enzyme-bound 3-FHB and 4-FHB, respectively.

References

Barnett, J. E. G. (1972) in *Carbon-Fluorine Compounds, Chemistry, Biochemistry and Biological Activities, Ciba Found. Symp.*, pp 95–110, Associated Scientific Publishers, New York, N.Y.

Cohen, T., Dietz, A. G., Jr., & Miser, J. R. (1977) *J. Org. Chem. 42,* 2053.

Deulofeu, V., & Guerrero, T. J. (1955), *Organic Syntheses,* Collect. Vol. III, p 586, Wiley, New York, N.Y.

Dunker, M. F. W., & Starkey, E. B. (1939) *J. Am. Chem. Soc. 61,* 3005.

Dwek, R. A. (1972) in *Carbon-Fluorine Compounds, Chemistry, Biochemistry, and Biological Activities, Ciba Found. Symp.*, pp 239–270, Scientific Publishers, New York, N.Y.

English, J., Mead, J. F., & Niemann, C. (1940) *J. Am. Chem. Soc. 62,* 350.

Fujisawa, H., & Hayaishi, O. (1968) *J. Biol. Chem. 243,* 2673.

Fujisawa, H., Hiromi, K., Uyeda, M., Nozaki, M., & Hayaishi, O. (1971) *J. Biol. Chem. 246,* 2320.

Fujisawa, H., Hiromi, K., Uyeda, M., Okuno, S., Nozaki, M., & Hayaishi, O. (1972a) *J. Biol. Chem. 247,* 4422.

Fujisawa, H., Uyeda, M., Kojima, Y., Nozaki, M., & Hayaishi, O. (1972b) *J. Biol. Chem. 247,* 4414.

Fujiwara, M., & Nozaki, M. (1973) *Biochim. Biophys. Acta 327,* 306.

Gutfreund, H. (1972) in *Enzymes: Physical Principles,* p 206, Wiley-Interscience, London.

Hamilton, G. A. (1974) in *Mechanisms of Oxygen Activation* (Hayaishi, O., Ed.) pp 450–451, Academic Press, New York, N.Y.

Lipscomb, J., Howard, J., Lorsback, T., & Wood, J. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 923 Abstr.

Lowry, O. H., Rosebrough, N. T., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193,* 265.

May, S. W., & Phillips, R. S. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol., 36,* 2957, Abstr.

Minor, J. T., & Vanderwert, C. A. (1952) *J. Org. Chem. 17,* 1425.

Rouche, H. (1921) *Bull. Sci. Acad. R. Belg.,* 534.

Scatchard, G., Coleman, J. S., & Shin, A. L. (1957) *J. Am. Chem. Soc. 79,* 12.

Stanier, R. Y., & Ingraham, J. L. (1954) *J. Biol. Chem. 210,* 799.

Tyson, C. (1975) *J. Biol. Chem. 250,* 1765.

Wells, M. C. C. (1972) Ph.D., University of Texas at Austin, University Microfilms, Ann Arbor, Mich.

Wood, J. M., Lipscomb, J. D., Que, L., Jr., Stephens, R. S., Orme-Johnson, W. H., Munck, E., Ridley, W. P., Dizikes, L., Cheh, A., Francia, M., Frick, T., Zimmerman, R., & Howard, J. (1977) in *Biological Aspects of Inorganic Chemistry,* p 261, Wiley, New York, N.Y.

Yoshida, R., Hori, K., Fujiwara, M., Saeki, Y., Kagamiyama, H., & Nozaki, M. (1976) *Biochemistry 15,* 4048.

Zaborsky, O. R., Hou, C. T., & Ogletree, J. (1975) *Biochim. Biophys. Acta 386,* 18.

Plant DNA-Dependent RNA Polymerases: Subunit Structures and Enzymatic Properties of the Class II Enzymes from Quiescent and Proliferating Tissues†

Tom J. Guilfoyle* and Jerry J. Jendrisak

ABSTRACT: Class II DNA-dependent RNA polymerases were purified from soybean tissues of different physiological states: (1) from seed embryo tissue, representative of a quiescent, low metabolic state and (2) from auxin-treated hypocotyl tissue, representative of a highly proliferative and metabolically active state. Dodecyl sulfate, polyacrylamide gel electrophoresis indicates that RNA polymerase II from embryonic tissue consists largely (90–95%) of the form IIA enzyme, the largest subunit having a molecular weight of 215 000. RNA polymerase II from hypocotyl tissue is exclusively a form IIB enzyme, the largest subunit having a molecular weight of 180 000. Polypeptides common to RNA polymerases IIA and IIB have the following molecular weights: 138 000; 42 000; 27 000; 22 000; 19 000; 17 600; 17 000; 16 200; 16 100; and 14 000. Peptide mapping in the presence of dodecyl sulfate suggests that the 215 000 and 180 000 subunits possess similar peptide fragments. Plant embryo tissues do not contain protease activity capable of cleaving the 215 000 subunit to the 180 000 subunit, but proliferating plant tissues do contain such an activity. Mixing experiments indicate that appreciable amounts of RNA polymerase IIB are not being artifactually produced during protein purification.

DNA-dependent RNA polymerase II enzymes have been purified to homogeneity from a number of plant and animal tissues and apparently exist in two or three forms which differ from one another in the molecular weight of their largest subunits (for reviews, see: Roeder, 1976; Chambon, 1975). The two major types of class II RNA polymerase, IIA and IIB, possess largest subunits of approximately 215 000 and 180 000, respectively. It has been suggested that RNA polymerase IIA gives rise to IIB enzyme via proteolytic cleavage of the 215 000 polypeptide (Dezelée et al., 1976; Greenleaf et al., 1976;

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Guilfoyle & Key, 1977b). In addition, it has been demonstrated that certain cells and tissues possess a protease activity capable of converting the 215 000 polypeptide to a 180 000 polypeptide in vitro (Dezelee et al., 1976; Greenleaf et al., 1976). Whether this proteolytic cleavage plays a functional role in regulating transcription by RNA polymerase II or is simply an artifact produced during enzyme purification remains to be elucidated.

