Protein Which Interacts with a Stimulatory Factor of RNA Polymerase II of Ehrlich Ascites Tumor Cells†

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ABSTRACT: When partially purified Ehrlich ascites tumor RNA polymerase II was further purified on a column of phosphocellulose, stimulation of its catalysis of RNA synthesis by stimulatory factor S-II was greatly decreased. This decrease in sensitivity to the stimulatory factor was reversible: the enzyme eluted from phosphocellulose became sensitive to the factor when mixed with a protein fraction eluted from the phosphocellulose at high salt concentration. Evidence was obtained that this protein, named helper protein, binds to the enzyme eluted from phosphocellulose, causing it to recover sensitivity to stimulatory factor S-II.

In differentiated eukaryotic cells only limited genome sequences are transcribed, resulting in the expression of cellular individuality. RNA polymerase II is believed to be the only enzyme responsible for the transcription of such genes, but it is unknown how the enzyme selects the specific genes in needs. There are many reports on isolation of proteins affecting the activity of RNA polymerase II in vitro (Stein and Hausen, 1970; Lentfer and Lezius, 1972; Natori, 1972; Seifart et al., 1973; Lee and Dahmus, 1973; Sugden and Keller, 1973; Shea and Kleinsmith, 1973; Natori et al., 1974; Kostraba et al., 1975; Kostraba and Wang, 1975; Chuang and Chuang, 1975; Stewart and Kruger, 1976). These proteins may participate in the regulation of gene expression.

Recently we purified a protein factor named S-II from Ehrlich ascites tumor cells that stimulates the activity of RNA polymerase II on native DNA as template (Sekimizu et al., 1976). This factor had the following characteristics. (1) It stimulated RNA polymerase II, but not RNA polymerase I or *E. coli* RNA polymerase. (2) It showed template specificity: homologous DNA was a good template, whereas calf thymus DNA was moderate and no stimulation was obtained when poly[d(AT)] was used as template. (3) The RNA synthesized in the presence of this factor was heterogeneous, but larger than control RNA. (4) The amount of the initiation complex formed in vitro was several fold greater when this factor was added, indicating that the factor enhances the formation of the initiation complex (Sekimizu et al., 1977).

These results suggest that this factor plays significant roles in regulation of transcription, especially in the selection of specific sites for transcription.

This paper describes the presence in the partially purified preparation of RNA polymerase II of a specific protein which interacts with stimulatory factor S-II. This protein, named helper protein, could be separated from RNA polymerase II on a column of phosphocellulose, leaving core RNA polymerase II, which did not respond to S-II. Complete enzyme that was sensitive to S-II could be reconstituted from the core enzyme and helper protein.

Materials and Methods

Cells. Male ddY mice were each inoculated with 3×10^6 Ehrlich ascites tumor cells and the ascitic fluid was harvested 7 days later. Cells were collected by centrifugation for 10 min at 150*g*. The cells harvested from 5 to 10 mice were combined and suspended in 27 mL of deionized water to disrupt erythrocytes. Then 3 mL of 10 × PBS[1] was added and the mixture was centrifuged. The resulting white pellet of cells was washed three times with PBS and stored at −80 °C.

Preparation and Assay of RNA Polymerase II. DNA-dependent RNA polymerase II was partially purified from 7-day cultures of Ehrlich ascites tumor cells, as described before (Natori et al., 1973a). The procedure involved the following steps: solubilization of the enzyme, ammonium sulfate precipitation, DEAE-cellulose chromatography, ammonium sulfate precipitation, and centrifugation on a glycerol gradient. This enzyme was named glycerol gradient enzyme.

