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THE AUTOPHOSPHORYLATION REACTION IN THE MECHANISM OF ACTIVATION OF PIG BRAIN CYCLIC AMP-DEPENDENT PROTEIN KINASE

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Autophosphorylation of cyclic AMP-dependent protein kinase (ATP: protein phosphotransferase, EC 2.7.1.37) was shown to occur via an intramolecular mechanism: the regulatory subunit undergoes phosphorylation only within the holoenzyme. The phospho form of the catalytic subunit has the capacity to phosphorylate the regulatory subunit. The phosphotransferase reaction and the reaction of autophosphorylation were found to proceed with the involvement of the same active site. The activation constant of phospho- and dephosphoprotein kinase under the influence of cyclic AMP and the dissociation constant of the cyclic AMP complex with phospho- and dephospho forms of the holoenzyme were estimated. Autophosphorylation was demonstrated to lead to almost complete dissociation of the holoenzyme under the influence of cyclic AMP. Circular dichroism spectra of the phosphorylated and non-phosphorylated forms of protein kinase were studied. The relative content of the secondary structure elements in proteins was estimated and conformational changes were detected in the enzyme upon its interaction with cyclic AMP. The *anti*-conformation of the cyclic nucleotide fixed in the complex with the phospho form of the regulatory subunit is suggested.

Introduction

At the present time the activation of cyclic AMP-dependent protein kinases is believed to involve the formation of an intermediate complex of the cyclic nucleotide with a tetramer of the holoenzyme (R_2C_2), followed by dissociation of the latter into the catalytic subunit (C) and a dimer of the regulatory (R) subunit which is bound to cyclic AMP (cAMP) [1,2]:

$$R_2C_2 + 2\ cAMP \rightleftharpoons [R_2C_2\text{-(cyclic AMP)}_2] \rightleftharpoons R_2\text{-(cyclic AMP)}_2 + 2\ C$$

The last stage of the reaction is rate-limiting [3]. The above scheme describes common features which are characteristic of the activation of all cyclic AMP-dependent protein kinases, whilst in each individual case the detailed mechanism may be different. Thus, two main types of protein kinase which differ in some structural and physico-chemical properties [4–6] can be primarily distinguished by details of the dissociation mechanism. This is, in particular, apparent in the modulations of this process in the course of the autophosphorylation reaction [6].

In this study, the role of autophosphorylation in the mechanism of activation of cyclic AMP-dependent protein kinase from pig brain (type II protein kinase) is discussed.

Materials and Methods

The following reagents were used: cyclic AMP and ATP from Sigma (U.S.A.), [^{3}H]-cyclic AMP (30 Ci/mol) and [γ-^{32}P]ATP (0.1 Ci/mol) from Amersham (U.K.), bovine serum albumin and dithiothreitol from

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Pierce (U.S.A.), β-mercaptoethanol from Fluka (Switzerland). Histone H1 was isolated from calf thymus by the method of Johns [7].

The holoenzyme of protein kinase was obtained using the method described earlier [8], and the catalytic and regulatory subunits according to our earlier papers [8–10] and using a DEAE-cellulose column in the presence of cyclic AMP (see below).

The phospho form of the catalytic subunit was obtained by incubation of the homogeneous catalytic subunit with ATP and [γ-^{32}P]ATP under the conditions indicated by Kochetkov et al. [11,12].

Protein concentration was determined by the method of Lowry et al. [13] using bovine serum albumin as standard. The phosphotransferase and cyclic AMP-binding activities of the enzymes were assayed by the methods of Nesterova et al. [8].

The autophosphorylation reaction of the holoenzyme was conducted in an incubation mixture (total volume 0.1 ml) containing 0.1 M Tris-HCl (pH 8.7)/10 mM Mg^{2+}/2 mM dithiothreitol/80 mM NaCl/0.1–0.3 μM holoenzyme/125 μM ATP or [γ-^{32}P]ATP and, in some cases, cyclic AMP. Incubation was carried out for 30 min at 4°C and then for 30 min at 30°C. The reaction was terminated by rapid cooling of the mixture to 4°C. The mixture was dialysed against 10 mM Tris-HCl buffer (pH 7.2)/50 mM NaCl/2 mM EDTA/1 mM dithiothreitol (buffer A). Phosphorylation of the individual regulatory subunit of protein kinase was performed under the same conditions, using the catalytic subunit or its phosphorylated form [11,12]. In the latter case, ATP and NaCl were omitted from the reaction mixture and the ratio R/C was 1.

