MxA protein is a member of the dynamin family of G-proteins [2]. Other members of this family include the endocytic protein dynamin [3] and the interferon γ-induced guanylate-binding protein [4]. All these proteins are modular proteins. They contain conserved sequence elements separated by loops of varied sequence. The conserved sequences constitute well-defined structural domains that are also present in various other signaling proteins. These domains have been shown by X-ray crystallography and other techniques [5,6] to form compact structures in three dimensions. The assortment of domains is held together by loops that show considerable variations between members of the family. For both the classical heterotrimeric transducin-like G-proteins and for the small monomeric Ras-like G-proteins it has been shown that conformational changes between the GTP- and GDP-bound forms of the G-protein are limited to a few discrete changes. These regions are typically known as the switch regions [7,8] as their conformational plasticity imparts the G-proteins the ability to function as molecular switches.

We have subjected the 76 kDa MxA protein to limited proteolysis using the proteases trypsin, papain and proteinase K in order to probe its conformations in the nucleotide-free, GDP-bound and GTP-bound states. The fragments generated by limited proteolysis have been visualized as characteristic patterns on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

2. Materials and methods

2.1. Materials

Trypsin, soybean trypsin inhibitor (STI) and phenylmethyl sulfonyl fluoride (PMSF) were from Life Technologies, USA. Papain, GDP and imidoguanosine triphosphate (GppNHp) were from Roche Biochemicals, Germany. Proteinase K was from Bangalore Genei, India. All other chemicals were of analytical grade and were obtained from either Sigma or Amersham.

2.2. Expression of MxA protein

The construct expressing hexahistidine-tagged MxA, pQE9-MxA, was generously provided by Prof Otto Haller (Freiburg, Germany). The purification of MxA was performed exactly as described by Pitossi et al. [9].

2.3. Limited proteolysis of MxA

Trypsinization was performed as follows. 180 μg of MxA was incubated with 2 mM guanine nucleotide (GDP or GppNHp) on ice for 10–15 min. After binding of nucleotide a 'zero-minute' time point (18 μg of MxA) was removed. The nucleotide-bound protein was subjected to proteolysis by trypsin (300 ngram) on ice and aliquots of the reaction mixture were taken out after 5, 10, 20, 30, 60 and 90 min of incubation. These aliquots (representing 18 μg of starting material) were added directly into 'termination buffer' (1× SDS–PAGE sample buffer containing 30 mM EDTA and 5 μg of STI). The samples were then stored in liquid nitrogen until just before visualization by 13.5% acrylamide SDS–PAGE. Digestion by papain was done for 1 h on ice with a 1:4000 protease to protein ratio. Bands were visualized on 10% acrylamide SDS–PAGE by Coomassie blue staining. For proteolysis

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Abbreviations: GDP, guanosine diphosphate; GTP, guanosine triphosphate; GppNHp, imidoguanosine triphosphate (a non-hydrolyzable analogue of GTP); PMSF, phenylmethyl sulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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by proteinase K, nucleotide-free MxA and MxA incubated with 2 mM guanine nucleotide (either GDP or GppNHp) on ice for 30 min was used for the analysis. Proteinase K at a 1:1400 dilution (by weight) was then added to the above mixture. Reactions representing different points in the time-course of such a reaction were quenched by addition into 1.6× SDS–PAGE sample buffer supplemented with the serine protease-specific inhibitor, 4 mM PMSF, followed by rapid freezing in liquid nitrogen. Proteinase K is not inactivated by metal ion chelating agents such as EDTA. The samples were then analyzed on 10% acrylamide SDS–PAGE.

3. Results and discussion

We subjected the nucleotide-free and nucleotide-bound forms of MxA to limited proteolysis on ice using a very low substrate to protease ratio. The rationale of the experiment was that the folded conformation of MxA-GDP would be such that some regions of MxA would be accessible to the protease while others would be hidden. When this same protein would be bound to GTP (or to GppNHp, the non-hydrolyzable analogue of GTP) the altered conformation would lead to the exposure of some sites which were hidden in the GDP-bound structure and the concealment of others. This difference in accessible cleavage sites in the different nucleotide-bound forms of the protein would be visualized as distinct proteolytic fragments seen on SDS–PAGE.

3.1. Limited proteolysis by trypsin

We initially established conditions for limited tryptic digestion of the dynamin family protein, MxA. Trypsin is a pancreatic serine protease that is active only against those peptide bonds in protein molecules that have carboxyl groups donated by arginine and lysine. Trypsin is thus widely employed as a reagent for the orderly and unambiguous cleavage of protein molecules. Complete digestion of a (denatured) protein with trypsin is thus expected to result in the formation of relatively small peptides. The digestion of protein in its native conformation, however, would result in formation of larger stable peptides due to the inability of the protease to access some of the hidden/buried sites.

