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The Rubisco large subunit binding protein

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Newly synthesized Rubisco large subunits made by isolated intact chloroplasts from *Pisum sativum* are bound non-covalently to another protein, termed the Rubisco large subunit binding protein. This protein is implicated in the assembly of Rubisco in higher plant chloroplasts. The binding protein has been purified from *Pisum sativum* in the form of an oligomer of relative molecular mass (M_r) about 720000. Analysis on polyacrylamide gels containing sodium dodecyl sulphate reveals equal amounts of two different types of subunit, termed alpha (M_r about 61000) and beta (M_r about 60000); thus the oligomer has the composition $\alpha_6\beta_6$. The alpha and beta subunits have been separated; their amino-terminal sequences are different, and antibodies raised against one subunit do not cross-react with the other subunit. Antibodies raised against the binding protein do not cross-react with the Rubisco large subunit, but do cross-react with polypeptides of M_r about 60000 in extracts of chloroplasts from wheat, barley and tobacco, and in extracts of leucoplasts from castor-bean endosperm.

The binding protein is made as a higher-molecular-mass precursor whan leaf polysomes are translated in a wheatgerm extract containing chloramphenicol, but is not synthesized by isolated intact chloroplasts. Thus the binding protein subunits are synthesized by cytoplasmic ribosomes and hence are likely to be encoded by nuclear genes. Etiolated *Pisum* plants contain binding protein, but exposure to light does not cause the same dramatic increase in amount that is seen in the case of Rubisco.

Treatment of stromal extracts with Mg–ATP in the range 0.1–5.0 mM causes dissociation of the binding protein oligomer into monomeric subunits; CTP, GTP, UTP, AMP and cyclic AMP do not have this effect. Mg^{2+} is required for dissociation but can be replaced by Ca^{2+}. Newly synthesized large subunits are released when the binding protein oligomer is dissociated, but re-attach when the dissociation is reversed by removal of ATP.

Introduction

In most eukaryotes the large subunits of ribulose bisphosphate carboxylase–oxygenase (Rubisco) are synthesized within the chloroplast, while the small subunits are synthesized in precursor form by cytoplasmic ribosomes (Ellis & Gatenby 1984). The precursor of the small subunit is transported across the chloroplast envelope into the stromal compartment, where a specific endoprotease removes the amino-terminal extension (Robinson & Ellis 1985). Mature small subunits assemble with large subunits to form the oligomeric holoenzyme in the stroma (Smith & Ellis 1979).

Synthesis of the large subunit by chloroplast ribosomes was demonstrated originally by electrophoretic analysis on polyacrylamide gels of the products of protein synthesis by isolated intact chloroplasts from *Pisum sativum* incubated with radioactively labelled amino acids (Blair & Ellis 1973). It was noted in these early experiments that the newly synthesized labelled large subunits are soluble in aqueous media. This may seem unsurprising in view of the high aqueous solubility of the Rubisco holoenzyme, which occurs at concentrations of up to 300 mg ml^{-1} in

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the stromal compartment. However, analysis of the products of protein synthesis by isolated intact chloroplasts on non-denaturing polyacrylamide gels showed that the bulk of the newly synthesized large subunits are not assembled into holoenzyme molecules but occur in some aggregated form of high molecular weight (Blair & Ellis, 1973; Ellis 1977). Subsequent work demonstrated that this aggregate consists of newly synthesized large subunits bound non-covalently to another chloroplast protein, and it is this protein that we have termed the Rubisco large subunit binding protein. Since large subunits isolated from higher plant Rubisco are insoluble in aqueous media in the absence of detergents, the hypothesis was advanced that the function of this large subunit binding protein is to keep the newly synthesized large subunits in a soluble form suitable for assembly with small subunits entering from the cytoplasm (Barraclough & Ellis 1980; Ellis *et al.* 1980; Ellis 1981).

Consistent with this hypothesis are the reports from Roy's laboratory that large subunits, newly synthesized by isolated *Pisum sativum* chloroplasts, will transfer from the binding protein to the Rubisco holoenzyme when stromal extracts are treated with Mg–ATP (Milos & Roy 1984; Milos *et al.* 1985). However, it has not been established that the combination of large subunits with the binding protein is an obligatory step in the assembly of Rubisco. It is not usual for the assembly of oligomeric proteins to require the presence of another protein, but there is a precedent for the existence of an obligatory assembly protein. The correct assembly of nucleosomes from DNA and histones in *Xenopus* extracts requires the presence of another soluble nuclear protein, termed nucleoplasmin. This protein acts as a 'molecular chaperone' in that it permits only the correct interactions between the DNA and histones and does not itself form part of the nucleosome (Laskey & Earnshaw 1980). Our working hypothesis is that the Rubisco large subunit binding protein is a 'molecular chaperone' that ensures the correct assembly of the large subunits with the small subunits of Rubisco.

Other hypotheses about the significance of the binding protein can however, be proposed. The binding of newly synthesized large subunits to another chloroplast protein might be an artefact, even though this binding is highly specific and occurs with high affinity (Hemmingsen & Ellis 1986). Alternatively, the binding protein may not be required for the assembly of Rubisco but may act simply to store large subunits until small subunits appear within the chloroplast. If the supply of large subunits is always evenly matched by the supply of small subunits, as it might be in cyanobacteria synthesizing these subunits from cotranscribed genes, the binding protein may be unnecessary in such organisms. We cannot distinguish between these possibilities at the present time. However, if the binding protein *is* required for the assembly of Rubisco in higher plants, a most important consequence follows.

Other articles in this symposium stress the vital role of Rubisco in controlling plant productivity, and emphasize the need to develop improved forms of the enzyme by genetic engineering techniques. Attempts to produce mutant forms of higher plant Rubisco by expressing cloned genes in bacteria have so far been hampered by the poor solubility and lack of enzymic activity of the large subunits, which fail to assemble with small subunits (see Bradley *et al.* and Somerville, this symposium). It is only with large subunits from cyanobacteria and photosynthetic bacteria where progress has been made, and with these organisms there do not appear to be the same problems of solubility and assembly. Thus the emphasis of our research at Warwick is on the characterization of the binding protein so that cloned cDNA sequences can be expressed in the same bacterial cells as the large and small subunit sequences. We regard this approach as the best test of the hypothesis that the assembly of enzymatically

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active Rubisco from higher plants requires the presence of the binding protein. In this way we hope to remove the blockage that is currently hampering attempts to produce mutated forms of Rubisco from higher plants.

The remainder of this article will summarize our research on the Rubisco large subunit binding protein from *Pisum sativum*.

