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Evidence for concurrent donor and acceptor side photoinduced degradation of the D1-protein in isolated reaction centres of Photosystem II

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By varying the period of illumination or by changing the concentration of the quinone electron acceptor, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), it has been possible to demonstrate, using immunoblottimg, that the photoinduced degradation of the D1-protein follows two distinct pathways in isolated Photosystem II (PS II) reaction centres. During the initial illumination period, with an intermediate level of DBMIB present (0.2 mM), a 23 kDa fragment containing the N-terminus of the D1-protein appears. Further illumination facilitates the accumulation of C-terminal fragments of apparent molecular masses 24 kDa and 16 kDa. The 24 kDa C-terminal fragment is not observed in the absence of DBMIB or when this quinone is present at high levels (1 mM). In the latter condition, the photoinduced degradation of the D1-protein is significantly protected and only the 23 kDa N-terminal fragment is produced. The immuno-detected band at 16 kDa is weaker than those of the higher molecular mass breakdown fragments and its appearance seems to follow conditions similar, but not identical, to those needed to generate the 24 kDa C-terminal fragment. A closer examination of the 16 kDa band revealed that it consists of both C-terminal and N-terminal portions of the D1-protein, suggesting it arises from a cleavage about midway in the protein. We conclude that both donor and acceptor side photoinhibition can occur in the isolated PS II reaction centre, the balance between them being controlled by the extent of the illumination and the level of DBMIB present. The 23 kDa N-terminal fragment is generated during acceptor side photoinhibition, while the 24 kDa C-terminal fragment is induced by damage on the donor side.

Introduction

The D1-protein of the Photosystem II (PS II) reaction centre complex turns over very rapidly in the light [1]. This characteristic of the protein has been linked with photoinhibition, where the protein is degraded and re-synthesised as part of a cycle resulting from photochemically induced damage within the PS II reaction centre [2,3]. During the light-dependent turn over of the D1-protein in vivo, an N-terminal 23.5 kDa fragment of the D1-protein has been detected [4], which seems to be produced by cleavage near to the highly conserved QEEET-motif [5] within the hydrophilic loop connecting the fourth and fifth putative transmembrane segments. This region of the protein is involved in the binding of the second stable electron acceptor, Q_B. Over-reduction of quinone acceptors [6–10], the PQ/PQH_2 ratio and occupancy of the Q_B site by PQ [11], are considered to be three important factors in controlling the degradation rate of the D1-protein. Other lines of evidence suggest that photoinhibitory light may affect the electron donor side of PS II [12–15]. A very rapid degradation of the D1-protein, together with loss of photochemical activity, occurs when thylakoids or other PS II preparations are exposed to light after inactivation of the water splitting reactions [16–18]. When the isolated PS II reaction centre is illuminated in the presence of a suitable electron acceptor (a condition under which the highly oxidising radicals of the donor side are expected to be accumulated), D1-protein breakdown fragments, having apparent molecular masses of 24, 16 and 10 kDa,

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Abbreviations: Chl, chlorophyll; Cyt, cytochrome; anti-D1n and anti-D1c, polyclonal antibodies with specificity towards the N-terminus and C-terminus of the D1-protein; DBMIB, dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PQ, plastoquinone-9; P680, primary donor of Photosystem II; Q_A, first stable electron acceptor of Photosystem II; Q_B, second stable electron acceptor of Photosystem II.

have been detected by immunoblotting with monospecific polyclonal antibodies [19]. Subsequent studies involving phosphorylation, proteolytic mapping and immunoblotting with C-terminal and N-terminal specific polyclonal antibodies showed that the 24 and 16 kDa fragments contained the C-terminus of the D1-protein [20]. In the same study, a 22 kDa fragment was also observed and attributed to the C-terminus, as well. These findings have led to the proposal that two different mechanisms of photoinhibition, i.e., acceptor and donor side, may actually trigger different pathways for degradation of the D1-protein [21]. Further experimental support for this view has recently been obtained by De Las Rivas et al. [22] who showed that by illuminating a PS II core preparation under conditions expected to result either in donor or acceptor side photoinhibition, different patterns for D1 degradation were found. A similar result has also been obtained using isolated reaction centres [23,24]. In this work, a 23 kDa fragment containing the N-terminus of the D1-protein was observed due to acceptor side photoinhibition, which is the condition when radical pair recombination occurs and the P680 triplet state is formed [10]. This D1-degradation product was only observed under aerobic conditions, lending support to the idea that singlet oxygen formed via the P680 triplet state plays a key role in photoinhibitory damage [10]. When an electron acceptor was present, such as 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), and donor side photoinhibition occurred, the 23 kDa N-terminal fragment was replaced by a 24 kDa C-terminal fragment.