In the present study, we have purified soluble RNA polymerase II from quiescent soybean embryonic axes and highly proliferative soybean hypocotyl tissue (induced by 2,4-dichlorophenoxyacetic acid) to determine which form, IIA or IIB, is dominant in each case and whether any other subunit differences exist in quiescent and rapidly proliferating tissue. We have compared the subunit molecular weights, subunit charges, and α-amanitin sensitivities of soybean RNA polymerase IIA and IIB. In addition, we have compared the peptide maps of the largest subunits of IIA and IIB enzymes which were generated by limited proteolysis (Cleveland et al., 1977). Finally, we have shown that proliferating plant tissues possess a protease activity which is absent in quiescent tissues. This protease cleaves the 215 000 subunit of IIA enzyme to a 180 000 polypeptide in vitro; however, mixing experiments with quiescent and proliferating tissues indicate that this proteolytic conversion does not appear to occur extensively during our purification procedures.

Experimental Procedures

Materials. Soybean (*Glycine max*) embryonic axes were obtained from Edible Soy Products, Inc., Hudson, Iowa. The embryonic axes were purified away from cotyledonary tissue by adjusting the density of a carbon tetrachloride–cyclohexane solution until contaminating cotyledonary tissues float away from the embryonic axes. Soybean seed (*Glycine max* var. Evans) was germinated in moist vermiculite in the dark for 5 days at 30 °C. Forty-eight hours prior to harvest of the mature hypocotyls, the seedlings were sprayed to run-off with a 2.5×10^{-3} M solution (pH 6.0) of 2,4-dichlorophenoxyacetic acid which induces cell proliferation in mature hypocotyls.

Wheat germ prepared from hexaploid wheat (*Triticum aestivum*) was obtained from General Mills, Vallejo, Calif. Cauliflower (*Brassica oleracea* var. *botrytis*) inflorescences were purchased from a local market. Acrylamide, methylenebis(acrylamide), ammonium persulfate, tetraethylmethylenediamine, sodium dodecyl sulfate, Coomassie brilliant blue R-250, and agarose were purchased from Bio-Rad. Enzyme grade ammonium sulfate and UltraPure urea were purchased from Schwarz/Mann. Tris base, glycine, dithiothreitol, 2-mercaptoethanol, nucleoside triphosphates, calf thymus DNA (type IV), and phenylmethanesulfonyl fluoride were obtained from Sigma. Ethylene glycol and glycerol were Baker Analyzed Reagents. DEAE-cellulose (DE-52) and phosphocellulose (P-11) were obtained from Whatman. Polymin P (polyethylenimine) was obtained from BASF, Rhein, West Germany. [5-^{3}H]UTP (specific activity, 18 Ci/mmol) was purchased from New England Nuclear. α-Amanitin was purchased from Henley and Co., New York, N.Y. Poly(dA-dT) was from Grand Island Biological Co., New York, N.Y. *Staphylococcus aureus* V8 protease was obtained from Miles. Soybean trypsin inhibitor was from Worthington. Highly purified soybean embryo DNA was a gift to Carolyn Silflow (Univeristy of Georgia, Athens, Ga.) and was prepared as described (Gurley, 1977). All other chemicals were analytical reagent grade.

Calf thymus DNA was dissolved (1 mg/mL) in a buffer consisting of 50 mM Tris-HCl (pH 7.2 at 25 °C), 1 mM ethylenediaminetetraacetic acid, and 100 mM NaCl and was purified by two successive extractions with water saturated phenol–*m*-cresol-8-hydroxyquinoline (Kirby, 1965). The purified calf thymus and soybean DNAs were precipitated with 2 volumes of 95% ethanol and dissolved (1 mg/mL) in the above buffer.

Buffers. Homogenizing buffer A was 50 mM Tris-HCl (pH 7.9 at 4 °C), 250 mM sucrose, 1 mM $MgCl_2$, and 15 mM 2-mercaptoethanol. Immediately prior to use, 0.01 volume of 100 mM phenylmethanesulfonyl fluoride in 100% dimethyl sulfoxide was added to homogenizing buffer A. Homogenizing buffer B was 50 mM Tris-HCl (pH 7.9 at 4 °C), 75 mM ammonium sulfate, 1 mM ethylenediaminetetraacetic acid, and 15 mM 2-mercaptoethanol. Chromatography buffer was 50 mM Tris-HCl (pH 7.9 at 4 °C), 1 mM ethylenediaminetetraacetic acid, 15 mM 2-mercaptoethanol, and 25% ethylene glycol. Storage buffer was 50 mM Tris-HCl (pH 7.9 at 4 °C), 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, and 50% glycerol.

RNA Polymerase II Purification. Soybean hypocotyls were homogenized with a Polytron PT20ST in 2 volumes of homogenizing buffer A or B and RNA polymerase II was purified by the method described by Jendrisak & Burgess (1975). Homogenization in either buffer gave a similar yield and overall purification. When homogenizing buffer A was used, the homogenate was filtered through eight layers of cheesecloth followed by filtration through Miracloth (Calbiochem). After filtration, Triton X-100 was added to a final concentration of 0.5% with rapid stirring, and the filtrate was then centrifuged at 10 000 rpm (Beckman JA-10 rotor) for 20 min. The supernatant was adjusted to 75 mM ammonium sulfate and Polymin P was added to a final concentration of 0.75% as described (Jendrisak & Burgess, 1975). All additional procedures with homogenizing buffer A or B were identical with those described by Jendrisak & Burgess (1975).