Discovery

The synthesis of radioactively labelled Rubisco large subunits by isolated intact chloroplasts from *Pisum sativum* proceeds at a faster rate than the assembly of these large subunits into the holoenzyme of Rubisco. Indeed, synthesis largely ceases before assembly begins. In addition the proportion of newly synthesized large subunits that enters the holoenzyme in isolated intact chloroplasts depends on the composition of the incubation medium. Assembly of labelled large subunits into holoenzyme is best seen in a medium containing sorbitol as the osmoticum; if the sorbitol is replaced by KCl, assembly is largely prevented, but synthesis is unaltered (Barraclough & Ellis 1980). Unassembled labelled large subunits remain in the supernatant fraction when the chloroplasts are osmotically lysed. It must be emphasized that these newly synthesized large subunits can be detected only by their radioactivity, since the amount that is made by isolated chloroplasts is far too small to be visualized by protein stains. However, analysis of the supernatant fraction on non-denaturing polyacrylamide gels reveals that these labelled large subunits comigrate with an abundant staining chloroplast protein, which behaves on these gels as if it had a relative molecular mass in the range $6–7 \times 10^5$ (Ellis 1977). It is important to realize that the labelled large subunits made by isolated chloroplasts comigrate exactly with the staining protein on these gels. Figure 1 shows a comparison of three tracks from a non-denaturing gel in which the shapes of the labelled large subunit bands are seen to match exactly the shapes of the stained protein bands. This exact comigration is also seen

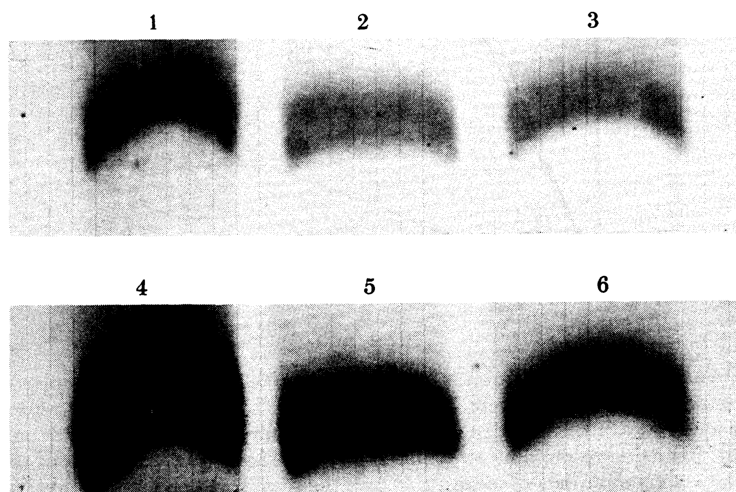

Figure 1. Exact comigration of staining protein bands with labelled Rubisco large subunit bands. Isolated intact chloroplasts were isolated from leaves of 7-day-old *Pisum sativum* plants and incubated in sorbitol resuspension medium for 30 min at 20 °C with [^{35}S]methionine and light, as described by Barraclough & Ellis (1980). Stromal extracts were electrophoresed on non-denaturing gels containing 50 g polyacrylamide per litre. The gel was stained with Coomassie blue, dried and radioautographed. The figure shows the staining band of protein from the upper region of the gel and the comigrating radioactivity due to newly synthesized Rubisco large subunits. Three tracks are shown, to demonstrate that the shape of the staining band always matches the shape of the radioactive band. Tracks 1–3, staining band; tracks 4–6, radioautograph of tracks 1–3.

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when non-denaturing gels which contain either different concentrations or a gradient of polyacrylamide are used.

The initial interpretation of these observations was that the stained protein is an aggregated form of unassembled large subunits made in the plant before the chloroplasts were isolated, so that comigration of newly synthesized labelled large subunits with this protein is to be expected (Ellis 1977). Roger Barraclough found that this interpretation is incorrect; analysis on two-dimensional polyacrylamide gels, in which the second dimension contains sodium dodecyl sulphate (SDS), shows that the staining protein band has a higher subunit relative molecular mass (60000) than the labelled Rubisco large subunits (52000). This 60 kDa protein is not itself labelled when isolated intact chloroplasts are incubated with radioactive amino acids. It was concluded that newly synthesized Rubisco large subunits made by isolated chloroplasts are bound non-covalently to another chloroplast protein that occurs as an oligomer of 60 kDa subunits. The hypothesis was advanced that this combination of large subunits with the binding protein is required for the assembly of the Rubisco holoenzyme (Barraclough & Ellis 1980).

Determination of the percentage of newly synthesized large subunits that become attached to the binding protein during the incubation of isolated chloroplasts shows that this value approaches 100% at the early stages of the incubation; the percentage drops with time as assembly proceeds, but it is never less than 60% (figure 2). The shapes of the curves shown

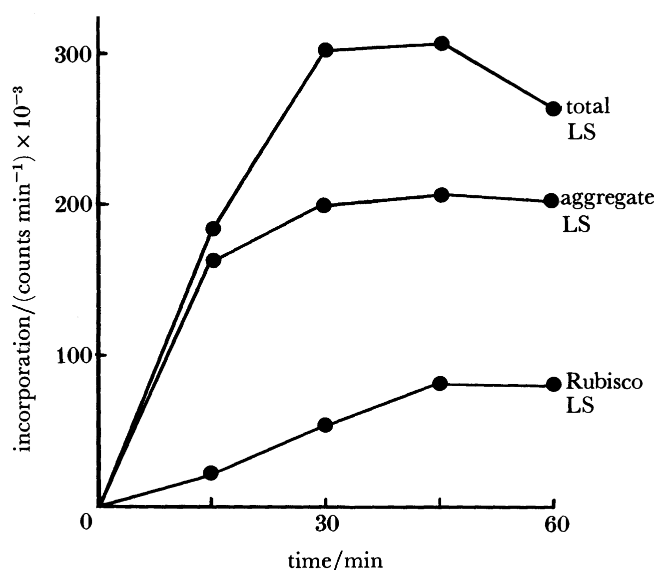

FIGURE 2. Association of newly synthesized Rubisco large subunits with binding protein. Chloroplasts were incubated as described in figure 1, and samples were removed at intervals. Stromal extracts were prepared by osmotic lysis and electrophoresed on either non-denaturing 5 $\mathrm{g\,l^{-1}}$ polyacrylamide gels or SDS–polyacrylamide gels and radioautographed. Bands corresponding to Rubisco large subunit were excised from the SDS gels and their radioactivity measured by scintillation counting to give the total Rubisco large subunit synthesized (total LS). Bands corresponding to the binding protein and to the Rubisco holoenzyme were excised from the non-denaturing gels and counted to give the proportion of newly synthesized large subunits associated with the binding protein (aggregate LS) and the Rubisco holoenzyme (Rubisco LS).

in figure 2 are consistent with a precursor–product relation in which large subunits entering the holoenzyme originate from combination with the binding protein, but they do not prove this relation.