In this paper we show that, by performing a time-course of illumination in the presence of the electron acceptor DBMIB, both the 23 kDa N-terminal and 24 kDa C-terminal fragments are generated and that there is a distinct difference in the kinetics of their appearance.

Material and Methods

Isolation of reaction centres of PS II both from spinach and wheat PS II membranes was carried out as described in Ref. 25. Illumination of isolated PS II reaction centres was performed at 50 μg Chl/ml in 50 mM Tris-HCl (pH 8.0), 2 mM dodecyl β-D-maltoside and 0.2 mM DBMIB at 20°C (if not otherwise stated). The illumination intensity was 4 500 $\mu E\,m^{-2}\,s^{-1}$ white light from an incandescent lamp passed through a heat absorbing filter. The procedures for performing SDS-PAGE and immunoblotting were according to Ref. 26. Properties of polyclonal antisera against D1 have been described elsewhere [20,26]. Trypsin cleavages were performed as previously described [27] on two-dimensional gels. Lys-C is an endopeptidase which specifically cleaves the peptide bond formed by the carboxylic acid group of lysine and was obtained from Sigma.

Results

Fig. 1 shows the result of an immunoblot with anti-D1 antiserum of isolated PS II reaction centres illuminated in the presence of 0.2 mM DBMIB for different periods of time, ranging from 5 to 120 min. Three fragments at 24, 23 and 16 kDa were produced, whose appearance is characterised by different kinetics. The 23 kDa fragment is the first to appear, being detected after 5 min of illumination (lane 2). Its amount increases during the following 20–30 min (lanes 3–5), and then disappears from the blot at longer times (lanes 6–8). The 16 kDa breakdown product is detected after 5–10 min of illumination (lanes 2 and 3) but, unlike the 23 kDa band, it is photoaccumulated over the course of the illumination period (lanes 4–8). The 24 kDa fragment starts to appear after 20–30 min of illumination and, like the 16 kDa fragment, is photoaccumulated with increasing exposure time (lanes 5–8). These fragments have previously been attributed to the C-terminal portion of the D1-protein [20] and a possible precursor-product relationship between the 23 kDa and the 16 kDa fragments has been hypothesised for similar studies using isolated PS II cores [28]. However, due to the fact that the 23 kDa fragment appears before the 16 kDa fragment, any strict relationship between them is ruled out.

In order to characterise further the origin of the various fragments we have employed limited proteolysis using the endopeptidases, trypsin and Lys-C (see Materials and Methods). With trypsin, the method used is based on proteolysis with two-dimensional gels

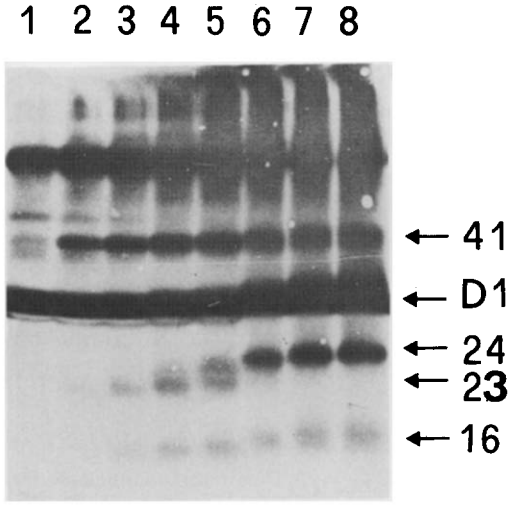

Fig. 1. Immunoblot with anti-D1 polyclonal antiserum showing a time-course for the appearance of D1 breakdown products. Reaction centres of PS II were illuminated in the presence of 0.2 mM DBMIB with 4 500 $\mu E\,m^{-2}\,s^{-1}$ white light at a chlorophyll concentration of 100 μg/ml. Lanes 1 to 8 are samples illuminated respectively for 0, 5, 10, 20, 30, 60, 90 and 120 min. Each gel lane was loaded with 2 μg Chl.