The glycerol gradient enzyme was further purified on a column of phosphocellulose (1.5 × 8 cm, Whatman P-11), equilibrated with 0.05 M Tris-HCl buffer, pH 7.9, containing 0.0001 M EDTA, 0.001 M dithiothreitol, and 25% glycerol. The enzyme activity was eluted as a single peak at a salt concentration of 0.25 M, when the column was developed with 60 mL of a linear gradient of 0.1 to 0.5 M NaCl in the buffer described above. This enzyme was named phosphocellulose enzyme. These preparations of RNA polymerase II had specific activities of 3000 and 36 000 units per mg of protein, respectively, and were completely inhibited by 1 μg per mL of α-amanitin. One unit of enzyme activity was defined as the amount catalyzing incorporation of 1 pmol of UMP into the acid-insoluble fraction under the standard assay canditions. The standard assay medium contained, in a total volume of 0.25 mL, 10 μmol of Tris-HCl, pH 7.9, 0.75 μmol of $MnCl_2$, 1.15 μmol of $MgCl_2$, 12.5 μmol of $(NH_4)_2SO_4$, 0.017 μmol of EDTA, 1 μmol of β-mercaptoethanol, 0.0625 μmol each of ATP, GTP, and CTP, 0.00625 μmol of UTP, 0.5 μCi of [^{3}H]UTP (20 Ci/mmol), 5 μg of purified Ehrlich ascites tumor DNA, and 10–20 units of RNA polymerase II. The mixture was incubated for 60 min at 37 °C, and then samples were chilled in ice and 0.25 mL of cold 10% Cl_3CCOOH solution was added. The volume was increased by adding 1 mL of cold 5% Cl_3CCOOH solution, and after 15 min each sample was applied to a Whatman GF-C glass fiber filter. The filter was washed with 60 mL of cold 5% Cl_3CCOOH solution containing 0.01 M sodium pyrophosphate and then with 1 mL of 95%

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[1] Abbreviations used: DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; Tris, tris(hydroxymethyl)aminomethane; CM, carboxymethyl, $NaDodSO_4$, sodium dodecyl sulfate.

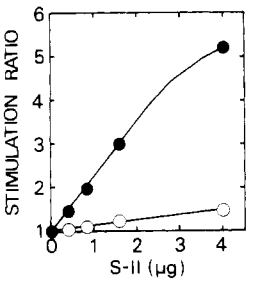

FIGURE 1: Effects of stimulatory factor S-II on the activities of the glycerol gradient and phosphocellulose enzyme. RNA synthesis was examined using 15 units of glycerol gradient enzyme (●) or phosphocellulose enzyme (○) under the standard conditions with increasing amounts of stimulatory factor S-II. Protein was determined by the method of Lowry et al. (1951). Stimulation was calculated on the basis of RNA synthesis without the factor.

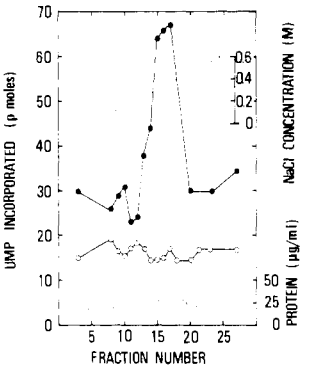

FIGURE 2: DEAE-cellulose chromatography of helper protein. Column size, 1.5 × 1.7 cm. Flow rate, 20 mL/h. Fraction size, 2 mL. Sample, about 4.6 mL of helper protein fraction from phosphocellulose was dialyzed extensively against 0.05 M Tris-HCl buffer, pH 7.9, containing 0.01 M $MgCl_2$, 0.0001 M EDTA, 0.001 M dithiothreitol, and 25% glycerol and then applied on a column of DEAE-cellulose equilibrated with the same buffer. The column was developed with 40 mL of a linear gradient of 0 to 0.8 M NaCl. Each fraction was dialyzed against 0.01 M Tris-HCl buffer, pH 7.9, containing 0.01 M KCl, 0.01 M $MgCl_2$, 0.0001 M EDTA, 0.003 M dithiothreitol, and 50% glycerol. After dialysis, 100 μL of each fraction was assayed for helper protein activity in the presence (●) or absence (○) of 50 units of S-II using 10 units of phosphocellulose enzyme under the standard conditions. (---, Bottom) Amounts of protein; (---, top) concentration of NaCl.

ethanol and dried under an infrared lamp. The radioactivity on the filter was counted in a Packard liquid scintillation spectrometer.