We thus systematically varied the amount of protease used to digest a fixed amount of substrate. The MxA substrate was verified in independent experiments to be active in terms of being able to hydrolyze (and hence bind) GTP. We assayed different protease to protein ratios such as 1:50, 1:100, 1:200, 1:300, 1:400, 1:500 and 1:600. Reactions were simultaneously carried out either at 37°C, at room temperature (~25°C) or on ice. Analysis of the products of the proteolysis reactions by 13.5% acrylamide SDS–PAGE indicated that digestion on ice using a protease to protein ratio of 1:600 gave the best resolution of proteolytic products. This was the relative concentration used in subsequent experiments aimed at establishing the digestion patterns for the different nucleotide-bound forms of MxA.

Distinct proteolytic patterns were observed for the GDP- and GppNHp-bound forms of MxA (Fig. 1A,B). In both the GDP- and GppNHp-bound forms the 76 kDa MxA protein showed the appearance of an ~66 kDa band within 5 min of proteolysis; also seen was a band at ~60 kDa. The 66 kDa band kept accumulating at the expense of the parent 76 kDa band in both the MxA-GDP and MxA-GppNHp digests. The

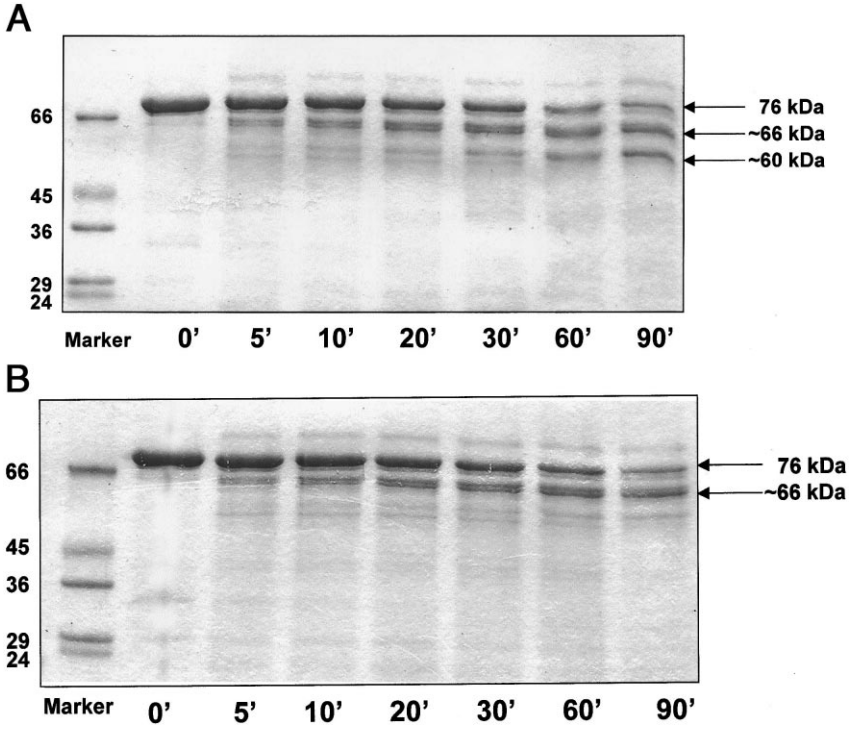

Fig. 1. Limited proteolysis of the GDP-bound and the GppNHp-bound forms of MxA using trypsin. Proteolysis was carried out on ice for the times indicated in the figures under each lane. The products of proteolysis were resolved by 13.5% acrylamide SDS–PAGE and visualized by staining with Coomassie blue. Uncut MxA (18 μg) before the addition of trypsin is shown in each panel in the lane marked 0′. Panel A shows the proteolytic profile of MxA-GDP. Proteolytic fragments are observed at 66 kDa and 60 kDa. The lower band remained stable even after 90 min of incubation with the protease. Panel B shows the profile obtained for MxA-GppNHp. A single stable proteolytic fragment at 66 kDa was observed.

intensity of the 60 kDa band did not increase appreciably during the first 20 min of digestion. After the 20 min time point there was a dramatic divergence between the two patterns. The MxA-GDP digest showed (Fig. 1A) an accumulation of both the 66 kDa and the 60 kDa band, resulting in the formation of a signature triplet at steady state (90 min) comprising bands at 76, 66 and 60 kDa. On the other hand, the MxA-GppNHp digest showed (Fig. 1B) the accumulation of only the 66 kDa band with a concomitant decrease in the parent 76 kDa band. There was no significant intensity change for the 60 kDa band even after 90 min of digestion.

In summary, limited trypsinization of MxA-GDP and MxA-GppNHp resulted in the accumulation of different products. Whereas a 66 kDa proteolytic band was a common product of the two conformations of MxA, the formation of a shorter 60 kDa band was seen only in the case of MxA-GDP. Interestingly, we also observe that the proteolytic pattern of nucleotide-free MxA is identical to that of MxA-GDP. This clearly indicates that there is a significant conformational change in MxA upon the exchange of bound nucleotide from GDP to GTP (represented here by GppNHp).