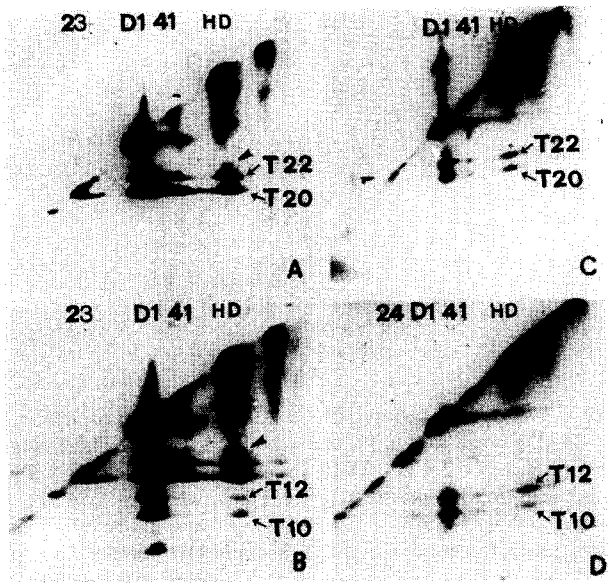

Fig. 2. Immunoblot with anti-D1n (A and C) and anti-D1c (B and D) antisera of PS II reaction centres illuminated for 10 (A and B) and 90 (C and D) min using the same conditions as described in the legend of Fig. 1. Polypeptides were resolved by SDS-PAGE and a gel lane was loaded onto a second identical gel whose stacking contained trypsin at a concentration of 100 μg/ml. After digestion of polypeptides, gel electrophoresis was carried out as usual and proteins electroblotted onto nitrocellulose membrane for immunodetection. In the case of B, the anti-D1n blot shown in A was exposed to additional blotting with anti-D1c.

as described in Ref. 27. Trypsin is known to cleave the D1-protein mainly at two sites, i.e., arg-225 and arg-238, giving rise to two N-terminal fragments of apparent molecular mass 20 and 22 kDa, respectively, called T20 and T22 [29]. This conclusion has been confirmed recently and, in addition, their respective C-terminal products identified as fragments with apparent molecular masses of 12 and 10 kDa (hereafter called T12 and T10). Moreover, a 24 kDa N-terminal fragment (T24) and a 7 kDa C-terminal fragment (T7) is also produced by trypsin treatment, presumably by cleavage at arg-257. However, the immunodetectable level of these fragments was poor and variable. Isolated PS II reaction centres were illuminated in the presence of 0.2 mM DBMIB for either 10 or 90 min to generate the 23 and 24 kDa fragments, respectively. These breakdown products were then subjected to limited proteolysis with trypsin and western blotting using selective anti-D1n or anti-D1c polyclonal antibodies [20,26]. In Fig. 2A the result of an immunoblot with anti-D1n of trypsinised PS II reaction centres that had been illuminated for 10 min is shown. Bands corresponding to D1, the D1/D2 heterodimer and to the previously characterised 41 kDa band [27], are detected along the diagonal of the gel, together with a 23 kDa photoinduced D1-protein breakdown product. Tryptic products are detected as off-diagonal spots. T24 (arrow in Fig. 2A and B), T22 and T20 were detected from proteolysis of D1, the heterodimer and the 41 kDa band. More interestingly, a 20 kDa fragment (T20) is also detected from proteolysis of the 23 kDa photoinduced fragment. When the same blot (i.e., Fig. 2A) was further reacted with the anti-D1c polyclonal (Fig. 2B), T12 and T10 fragments also became evident from proteolysis of D1, and the heterodimer, but not from the 23 kDa fragment, indicating the latter does not contain the C-terminal portion of the D1-protein. The T7 band was also detected in the D1 lane but, presumably because of lower levels, this trypsin fragment was not detected in the heterodimer lane. For the same reason, the T12, T10 and T7 bands were not observed in the 41 kDa lane of Fig. 2B, although T12 and T10 can just be seen in the corresponding lane of Fig. 2D.