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Purification and properties

The Rubisco large subunit binding protein has been purified from *Pisum sativum* chloroplasts by conventional procedures (Hemmingsen & Ellis 1986); a protein with the same properties has also been purified from *Hordeum vulgare* chloroplasts (R. A. Johnson & R. J. Ellis, unpublished). Analysis of the products of partial digestion by protease reveals no similarity between the products obtained from Rubisco large subunits and those of the binding protein from *Pisum sativum* (figure 3). Polyclonal antibodies raised against the *Pisum* binding protein do not

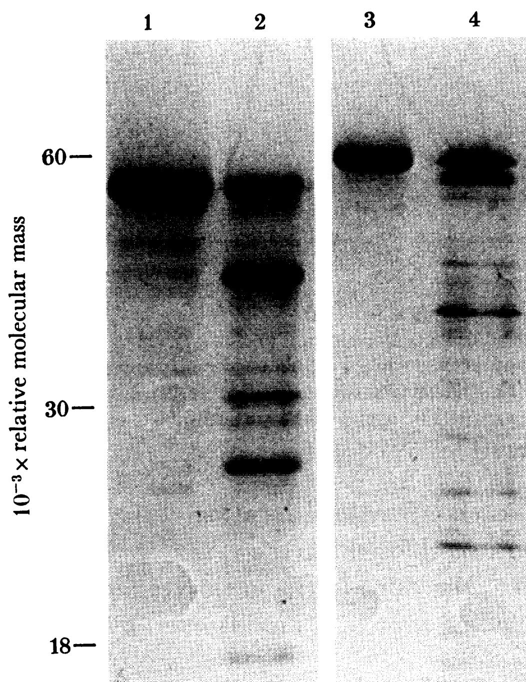

Figure 3. Partial protease digestion comparison of Rubisco large subunit and binding protein. Rubisco large subunit and binding protein bands were excised from SDS–polyacrylamide gels of the proteins purified from *Pisum sativum* and treated with V8 protease from *Staphylococcus aureus*, as described by Cleveland *et al.* (1977). The figure shows the Coomassie-blue-stained SDS gel tracks of the digestion products. Track 1, Rubisco large subunit, undigested; track 2, Rubisco large subunit incubated in the well with 0.01 μg protease; track 3, binding protein undigested; track 4, binding protein incubated in the well with 0.01 μg protease. Marker proteins were run to provide the calibration of relative molecular masses.

cross-react with the Rubisco large subunit from *Pisum*, nor do anti-Rubisco antibodies cross-react with the binding protein. These observations confirm that the binding protein is not an aggregated form of Rubisco large subunits but is a distinct protein. There is a slight cross-reaction of anti-binding protein antibodies with the Rubisco small subunit; if the binding protein and small subunits bind to large subunits at some common sites, they may possess domains that are co-recognized by a subset of antibodies raised against the binding protein.

The apparent relative molecular mass of the purified *Pisum* binding protein is 720000 determined on calibrated Sephacryl columns. Analysis on SDS–polyacrylamide gels reveals the presence of two subunits, which migrate so close together that they can be resolved only at high ratios of acrylamide to bis-acrylamide (Hemmingsen & Ellis 1986). The apparent relative molecular masses of these subunits are about 61000 (the alpha subunit) and 60000 (the beta

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subunit). Densitometric scanning of silver-stained gels shows that these two types of subunit are present in equal amounts (figure 4). Solid-phase Edman degradation of the amino-terminal sequences of the separated alpha and beta subunits shows that these sequences are totally dissimilar for at least the first 20 residues. Antisera raised against the separated subunits show

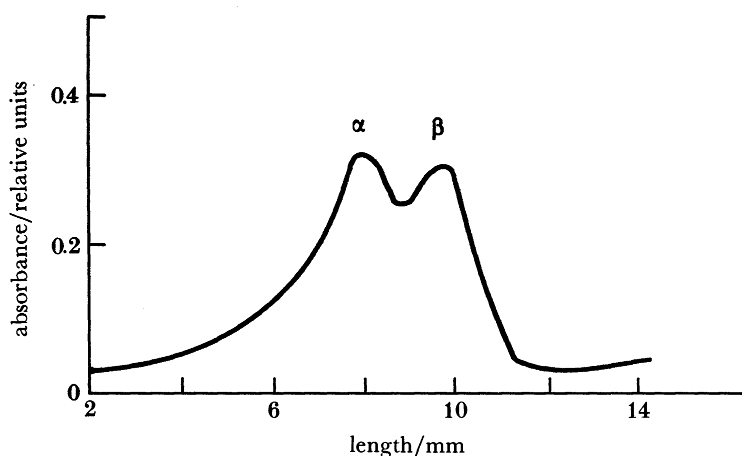

FIGURE 4. Relative content of the two subunits of the binding protein. Purified binding protein was electrophoresed on an SDS–polyacrylamide gel containing 150 g l^{-1} polyacrylamide and 1.5 g l^{-1} bis-acrylamide as described by Hemmingsen & Ellis (1986). The gel was stained with silver and scanned with a Joyce–Loebl densitometer; the figure shows the densitometer scan. The alpha and beta subunits are present in equal amounts. The same result is seen if the gel is stained with Coomassie blue.

little or no cross-reaction with each other. Identification of the subunits, on two-dimensional O'Farrell gels, of extracts from *Pisum* chloroplasts shows that the alpha subunit has an apparent isoelectric point of about pH 5.5; the value for the beta subunit is about pH 6.0. We propose from these findings that the binding protein is an oligomer of two types of subunit with the composition $\alpha_6\beta_6$.

Antibodies to *Pisum* binding protein react strongly with proteins of M_r about 60000 when extracts from tobacco, wheat and barley chloroplasts, and from castor-bean endosperm leucoplasts, are analysed on SDS–polyacrylamide gels by immunoblotting. The binding protein is thus likely to be a common component of plastids that contain Rubisco.

SYNTHESIS

The subunits of the binding protein are not labelled when isolated intact chloroplasts are incubated with radioactive amino acids. When polysomes from *Pisum* leaves are run off in a wheatgerm translation system in the presence of [^{35}S]methionine and chloramphenicol, a ^{35}S-labelled protein can be precipitated by antibodies to the binding protein. This labelled protein has a slightly higher apparent molecular mass than the binding protein subunits when analyzed on SDS–polyacrylamide gels (Hemmingsen & Ellis 1986). These results are consistent with the behaviour expected for chloroplast proteins that are encoded in nuclear genes and synthesized by cytoplasmic ribosomes as higher-molecular-mass precursors.