In order to determine the amount of radioactivity incorporated into the protein, the incubation mixture, after cooling, was applied to Whatman 3 MM paper filters. The filters were treated as described earlier [8] in the case of the phosphotransferase assay.

Separation of the subunits was performed by chromatography on a DEAE-cellulose column (0.8 × 5 cm), eluting at 10 ml/h with 0.9-ml fractions. The samples were incubated in buffer A, containing 5 μM cyclic AMP, for 2 h and then applied to the column. The column was then washed with 15 ml buffer A, containing 50 μM cyclic AMP, followed by 15 ml of the same buffer without cyclic AMP, and finally with a linear gradient of 50–400 mM NaCl in the same buffer (also without cyclic AMP). The subunits of protein kinase obtained were dialysed.

Activation constants (K_a) of the enzyme in the presence of cyclic AMP were estimated as the cyclic AMP concentration in the incubation mixture at which the phosphotransferase reaction rate is half-maximal. Dissociation constants (K_d) of the cyclic AMP complex with the holoenzyme were estimated from the Scatchard plot of the results on cyclic [^{3}H]AMP binding to the enzyme according to the following equation [14]:

$$r/A = n/K_d - r/K_d$$

where r is the relative concentration of the binding sites, n is the number of binding sites/protein molecule and A is the concentration of the free ligand.

CD spectra were recorded on a Dichrograph III (Jobin-Yvon, France) at 20°C. Each spectrum was recorded three times and the results were averaged. As solvent, 0.1 M Tris-HCl buffer (pH 8.0), containing 0.1 M NaCl and 1 mM dithiothreitol was used. Protein kinase was incubated with cyclic AMP for 30 min at 20°C.

The relative content of α-, β-, and unordered structures in proteins was estimated by the method of Greenfield and Fasman [15,16] using spectra of the model structures of poly(L-lysine) as standards [16].

Results and Discussion

After incubation of the homogeneous holoenzyme of cyclic AMP-dependent protein kinase with [γ-^{32}P]ATP in the presence of dithiothreitol and Mg^{2+} and subsequent precipitation of the protein with 10% trichloroacetic acid, incorporation of the radioactive phosphate into the acid-insoluble fraction was observed. The reaction was terminated after several minutes. In the absence of Mg^{2+}, the reaction proceeded very slowly.

In the holoenzyme of the protein kinase both the catalytic and regulatory subunits can be phosphorylated [6,17]. In particular, the phospho form of the catalytic subunit of the pig brain protein kinase has been obtained. This is the enzyme intermediate in the phosphotransferase reaction, and a histidine

residue has been identified as the immediate acceptor of γ-phosphate [11,12]. However, it seems unlikely that this phospho form of histidine is the final product in the autophosphorylation reaction terminated by 10% trichloroacetic acid precipitation, in view of the rapid hydrolysis of the phosphoimidazole bond at low pH values.

Analysis of the ^{32}P-labelled phosphoholoenzyme by electrophoresis in the presence of 0.1% sodium dodecyl sulphate (SDS) shows a peak of ^{32}P radioactivity coinciding in mobility with the protein kinase component with molecular weight of 53 000, i.e., within the holoenzyme, the regulatory subunit of protein kinase is subjected to phosphorylation.

The increase in [γ-^{32}P]ATP concentration in the incubation mixture leads to incorporation of up to 1.6 mol phosphate/mol holoenzyme (Fig. 1). Since there are two molecules of the regulatory subunit in the holoenzyme and the endogenous phosphate is present in the initial protein kinase preparation [1,18], one can assume that each monomer of the regulatory subunit after phosphorylation contains 1 mol phosphate.

The linear dependence of the autophosphorylation rate on the protein kinase concentration (Fig. 2) indicates that the reaction is an intramolecular process and occurs only within a tetrameric complex. Addition to the native holoenzyme of the homogeneous individual subunits did not affect the amount of radioactive phosphate incorporated into the protein. When the amount of free subunits in the incubation medium was varied, radioactivity detected in the trichloroacetic acid-insoluble fraction of the protein was also directly proportional only to the amount of the newly-formed holoenzyme.

