Studies on the Structural Requirements of Substrate Protein for Protein Methylase II*

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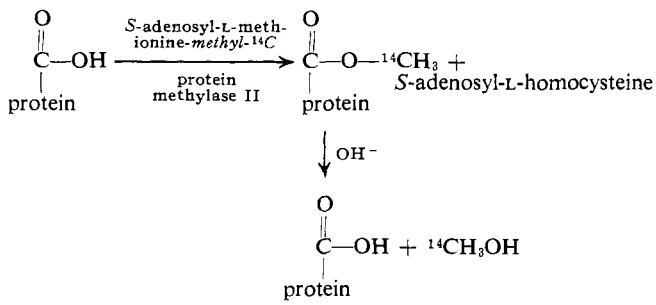
ABSTRACT: Structural requirements of proteins necessary for acceptance of methyl groups from *S*-adenosyl-L-methionine by the action of protein methylase II have been investigated using pancreatic ribonuclease as the model substrate. Chemical modification of the free carboxyl groups of ribonuclease by conversion to the glycine methyl ester amide decreased the methyl-acceptor activity by 85%, demonstrating that protein methylase II methylates the free carboxyl groups. When the ester bond was broken by treatment of the modified ribonuclease with mild alkali, full methyl-acceptor activity was recovered. When the structure of ribonuclease was modified either by oxidation with performic acid or reduction with 2-mercaptoethanol, the initial rate of methylation of the product was greatly enhanced. These results indicate that the reactive carboxyl groups are easily accessible to protein methylase II. Furthermore, pretreatment of the oxidized ribonuclease with either trypsin or chymotrypsin did not influence the ability of the protein to accept the methyl group. This suggested that the whole molecule of ribonuclease is not required; only a reasonable portion of the total peptide chain is adequate as a substrate for protein methylase II.

Calf spleen contains an enzyme which methylates added proteins such as ovalbumin, pepsin, or human serum albumin in the presence of *S*-adenosyl-L-methionine (Liss *et al.*, 1969). The incorporated methyl group becomes volatile on either acid or alkaline hydrolysis of the methylated proteins, and the liberated methyl group has been identified as methyl alcohol. We have recently purified this enzyme from calf thymus to homogeneity by both polyacrylamide gel electrophoresis and analytical centrifugation (Kim and Paik, 1970 and 1971a), and have designated it as protein methylase II. The best substrate is a calf thymus cytosol protein which had been treated with 0.05 N NaOH. The mechanism we proposed for protein methylase II is depicted in Scheme I.

SCHEME I



We assume that the carboxyl groups of the substrate are methylated because the product, like other carboxylic methyl esters, is easily hydrolyzed in aqueous alkaline solution, liberating the methyl group as methyl alcohol (Kim and Paik, 1970). Since we developed recently a new convenient assay technique for protein methylase II activity (Kim and Paik, 1971b), we have investigated further some of the structural requirements of substrate proteins.

Materials and Methods

Materials. Pancreatic ribonuclease (five-times recrystallized), trypsin, soybean trypsin inhibitor, chymotrypsin, and urea were obtained from Sigma Chemical Co., and *S*-adenosyl-L-methionine *methyl*-^{14}C (specific activity 28.9 mCi/mmole in dilute acid solution) from International Chemical and Nuclear Corp. 1-Benzyl-3-dimethylaminopropylcarbodiimide and glycine methyl ester were purchased from Aldrich Chemical Co., Milwaukee, Wis., and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide from the Ott Chemical Co., Muskegon, Mich. The remaining reagents were purchased from various local sources. Protein methylase II was purified from frozen calf thymus as described previously (Kim and Paik, 1971a) and was kept in 50% glycerol at −15°. The activity of the purified protein methylase II used in the present experiments was approximately 5300 μμmoles of *S*-adenosyl-L-methionine-*methyl*-^{14}C used per min/mg of enzyme protein when oxidized ribonuclease was used as methyl acceptor.