Because the accumulation of Rubisco by leaves of *Pisum sativum* is greatly stimulated by light, acting at the level of transcription (Gallagher & Ellis 1982), studies have been performed to ascertain the effect of light on the accumulation of the Rubisco large subunit binding protein.

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Rocket immunoelectrophoresis reveals that etiolated *Pisum* plants contain low but detectable amounts of both Rubisco and binding protein. When the etiolated plants are illuminated with continuous white light, the amount of each protein per shoot increases, but the increase in Rubisco is greater than the increase in the binding protein. Comparison of etiolated plants with plants grown for the same time under a 12 h photoperiod shows that the content of Rubisco per shoot is increased 30-fold by growth in the light, but that the content of binding protein is increased only 7-fold (Lennox & Ellis 1986). Thus the expression of the nuclear genes for the Rubisco large subunit binding protein in *Pisum sativum* is not subject to the same high degree of photoregulation as the genes for Rubisco.

Dissociation by Mg–ATP

The addition of Mg–ATP to stromal extracts of *Pisum* chloroplasts causes dissociation of the binding protein oligomer (Bloom *et al.* 1983); we have confirmed this observation, and shown that this dissociation is not accompanied by the stable phosphorylation or adenylation of the binding protein subunits (Hemmingsen & Ellis 1986).

More recently, the dissociation of the binding protein has been studied by means of electrophoresis on non-denaturing gels containing a gradient of polyacrylamide concentration. Such gels exhibit a much greater resolving power than the single-concentration polyacrylamide gels used previously, and allow the detection of specific proteins by immunoblotting. A new staining band of protein, of apparent M_r about 60000, appears on the gel when stromal extracts are treated with Mg–ATP concentrations as low as 0.5 mM (figure 5, tracks 3–5). Accompanying the appearance of this new protein is a decrease in the staining intensity of the binding protein oligomer band running with a relative molecular mass of 720000. When a gel such as that shown in figure 5 is immunoblotted with antibodies against the binding protein, the 60 kDa protein band is found to react strongly with the antiserum (figure 6). Immunoblotting is more sensitive than staining, and reveals the presence of some 60 kDa protein even in dialysed stromal extracts to which no Mg–ATP has been added (figure 6, track 6). Concentrations of Mg–ATP as high as 5 mM do not cause complete dissociation (figures 5 and 6, track 5). Use of antibodies to the separated subunits suggests that both subunits are present in the 60 kDa region of the gel (not shown).

The dissociation by Mg–ATP is highly specific for this nucleotide; CTP, UTP, GTP, AMP and cyclic AMP at 1–5 mM, with Mg^{2+} present at the same concentrations, do not cause dissociation. Figure 6 (tracks 7 and 8) shows the inability of Mg–GTP to cause dissociation. Mg^{2+} ions are required for dissociation by ATP but can be replaced by Ca^{2+}. If the ATP concentration is lowered by allowing protein synthesis to occur in the stromal extracts, the dissociation is reversed and the oligomeric form of the binding protein increases progressively with time (figure 7, tracks 5–8). This reversal of dissociation is not seen if the ATP is removed by dialysis; this result suggests that dialysable factors may be required for reversal. Newly synthesized large subunits are released from the binding protein on dissociation, but re-attach when the dissociation is reversed. The released large subunits are not bound to the monomeric subunits, as judged by analysis on gradient non-denaturing gels, and run near the top of sucrose density gradients (Hemmingsen & Ellis 1986). We have not so far been able to observe the transfer of large subunits to the Rubisco holoenzyme to the extent reported by Milos & Roy (1984) and Milos *et al.* (1985).

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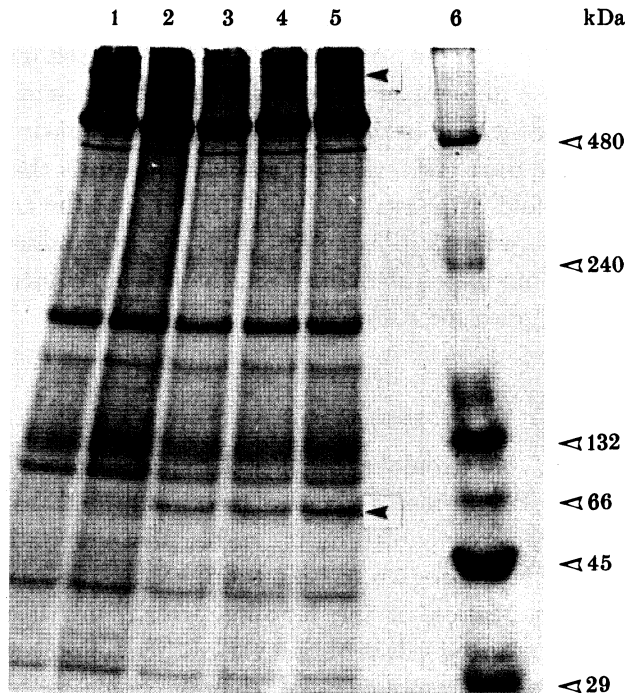

FIGURE 5. Dissociation of the binding protein to its monomeric subunits by Mg–ATP. Stromal extracts of *Pisum sativum* chloroplasts were dialysed for 3 h to lower the content of endogenous ATP, and then incubated for 30 min at 0 °C with Mg–ATP at the concentrations indicated. The extracts were electrophoresed on a non-denaturing gel containing a 40–300 g l^{-1} gradient of polyacrylamide concentration. The gel was stained with Coomassie blue. The upper arrow indicates the binding protein oligomer (720 kDa) while the lower arrow indicates the binding protein subunits (60 kDa). Track 1, no added Mg–ATP; track 2, 0.1 mM Mg–ATP; track 3, 0.5 mM Mg–ATP; track 4, 1.0 mM Mg–ATP; track 5, 5.0 mM Mg–ATP; track 6, molecular mass markers.

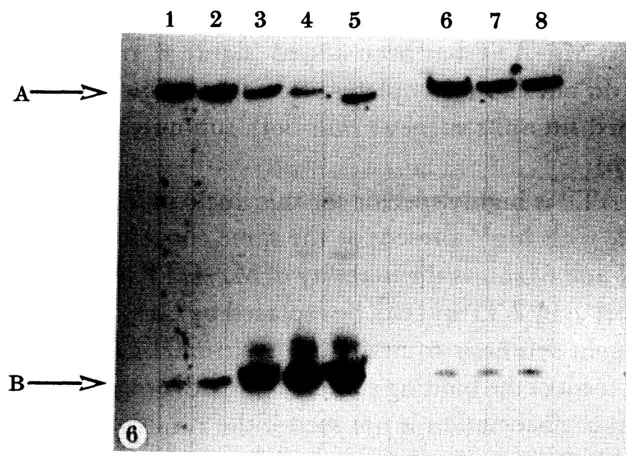

FIGURE 6. Immunological identification of monomeric subunits produced by the dissociation of the binding protein by Mg–ATP. Stromal extracts were treated with Mg–ATP and analysed as described in figure 5, but the gel was then immunoblotted with ^{125}I-labelled antibodies to the binding protein, as described by Hemmingsen & Ellis (1986). The figure shows the resulting radioautograph. Tracks 1–5 as in figure 5; track 6, no addition of nucleotide; track 7, 1.0 mM Mg–GTP; track 8, 5.0 mM Mg–GTP. Arrows: A, binding protein oligomer; B, binding protein monomers.