The intramolecular character of the autophosphorylation reaction is also shown by the inhibitory effect of cyclic AMP on this reaction (Fig. 3). The increase in cyclic AMP concentration in the incubation medium leads to a decrease in the rate of autophosphorylation. This correlates well with the conventional scheme of the mechanism of cyclic AMP interaction with protein kinase: binding of cyclic AMP is accompanied by dissociation of the holoenzyme into subunits and, thus, causes a decrease in the autophosphorylation rate.

The autophosphorylation reaction can be subdivided into a number of discrete stages: in the first stage, binding of ATP at the active site of catalytic subunit within the holoenzyme takes place; then hydrolysis of the phosphoether bond of the ATP molecule occurs, with the formation of an intermediate phospho form of the enzyme (containing a phosphohistidine residue in the catalytic subunit), and finally the proper autophosphorylation reaction of the regulatory subunit proceeds. This consists of the transfer of the phosphate residue from the catalytic subunit to the regulatory one (serine and/or threonine residues being phosphorylated).

The last stage can be observed during incubation of the phospho form of the catalytic subunit containing radioactive phosphate [11,12] with the

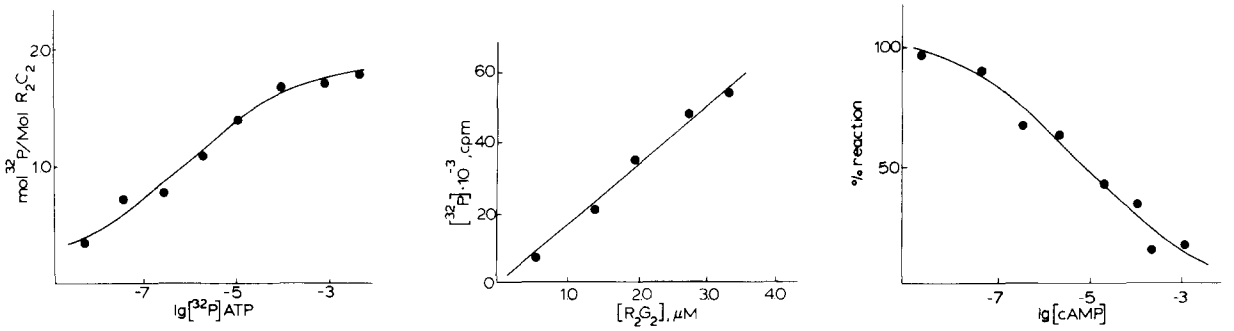

Fig. 1. Kinetics of autophosphorylation of protein kinase. Concentration of holoenzyme 0.4 μM. (Left-hand figure).

Fig. 2. Rate of autophosphorylation reaction as a function of holoenzyme concentration. (Centre figure).

Fig. 3. Effect of cyclic AMP on autophosphorylation reaction of protein kinase. (Right-hand figure).

regulatory subunit and subsequent analysis of the components upon separation on a DEAE-cellulose column (Fig. 4). The elution profile of the catalytic subunit phospho form on DEAE-cellulose does not differ from that of the non-phosphorylated preparation; peaks of the phosphotransferase activity and ^{32}P-radioactivity being coincident (Fig. 4a). After incubation of the ^{32}P-labeled phospho form of the catalytic and regulatory subunits under the conditions of the autophosphorylation reaction, followed by separation of the products obtained (Fig. 4b), ^{32}P-radioactivity was detected only in the fractions which possessed the cyclic AMP-binding activity, i.e., those corresponding to the regulatory component of protein kinase. The almost complete absence of ^{32}P label in the fractions of the catalytic subunit indicates the transfer of the phosphate residue from phosphohistidine of the catalytic subunit to the regulatory subunit. This reaction occurs as shown above, within the newly-formed holoenzyme. This is also supported by the traces of ^{32}P-radioactivity found in the peak of the DEAE-cellulose elution profile which corresponds to that of the holoenzyme (fractions 35–45 Fig. 4b).