Soybean embryonic axes were pulverized with a Wiley Mill and homogenized in a Waring blender in homogenizing buffer B. Soybean embryo RNA polymerase II as well as wheat germ and cauliflower inflorescence RNA polymerase II were purified by the method of Jendrisak & Burgess (1975).

DNA–agarose chromatography was utilized as a final step of purification to remove trace polypeptide contaminants from the Form IIA enzymes. DNA–agarose was prepared from wheat germ DNA (Jendrisak & Becker, 1973) by the method of Schaller et al. (1972). Prior to DNA–agarose chromatography, RNA polymerase preparations were dialyzed for 16 h at 4 °C against 50 volumes of chromatography buffer containing 50 mM ammonium sulfate. These were applied to 2-mL columns of DNA–agarose (4 mg of DNA/mL packed resin) equilibrated with chromatography buffer containing 75 mM ammonium sulfate. After applying a sample, the column was washed with 20 column volumes of chromatography buffer containing 75 mM ammonium sulfate, and RNA polymerase II was eluted from the column with chromatography buffer containing 300 mM ammonium sulfate. Purified enzymes were concentrated by dialysis against storage buffer and were frozen at −70 °C. All purification procedures were carried out at approximately 4 °C. RNA polymerase activities were assayed throughout purification as described by Guilfoyle et al. (1975), unless indicated otherwise in the figure and table legends.

Mixing Experiments. One hundred grams of wheat germ or soybean embryos was homogenized with 1000 g of soybean hypocotyl or cauliflower inflorescence tissue in 2000 mL of homogenizing buffer B. These quantities of tissue were chosen to yield approximately equal amounts of RNA polymerase II from each tissue as determined from previous purification data.

TABLE I: Summary of the Purification of RNA Polymerase II from 1-kg Amounts of Soybean Embryonic Axes and Soybean Hypocotyls.[a]

Fraction	Protein (mg)		Spec act. (units/mg)		Yield (%)	
	Embryo	Hypocotyl	Embryo	Hypocotyl	Embryo	Hypocotyl
1. Crude extract	238 000	30 500	0.048	0.078	100	100
2. $(NH_4)_2SO_4$ precipitate	1 550	628	8.3	5.0	113	132
3. DEAE-Cellulose	126	7.8	69	250	76	82
4. Phosphocellulose	17.7	1.8	380	520	50	39
5. DNA agarose	10.2		550		49	

[a] Fraction 2 represents the $(NH_4)_2SO_4$ precipitate of the Polymin P eluate (Jendrisak & Burgess, 1975). A unit is 1 nmol of UTP incorporated into RNA per 30 min at 30 °C using the assay method of Guilfoyle et al. (1975).

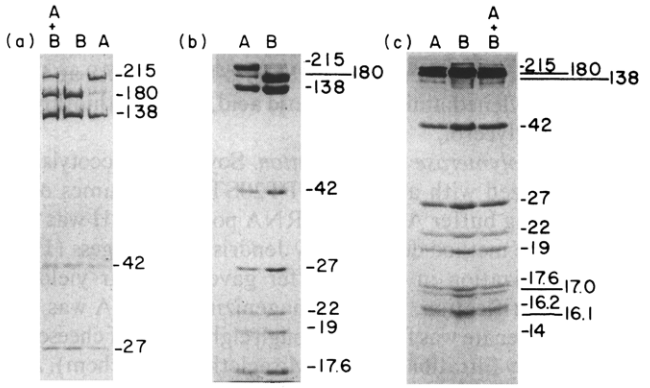

FIGURE 1: Polypeptide subunits of soybean RNA polymerases IIA and IIB separated on (a) 7.5%, (b) 10%, and (c) 15% polyacrylamide gels containing dodecyl sulfate. Enzymes were subjected to electrophoresis individually or as mixtures (2–10 μg amounts) on 0.75-mm thick slab gels. Migration was toward the anode from top to bottom. The scales to the right of the figures indicate subunit molecular weights in kilodaltons. Details of electrophoresis, staining, and destaining are described in the text.

In the mixing experiments, enzymes were purified to homogeneity utilizing the methods of Jendrisak & Burgess (1975) with the addition of DNA–agarose chromatography.

Peptide Mapping. Peptide cleavage in the presence of dodecyl sulfate and analysis by polyacrylamide gel electrophoresis were performed essentially by the method of Cleveland et al. (1977).

Plant Protease Activity. One gram of soybean embryos or wheat germ and 10 g of soybean hypocotyl or cauliflower inflorescence were homogenized individually in 20 mL of homogenizing buffer B with a Polytron PT20ST. The homogenates were filtered through one layer of Miracloth and centrifuged for 3 h at 50 000 rpm with a Beckman Ti 75 rotor. Supernatants were collected and adjusted to a protein concentration of 0.5 mg/mL with homogenizing buffer B. Ten-microliter portions were assayed for protease activity by incubation at 30 °C for 2 h using soybean embryo or wheat germ RNA polymerase IIA (3 μg) as substrate. Following the incubation period, dodecyl sulfate and 2-mercaptoethanol were both added to final concentrations of 2% and samples were immediately placed in a boiling water bath for 1 min. These samples were subjected to polyacrylamide slab gel electrophoresis in the presence of dodecyl sulfate, carried out on 10% gels as described below.

Polyacrylamide Gel Electrophoresis. One-dimensional gel electrophoresis in the presence of dodecyl sulfate was performed on 0.75-mm thick slab gels by the method of Laemmli (1970). Two-dimensional gel electrophoresis with the first dimension in 8 M urea and the second dimension in sodium dodecyl sulfate was performed as described by Jendrisak & Burgess (1977). Polyacrylamide gels were stained with Coomassie brilliant blue R and destained as previously described (Burgess & Jendrisak, 1975). Subunit molecular weights and molar ratios were determined as described previously (Jendrisak & Burgess, 1977).