Preparation of Stimulatory Factor S-II and Assay of the Stimulation of RNA Synthesis. Stimulatory factor S-II was purified from Ehrlich ascites tumor cells as described previously and the fraction with activity from the CM-cellulose was used throughout the experiments (Sekimizu et al., 1976). The purity of this fraction was about 90% and the specific activity was 10 000–30 000 units per mg of protein. Stimulation of RNA synthesis was assayed under the standard assay conditions and the incorporation of UMP into the acid-insoluble fraction was compared with that in reaction mixture without the test fraction. One unit of stimulatory activity was defined as the amount which enhanced the activity of 10 units of RNA polymerase II to 11 units under these conditions.

Reconstitution of S-II Sensitive RNA Polymerase II from the Phosphocellulose Enzyme and Helper Protein. The reaction mixture contained, in a total volume of 0.25 mL, 10 μmol of Tris-HCl, pH 7.9, 0.025 μmol of EDTA, 0.25 μmol of dithiothreitol, 25% glycerol, 6 μg of phosphocellulose enzyme (220 units), and 120 μg of helper protein. After incubation for 30 min at 0 °C, the mixture was layered on top of 5 mL of a gradient of 5–20% sucrose containing 0.05 M Tris-HCl, pH 7.9, 0.005 M $MgCl_2$, 0.1 M $(NH_4)_2SO_4$, 0.0001 M dithiothreitol, 0.0001 M EDTA, 0.5 mg per mL of BSA, 0.2% Nonidet P-40, and 25% glycerol, and the gradient was centrifuged for 22 h at 56 000 rpm in a Hitachi RPS-65T rotor. Fractions were collected from the bottom of the tube and RNA polymerase activity in each fraction was assayed in the presence and absence of an excess amount of stimulatory factor S-II under the standard assay conditions.

Analysis of the Molecular Size of RNA Synthesized. The procedure used was exactly as described before (Natori et al., 1973b). RNA samples synthesized in vitro were layered on top of a 12-mL gradient of 7–25% sucrose in 0.01 M Tris-HCl buffer, pH 7.4, containing 0.01 M NaCl and 0.01 M EDTA and centrifuged for 16 h at 34 000 rpm in a Hitachi RPS-40T rotor. *E. coli* RNA labeled with [^{14}C]uracil was used as an internal marker.

Results

Separation of Helper Protein from Glycerol Gradient Enzymes. When partially purified RNA polymerase II (glycerol gradient enzyme) was further purified on a column of phosphocellulose, the activity of RNA polymerase was recovered as a single peak at an NaCl concentration of 0.25 M and the specific activity was increased from 3000 to 36 000 units per mg of protein. The recovery of RNA polymerase activity at this step was always 70–90%. However, this phosphocellulose enzyme was stimulated much less than the glycerol gradient enzyme by stimulatory factor S-II, as shown in Figure 1. In this experiment when the same units of enzymes were used, activity of the phosphocellulose enzyme was enhanced only 1.5-fold, whereas that of the glycerol gradient enzyme was enhanced more than 5-fold. Stimulation of the phosphocellulose enzyme by S-II varied with different preparations of enzyme, but it was always less than that of the glycerol gradient enzyme.

These results show that a protein fraction which is necessary for S-II to express its function was separated from the glycerol gradient enzyme during phosphocellulose column chromatography. To test this possibility, we tested the effects of various protein fractions from the phosphocellulose column on the sensitivity of the phosphocellulose enzyme to S-II and found that a protein fraction eluted from phosphocellulose with about 0.5 M NaCl markedly enhanced the effect of S-II on the phosphocellulose enzyme. This protein was tentatively named helper protein. The activity of helper protein to enhance the effect of S-II was completely inactivated on heating for 15 min at 60 °C, indicating that this substance is protein in nature. For simplicity, in later work helper protein was washed off the column with 0.6 M NaCl as soon as RNA polymerase II had been eluted. Preparations of phosphocellulose enzyme were usually not completely insensitive to S-II, indicating that helper protein was not removed completely from glycerol gradient enzyme by phosphocellulose column chromatography.