Enzymatic Assay. The ingredients of the incubation mixture for the assay of protein methylase II activity were essentially the same as previously described (Kim and Paik, 1970); 0.5 ml of the total incubation mixture contained 0.1 ml of citrate–phosphate buffer (pH 6.0), 1.0 μmole of EDTA, 6 μmoles of 2-mercaptoethanol, 4.78 mμmoles of *S*-adenosyl-L-methionine-*methyl*-^{14}C (3.5×10^5 cpm), 3 μg of purified protein methylase II, and 2 mg of ribonuclease. To prepare the citrate–phosphate buffer of pH 6.0, 1 part of 0.5 M disodium phosphate solution was mixed with 0.6 part of 0.25 M citric acid solution. A control tube in which protein methylase II was added after stopping the reaction was run simultaneously, and the values were corrected for the control. The reaction was carried out at 37° for 15 min. Depending on the need, however, one of the following two assay methods was used to measure

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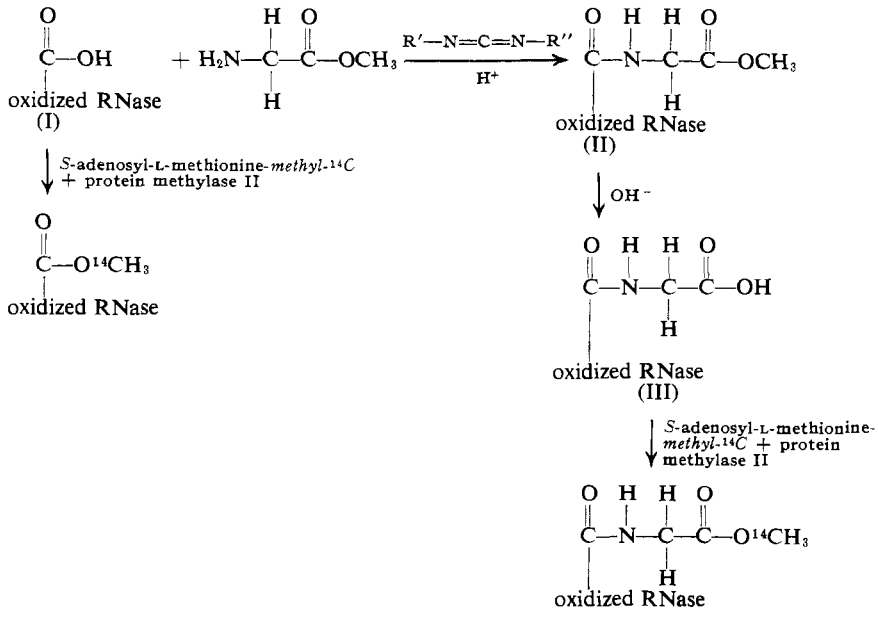
SCHEME II



the extent of transmethylation: first, the trichloroacetic acid precipitation method. When the methyl-acceptor substrates were trichloroacetic acid insoluble, the unreacted *S*-adenosyl-L-methionine-*methyl*-^{14}C and phospholipids were removed by washing the residue successively with 15% trichloroacetic acid and ethanol, and the radioactivity remaining in the trichloroacetic acid precipitate was measured (Kim and Paik, 1970). However, when a trichloroacetic acid soluble substrate such as the trypsin digest was used, the recently developed distillation method (Kim and Paik, 1971b) was employed. This second method exploits the fact that the incorporated methyl groups exist as carboxymethyl esters, which are easily hydrolyzed in mild alkaline solution, and the methyl alcohol formed is distillable. The reaction was stopped by rapid addition of 0.3 ml of 0.5 M phosphate buffer at pH 7.8 into 0.5 ml of the incubation mixture, immediately followed by plunging the tubes into acetone cooled with Dry Ice. The tubes were thawed just before the distillation, with 0.4 ml of cold methanol added as carrier.

Oxidation of Ribonuclease. Oxidation of pancreatic ribonuclease with performic acid was conducted essentially as described by Hirs (1956). Performic acid was prepared by mixing 9.5 ml of formic acid (90.7% Fisher Chemical Co.) and 0.5 ml of H_2O_2 (30%, Fisher Chemical Co.), and was allowed to stand at room temperature for 2 hr in a stoppered flask. The oxidation of ribonuclease was carried out in a flask with a side arm. In the main compartment of the flask 400 mg of ribonuclease in 10 ml of formic acid was added; and in the side arm 5.0 ml of freshly prepared performic acid. The flask was cooled for 30 min in ice and the contents of the side arm tipped into the main compartment. After 2.5 hr in ice, the contents were transferred quantitatively into 800 ml of precooled distilled water, and the diluted solution was immediately lyophilized. The lyophilized sample was dissolved in 160 ml of water and was again lyophilized.