Results

Purification of Soybean RNA Polymerase II Enzymes. Table I summarizes the purification of soluble RNA polymerase II from soybean embryonic axes and rapidly proliferating soybean hypocotyl tissue. Approximately 20 mg of RNA polymerase II is present per kg of ungerminated embryonic axes (assuming a recovery of 50%; see Table I), while approximately 4 mg of RNA polymerase II is present per kg of proliferating hypocotyl tissue (assuming a recovery of 40%; see Table I). The fivefold higher level of enzyme (based on fresh weight) present in embryonic axes is probably due primarily to the dehydrated state of this tissue. This high recovery and overall purification indicate that the method originally described for the purification of RNA polymerase II from wheat germ by Jendrisak & Burgess (1975) may be utilized as a routine procedure for the purification of plant RNA polymerase II enzymes. Although homogeneity of RNA polymerase II can sometimes be achieved following phosphocellulose chromatography, in other cases contaminating polypeptides of approximately 100 000 are present at this stage of purification (Guilfoyle & Key, 1977a,b). An additional chromatographic step utilizing DNA–agarose may then be required to achieve final purification. The presence of the protease inhibitor, phenylmethanesulfonyl fluoride, does not alter the type (IIA or IIB) or amount of RNA polymerase II recovered from quiescent embryo or rapidly growing hypocotyl tissues.

Subunit Structures of RNA Polymerase II from Soybean Embryonic Axis and Soybean Hypocotyl. The polypeptides associated with soybean RNA polymerase II purified from embryonic axes and hypocotyl tissue are compared by gel electrophoresis on 7.5%, 10%, and 15% polyacrylamide gels containing dodecyl sulfate (Figure 1). Electrophoresis on 7.5% and 10% gels shows that the largest subunits associated with the RNA polymerase II enzymes purified from embryonic axes and proliferating hypocotyls are significantly different in molecular weight. The enzyme purified from embryonic axes possesses two predominant high molecular weight subunits of 215 000 and 138 000 with a minor component of 180 000. This RNA polymerase II enzyme is approximately 90% IIA and 10% IIB. In contrast to the embryo RNA polymerase II, the enzyme purified from proliferating hypocotyl tissue possesses high molecular weight subunits of 180 000 and 138 000 and is exclusively a IIB enzyme.

Although electrophoresis on 7.5% and 10% polyacrylamide gels resolves several smaller polypeptides associated with RNA polymerase IIA and IIB enzymes, electrophoresis on 15%

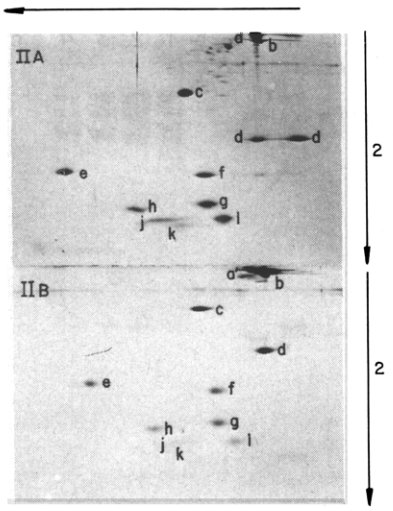

FIGURE 2: Polypeptide subunits of soybean RNA polymerases IIA and IIB separated on a two-dimensional polyacrylamide gel electrophoresis system. The first dimension (horizontal) consisted of electrophoresis of individual enzymes on 7.5% polyacrylamide cylindrical gels containing 8 M urea at pH 8.7, and the second dimension (vertical) consisted of electrophoresis of polypeptides from the urea gel onto a 1.5-mm thick, 15% polyacrylamide slab gel containing dodecyl sulfate. Approximately 50 μg of each enzyme was subjected to analysis. Directions of migration are indicated in the figure. Letters correspond to the polypeptide subunits listed in the text. Additional details of this gel system are presented elsewhere (Jendrisak & Burgess, 1977).

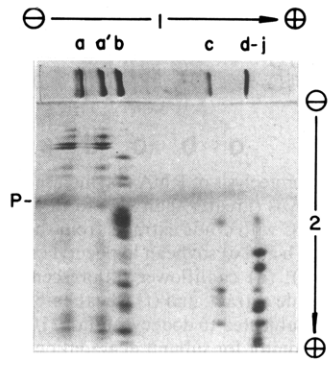

FIGURE 3: Peptide mapping of soybean RNA polymerases IIA and IIB high molecular weight subunits by limited proteolysis in dodecyl sulfate and analysis by gel electrophoresis. The first dimension (1) represents a track from a 0.75-mm thick, 5% polyacrylamide, dodecyl sulfate slab gel to which was applied an equimolar mixture of soybean RNA polymerases IIA and IIB (25 μg of total protein). This was stained in order to visualize the positions of the RNA polymerase II subunits which are indicated by letter. An identical track was equilibrated in dodecyl sulfate sample buffer and was positioned on top of the stacking gel of a 15% polyacrylamide, dodecyl sulfate, 1.5-mm thick slab gel. Sample buffer (0.5 mL) containing 100 μg/mL of *Staphylococcus aureus* V8 protease was layered over the sample gel and electrophoresis carried out at 12.5 mA until the marker dye reached the bottom. Migration of the protease is indicated by the letter P. The gel was stained with Coomassie blue as described in the text. Directions of migration (toward the anode in both dimensions) are indicated by the arrows.

polyacrylamide gels is necessary to totally resolve the low molecular weight subunits associated with these enzymes. Figure 1c shows that RNA polymerase IIA and IIB enzymes possess identical low molecular weight polypeptides of 42 000, 27 000, 22 000, 19 000, 17 600, 17 000, 16 200, 16 100, and 14 000. The molar ratio of each low molecular weight subunit is near unity except for the 27 000 polypeptide which is present at a molar ratio of approximately 2 in both RNA polymerase IIA and IIB and the 14 000 subunit which is present at less than stoichiometric amounts in both enzymes. To facilitate discussion, the subunits associated with RNA polymerases IIA and IIB will sometimes be referred to by letters: 215 000 (a); 180 000 (a′); 138 000 (b); 42 000 (c); 27 000 (d); 22 000 (e); 19 000 (f); 17 600 (g); 17 000 (h); 16 200 (i); 16 100 (j); 14 000 (k).