Helper protein may be single protein, because when the helper protein fraction from phosphocellulose was purified further on a column of DEAE-cellulose, it was eluted as a single peak, as shown in Figure 2. In this experiment, two assays were performed on each fraction, one with S-II and the other without S-II. The activity of helper protein could only be detected when S-II was present in the reaction mixture: none of the fractions had any affect on the activity of the phosphocellulose enzyme in the absence of S-II. Thus, helper protein was essential for the sensitivity of the phosphocellulose enzyme to S-II. The dose–response curve of helper protein is shown in Figure 3: stimulation of the phosphocellulose enzyme by S-II depended upon the amount of helper protein, and when excess

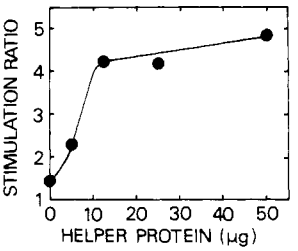

FIGURE 3: Effect of helper protein on stimulation of RNA synthesis. RNA synthesis was examined using 18 units of phosphocellulose enzyme and 60 units of S-II under the standard conditions with increasing amounts to helper protein obtained by phosphocellulose chromatography. Stimulation of RNA synthesis was calculated on the basis of RNA synthesis without S-II and helper protein.

TABLE I: Effects of Helper Protein on Two Preparations of RNA Polymerase.[a]

	Helper protein	UMP incorp (pmol)
Glycerol gradient enzyme	+	57
	−	53
Phosphocellulose enzyme	+	42
	−	18

[a] RNA synthesis was examined using 10 units of glycerol gradient enzyme or phosphocellulose enzyme with 47 units of stimulatory factor S-II under the standard conditions. Twenty-five micrograms of helper protein was added to the reaction mixture when indicated.

helper protein was added the activity was restored to almost the level of glycerol gradient enzyme. Helper protein was only effective with the phosphocellulose enzyme and it did not affect the activity of the glycerol gradient enzyme, as shown in Table I, indicating that the glycerol gradient enzyme contains sufficient helper protein.

Reconstitution of S-II Sensitive Enzyme from S-II Insensitive Enzyme and Helper Protein. Next we examined whether S-II sensitive RNA polymerase II could be reconstituted from the phosphocellulose enzyme and helper protein. A mixture of the phosphocellulose enzyme and excess helper protein was incubated for 30 min at 0 °C, and then layered on top of a 5–20% sucrose gradient and centrifuged as described in the Materials and Methods; then the RNA polymerase activity in each fraction was assayed in the presence and absence of stimulatory factor S-II. Preparations of phosphocellulose enzyme and glycerol gradient enzyme were used as controls. The result in Figure 4 shows that S-II alone stimulated the phosphocellulose enzyme about 2-fold, whereas when it was preincubated with the helper protein it caused up to 3.5-fold stimulation, giving the same level of activity as that of glycerol gradient enzyme. Helper protein remained at the top of the sucrose gradient under these conditions, as shown in Figure 5. Therefore, it was concluded that enzyme with a similar structure to that of the glycerol gradient enzyme, which is sensitive to S-II, could be reconstituted by mixing the phosphocellulose enzyme with helper protein. As is evident from Figure 4, the glycerol gradient enzyme and phosphocellulose enzyme with and without S-II sedimented to nearly the same position in the sucrose gradient, indicating that addition of helper protein to phosphocellulose enzyme did not alter the molecular weight significantly. The molecular weight of helper protein was estimated to be about 50 000 by gel filtration through Bio-Gel P-100.

Mode of Action of S-II on Reconstituted Enzyme. The S-II sensitivities of reconstituted enzyme recovered from the sucrose