A very different result was obtained when reaction centres were illuminated for 90 min. The anti-D1n antibody (Fig. 2C) does not recognise any off-diagonal spot in the 23 kDa region, whilst the usual T22 and T20 are detected as trypsin induced degradation products of the D1, the heterodimer and the 41 kDa band. In contrast, the anti-D1c antibody detected the 24 kDa breakdown fragment and showed that it could be degraded by trypsin to produce the low molecular mass C-terminal bands which were also detected from trypsin mediated proteolysis of D1, the heterodimer and the 41 kDa band (Fig. 2D). Thus, these results indicate that the 23 kDa fragment formed during the first period of photoinhibition contains the N-terminus of the D1-protein and further confirms the C-terminal origin of the 24 kDa fragment which is photoaccumulated over longer times.

To further verify the N-terminal origin of the 23 kDa fragment, it was isolated from PS II reaction centres of wheat that had been exposed to mild photoinhibitory treatment in the presence of 0.2 mM DBMIB. Wheat was chosen for this experiment because it has a unique lysine residue at position 238 in its D1-protein rather than the arginine found in other species. The isolated fragment was therefore subjected to Lys-C proteolysis. In Fig. 3 an immunoblot with anti-D1n antiserum shows that the 23 kDa fragment can be digested with Lys-C to give a product (indicated by dark arrow) having an apparent molecular mass of about 1 to 1.5 kDa less than the photoinduced fragment (compare lanes 1 and 2) and identical to that obtained from Lys-C digestion of the entire protein (lane 3). This finding is consistent with the view that the photocleavage site is located on the C-terminal side of residue 238 [5,22], the effect of Lys-C being that of removing a short stretch of amino acids from this photoinduced product. Our data therefore indicate that degradation of the D1-protein during the first minutes of illumination occurs by cleavage in the loop connecting the transmembrane segments IV and V, which generates 23 kDa N-terminal and 10 kDa C-terminal

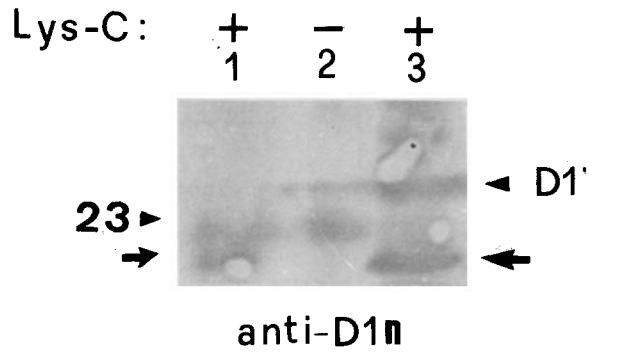

Fig. 3. Further evidence that the 23 kDa breakdown fragment is N-terminal. Wheat PS II reaction centres were illuminated for 10 min in the presence of 0.2 mM DBMIB as described in Fig. 1. The 23 kDa breakdown fragment was isolated by SDS-PAGE and its Lys-C proteolytic products probed with the anti-D1n polyclonal. Lane 1, cleaved 23 kDa fragment; lane 2, uncleaved fragment; lane 3, cleaved D1-protein. Bold arrow shows the Lys-C induced fragment corresponding to the N-terminal region of the D1-protein up to residue 238.

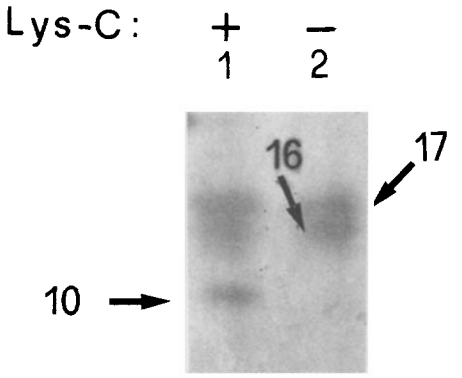

Fig. 4. Two different photoinduced D1 fragments are present in the 15–16 kDa range. Wheat PS II reaction centres were illuminated in the presence of 0.2 mM DBMIB as described in the legend of Fig. 1. After acid-free Coomassie staining, bands were cut from the gel, proteolysed with Lys-C on a second gel and digestion products probed with a mixture of anti-D1n and anti-D1c polyclonal antisera. Lane 1, digested fragments; lane 2, undigested fragments.

fragments [22,23]. Later, cleavages occur at the lumenal-exposed loops. Cleavage at the hydrophilic loop connecting the first and second transmembrane segments generates the 24 kDa C-terminal and a 9 kDa N-terminal fragment [20,22].

