Physicochemical Characterization of Ribonucleoside Diphosphate Reductase from *Escherichia coli*

Lars Thelander

From the Medical Nobel Institute, Department of Biochemistry, Karolinska Institutet, S-104 01 Stockholm, Sweden

**SUMMARY**

Ribonucleotide reductase from *Escherichia coli* consists of two nonidentical subunits, proteins B1 and B2. Affinity chromatography resulted in a B1 preparation which appeared homogeneous during polyacrylamide gel electrophoresis and ultracentrifugation. The molecular weight was 100,000 by sedimentation equilibrium centrifugation using a partial specific volume of 0.706 ml per g at 4°C. Protein B1 was composed of two polypeptide chains of similar or identical size, both having isoleucine as the COOH-terminal residue. In contrast, the NH2-terminal residues were different: glutamic acid and aspartic acid. This difference in amino acid composition was shown to reach further into the polypeptide chains. From these data the subunit structure of protein B1 seems to be of the type \( \alpha \alpha' \). The \( \alpha \alpha' \) complex starts to dissociate in the absence of magnesium ions, and dissociation also occurred on oxidation of protein B1 in air. The latter dissociation was followed by an inactivation of the protein and was a result of oxidation of cysteine residues to cystines. Both dissociation and inactivation were reversed to a great extent by incubation in dithiothreitol.

A homogeneous preparation of protein B2 had a molecular weight of 78,000 using a partial specific volume of 0.683 ml per g at 4°C. This protein also was composed of two polypeptide chains of similar or identical size, but in this case both chains had the same NH2-terminal residue (alanine) and COOH-terminal residues (leucine). Analysis of tryptic peptides further supported the identity of the two chains. The subunit structure of protein B2 can therefore be designated \( \beta \beta' \).

In the presence of the stimulatory effector dTTP proteins B1 and B2 formed a complex with a \( s_{20, w} \) of 10.1 S and a molecular weight of 245,000. This indicates that the active form of ribonucleotide reductase consists of a 1:1 complex between proteins B1 and B2 of the type \( \alpha \alpha' \beta \). In the presence of the inhibitory effector dATP, proteins B1 and B2 form heavier complexes, the type of which are strongly influenced by the presence of sucrose.

The biosynthesis of deoxyribonucleotides, a necessary reaction for DNA synthesis, occurs mainly as the direct reduction of the corresponding ribonucleotides (1). In *Escherichia coli* this reaction is catalyzed by the enzyme ribonucleoside diphosphate reductase together with thioredoxin and thioredoxin reductase which function in the supply of electrons from NADPH (2, 3). Early in the study of ribonucleotide reductase it was observed that this protein consists of two components, designated proteins B1 and B2, neither of which possessed enzymatic activity by itself but generated full activity upon combination in the presence of Mg++. The ribonucleotide reductase reaction can be summarized as follows:

\[
\text{Ribonucleoside diphosphate} + \text{thioredoxin-(SH)}_n \xrightarrow{\text{proteins B1 + B2}} \text{deoxyribonucleoside diphosphate + thioredoxin-S}_2 \quad (1)
\]

\[
\text{Thioredoxin-S}_2 + \text{NADPH} + \text{H}^+ \xrightarrow{\text{thioredoxin reductase}} \text{thioredoxin-(SH)}_2 + \text{NADP}^+ \quad (2)
\]

Studies in sucrose gradients indicated that the active ribonucleotide reductase complex (9.7 S) contains equimolar amounts of proteins B1 and B2 (4). The reduction of all four different ribonucleoside diphosphates is catalyzed by the same enzyme. The substrate specificity is regulated by allosteric effectors (dATP, ATP, dTTP, dGTP) which are specifically bound to protein B1, which has four binding sites for dATP and ATP and two sites for dTTP and dGTP (5). Binding of positive effectors (dTTP, dGTP, and ATP) to the ribonucleotide reductase complex does not significantly change its sedimentation behavior, while binding of the negative effector dATP results in an inactive enzyme complex with a sedimentation coefficient of 15.5 S (4). Protein B1 also binds the nucleoside diphosphate substrates.