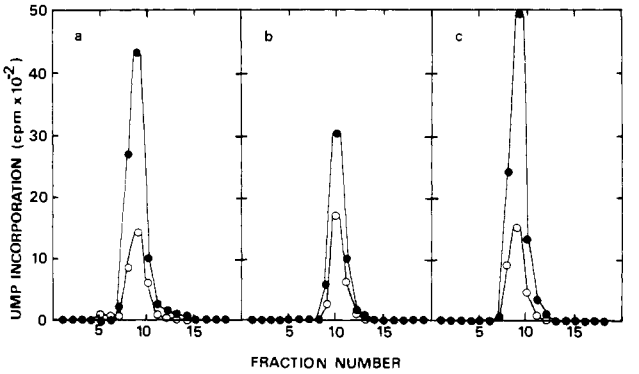

FIGURE 4: Reconstitution of S-II sensitive RNA polymerase II from the phosphocellulose enzyme and helper protein. RNA polymerase II which is sensitive to S-II was reconstituted from 220 units of phosphocellulose enzyme and 120 μg of helper protein, obtained by phosphocellulose chromatography. Reconstituted enzyme was analyzed by sucrose density gradient centrifugation. Procedures are described in detail in the Materials and Methods. Each fraction from the sucrose gradient was assayed for RNA polymerase activity in the presence (●) or absence (○) of 55 units of S-II. As controls, the same units of glycerol gradient enzyme and phosphocellulose enzyme were run simultaneously. (a) Glycerol gradient enzyme; (b) phosphocellulose enzyme; (c) reconstituted enzyme.

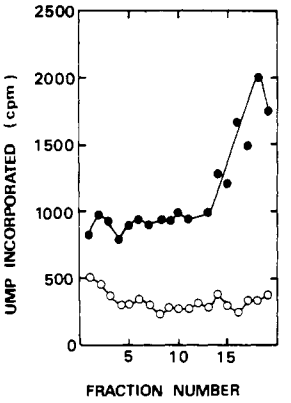

FIGURE 5: Sedimentation profile of helper protein on sucrose density gradient centrifugation. Helper protein (120 μg) was analyzed by sucrose density gradient centrifugation. The experimental conditions were as described in the legend to Figure 4. After fractionation, helper protein activity was assayed in the presence (●) or absence (○) of 55 units of S-II using 12 units of phosphocellulose enzyme under the standard conditions.

gradient and of glycerol gradient enzyme were compared using the same units of enzyme activity. As shown in Figure 6, the stimulations of these two enzymes increased in the same way with increase in the amount of S-II added to the reaction mixture and both were significantly higher than that of the phosphocellulose enzyme.

A characteristic of S-II is that it increases the size of the product RNA. Therefore, the RNA synthesized by the phosphocellulose enzyme under various conditions was analyzed. As shown in Figure 7, the RNA synthesized in the presence of S-II was larger than control RNA. However, when helper protein was present in addition to S-II, although the amount of product increased greatly, its molecular size was not changed. No increase in size or amount of product was detected when helper protein alone was added to the reaction mixture. These results indicate that phosphocellulose enzyme contains a small amount of helper protein and causes production of larger RNA molecules when S-II is present. However, the small amount of helper protein clearly restricted production of large RNA molecules in this system, because production

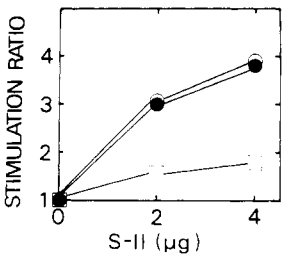

FIGURE 6: Effect of stimulatory factor S-II on the activity of reconstituted RNA polymerase II. RNA synthesis was examined using 5 units of reconstituted enzyme (●), glycerol gradient enzyme (○), or phosphocellulose enzyme (□) under the standard conditions with increasing amounts of stimulatory factor S-II. All the enzyme samples were obtained by sucrose density gradient centrifugation. Stimulation was calculated on the basis of RNA synthesis without S-II.