Reduction of Protein. The procedure for reduction of pancreatic ribonuclease was described previously (Anfinsen and Haber, 1961). Ribonuclease (100 mg) was dissolved in 3 ml of 8 M urea–0.04 M Tris-HCl buffer at pH 9.1 and 0.1 ml of 2-mercaptoethanol. The solution was flushed through with nitrogen and was allowed to stand at room temperature for 4 hr. The pH was then adjusted to 3.5 with glacial acetic acid, and the entire solution was dialyzed against 2000 ml of 0.05 N acetic acid. Four changes of the dialyzing solution were made during 24-hr dialysis. The dialyzed sample was finally lyophilized.

Colorimetric Determination of the Ester Bond. The method for determination of ester by ferric–acethydroxamic acid complex was essentially as reported by Hestrin (1949) except that the test was carried out on a small scale in a Coleman cuvet (12 × 75 mm) containing 0.5 ml of the sample, to which was added 1 ml of alkaline hydroxylamine reagent. The hydroxylamine reagent was freshly prepared before use by mixing an equal volume of 2 M hydroxylamine hydrochloride solution and 3.5 N NaOH solution. After 1 min, 0.5 ml of 4 N HCl and 0.5 ml of 0.37 M ferric chloride in 0.1 N HCl were added. The density of the color was promptly measured at 540 mμ. It should be mentioned here that glycine methyl ester used as standard for the ferric–acethydroxamic acid reaction (see the legend in Table II) is not an ideal one, since the ester linked to protein might have a different color value. Chromogenicity of esters in the ferric–acethydroxamic acid reaction depends on the chain length of their acid radicals (Hestrin, 1949).

Chemical Modification of Carboxyl Groups in Protein. The activation of carboxyl groups in oxidized ribonuclease by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and the subsequent reaction of the activated carboxyl groups with glycine methyl ester were described (Hoare and Koshland, 1967); a solution of 4 ml containing 40 mg of performic acid oxidized ribonuclease, 3 ml of 10 M urea, and 0.668 g (5.32 mmoles) of glycine methyl ester hydrochloride was added to 12 ml of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in 7.5 M urea at pH 4.8. While the solution was mixed with a magnetic stirrer at room temperature, the pH of the solution was checked every 15 min, and was adjusted to 4–5 with dropwise addition of 0.5 N HCl. After the indicated period of time, the reaction was terminated by addition of 60 ml of 2 N acetate buffer at pH 4.8 and was dialyzed for 36 hr against three changes of 2000 ml of 0.001 N HCl in the cold room. The

TABLE I: Enzymatic Activity of Protein Methylase II with Chemically Modified Ribonuclease.

Substrate Protein	Amount of Substrate (mg)	Sp Act.[a]	% Act.	Gly/Mole of RNase[b]
Oxidized RNase	3.0	7000	100	3.1
	2.0	5550	79.2	
	1.0	3210	50.2	
Oxidized RNase, COOH blocked by EDC[c] treatment for 10 min	3.0	2290	32.7	
	2.0	1930	27.6	
	1.0	950	13.5	
Oxidized RNase, COOH blocked by EDC treatment for 30 min	3.0	1070	15.3	
	2.0	770	11.0	12.3
	1.0	467	6.7	
Oxidized RNase, COOH blocked by EDC treatment for 2 hr	3.0	1470	21.0	
	2.0	936	13.4	12.0
	1.0	433	6.2	
Oxidized RNase + 10 mM EDC	3.0	7240	103	

[a] Micromicromoles of *S*-adenosyl-L-methionine-*methyl*-^{14}C used per minute per milligram of enzyme protein. [b] Theoretical content of glycine in ribonuclease is 3 moles/mole of protein, based on a molecular weight of 13,700. [c] EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

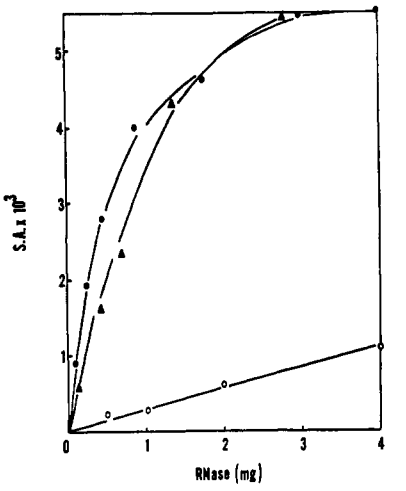


FIGURE 1: Effect of different concentrations of the native and the modified ribonuclease on protein methylase II activity. (○) Native RNase, (▲) RNase oxidized with performic acid, and (●) RNase reduced with 2-mercaptoethanol. To assay the protein methylase II activity, the trichloroacetic washing method was used. The rest of the experimental procedures are described under Methods.

samples were lyophilized. As shown in Scheme II, the compound just described has the structure II.