We conclude that phosphorylation of protein kinase substrates (in our case, histone H1), and the regulatory subunit, involves the same active site of the catalytic subunit, and phosphorylation of the regulatory subunit takes place within the holoenzyme through formation of the phospho form of the catalytic subunit. The functions of the catalytic subunit of protein kinase as regards ATP hydrolysis and the transfer of the phosphate group within the holoenzyme or its complex with cyclic AMP are preserved. The regulatory subunit regulates the access for the substrate proteins into the active site of the catalytic subunit, without affecting the mechanism of cleavage of the phosphoether bond in the ATP molecules.

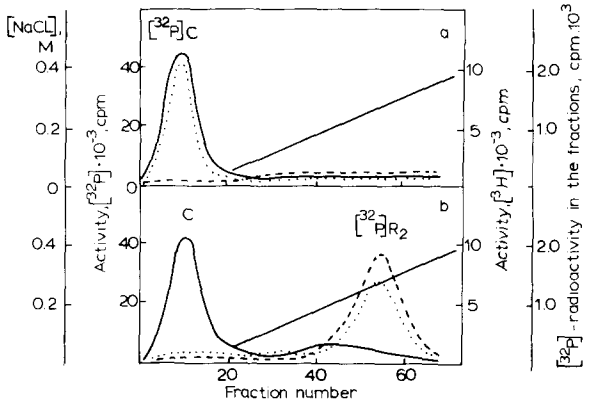

Fig. 4. DEAE-cellulose chromatography of catalytic and regulatory subunits of protein kinase. a, chromatography of ^{32}P-phospho form of catalytic subunit; b, separation of subunits after incubation of ^{32}P-phospho form of the catalytic subunit with the regulatory subunit. ———, phosphotransferase ^{32}P activity; - - - - - -, cyclic AMP-binding ^{3}H activity; ·····, ^{32}P content in fractions determined in 0.5-ml aliquots.

Phosphorylation of the regulatory subunit introduces no changes in the globular structure of the holoenzyme (Fig. 5) and does not activate the enzyme. However, in the presence of cyclic AMP, equimolar amounts of phosphorylated and non-phosphorylated protein kinase dissociate to form different quantities of free subunits; the phospho form of the proteins dissociates almost entirely (Fig. 6 a). Unlike the case with the phosphoholoenzyme, complete dissociation of the non-phosphorylated protein kinase was not achieved even at increased cyclic AMP concentration (50 M).

A Scatchard plot of the results obtained on cyclic AMP binding by both forms of protein kinase (Fig. 7) demonstrates that the phosphoholoenzyme is more sensitive to the cyclic nucleotide ($K_d = 10^{-9}$ M), i.e., has better capacity to bind cyclic AMP than the

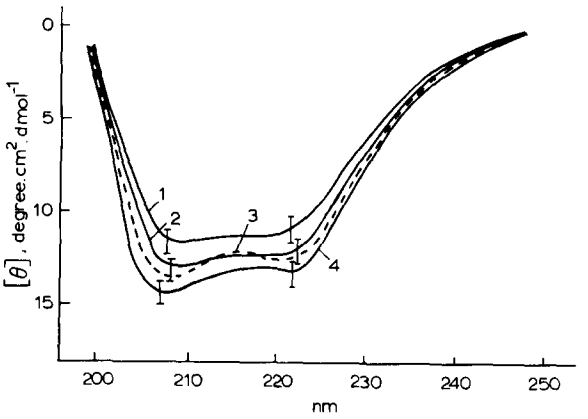

Fig. 5. CD spectra of protein kinase holoenzyme and its components. Regulatory subunit (complex with cyclic AMP): dephospho form – 1; phospho form – 2; holoenzyme (phospho and dephospho forms) – 3; catalytic subunit – 4.

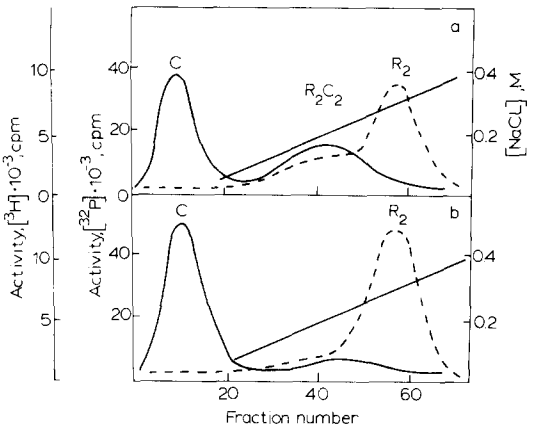

Fig. 6. DEAE-cellulose chromatography of dephospho- (a) and phosphoprotein kinase (b). ———, phosphotransferase ^{32}P activity; - - - - - -, cyclic AMP-binding ^{3}H activity.