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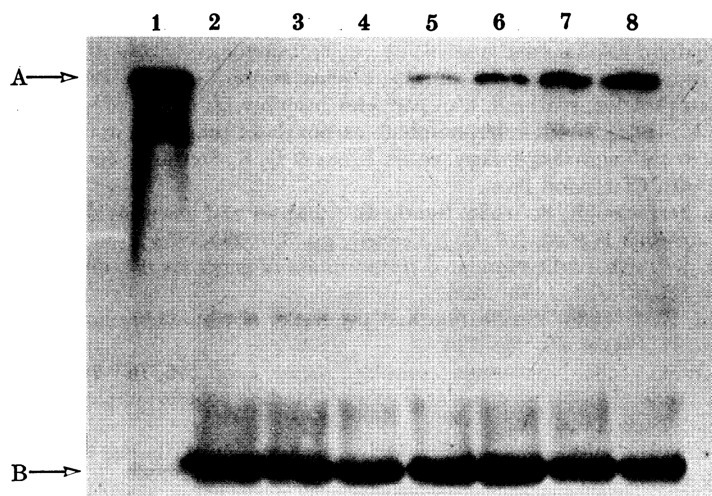

FIGURE 7. Reversible dissociation of the binding protein oligomer. Isolated *Pisum sativum* chloroplasts were lysed by resuspension in 10 mM Tris–HCl, 10 mM $MgSO_4$, pH 8.0, and the thylakoids were then removed by centrifugation. The supernatant extract was incubated with 2 mM ATP, 0.2 mM GTP, and 80 mM KCl at 25 °C for up to 120 min to allow protein synthesis by free chloroplast ribosomes to remove added ATP. Aliquots were analysed by electrophoresis under non-denaturing conditions on a 40–300 g l^{-1} polyacrylamide gel. Immunoblotting was carried out as described in figure 6. The figure shows the resulting radioautograph. Track 1, zero time, no added ATP; track 2, 15 min; track 3, 30 min; track 4, 45 min; track 5, 60 min; track 6, 75 min; track 7, 90 min; track 8, 120 min. Arrows A and B as in figure 6. Note the reappearance of the oligomeric form in tracks 5–8.

These observations suggest that the binding protein undergoes a reversible dissociation between the oligomeric form and the monomeric subunits, Mg–ATP causing the equilibrium to shift towards the monomeric subunits. This equilibrium can be represented as follows:

$$\text{LS–}\alpha_6\beta_6 \overset{\text{Mg–ATP}}{\rightleftharpoons} \text{LS} + 6\alpha + 6\beta,$$

where LS represents the Rubisco large subunit. Under *in vivo* conditions, the bulk of the binding protein will presumably be in the monomeric form, since the ATP concentration in the stroma is usually greater than 0.5 mM. When a stromal extract is prepared by osmotic lysis, the ATP concentration is reduced by a factor of at least 100, resulting in a shift to the oligomeric form to which newly synthesized large subunits are bound. Whether this complex of large subunits and binding protein oligomer is involved in the assembly of Rubisco holoenzyme remains to be determined.

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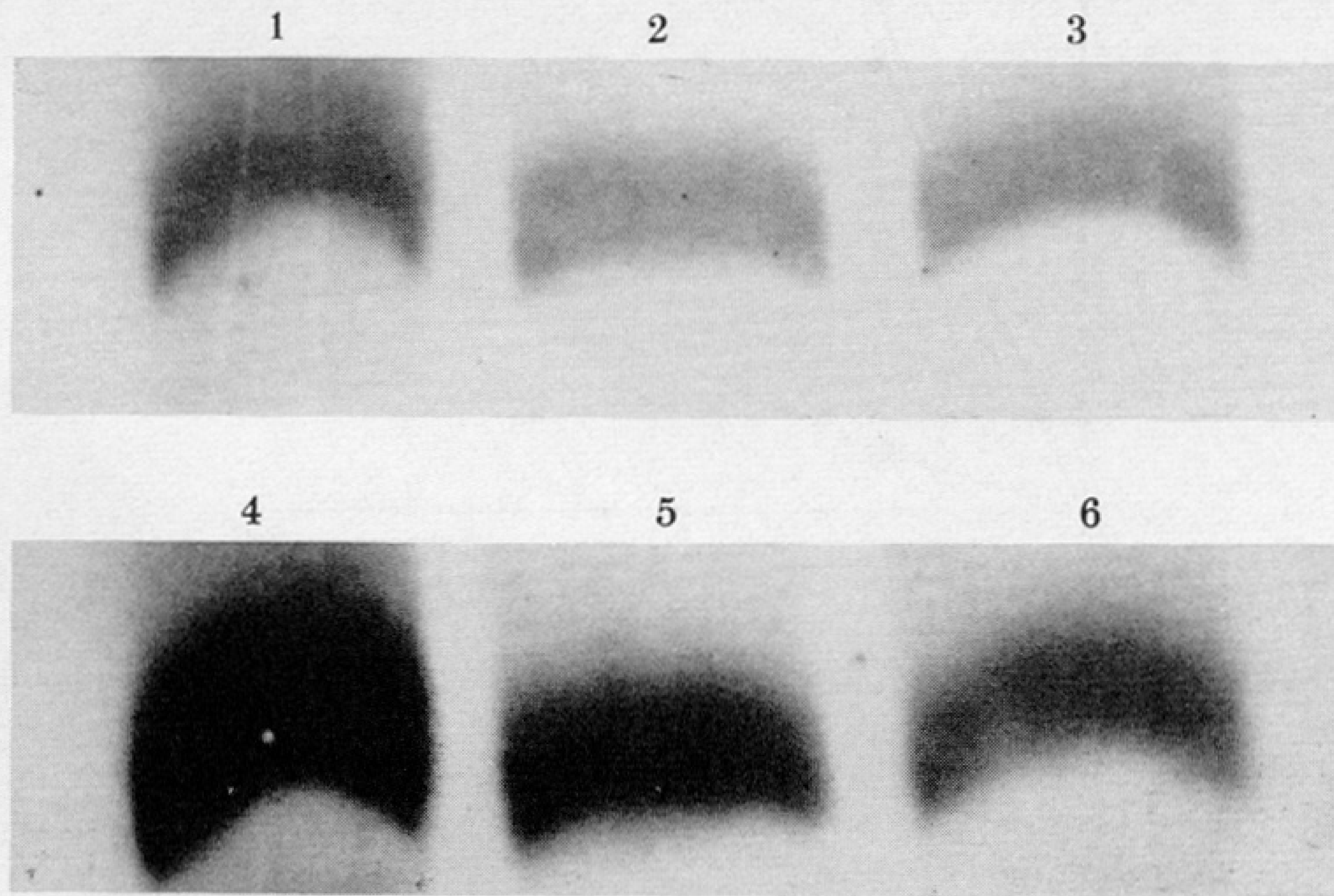