To determine whether the low molecular weight polypeptides associated with RNA polymerase IIA and IIB enzymes are identical in charge as well as molecular weight, we compared the subunit structures by two-dimensional polyacrylamide gel electrophoresis where polypeptides are separated largely on the basis of charge in the first dimension (8 M urea, pH 8.7, 7.5% polyacrylamide) and on the basis of molecular weight in the second dimension (dodecyl sulfate, 15% polyacrylamide). The high molecular weight subunits (215 000, 180 000, and 138 000) fail to migrate into the separating gel in the first dimension and are found at the stacking gel–separating gel interface. The low molecular weight (less than 45 000) polypeptides of embryo RNA polymerase II migrate identically with those of hypocotyl RNA polymerase II in the two-dimensional gel system (Figure 2) with the possible exception of the 27 000 polypeptide. In RNA polymerase IIA, some of the 27 000 polypeptide fails to migrate into the stacking gel in the presence of 8 M urea at pH 8.7, while some of the 27 000 subunit is also found at the top of the stacking gel–separating gel interface. However, in the IIB enzyme, the 27 000 subunit is found exclusively at the stacking gel–separating gel interface. In both RNA polymerase IIA and IIB enzymes, this polypeptide is basic in charge as evidenced by its failure to migrate into the separating gel in the presence of 8 M urea at pH 8.7. The heterogeneity in the 27 000 polypeptide could result from subunit modification (e.g., phosphorylation, adenylation, or ADP-ribosylation), but might also be an artifact of electrophoresis.

Peptide Mapping of RNA Polymerase II Subunits by Limited Proteolysis. To determine whether the largest subunit of soybean RNA polymerase IIA is structurally related to the largest subunit of IIB enzyme, we analyzed the peptide maps of the two subunits following limited proteolysis of the polypeptides with *S. aureus* V8 protease (Figure 3). We first subjected an equal mixture of soybean IIA and IIB enzyme to polyacrylamide gel electrophoresis in the presence of dodecyl sulfate as described in the legend of Figure 3. This effectively resolved the large subunits. The subunits were then electrophoresed from this gel into a second polyacrylamide gel in the presence of *S. aureus* V8 protease and dodecyl sulfate. The peptide maps generated by this procedure are nearly identical for the largest subunits of RNA polymerase IIA (subunit a) and IIB (subunit a′). The peptide maps of subunits b, c, and d–k which are common to IIA and IIB enzymes are highly different from the a and a′ peptide maps. This result indicates that subunits a and a′ possess similar peptide fragments and suggests that subunit a may be a precursor of subunit a′.

In Vitro Proteolysis of RNA Polymerase IIA. Since the above result suggested that subunit a is a precursor of subunit a′, we examined crude extracts from quiescent and proliferating tissues for protease activity which could cleave subunit a to the a′ subunit in vitro. Extracts of two quiescent tissues, soybean embryonic axes and wheat germ, and two proliferating tissues, soybean hypocotyl and cauliflower inflorescence, were assayed for protease activity using either wheat germ or soybean RNA polymerase IIA as substrate. Figure 4 shows the results obtained utilizing wheat germ RNA polymerase IIA as substrate. Neither quiescent tissue extract contained protease activity which cleaved subunit a to a′; however, both proliferative tissue extracts contained protease activity which

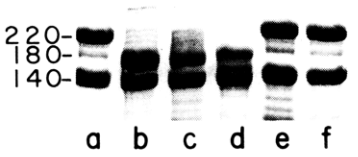

FIGURE 4: In vitro proteolysis of RNA polymerase IIA. RNA polymerase II from wheat embryos (greater than 95% form IIA enzyme) was incubated for 2 h at 37 °C with crude extracts from various plant tissues: (a) buffer A (control); (b and c) soybean hypocotyl crude extracts (two different preparations); (d) cauliflower inflorescence crude extract; (e) soybean embryo crude extract; and (f) wheat embryo crude extract. The proteins were then subjected to dodecyl sulfate, 10% polyacrylamide gel electrophoresis to monitor for subunit degradation. The scale to the right of the figure indicates subunit molecular weights in kilodaltons. Polypeptides which are observed below the 140 000 subunit are present in the crude extracts and are not products of proteolysis.

completely degraded subunit a to a′ during a 2-h incubation at 30 °C. Similar results were obtained utilizing soybean RNA polymerase IIA as substrate (e.g., only proliferative tissue extracts contained protease activity which degraded subunit a to a′). The in vitro proteolytic conversion of subunit a to a′ was not inhibited by 2 mM phenylmethanesulfonyl fluoride nor by various concentrations (10–1000 μg/mL) of soybean trypsin inhibitor. In addition, none of the other subunits (b–k) of wheat germ or soybean RNA polymerase IIA were proteolytically degraded in vitro (data not shown).

