Communication

No Effect of cAMP on Protein Synthesis in Reticulocyte Lysates*

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SUMMARY

The results of a series of experiments are interpreted to indicate that protein synthesis in reticulocyte lysates is not affected by the reticulocyte cAMP-dependent protein kinase. The catalytic subunit of this enzyme was isolated to apparent homogeneity. Also, the protein inhibitor of this protein kinase was isolated from muscle. Neither physiological concentrations of cAMP nor any of these protein components had a detectable effect on protein synthesis in reticulocyte lysates in the presence or absence of exogenous heme. Phosphorylation of the smallest subunit of eukaryotic initiation factor 2 or the 90,000 to 100,000-dalton peptide associated with eukaryotic initiation factor 2 kinase activity were not affected by the activity of the cAMP-dependent protein kinase under conditions in which exogenous heme has a pronounced effect on these reactions.

Protein synthesis in rabbit reticulocytes and their cell-free lysates is controlled by the availability of hemin (1, 2). Gross and Rabiniowitz (3) isolated an inhibitor of polypeptide chain initiation from reticulocyte postribosomal supernatant incubated in the absence of hemin. This inhibitor, called the hemin-controlled repressor, was formed from an inactive proinhibitor of similar molecular weight. Later it was shown that HCR1 is associated with protein kinase activity for the smallest subunit of the peptide initiation factor eIF-2 (4–6). The mechanism by which this enzyme is regulated appears to be of crucial importance to an understanding of translational control in higher organisms.

Trachsel et al. (7) purified the heme-reversible form of HCR to homogeneity. They report that heme prevents activation of the purified kinase, apparently by direct interaction with the enzyme, which seems to be phosphorylated when it is in the active state. HCR itself is not affected by cAMP. However, Ochoa and his co-workers (6) concluded that a cAMP-dependent protein kinase or its catalytic subunit can promote the conversion from the inactive proinhibitor to active HCR. This conclusion was based on their observation that a commercial preparation of bovine heart cAMP-dependent protein kinase or its catalytic subunit would inhibit protein synthesis in lysates of rabbit reticulocytes. However, Horak and Koschel (9) found no effect on protein synthesis in intact cells when intracellular cAMP was raised to very high levels following exposure to catecholamines.

In this communication, we report that cAMP, a homogeneous preparation of the catalytic subunit of cAMP-dependent protein kinase isolated from reticulocytes, or the inhibitor of this kinase (10) has no detectable effect on protein synthesis in reticulocyte lysates.

EXPERIMENTAL PROCEDURES

The preparation of the catalytic subunit of cAMP-dependent kinase will be described elsewhere in detail. Briefly, the postribosomal supernatant from rabbit reticulocytes was chromatographed on DEAE-cellulose, fractionated by ammonium sulfate precipitation, and then successively chromatographed on hydroxypapitate, DEAE-cellulose in the presence of cAMP, phosphocellulose, histone-Sepharose, and finally Sephadex G-75.

The HCR from rabbit reticulocytes was partially purified from the postribosomal supernatant. It co-chromatographed with the cAMP-dependent protein kinase up to the hydroxypapitate step described above. To measure HCR activity in the reticulocyte lysate, this fraction was made 5 mM in N-ethylmaleimide, incubated for 5 min at 37°C, then made 5 mM in dithioerythritol, and incubated for 5 min on ice before it was added to the lysate system.

Protein synthesis in rabbit reticulocyte lysates was measured by [3H]leucine (40 Ci/mmol) incorporation as described (6) except that reaction mixtures contained 10 µl of lysate in a final volume of 50 µl. Incubation was for 30 min at 37°C. Reactions were stopped by the addition of 100 µl of 1 N NaOH and 100 µl of 3% H2O2. After incubation for 5 min at 37°C, the samples were made 5% in trichloroacetic acid and incubated at 95°C for 2 min. The precipitate formed was collected on glass fiber filters (Schleicher and Schull, No. 259), washed, and counted (6). Leucine incorporation is nearly linear for 30 min under the conditions used.

The inhibitor of cAMP-dependent protein kinases was prepared from rabbit hind leg muscle by a procedure described by Walsh et al. (10) with modifications according to Cohen et al. (11). The muscle tissue was homogenized with 2% volumes of 5 mM EDTA and centrifuged (Sorvall, rotor GS5; 10 min at 9,500 rpm), and the supernatant was adjusted to pH 6.1 with 1 M acetic acid. After 10 min on ice, centrifugation was repeated for 30 min. The resulting supernatant was raised to pH 7.0 with 6 M NH4OH, then heated for 20 min in a boiling water bath, cooled on ice, and filtered. The inhibitor fraction was precipitated by the addition of 50% trichloroacetic acid to give a final concentration of 15%. Precipitated protein was collected by centrifugation and resuspended and dialyzed against 10 mM Tris-HCl (pH 7.5). About 8 mg of the inhibitor fraction was loaded on a column (0.9 × 55 cm) containing Sephadex G-75 that was equilibrated in 10 mM Tris-HCl (pH 7.5). Fractions were assayed for activity that would inhibit phosphorylation of histones by a crude fraction of reticulocyte cAMP-dependent protein kinase in the presence of 5 mM cAMP.

Protein kinase activity was assayed and 32P-labeled proteins were analyzed by gel electrophoresis in 10% polyacrylamide in the presence of sodium dodecyl sulfate followed by autoradiography as described (12). Specific radioactivity of [γ-32P]ATP is given in the appropriate legends; cAMP’ was added where indicated.