FIGURE 1. Exact comigration of staining protein bands with labelled Rubisco large subunit bands. Isolated intact chloroplasts were isolated from leaves of 7-day-old *Pisum sativum* plants and incubated in sorbitol resuspension medium for 30 min at 20 °C with [^{35}S]methionine and light, as described by Barraclough & Ellis (1980). Stromal extracts were electrophoresed on non-denaturing gels containing 50 g polyacrylamide per litre. The gel was stained with Coomassie blue, dried and radioautographed. The figure shows the staining band of protein from the upper region of the gel and the comigrating radioactivity due to newly synthesized Rubisco large subunits. Three tracks are shown, to demonstrate that the shape of the staining band always matches the shape of the radioactive band. Tracks 1–3, staining band; tracks 4–6, radioautograph of tracks 1–3.

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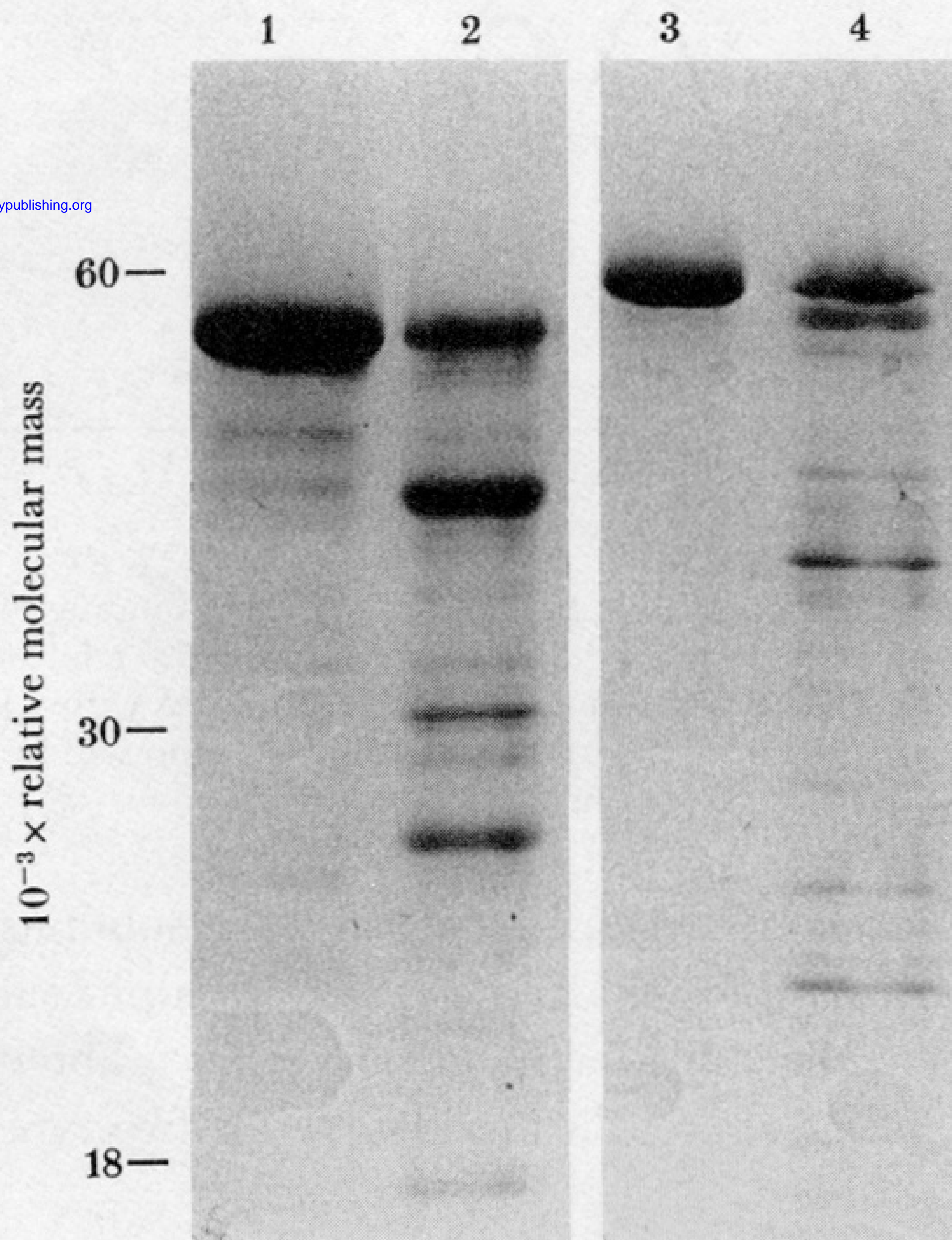

FIGURE 3. Partial protease digestion comparison of Rubisco large subunit and binding protein. Rubisco large subunit and binding protein bands were excised from SDS–polyacrylamide gels of the proteins purified from *Pisum sativum* and treated with V8 protease from *Staphylococcus aureus*, as described by Cleveland *et al.* (1977). The figure shows the Coomassie-blue-stained SDS gel tracks of the digestion products. Track 1, Rubisco large subunit, undigested; track 2, Rubisco large subunit incubated in the well with 0.01 μg protease; track 3, binding protein undigested; track 4, binding protein incubated in the well with 0.01 μg protease. Marker proteins were run to provide the calibration of relative molecular masses.