dephosphoprotein kinase ($K_d = 7.5 \cdot 10^{-9}$ M). There was no notable cooperativity in the process of cyclic AMP binding; being saturated with the cyclic nucleotide, both forms bind 2 mol cyclic AMP/mol holoenzyme (R_2C_2). The calculated dissociation constants of protein kinases under the action of cyclic AMP correspond to a higher degree of activation of the phosphoholoenzyme by cyclic AMP, especially at low concentrations of the cyclic nucleotide. The K_a value for the phospho form was $2 \cdot 10^{-8}$ M, and that for the dephosphoholoenzyme $8 \cdot 10^{-8}$ M [19].

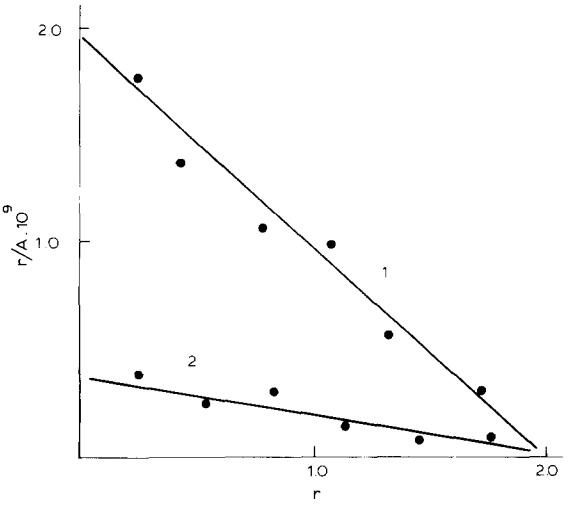

Fig. 7. Cyclic AMP binding to phosphorylated (1) and unphosphorylated (2) forms of protein kinase.

Thus, phosphorylation of protein kinase brings about significant changes in the functional properties of the enzyme, in particular changes in the affinity of the holoenzyme for cyclic AMP, in the capacity of the subunits to dissociate under the influence of the cyclic nucleotide and to reassociate in its absence [19,20]. It is natural to suppose that such changes in the protein kinase properties are caused by certain transformations in the structure of both free subunits and their complex, namely the holoenzyme.

The analysis of the CD spectra of protein kinase and its components shows (Fig. 5) that the catalytic subunit, regulatory subunit and the holoenzyme contain approx. equal amounts of α- and β-structures (catalytic subunit, 35 ± 3 and 20 ± 5; regulatory subunit, 25 ± 3 and 30 ± 5 for α- and β-structures, respectively) and a relatively high amount of an unordered structure (45 ± 5%). The data obtained for the catalytic subunit are in good correlation with the relative content of α- and β-structures determined for the catalytic subunit of cyclic AMP-dependent bovine liver protein kinase where α-helical segments constitute 29%, β-structure 20% and the unordered structure 51% [21].

Comparison of CD spectra for phosphorylated and unphosphorylated forms of proteins demonstrates that the phosphoprotein kinase spectrum differs from that of the dephospho form only within the limits of the experimental error (Fig. 5, 3); in the case of modification of the regulatory subunit, the picture observed is different (Fig. 5, 1 and 2). The α- and β-contents of the phosphorylated regulatory subunit of protein kinase exceed the respective amount for the unmodified protein. In the latter case, the complex of the regulatory subunit with the cyclic nucleotide is already formed. Thus, it follows that phosphorylation of the regulatory subunit causes conformational changes in this subunit.

At present, the phosphorylated site of the regulatory subunit of protein kinase is believed to be located close to the cyclic AMP-binding site which is formed partly by hydrophobic aromatic amino acid residues of the protein [22]. Therefore, all the effects of phosphorylation-dephosphorylation and cyclic nucleotide binding can be more readily detected in CD spectra of the protein, mainly in the region of absorption of aromatic residues.