In this and previous studies [20,22,28], a 16 kDa C-terminal fragment has also been detected. If the generation of this fragment involves a cleavage independent of the cleavages shown above then, in principle, an approx. 16 kDa N-terminal fragment should also be generated. Therefore, PS II reaction centres isolated from wheat were illuminated in the presence of 0.2 mM DBMIB for 30 min and subjected to SDS-PAGE. After Coomassie staining, the region of the gel corresponding to the 15–18 kDa region was cut out and subjected to Lys-C digestion on a second gel. After blotting, the filter was reacted with a mixture of anti-D1n and anti-D1c polyclonals. The results obtained are displayed in Fig. 4 and show the presence of two bands (lane 2), one of which was found to be sensitive to Lys-C (lane 1). From this we conclude that two bands exist at the 16–17 kDa region with one containing N-terminus of the D1-protein (Lys-C insensitive) and the other the C-terminus (Lys-C sensitive). We therefore suggest that a third cleavage site exists positioned about half-way into the protein, probably in the loop joining putative transmembrane segments III and IV on the electron donor side of the reaction centre.

Above, we have clearly shown that, in the presence of 0.2 mM DBMIB, photoinduced cleavages can occur both on the 'acceptor' and 'donor' side of the D1-protein. The kinetics of the appearance of the degradation products are, however, different. We have therefore investigated the effect of various concentrations of DBMIB on the pattern of D1-protein degradation. Fig. 5 shows an experiment in which isolated PS II reaction centres were illuminated with photoinhibitory light for 30 min in the presence of DBMIB at concentrations ranging from 0 to 1 mM. Since the reaction centre concentration was constant, then the ratio between DBMIB and P680 was being varied. It can be seen that, at lower levels of DBMIB, the C-terminal photoinduced fragments, both at 16 and 24 kDa, are clearly observed. In contrast, the 23 kDa N-terminal fragment is most clearly observed at high DBMIB concentrations

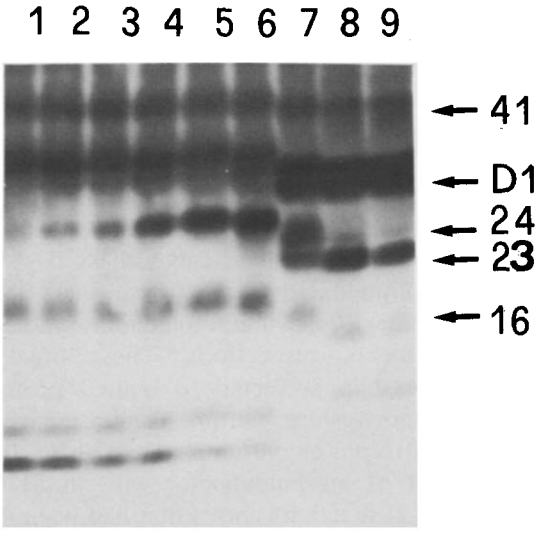

Fig. 5. Appearance of 24 kDa C-terminal and 23 kDa N-terminal breakdown fragments depend on the DBMIB/P680 ratio. Isolated PS II reaction centres were illuminated for 30 min with the following DBMIB concentrations: 0 μM (lane 1); 5 μM (lane 2); 10 μM (lane 3); 25 μM (lane 4); 50 μM (lane 5); 100 μM (lane 6); 250 μM (lane 7); 500 μM (lane 8); 1000 μM (lane 9). Western blotting was performed using anti-D1 polyclonal antiserum. Chlorophyll, 50 μg ml^{-1}; other conditions as for Fig. 1.

(particularly lanes 7, 8 and 9). Also to be noted is that degradation of the D1-protein, as indicated both by a decrease in its amount detected by immunoblotting and by an upward shift in its apparent molecular mass, is significantly reduced when high levels of DBMIB are present. Concomitant with this apparent protection at higher DBMIB concentrations is a decrease in the formation of the 41 kDa D1/cyt *b*-559 α-subunit adduct [27].