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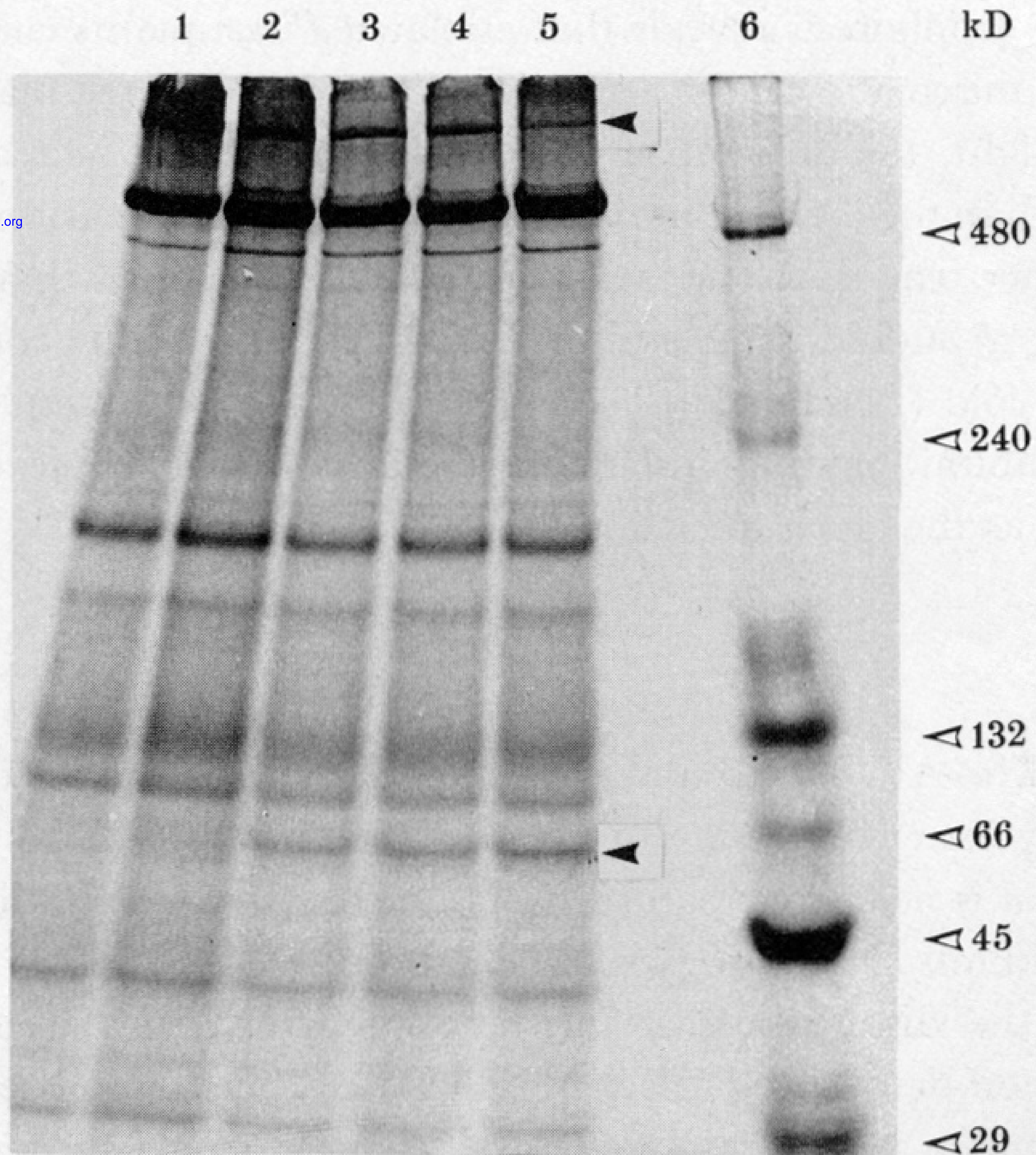

FIGURE 5. Dissociation of the binding protein to its monomeric subunits by Mg–ATP. Stromal extracts of *Pisum sativum* chloroplasts were dialysed for 3 h to lower the content of endogenous ATP, and then incubated for 30 min at 0 °C with Mg–ATP at the concentrations indicated. The extracts were electrophoresed on a non-denaturing gel containing a 40–300 g l^{-1} gradient of polyacrylamide concentration. The gel was stained with Coomassie blue. The upper arrow indicates the binding protein oligomer (720 kDa) while the lower arrow indicates the binding protein subunits (60 kDa). Track 1, no added Mg–ATP; track 2, 0.1 mM Mg–ATP; track 3, 0.5 mM Mg–ATP; track 4, 1.0 mM Mg–ATP; track 5, 5.0 mM Mg–ATP; track 6, molecular mass markers.

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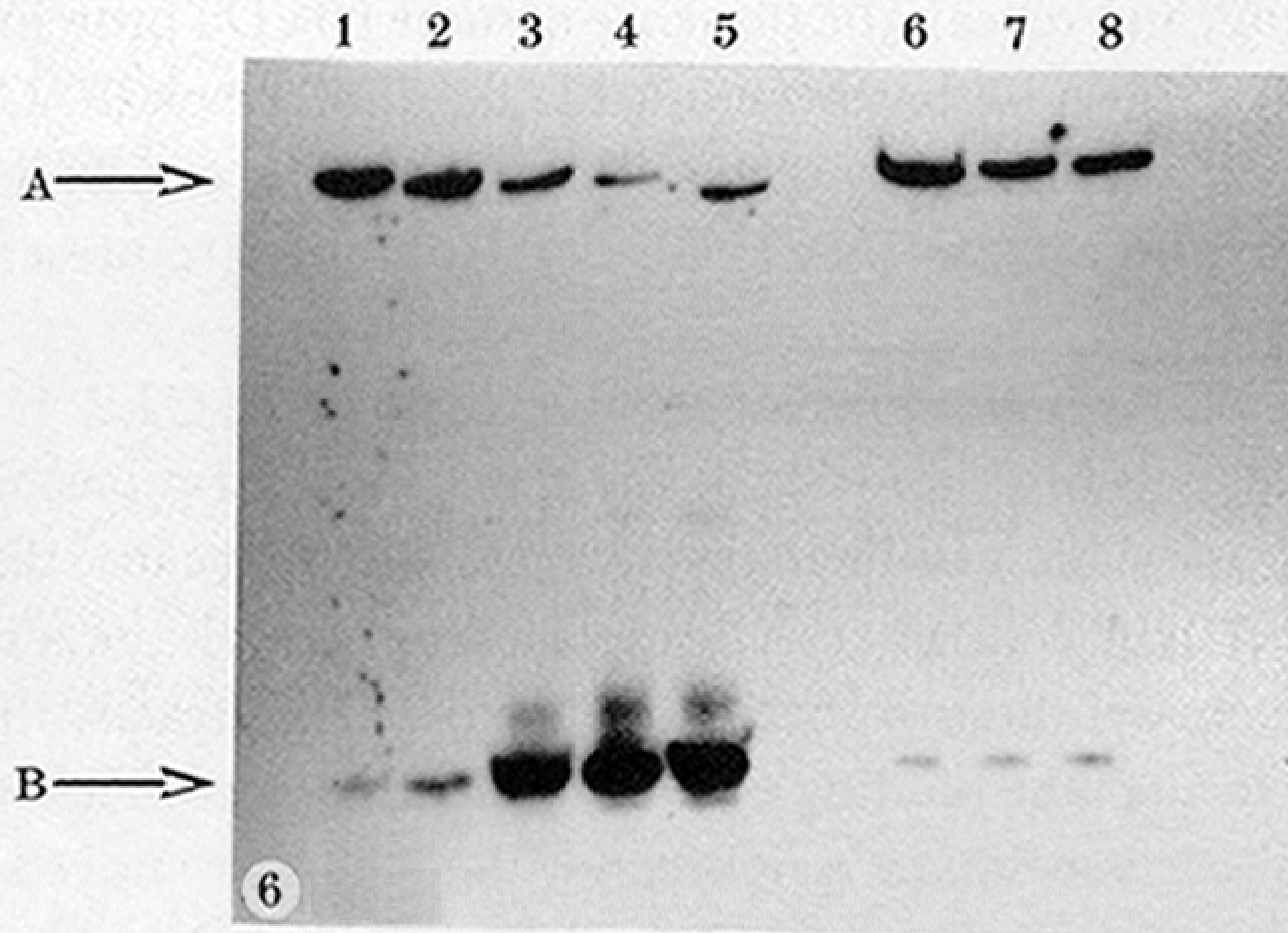