Mixing Experiments with Quiescent and Proliferative Tissues. Since the proliferative tissues, soybean hypocotyl and cauliflower inflorescences, possess protease activities capable of converting the a subunit to the a′ subunit in vitro and yield only RNA polymerase IIB when the enzymes are purified (Guilfoyle, 1976; Guilfoyle & Key, 1977b), we designed experiments to test whether proteolytic cleavage of subunit a to a′ was occurring during our purification procedure. Mixtures of soybean embryonic axes and soybean hypocotyl, wheat germ and soybean hypocotyl, or wheat germ and cauliflower inflorescence were homogenized in proportions which were calculated from previous purification data to yield equal quantities of RNA polymerase II from each tissue. If RNA polymerase IIA is converted to IIB enzyme during our purification procedure, then soybean embryo or wheat germ RNA polymerase IIA should be converted to the IIB enzyme when purified in the presence of tissues that yield only IIB enzyme. The advantage in using wheat germ in mixing experiments is that the low molecular weight subunits of RNA polymerase II can be distinguished from the analogous subunits in both soybean and cauliflower on dodecyl sulfate, polyacrylamide gels. This serves as a check that our purification procedure did not favor the selective purification of enzyme from one tissue.

Figure 5 shows that mixtures of wheat germ and soybean hypocotyl or wheat germ and cauliflower inflorescence tissues yield nearly equal quantities of RNA polymerases IIA and IIB and nearly equal quantities of RNA polymerase II from each tissue. This result indicates that enzyme IIB does not arise as an artifact of subunit proteolysis during purification. Similar results were obtained using mixtures of soybean embryonic axes and soybean hypocotyl tissues.

Enzymatic Properties of Soybean RNA Polymerases IIA and IIB. Both soybean RNA polymerases IIA and IIB were titrated with α-amanitin to determine whether each enzyme is equally sensitive to inhibition by the fungal toxin. Figure 6 shows that both enzymes display nearly identical inhibition curves with α-amanitin and 50% inhibition is obtained in each case at approximately 0.05 μg/mL α-amanitin. These inhibition curves are similar to those described for other plant class

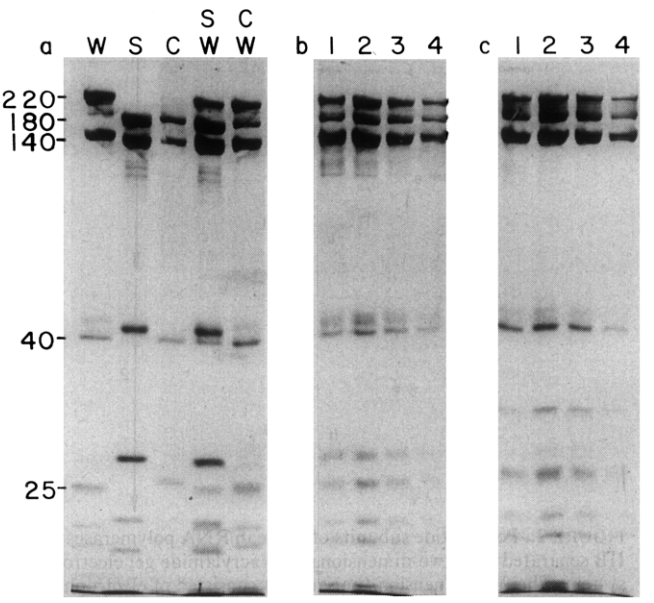

FIGURE 5: The extent of RNA polymerase IIA proteolysis during enzyme purification as examined by dodecyl sulfate, 10% polyacrylamide gel electrophoresis. (a) Standards of purified RNA polymerases (5–10-μg amounts) from wheat (W) embryos, soybean (S) hypocotyls, and cauliflower (C) inflorescences were subjected to electrophoresis individually and as mixtures of two enzymes. (b) Class II RNA polymerases were purified from a mixture of soybean hypocotyls and wheat embryos. The gels contain aliquots of successive fractions resulting from DNA agarose chromatography, the final purification step. (c) Class II RNA polymerases were purified from a mixture of cauliflower inflorescences and wheat embryos. The gels contain aliquots of successive fractions resulting from DNA agarose chromatography, the final purification step. Additional details are presented in the text.

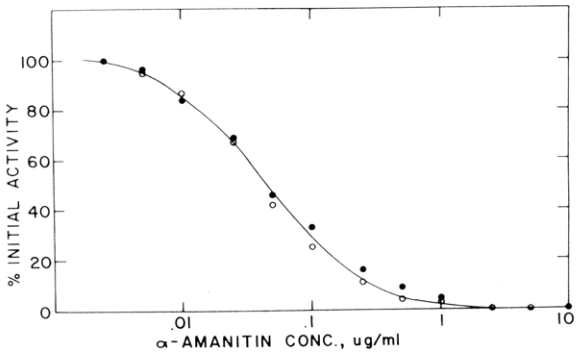

FIGURE 6: Effect of α-amanitin on the activities of soybean RNA polymerases IIA (●) and IIB (○). RNA polymerase activities were determined with 5 mM $MgCl_2$, 25 mM $(NH_4)_2SO_4$, and heat denatured soybean DNA (10 μg/mL) in the presence of the indicated concentration of α-amanitin.

II enzymes including wheat, rye, cauliflower, and maize (Guilfoyle, 1976; Jendrisak & Guilfoyle, 1978).

RNA polymerases IIA and IIB display nearly identical divalent cation optima. Utilizing native soybean DNA (10 μg/mL) as template and 25 mM ammonium sulfate, the $MgCl_2$ and $MnCl_2$ optima for both RNA polymerases IIA and IIB are 2–10 and 2 mM, respectively. For each enzyme, a relatively broad optimum is obtained with $MgCl_2$ and a relatively sharp optimum is obtained with $MnCl_2$. The ionic strength optima for RNA polymerases IIA and IIB using ammonium sulfate were determined in the presence of either $MgCl_2$ or $MnCl_2$ on three different templates including native soybean DNA, heat denatured soybean DNA, and poly(dA-

dT). Similar optima were found for the two forms of the enzyme on each template with either divalent cation (data not shown).