In order to prepare oxidized ribonuclease–glycine (III in Scheme II), II was hydrolyzed at pH 9.0 at 100° for 30 min. The hydrolysis of glycine methyl ester which had been incorporated into the oxidized RNase was not complete at a temperature lower than 100°. The degree of modification of carboxyl groups in the protein was estimated by measuring the amounts of glycine in the acid hydrolysate by an amino acid analyzer (Spackman *et al.*, 1958) and the content of ester by the ferric–acethydroxamic acid reaction (Hestrin, 1949). The concentration of protein was determined by the method of Lowry *et al.* (1951). Specific activity of the protein methylase II represents nanomoles of *S*-adenosyl-L-methionine-*methyl*-^{14}C used per minute per milligram of enzyme protein.

Results

Effect of Oxidation and Reduction of the Substrate Protein. The effect of changes in the structure of the protein on the availability of sites capable of accepting methyl groups from *S*-adenosyl-L-methionine through the action of the methylating enzyme is illustrated in Figure 1. Both oxidation and reduction of ribonuclease greatly increased its capability for methylation.

Effect of Blocking the Carboxyl Groups. In order to further test the hypothesis that the free carboxyl groups in the substrate protein were methylated, glycine methyl ester was introduced into the COOH of oxidized ribonuclease molecule through carbodiimide for different periods of time. It has been reported that treatment of ribonuclease with 1-benzyl-3-dimethylaminopropylcarbodiimide in 7.5 M urea at pH 4.75 modifies all 10 carboxyl groups in 30 min (Hoare and Koshland, 1967). However, due to the low solubility of the activating reagent, the modification was not complete. Therefore, we resorted to the modification of the ribonuclease with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. As shown in Table I, practically all of the 10 carboxyl groups were blocked by glycine methyl ester. By blocking most of the carboxyl groups in ribonuclease, the efficiency of the oxidized ribonuclease as a methyl acceptor decreased by approximately 85% (79.2% activity for the oxidized protein *vs.* 13.4 and 11.0% for the carboxyl-blocked protein at 2 mg of substrate protein). We were unable to depress completely the substrate activity of the protein and cannot offer any explanation for this. The possibility that contaminating 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide could inhibit the protein methylase II activity can be eliminated by the results shown in the last line in the table. Changes in the per cent of activity depending on the substrate concentration are due to the effect of substrate concentration on the specific activity of protein methylase II, illustrated in Figure 1.

It was of interest to examine whether the carboxyl group of glycine, which had been introduced on the carboxyl group of ribonuclease as glycine methyl ester, can serve as methyl acceptor after the methyl group has been removed. Therefore, II in Scheme II was hydrolyzed in mild alkaline solution as described under Methods. As shown in Table II, oxidized RNase–glycine methyl ester (II) has a strong ferric–acethydroxamic acid reaction, and the amount of glycine per mole of ribonuclease indicates that all of the carboxyl groups in the ribonuclease were modified (see also Table I). However, when the II was hydrolyzed to remove the methyl group, III gave an almost equal amount of the ferric–acethydroxamic acid reaction as the oxidized RNase (I), indicating that all of the methyl esters has been hydrolyzed. The amount of glycine found per mole of ribonuclease indicated that the

TABLE II: Substrate Efficiency of Modified Oxidized Ribonuclease for Protein Methylase II.

Substrate Protein	nmoles of $^{14}CH_3$ Transferred/min[a]	Ferric–Acethydroxamic Acid Complex/Mole of RNase[a]	Glycine per RNase
Oxidized RNase–glycine methyl ester (II)[b]	4.9	29.5	11.4
Oxidized RNase–glycine (III)	23.6	4.6	12.1
Oxidized RNase (I)	23.1	3.5	2.8

[a] Glycine methyl ester was used as a standard for ferric–acethydroxamic acid reaction since standard protein–methyl ester is not available. The molecular weight of pancreatic RNase is taken as 13,700. The protein methylase II assay was carried out with 3 mg of the substrate and 3 μg of purified protein methylase II in a standard incubation mixture by the distillation method. [b] For the interrelationships between I, II, and III, see Scheme II. The introduction of glycine methyl ester into the oxidized RNase was performed by the method described under Methods by reacting the protein with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide for 30 min.