FIGURE 6. Immunological identification of monomeric subunits produced by the dissociation of the binding protein by Mg–ATP. Stromal extracts were treated with Mg–ATP and analysed as described in figure 5, but the gel was then immunoblotted with ^{125}I-labelled antibodies to the binding protein, as described by Hemmingsen & Ellis (1986). The figure shows the resulting radioautograph. Tracks 1–5 as in figure 5; track 6, no addition of nucleotide; track 7, 1.0 mM Mg–GTP; track 8, 5.0 mM Mg–GTP. Arrows: A, binding protein oligomer; B, binding protein monomers.

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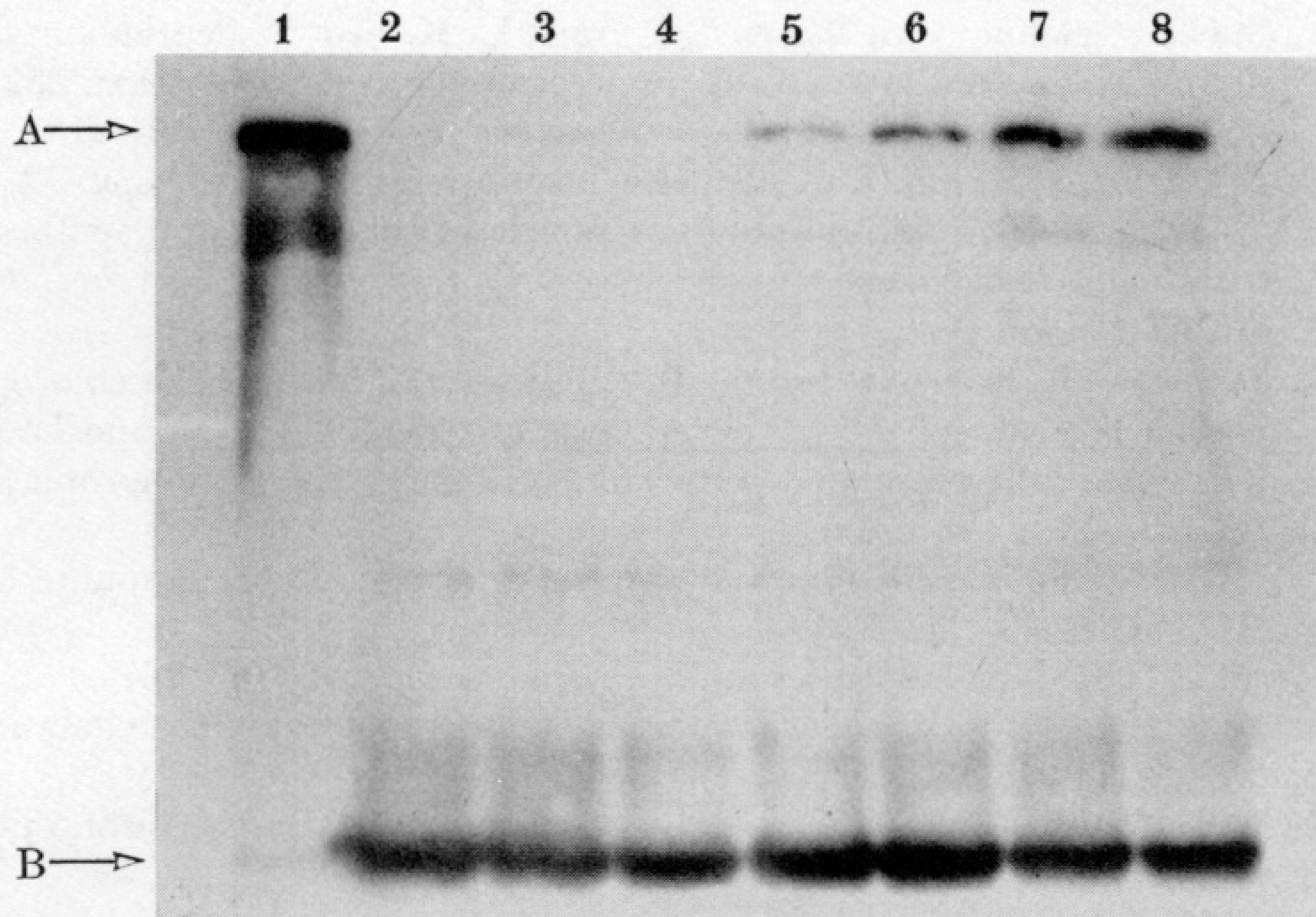

FIGURE 7. Reversible dissociation of the binding protein oligomer. Isolated *Pisum sativum* chloroplasts were lysed by resuspension in 10 mM Tris–HCl, 10 mM $MgSO_4$, pH 8.0, and the thylakoids were then removed by centrifugation. The supernatant extract was incubated with 2 mM ATP, 0.2 mM GTP, and 80 mM KCl at 25 °C for up to 120 min to allow protein synthesis by free chloroplast ribosomes to remove added ATP. Aliquots were analysed by electrophoresis under non-denaturing conditions on a 40–300 g l^{-1} polyacrylamide gel. Immunoblotting was carried out as described in figure 6. The figure shows the resulting radioautograph. Track 1, zero time, no added ATP; track 2, 15 min; track 3, 30 min; track 4, 45 min; track 5, 60 min; track 6, 75 min; track 7, 90 min; track 8, 120 min. Arrows A and B as in figure 6. Note the reappearance of the oligomeric form in tracks 5–8.

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