One additional observation noted during enzymatic characterization of RNA polymerase IIA and IIB deserves comment. Commercial preparations of DNA may contain proteases capable of cleaving RNA polymerase during the assay for RNA polymerase activity (Jendrisak, unpublished). These DNA associated proteases are capable of degrading subunits a, a′, and b during in vitro assays without altering the specific activity of the enzymes (Jendrisak, unpublished). Thus when comparing RNA polymerase IIA and IIB activities on templates in vitro, caution must be exercised (i.e., templates and enzyme preparations must be shown to be free of protease activity).

Discussion

This study shows that the subunit structures of soybean RNA polymerases IIA and IIB differ only in the molecular weight of their largest subunits which are 215 000 for IIA and 180 000 for IIB. Soybean RNA polymerases IIA and IIB have 10 subunits in common with the following molecular weights: 138 000, 42 000, 27 000, 22 000, 19 000, 17 600, 17 000, 16 200, 16 100, and 14 000. The number of and molecular weights of the putative RNA polymerase IIA and IIB subunits differ somewhat from previously published data (Guilfoyle & Key, 1977b) due to improvement in the resolution of polypeptides on dodecyl sulfate, polyacrylamide gels (Jendrisak & Burgess, 1977).

Our results indicate that with the exceptions of the alteration in the molecular weight of the largest subunit and the possible alteration in molecular charge of the 27 000 subunit, the remaining subunits of soybean RNA polymerase II are unchanged during totally different growth states. In one case, the ungerminated embryonic axes, the tissue is in a quiescent state and the rate of transcription is negligible (Guilfoyle, unpublished; Spiegel et al., 1975). In the second case, the auxin-treated soybean hypocotyl, the tissue is in a highly proliferative state and the rate of transcription is very high (Guilfoyle & Key, 1977a). Although some minor modification in subunit structure (e.g., phosphorylation, adenylation, or ADP-ribosylation) might not be detected in this study, it is evident that extensive subunit modification does not occur.

In a preliminary report (Guilfoyle & Key, 1977b), it was suggested that the alteration in the largest subunit might play some role in regulating transcription by RNA polymerase II. Since a 215 000 polypeptide is associated with RNA polymerase II purified from quiescent tissue and a 180 000 subunit is associated with RNA polymerase II from rapidly proliferating tissue, it is possible that RNA polymerase IIA is a storage or precursor form of the enzyme which is proteolytically cleaved before it becomes active in transcription. An alternative possibility is that actively proliferating soybean hypocotyl tissue possesses a protease activity capable of artifactually converting the 215 000 polypeptide to the 180 000 polypeptide during purification procedures. A final possibility is that the 215 000 and 180 000 polypeptides are products of different genes and do not share a precursor–product relationship.

Our results support the following relationship between the 215 000 and 180 000 subunits. The similarity of the peptide maps of the two subunits indicates that the a and a′ subunits are structurally related and suggests that they may be products of the same gene. Although proliferating or metabolically active tissues possess protease activity capable of converting the 215 000 to the 180 000 polypeptide in vitro, this proteolysis does not appear to occur extensively during our purification procedure as supported by mixing experiments. Thus, our results suggest that proteolytic cleavage of the 215 000 subunit may occur in vivo, but what if any role this plays in the regulation of transcription remains to be elucidated.

Our results are similar to those of Krebs & Chambon (1976) who suggested that proteolytic conversion of the largest subunits of hen liver RNA polymerase II does not occur during routine purification procedures, but, in addition, our results rule out the possibility that proteolysis could occur during isolation of nuclei since our purification procedure does not require this step. Our results differ from those found with yeast (Dezelée et al., 1976) and *Drosophila* class II RNA polymerases (Greenleaf et al., 1976). In each case, it was concluded that RNA polymerase IIB arises artifactually via proteolytic conversion of IIA during purification of the enzymes. However, neither of these studies demonstrated that proteolysis occurs only as an artifact and that such a conversion does not also occur in vivo. The mere presence of a protease activity in a tissue which is capable of converting RNA polymerase IIA to IIB in vitro does not necessarily indicate that this proteolysis occurs during purification of the enzymes. In addition, the yeast studies are complicated by the fact that several yeast RNA polymerase II subunits are sensitive to proteolytic attack in addition to the 215 000 polypeptide.

The conservation of the 215 000 polypeptide and its proteolytically derived product, the 180 000 polypeptide, is striking. This conservation in structure ranges from lower eukaryotes such as yeast to higher animals and plants and may be indicative of a structure required for some aspect of transcriptional activity or its regulation.

References

Burgess, R. R. & Jendrisak, J. J. (1975) *Biochemistry 14,* 4634–4638.

Chambon, P. (1975) *Annu. Rev. Biochem. 44,* 613–638.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W., & Laemmli, U. K. (1977) *J. Biol. Chem. 252,* 1102–1106.

Dezelée, S., Wyers, F., Sentenac, A., & Fromageot, P. (1976) *Eur. J. Biochem. 65,* 543–552.

Greenleaf, A. L., Haars, R., & Bautz, E. K. F. (1976) *FEBS Lett. 71,* 205–208.

Guilfoyle, T. J. (1976) *Plant Physiol. 58,* 453–458.

Guilfoyle, T. J., & Key, J. L. (1977a) in *Nucleic Acid and Protein Synthesis in Plants* (Bogorad, L., & Weil, J., Eds.) pp 37–63, Plenum Press, New York, N.Y.

Guilfoyle, T. J., & Key, J. L. (1977b) *Biochem. Biophys. Res. Commun. 74,* 308–313.

Guilfoyle, T. J., Lin, C. Y., Chen, Y. M., Nagao, R. T., & Key, J. L. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 69–72.

Gurley, W. B. (1977) Ph.D. Thesis, University of Georgia, Athens, Georgia.

Jendrisak, J. J., & Becker, W. M. (1973) *Biochim. Biophys. Acta 319,* 48–54.