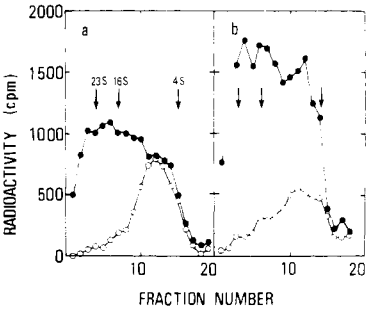

FIGURE 7: Analysis of the molecular size of RNA synthesized in vitro. RNA was synthesized using 20 units of phosphocellulose enzyme in the presence (●) or absence (○) of 40 units of stimulatory factor S-II under the standard conditions. After incubation, 2.5 μg of RNase-free DNase was added and incubation was continued for 15 min more to digest template DNA. Then a final concentration of 0.1% $NaDodSO_4$ was added and the mixture was dialyzed against 0.01 M Tris-HCl buffer, pH 7.9, containing 0.01 M $MgCl_2$ for 90 min. The dialyzate was applied to a sucrose gradient and centrifuged as described in the Materials and Methods. After centrifugation fractions were collected from the bottom of the tube and acid-insoluble radioactivity was measured. (a) Reaction mixture without helper protein; (b) reaction mixture containing 25 μg of helper protein.

increased significantly when helper protein was added exogenously.

Discussion

In this work we found that, when the glycerol gradient enzyme was further purified on a column of phosphocellulose, its sensitivity to a specific stimulatory factor S-II decreased markedly. A similar observation was made by Dahmus with his stimulatory factor HLF_3 of Novikoff ascites tumor cells (Dahmus, 1976). It is well known that σ factor of *E. coli* RNA polymerase is separated by column chromatography on phosphocellulose (Burgess et al., 1969). In this work, we also found that a specific protein fraction, named helper protein, which is essential for the phosphocellulose enzyme to respond to S-II, was separated by phosphocellulose column chromatography. This helper protein had no affect on the glycerol gradient enzyme, indicating that the latter contains sufficient helper protein. Moreover, when the phosphocellulose enzyme was analyzed by sucrose density gradient centrifugation after preincubating it with helper protein, reconstituted RNA polymerase II was obtained which was as sensitive to S-II as glycerol gradient enzyme.

These observations strongly suggest that the helper protein is a component of RNA polymerase II. However, we could not identify the band of helper protein on polyacrylamide gel electrophoresis because the preparations of RNA polymerase II and helper protein were still impure. We found that stimulatory factor S-II enhanced the formation of the initiation complex with RNA polymerase II and homologous DNA. Since stimulation by S-II decreased when the helper protein was removed from RNA polymerase II, this protein may function in the initiation step of transcription by interacting with S-II.

RNA polymerase I may not contain helper protein, because it was not stimulated by S-II (Sekimizu et al., 1976). It seems difficult to remove the helper protein completely from the glycerol gradient enzyme, because the phosphocellulose enzyme was still stimulated by S-II to some extent, though much less than the glycerol gradient enzyme. The functional difference between free and combined helper protein is unknown.

It is tempting to think that regulatory proteins participate in eukaryotic transcription, especially in the initiation step and influence RNA polymerase II so that it selects the necessary genes for transcription. In attempts to find these regulatory proteins, we have been looking for proteins which enhance transcription in vitro and have so far purified one protein named S-II which specifically stimulates the activity of RNA polymerase II. The mechanism of stimulation of RNA synthesis by this protein is unknown, but the present results show that a component of RNA polymerase II, named helper protein, interacts with S-II. In previous studies, 6–10 subunits have been suggested to eucaryotic RNA polymerase II, depending upon the preparation (Kedinger et al., 1974; Schwartz and Roeder, 1975; Greenleaf and Bantz, 1975), although the functions of these subunits are unknown. Helper protein could be one of these subunits. Further studies on S-II and helper protein should provide information on the regulation of eucaryotic transcription.

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Adenosine 3′:5′-Monophosphate Dependent Protein Kinase from Bovine Heart. Characterization of the Catalytic Subunit†