RESULTS AND DISCUSSION

The cAMP-dependent protein kinase(s) co-chromatographs through the first steps of purification in the preparation of the HCR from rabbit reticulocytes. However, these activities were separated on DEAE-cellulose in the presence of cAMP. This compound causes the cAMP-dependent protein kinase(s) to dissociate into its regulatory and catalytic subunit. The latter

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1 The abbreviations used are: HCR, hemin-controlled repressor; eIF-2, eukaryotic initiation factor 2.
does not adsorb to DEAE-cellulose under the conditions used (cf. Ref. 13). The catalytic subunit was further purified to apparent homogeneity as outlined under “Experimental Procedures.” Its molecular weight was estimated to be 43,000 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (cf. Fig. 1). As shown in Fig. 1, this enzyme phosphorylates histones and a protein of the 40 S ribosomal subunits. This ribosomal protein has an apparent molecular weight of 32,000 and may be S6 (nomenclature of Lin and Wool, Ref. 14; cf. Ref. 15). Phosphorylation by the catalytic subunit of this protein kinase is greatly reduced by the low molecular weight protein inhibitor of CAMP-dependent protein kinases described by Walsh et al. (10). The effect of this inhibitor, isolated from rabbit muscle, on phosphorylation of histones and the ribosomal protein by the catalytic subunit of the CAMP-dependent protein kinase isolated from reticulocyte also is shown in Fig. 1. The enzyme does not phosphorylate the initiation factor eIF-2 (Fig. 1).

It has been suggested that in reticulocytes the CAMP-dependent protein kinase that phosphorylates the smallest subunit of eIF-2 (an integral part of the HCR system) may be activated by a CAMP-dependent protein kinase (8, 16, 17). Evidence was presented indicating that the catalytic subunit of bovine heart CAMP-dependent protein kinase inhibits protein synthesis in reticulocyte lysates (8). In contrast to these results, the CAMP-dependent protein kinase from reticulocytes appears to have no effect on \[^{14}C\]leucine incorporation in reticulocyte lysates as demonstrated by the data presented in Fig. 2. When added to a hemin-supplemented (Fig. 2B) or a hemin-deficient (Fig. 2E) lysate, the reticulocyte catalytic subunit does not affect protein synthesis over a range of 0.3 to 7 \(\mu\)g/50-\(\mu\)l lysate reaction mixture. Also shown in Fig. 2B is the inhibition of \[^{14}C\]leucine incorporation caused by exogenously added HCR. Protein synthesis in reticulocyte lysates incubated in the absence (Fig. 2D) or presence (Fig. 2A) of hemin is not influenced by CAMP over a range of 10\(^{-7}\) to 10\(^{-4}\) M. Edeine, a small peptide antibiotic known to interfere with peptide initiation (18), reduces \[^{14}C\]leucine incorporation to a basal level thought to reflect completion of nascent peptide chains (Fig. 2A).

If a CAMP-dependent protein kinase were activated in the absence of hemin, as suggested (8), the addition of the CAMP-dependent kinase inhibitor to such a lysate system would be expected to prevent HCR-dependent inhibition of protein synthesis. However, we have been unable to detect an effect of the inhibitor either in the presence or absence of exogenous heme. Typical results are presented in Fig. 2, C and F. We conclude that a CAMP-dependent protein kinase is not involved in the activation of HCR in reticulocytes. We have no explanation for the discrepancies between the results presented here and those reported in Ref. 8.

Gross and Rabinovitz (19) demonstrated that the inactive proinhibitor was converted first to what they called the “reversible repressor” that could be converted back to an inactive form in the presence of hemin. Only prolonged absence of heme would create the irreversible inhibitor that no longer responded to hemin. The reversible inhibitor may be purified in the presence of compounds that protect —SH groups (7, 20). As shown in Fig. 3, an HCR preparation from the hydroxyapatite step containing the reversible inhibitor will phosphorylate the smallest subunit of eIF-2 (Track 1) and, upon addition of CAMP, a protein (32,000 daltons) of the 40 S ribosomal subunit (Track 3). The phosphorylation of the ribosomal protein is due to CAMP-dependent protein kinase present in this preparation. Note that in those samples with the 40 S ribosomal protein as substrate, a 3-fold higher amount of the enzyme fraction was used to obtain a comparable extent of phosphorylation. Addition of hemin (33 \(\mu\)M) to an incubation containing this hydroxyapatite fraction will strongly reduce the phosphorylation of the eIF-2 subunit (Track 4) but

![Fig. 1. Substrate specificity and inhibition of the catalytic subunit of reticulocyte CAMP-dependent protein kinase.](image-url)
activity and phosphorylation of the 38,000-dalton subunit of eIF-2 (7, 20). Hemin was shown to prevent phosphorylation of a 65,000-dalton peptide assumed to be the eIF-2 kinase (7). Similar results are presented above in Fig. 3, Tracks 4 to 6. The fraction used in the experiment for Fig. 3 also contained cAMP-dependent protein kinase activity that, in the presence of cAMP, will phosphorylate its substrates. However, no phosphorylation of peptides in the 95,000- to 100,000-dalton range is seen under these conditions (Fig. 3, Track 6). These results provide a direct demonstration that the cAMP-dependent protein kinase that carries out phosphorylation of eIF-2 is not phosphorylated by the CAMP-dependent kinase in reticulocytes.

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