TABLE III: Effect of Proteolysis of Ribonuclease as Substrate for Protein Methylase II.

Pretreatment of the Oxidized RNase	nmoles of $^{14}CH_3$ Transferred		Rel Ninhydrin Color Value (%)
	Assay	Control[a]	
None	26.0		100
Heated at 100° for 30 min	29.0		
Trypsin	28.4	24.4	179
Trypsin + chymotrypsin	20.8	25.6	394
Pronase	5.4	22.0	400

[a] Control tubes contained an equal amount of inactivated proteolytic enzyme and the incubation was carried out as in the assay. Protein methylase II methylates the inactivated proteolytic enzymes and the following "blank" values were subtracted from the values given in the table; 1.4 nmoles of $^{14}CH_3$/min, 3.3, and 3.5 for trypsin, chymotrypsin, and Pronase, respectively. Protein methylase II (3 μg) and oxidized RNase (2 mg) were used and assayed by the distillation method. Tryptic digestion of the oxidized RNase was carried out as follows: 28 mg of the oxidized RNase was incubated with 0.38 mg of trypsin in 1.5 ml of 0.07 M phosphate buffer at pH 7.0 for 4 hr at 37°. At the end of the digestion, 0.75 mg of soybean trypsin inhibitor was added and the pH of the suspension was adjusted to 6.0. A portion containing 2 mg of the oxidized RNase was used for the assay for protein methylase II. Trypsin–chymotrypsin digestion of the oxidized RNase was performed by first incubating 26 mg of the oxidized RNase with 0.2 mg of trypsin in 1.9 ml of 0.02 M phosphate buffer at pH 7.0 for 3 hr at 37°. After the pH was readjusted to 7.0, 0.02 ml of chymotrypsin suspension (0.1 mg) was added into the incubation mixture, and the incubation was continued for another 3 hr. The reaction was terminated by adding 1.6 mg of trypsin inhibitor and by adjusting the pH to 2.2 with 2.5 N HCl to inactivate chymotryptic activity. Pretreatment of the oxidized RNase with Pronase was carried out as follows: 12.8 mg of the oxidized RNase was incubated with 1 mg of Pronase in 1.0 ml of 0.025 M phosphate buffer at pH 7.8 for 4 hr at 37°. The Pronase was inactivated by boiling the incubation mixture at 100° for 30 min and the precipitate formed was removed by centrifugation. In order to check the progress of proteolysis, a small aliquot of the incubation mixture was taken for a ninhydrin color reaction, and the digestion was continued until no further increase in the ninhydrin color was observed.

glycine was still attached to the protein molecule. Furthermore, efficiency of methyl acceptability was completely recovered by removing the methyl group from II. These results, therefore, strongly suggest that the carboxyl groups whether donated by dicarboxylic acids or glycine could serve equally well as methyl acceptor for the action of protein methylase II.

Effect of Enzymatic Hydrolysis of Ribonuclease. The following experiment was designed to test an important question, namely, does the protein have to remain completely intact in order to be able to accept the methyl group? Therefore the oxidized ribonuclease was pretreated with trypsin, chymotrypsin, or pronase, and after inactivating the proteolytic enzymes the digested ribonuclease was used for the assay of protein methylase II activity. As can be seen in Table III, neither pretreatment of the protein with trypsin nor with the combination of trypsin and chymotrypsin changed the availability of the protein as substrate to any significant extent. On the other hand, pretreatment of the oxidized ribonuclease with Pronase decreased the activity greatly. From the above results, it can be concluded that the whole molecule of ribonuclease is not necessary for its activity as the methyl acceptor for protein methylase II.

Discussion

It has been demonstrated in the present paper that when pancreatic ribonuclease is modified by either oxidation or reduction, it is a far better methyl acceptor than the native protein. This is most likely due to easier access of protein methylase II to the site of methylation. Predigestion of ribonuclease with trypsin or chymotrypsin did not impair the substrate capability of the protein for protein methylase II. Since the color value of the ninhydrin reaction increased (Table III), the ribonuclease molecule was definitely split into smaller peptides. These results strongly indicate that the whole molecule of the oxidized ribonuclease was not required for substrate activity. However, since the rate of methyl group transfer remained unchanged (Table III), the C-terminal carboxyl group is probably not an acceptor of the methyl group from *S*-adenosyl-L-methionine. The fact that pronase treatment of the oxidized ribonuclease decreased the substrate capability indicates that the length of the peptide chain should not be too short to serve as a methyl acceptor for protein methylase III. This is in conformity with the observation made previously that free carboxyl groups of amino acids, dipeptides of dicarboxylic amino acids, and homopolymers of amino acids did not serve as methyl acceptors for protein methylase II (Kim and Paik, 1971b).