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ABSTRACT: The catalytic subunit of adenosine 3′:5′-monophosphate dependent protein kinase has been isolated in pure form from bovine heart with a 37% yield. The monomeric species (mol wt 39 000) exhibits a strong tendency to aggregate at neutral pH and low ionic strength. It contains 7 tryptophans ($A_{280,1cm}^{1\%}$ = 14.9), no carbohydrate, and 1.7 equiv of acid-stable protein-bound phosphate. The amino terminus of the polypeptide chain is blocked. By isoelectric focusing, 3 major isozymes (pI = 7.01, 7.48, and 7.78) can be separated; they exhibit a similar phosphate content but might differ in their amino acid composition. The catalytic subunit contains three sulfhydryl groups which can be titrated by 5,5′-dithiobis(2-nitrobenzoic acid) with $t_{1/2}$ = 0.36, 3.5, and 110 min, respectively. Substitution of the most reactive thiol group does not inactivate the enzyme; inhibition results from the titration of the second group (presumably located close to the active site) and the third (buried) SH group. None of the cysteinyl side chains is directly involved in catalysis since the percyanylated protein retains 63% of the activity of the native enzyme. The percyano derivative is immune to iodoacetamide inactivation and exhibits an intact protein–substrate binding site, as probed by the use of the protein kinase inhibitor. Cleavage of the *S*-cyano catalytic subunit at pH 9.0 yields a fragment of mol wt 23 000, that originates from the amino end of the molecule, plus two overlapping peptides of mol wt 16 500 and 15 000 that appear to be located on the COOH-terminal half of the protein.

The effects of the cyclic nucleotides, produced upon stimulation of the corresponding cyclases, are mediated through the activation of protein kinases (ATP:protein phosphotransferases, EC 2.7.1.37) which transfer phosphoryl groups to seryl or threonyl side chains of various protein substrates (for reviews, see Walsh and Krebs, 1973; Rubin and Rosen, 1975).

Whereas the cGMP[1]-dependent protein kinase is activated without dissociation into lower molecular weight subunits (Gill et al., 1976; Lincoln et al., 1977), the cAMP-dependent protein kinases, upon binding of the nucleotide, dissociate into two catalytically active subunits (C) and a dimeric, cAMP-regulatory subunit complex (Hofmann et al., 1975).

It is thus possible to study separately the catalytic and the regulatory moieties of the molecule on both a structural and functional level. As a first step toward elucidating the primary structure of the catalytic subunit from bovine heart cAMP-dependent protein kinase (peak II), the enzyme has been isolated in pure form. Some of its properties were studied, with special reference to the several components obtained upon isoelectric focusing, and the reactivity of its thiol groups. Bovine heart was chosen because it is readily available; it contains a high concentration of the cAMP-dependent protein kinase (Hofmann et al., 1977) on which a considerable amount of information is already available (Rosen and Erlichman, 1975; Hofmann et al., 1975; Rangel-Aldao and Rosen, 1976a,b).

Materials and Methods

Histone IIA and protein markers were obtained from Sigma; ovalbumin and pepsin (3100 U/mg) were from Worthington. $^{32}P_i$, [^{14}C]KCN, and 2,5-diphenyloxazole were from New England Nuclear. 5,5′-Dithiobis(2-nitrobenzoic acid) (Nbs_2), citraconic anhydride, and all reagents used in the sequencer were from Pierce. [γ-^{32}P]ATP, ca. 780 mCi/mmol, was prepared by the procedure of Glynn and Chappell (1964). The protein inhibitor of cAMP-dependent protein kinases (PKI) was isolated from rabbit skeletal muscle as previously described (Demaille et al., 1977). The assays for protein kinase and for PKI were also carried out according to Damaille et al. (1977) except that dithiothreitol or 2-mercaptoethanol was omitted from the incubation mixture unless otherwise specified.

Isolation of the Catalytic Subunit. The catalytic subunit of the cAMP-dependent protein kinase (peak II) was prepared from bovine heart essentially as previously described (Beavo et al., 1974; Bechtel et al., 1977; Demaille et al., 1977) with the following minor modifications. (a) C subunit solutions were

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[1] Abbreviations used are: C subunit, the catalytic subunit of the adenosine 3′:5′-monophosphate dependent protein kinase; PKI, protein kinase inhibitor; cAMP, cyclic adenosine 3′:5′-monophosphate; cGMP, cyclic guanosine 3′:5′-monophosphate; EDTA, ethylenediaminetetraacetic acid; CM, carboxymethyl; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet; P_i, inorganic phosphate; Gdn·HCl, guanidine hydrochloride; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); Nbs, nitrothiobenzoate; Pth, phenylthiohydantoin.