3.2. Limited proteolysis by papain

Various dilutions of papain were employed in order to establish optimal conditions for proteolysis. Digestion on ice with a 1:4000 protease to protein ratio was the optimal condition for the proteolysis of nucleotide-free MxA in that stable bands were observed as end products of the 1 h digest. However, when the band patterns for papain proteolysis of the GDP- and GppNHp-bound forms of MxA were analyzed, it was observed (Fig. 2) that there was no obvious difference between them. In either case a stable doublet around 40 kDa was seen. It is thus concluded that proteolysis by papain is not discriminatory enough to distinguish between the conformations of MxA-GDP and MxA-GppNHp.

3.3. Limited proteolysis by proteinase K

Proteinase K is a serine protease that cleaves peptide bonds at the carboxylic sides of aliphatic, aromatic or hydrophobic amino acids. Using the same approach as described above for the case of trypsin it was determined that a protease to protein ratio of 1:1400, with the proteolysis being carried out on ice, constituted the optimal conditions for the limited proteolysis of MxA by proteinase K.

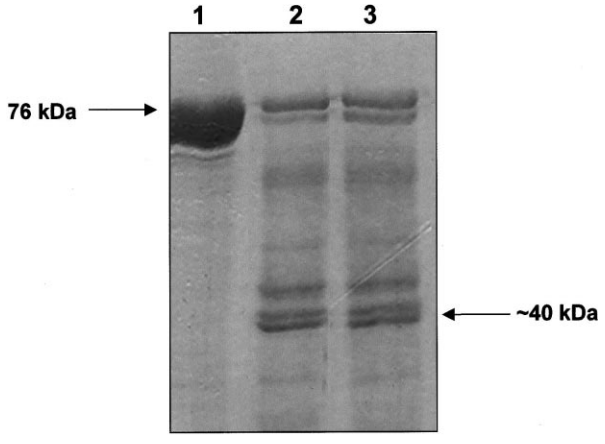

Fig. 2. Limited proteolysis of MxA by papain. Coomassie blue-stained 10% acrylamide SDS–PAGE showing the proteolytic profile obtained upon incubating MxA with papain for 60 min on ice. Uncut MxA before addition of papain (lane 1), GDP-bound MxA (lane 2) and GppNHp-bound MxA (lane 3). The proteolytic patterns for GDP-bound and GppNHp-bound MxA are seen to be identical.

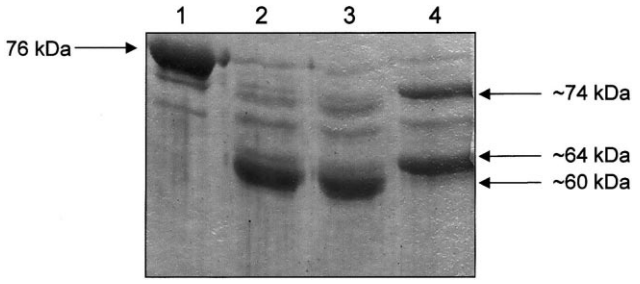

Fig. 3. Limited proteolysis of MxA by proteinase K. Coomassie blue-stained 10% acrylamide SDS–PAGE showing the products of MxA proteolysis upon incubation with proteinase K for 90 min on ice. Uncut MxA before addition of proteinase K (lane 1), proteolysis of nucleotide-free MxA (lane 2), GDP-bound MxA (lane 3) and GppNHp-bound MxA (lane 4). The identical proteolytic pattern obtained in the case of nucleotide-free and GDP-bound protein is completely different from that obtained for GppNHp-bound MxA protein.

Fig. 3 shows only the steady state patterns displayed by the nucleotide-free, GDP-bound and the GppNHp-bound forms of MxA after 90 min of proteolysis on ice by proteinase K.

Consistent with the observations made with trypsin, it is seen (Fig. 3) that the proteolytic pattern displayed by the proteinase K proteolysis of nucleotide-free or GDP-bound forms of MxA is the same. In either case the parent 76 kDa band is reduced to a stable fragment of $\sim$60 kDa. This is consistent with an earlier report [10] wherein proteinase K digestion of nucleotide-free MxA was reported to result in the formation of a stable 60 kDa fragment. However, upon binding the non-hydrolyzable GTP analogue GppNHp, the conformation of MxA undergoes a significant change as evidenced by the fact that proteinase K digestion now results in two stable fragments of $\sim$74 kDa and $\sim$64 kDa.

In summary, using limited proteolysis by a variety of proteases as a readout of MxA conformation we suggest that the conformation of MxA does not change significantly between the nucleotide-free and the GDP-bound form of MxA. There is, however, a significant conformational change in MxA upon binding the GTP analogue GppNHp. Thus, the proteolysis assays using trypsin and proteinase K are effective tools for probing the structural changes in MxA in its 'ON' (GTP-bound) and 'OFF' (GDP-bound) states. Such assays will be useful in studying the nucleotide-binding capabilities of mutant MxA proteins as well as in assessing the degree of conformational changes in mutant MxA molecules as a consequence of nucleotide association.

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