Discussion

In this paper we describe results obtained from a reinvestigation of the nature of photoinduced fragments of the D1-protein formed on illumination of isolated PS II reaction centres in the presence of DBMIB. Our results show that both N- and C-terminal photoinduced breakdown fragments of apparent molecular masses in the region of 23 to 24 kDa can be obtained under our experimental conditions. These results indicate that both donor and acceptor side cleavages of the D1-protein can occur in the isolated PS II reaction centre when subjected to illumination and are consistent with previous findings using PS II cores [22] as well as isolated reaction centres [23,24]. We should stress that our results are qualitative, since they are subject to differences in immunological cross-reactivity. Nevertheless, the use of C- and N-terminal specific antibodies has given us a tool, not only to detect the various D1-fragments, but also to allow speculation about their origin.

The fact that our anti-D1 antiserum, previously shown to be specific for the C-terminus of D1 [20], recognises the photoinduced 23 kDa N-terminal (e.g. as in Figs. 1 and 5) is explained by assuming that a major epitope for our polyclonal is located in the region between residue 238 and the photoinduced cleavage site. Therefore, this immunological response is lost after treating with trypsin or Lys-C which cuts at residue 238 (Arg in spinach but Lys in wheat). The same argument has also been presented in Ref. 5 with a polyclonal raised against the product of cloned *psb*A gene expressed in *E. coli* [30].

In models explaining the mechanism of photoinhibition [21,31], it is believed that acceptor and donor side photoinhibition lead to different patterns of degradation of the D1-protein. In the former, the generation of singlet oxygen as a consequence of recombination of the radical pair $P680^+Pheo^-$ in reaction centres having impaired Q_A function, is thought to be the step leading to proteolytic degradation of D1 [10], whilst in the latter the photoinduced accumulation of highly oxidising radicals is proposed as the event bringing about D1-protein degradation [19]. According to these ideas, we can conclude from our results that, when isolated PS II reaction centres are illuminated in the presence of DBMIB, both acceptor and donor side photoinhibition occur and that the balance between the two is dependent on the concentration of the added quinone. Although some experimental parameters such as temperature and pH have been shown to affect the degradation of the D1-protein [31,32], even in isolated PS II reaction centres [23,33], it is particularly noteworthy that the actual concentration of DBMIB is also a critical factor. We have shown that, depending on the concentration, DBMIB may elicit preferentially the production of 24 kDa C- (low concentration) or 23 kDa N-terminal fragments (high concentrations). How can the concentration of DBMIB be related to the mechanism of photoinhibition? One possibility is that reconstitution of the quinone at either or both Q_A and Q_B sites, may be responsible for this effect. DBMIB is thought to accept electrons from the reduced pheophytin within the isolated PS II reaction centre but it is not known if it occurs at either or both Q_A and Q_B sites [34]. Moreover, reconstitution of DBMIB with isolated PS II reaction centres catalyses a quinone-dependent cyclic electron flow around P680 in which cytochrome *b*-559 is reduced by DBMIB (in less that 1 μs, see Ref. 35) and oxidised by P680 (in ms, Ref. 35). But in addition to this, DBMIB may also mediate electron flow to molecular oxygen and therefore catalyse a non-cyclic pathway [36]. Therefore, to understand the observed pattern of photoinduced breakdown fragments of the D1-protein due to donor or acceptor side inhibition requires a more careful study of the relationship between the concentration of DBMIB and the photochemical activity of the isolated reaction centre. Such a study should help to explain why high concentrations of DBMIB protect the D1-protein from overall degradation and facilitate the appearance of the 23 kDa N-terminal fragment, as clearly shown in Fig. 6. Moreover, it is possible that the effect of DBMIB on the redox state of cytochrome *b*-559 may account for the considerable reduction in the appearance of the 41 kDa D1-cytochrome α-subunit adduct. It is also clear that, to obtain quantitative information about the levels of breakdown products and the true kinetics of their appearance, it will be necessary to supplement the western blotting approach presented here by specific labelling techniques, such as phosphorylation.

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