The fact that the methyl groups incorporated into protein become volatile on mild hydrolysis gives an important clue to the probability that the linkage between the methyl and carboxyl groups is an ester bond (Liss *et al.*, 1969; Kim and Paik, 1970). Therefore, modification of free carboxyl groups by introduction of glycine methyl ester through the reaction with a water-soluble carbodiimide should be useful for investigating the possible participation of carboxyl groups as methylation sites, since this modification of ribonuclease exclusively involves free carboxyl groups (Hoare and Koshland, 1967). It has been clearly demonstrated that methyl acceptor capability of ribonuclease decreased to about 15% of the control when modified by glycine methyl ester formation (Table I). Furthermore, when the methyl group in the RNase–glycine methyl ester was removed and the carboxyl groups of RNase–glycine became free, RNase recovered full activity as substrate. These results together with the results in Table III strongly suggest that the internal carboxyl groups in ribonuclease are the sites of methylation. However, it is still possible that the C-terminal carboxyl groups of lysine, arginine, and aromatic amino acids which are available by the action of trypsin and chymotrypsin are the only C-terminal carboxyl groups which do not accept the methyl group.

Oxidation of proteins with performic acid affects only two amino acids: cystine is changed to cysteic acid and methionine to the sulfone (Hirs, 1956). Since substrate activities of the reduced or oxidized ribonuclease toward protein methylase II are about equal within experimental error (Figure 1), it can be said that cystine and methionine residues of RNase are not involved in the methyl transferase reaction.

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Ionic Strength Dependent Dissociation and Association of Yeast Uridine Diphosphate Galactose 4-Epimerase Subunits*

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ABSTRACT: It has been determined by equilibrium ultracentrifugation and gel filtration chromatography in 6 M guanidine hydrochloride that the subunits of yeast uridine diphosphate galactose 4-epimerase are of 60,000 molecular weight. Dissociation of the native enzyme without loss of cofactor occurred in buffers of very low ionic strength at neutral pH. In 0.001 M Tris-HCl (pH 7.4) the enzyme sedimented as an equal mixture of 3S and 4S components; sedimentation equilibrium centrifugation indicated only a single component with 60,000 molecular weight. The apoenzyme sedimented as a single 3.7S protein. When the Tris concentration was increased to 0.1 M, the subunits reassociated to dimers but not to tetramers. Gel filtration chromatography in 0.001 M Tris-HCl buffer (pH 7.4) yielded partial fractionation into components differing in specific activity and fluorescence. The reactivation and reassociation to dimers and tetramers of the apoenzyme subunits were found to be dependent on the presence of pyridine nucleotide.

Uridine diphosphate galactose 4-epimerase (EC 5.1.3.2.) from yeast catalyzes the reversible epimerization of UDP-galactose[1] to UDP-glucose. The enzyme contains 1 mole of tightly bound pyridine nucleotide cofactor per 120,000 grams of protein (Darrow and Rodstrom, 1968). The apo enzyme is formed by removal of the cofactor by *p*-chloromercuribenzoate titration which results in total loss of catalytic activity. The addition of NAD partially restores activity to the enzyme (Maxwell *et al.*, 1958); however, the cofactor removed from the native enzyme is more effective in restoring catalytic activity than is NAD.

Darrow and Rodstrom (1966) have demonstrated that the enzyme can assume three distinct states of aggregation. In buffers of low cation concentration such as 0.01 M Tris-HCl (pH 7.5) the enzyme has a molecular weight of 125,000 (Darrow and Rodstrom, 1968) and a sedimentation coefficient of 6.5 S. In buffers of higher cation concentration, such as 0.1 M Tris-HCl (pH 7.5), the sedimentation coefficient increases

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[1] Abbreviations used are: UDP-galactose 4-epimerase, UDP-glucose 4-epimerase (EC 5.1.3.2); UDP-galactose, uridine 5′-(α-D-galactopyranosyl pyrophosphate); 5′-UMP, uridine 5′-monophosphate; ME, 2-mercaptoethanol; PMB *p*-chloromercuribenzoic acid; DTT, dithiothreitol; Gdn·HCl, guanidine hydrochloride.