The CD spectra of protein kinase in this region for

phosphorylated and unphosphorylated forms of the holoenzyme are given in Fig. 8. Phosphorylation of protein kinase essentially increases the CD amplitude of the protein in the region 250–300 nm; the shapes of these spectra still being similar.

A significant change in the CD spectra is observed when cyclic AMP is added to the holoenzyme (Fig. 9). In the differential CD spectra of protein kinase in the presence of the cyclic nucleotide (i.e., the spectrum of the holoenzyme in the presence of cyclic AMP minus the spectra of the free holoenzyme and cyclic AMP separately at the same concentration) this is expressed by the pronounced negative CD band at 260 nm and positive CD at 275–290 nm. It follows from the analysis of the spectra that the changes in the region 275–290 nm can be assigned to the change in the asymmetric environment of aromatic chromophores. Whereas negative dichroism at 255–270 nm is more likely to be due to the effect induced in the cyclic nucleotide chromophore which possesses its own optical activity in this region of the spectrum [23].

As seen in Fig. 9, the CD amplitude is directly dependent on whether the protein is phosphorylated or not. Differences in the CD effect for phospho and dephospho enzymes (Fig. 9, curves 1 and 2) can be accounted for by the different structures of the cyclic AMP-binding sites of these proteins. Since *syn-* and *anti*-conformations of the cyclic AMP molecules with respect to the C-N-glycoside bond have opposite CD effects at 260 nm (negative in the case of *anti*-conformation and positive for *syn*-conformation) [23], *anti*-conformation of the cyclic nucleotide fixed in

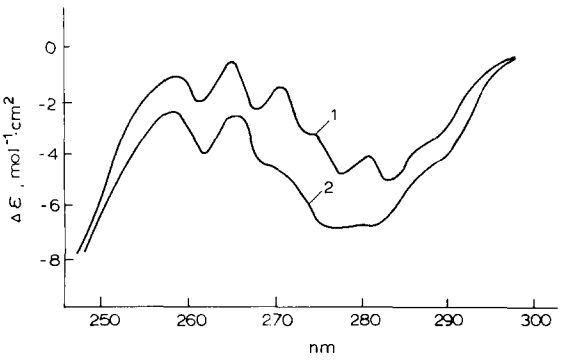

Fig. 8. CD spectra of dephospho- (1) and phosphoprotein kinase (2).

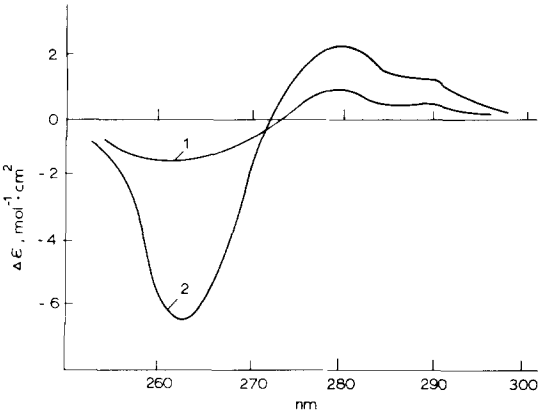

Fig. 9. Differential CD spectra of protein kinase (dephospho form – 1; phospho form – 2) in the presence of cyclic AMP. Protein concentration $5 \cdot 10^{-6}$ M; cyclic AMP concentration $5 \cdot 10^{-5}$ M. CD spectrum of holoenzyme in the presence of cyclic AMP minus spectra of the free holoenzyme and cyclic AMP separately at the same concentration.

the complex with the phosphoform of the regulatory subunit appears obvious.

All the above data lead us to the conclusion that autophosphorylation of the cyclic AMP-dependent protein kinase does not cause any essential changes in its secondary structure but affects the character of asymmetrical environment of aromatic residues of the protein. Interaction of cyclic AMP with the phosphorylated form of the cyclic AMP-dependent protein kinase is accompanied by the induced changes in the holoenzyme and leads to the complete dissociation of the protein kinase phospho form into subunits. The cyclic nucleotide is firmly fixed in the cyclic AMP-binding site in anti-conformation. The binding of cyclic AMP to the unphosphorylated holoenzyme leads to the formation of a relatively stable complex of the holoenzyme with the cyclic nucleotide which has a rather low degree of dissociation.

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