Jendrisak, J. J., & Burgess, R. R. (1975) *Biochemistry 14,* 4639–4645.

Jendrisak, J. J., & Burgess, R. R. (1977) *Biochemistry, 16,* 1959–1964.

Jendrisak, J. J., & Guilfoyle, T. J. (1978) *Biochemistry 17,* (in press).

Kirby, K. S. (1965) *Biochem. J. 96,* 266–269.

Krebs, G., & Chambon, P. (1976) *Eur. J. Biochem. 61,* 15–25.

Laemmli, U. K. (1970) *Nature (London) 227*, 680-685.
Roeder, R. G. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) pp 285-329, Cold Spring Harbor, New York, N.Y.
Schaller, H., Nüsslein, C., Benhoeffer, F. J., Rusz, C., & Nuitzschmann, J. (1972) *Eur. J. Biochem. 26*, 474-481.
Spiegel, S., Obendorf, F. L., & Marcus, A. (1975) *Plant Physiol. 56*, 502-507.

Nitrogenase: The Reaction between the Fe Protein and Bathophenanthrolinedisulfonate as a Probe for Interactions with MgATP†

Torbjørn Ljones‡ and R. H. Burris*

ABSTRACT: The reaction between the Fe(II) chelating agent, bathophenanthrolinedisulfonate, and the iron-sulfur cluster in the Fe protein of nitrogenase from *Clostridium pasteurianum* has been studied. This reaction is greatly accelerated by the presence of MgATP. Analysis of the relationship between reaction rate and concentration of MgATP supports a model in which both of two binding sites for MgATP on the Fe protein must be occupied before the protein undergoes a conformational change, allowing the iron-sulfur site to react rapidly with chelator. This model is also consistent with presently available data on equilibrium binding of MgATP to the Fe protein. MgADP inhibits the effect of MgATP on the chelator reaction in a manner which suggests that MgADP binds strongly to one of the MgATP sites and more weakly to the other. Loss of enzymic activity due to exposure to O_2 or 0 °C is accompanied by a decrease in the ATP-specific chelator reaction. Hence, this reaction was used to estimate the concentration of active iron-sulfur centers for the purpose of computing the extinction coefficient of the Fe protein, giving the value $\Delta\epsilon_{430nm}$(ox-red) = 6600 M^{-1} cm^{-1}.

Nitrogenase, the enzyme system that is responsible for biological nitrogen fixation, requires ATP for its catalysis and hydrolyzes the ATP to ADP and orthophosphate (for reviews, see Zumft & Mortenson, 1975; Winter & Burris, 1976; Orme-Johnson & Davis, 1977). There is experimental evidence that the Fe protein of nitrogenase interacts with MgATP, followed by detectable changes in physical and chemical properties of the protein. No observations supporting direct interaction between MgATP and the MoFe protein have so far been reported.

Tso & Burris (1973) used a gel equilibration method to study the binding of MgATP and MgADP to purified Fe protein and found that the protein possesses two binding sites for MgATP. MgADP binds to one of these sites. The EPR[1] spectrum of the Fe protein undergoes a change when MgATP binds (Orme-Johnson et al., 1972; Smith et al., 1973; Zumft et al., 1973), suggesting that MgATP binding alters the conformation of the Fe protein. Zumft et al. (1973) concluded, on the basis of EPR spectra of the Fe protein with different levels of MgATP, that the Fe protein binds 2 molecules of MgATP per protein molecule. The binding of MgATP lowers the redox potential of the Fe protein (Zumft et al., 1974; Ljones, Tso, Orme-Johnson, & Burris, cited in Burris & Orme-Johnson (1974) and in Orme-Johnson & Davis (1977)).

G. A. Walker & L. E. Mortenson (1973, 1974) have presented a simple and elegant approach to studies of the interaction between the Fe protein and MgATP. Earlier work had shown that iron-sulfur clusters in ferredoxins react with chelating agents for iron, thus disrupting the clusters (Malkin & Rabinowitz, 1967). This reaction is rather slow unless the ferredoxin is denatured, and both of the nitrogenase proteins display similar behavior. Walker & Mortenson discovered that addition of MgATP would dramatically increase the rate of reaction between the chelating agent 2,2′-bipyridyl and the Fe protein. The MoFe protein showed no such response to MgATP. All the requirements and characteristics of the interaction between the Fe protein and MgATP that have been inferred from other types of experiments were observed: requirement for Mg^{2+} in addition to ATP, inhibition by ADP, little or no response to other nucleoside triphosphates. These observations were interpreted by Walker & Mortenson as evidence that the binding of MgATP changes the conformation of the Fe protein such that the iron sulfur site becomes more accessible to reaction with chelators.

Walker & Mortenson (1974) did not measure initial velocities of the reaction with 2,2′-bipyridyl, thus precluding detailed kinetic analysis. Mortenson et al. (1975) later determined initial velocities in similar experiments and proposed a model for binding of MgATP to the Fe protein, but their model is not consistent with the data and model of Tso & Burris (1973). We have, therefore, further pursued the approach of Walker & Mortenson. On the basis of our chelator experiments we propose a model for interaction between the binding of MgATP and reactions at the iron-sulfur site of the Fe protein; this model is consistent both with the model of Tso

† From the Department of Biochemistry and Center for Studies of N_2 Fixation, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706. *Received October 31, 1977.* This investigation was supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, by Public Health Service Grant AI-00848 from the National Institute of Allergy and Infectious Diseases, by National Science Foundation Grant PCM-74-17604, and by a travel grant to T.L. from the Norwegian Research Council for Science and the Humanities.

‡ Permanent address: Department of Biochemistry, University of Bergen, N-5000 Bergen, Norway.

[1] Abbreviations used: Bes, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; EPR, electron paramagnetic resonance.

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