Protein B2 contains 2 atoms of non-heme bound iron per molecule and these are essential for enzymatic activity (6).

This paper describes (a) the purification of protein B1 to give a homogeneous preparation; (b) the characterization of this as well as a homogeneous preparation of protein B2 with respect to molecular weight, amino acid composition, and subunit structure; (c) the interaction between protein B1 and protein B2.

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1 U. Jennergren and P. Reichard, unpublished results.
EXPERIMENTAL PROCEDURE

Materials—The general preparation procedure for proteins B1 and B2 and enzyme assays were described earlier (7). The proteins were stored as ammonium sulfate precipitates in liquid nitrogen after the final purification step (chromatography on Sephadex G 200).

Dithiothreitol was obtained from Calbiochem A. G. Deoxyribonucleoside triphosphates were purchased from P-L Biochemicals, Inc., and purified before use by chromatography on DEAE-Sephadex (8). Guanidine hydrochloride (ultra pure) and N-dinitrophenyl-amino acids were obtained from Mann. Guanidine hydrochloride was used without further purification while the dinitrophenyl-amino acids were purified by chromatography on Celite (9, 10). Sodium dodecyl sulfate and iodoacetic acid were obtained from Eastman. Sodium dodecyl sulfate was recrystallized from ethanol and iodoacetic acid from ether-hexane prior to use. Carboxypeptidase A, treated with diisopropyl phosphorofluoridate, chymotrypsin, and trypsin were obtained from Worthington. N-Ethythymolmine (Fluka A. G.) was redistilled before use and stored over NaOH pellets at 4°. Sodium dodecyl sulfate and iodoacetic acid s were obtained and stored in liquid nitrogen prior to use.

Analytical Polyacrylamide Gel Electrophoresis—Electrophoresis was performed as described (7) using a single gel and a circulating continuous buffer system (0.05 M Tris-Cl, pH 8.74-0.002 M dithiothreitol). Gels were prepared by mixing a solution containing 0.25 mg of riboflavin and 25 ml of N,N,N',N'-tetramethylethylenediamine per 100 ml of 0.05 M Tris-Cl, pH 8.74, with an equal volume of the same buffer containing 9.5 g of acrylamide and 0.5 g of N,N'-methylenebisacrylamide per 100 ml. Polymerization was induced by light. Protein was applied to the gel columns in 100 ml of 0.01 M Tris-Cl, pH 8.74-0.01 M dithiobisethreitol containing 20% glycerol. The gels were fixed in sulfosalicylic acid and stained with Coomassie brilliant blue.

Analytical Ultracentrifugation—Analyses were made with a Spinco model E analytical ultracentrifuge equipped with the K11C temperature control unit, electronic speed control (not in all experiments), a monochromator, and a high intensity light source. Interference optics were aligned according to Richards et al. (11).

Molecular weight determinations of native proteins B1 and B2 were performed by the long column meniscus depletion sedimentation equilibrium technique as described by Chervenka (12). Prior to centrifugation the proteins were equilibrated with the appropriate buffers on columns of Sephadex G-25. Protein B1 was centrifuged for 13 hours at 4° and 11,000 rpm and protein B2 for 13 to 20 hours at 16,000 rpm and 4° or 20°. Protein concentrations were about 0.5 mg per ml. Interference optics were used and a water blank was run to correct for optical differences between solution and solvent channels (13).

Molecular weights of proteins B1 and B2 in guanidine hydrochloride were determined both by the method mentioned above and by the high speed sedimentation equilibrium method of Yphantis as described in detail elsewhere (13, 14). The plates (Kodak spectrographic plates, emulsion type II-G) were read in a Nikon profile projector model 6C T2.

Sedimentation velocity runs on dilute samples of protein B1 (about 0.3 mg per ml) were performed at 20° and 50,780 rpm using an An-H rotor and a 4° single sector cell with sapphire windows and a 12-mm Ke-F centerpiece. Sedimentation patterns were recorded on Kodak commercial fine grain CP 7 film (Estar base) using absorption optics and light with a wave length of 280 nm. The blackening of the film was measured with a Joyce-Loebl Mark III S microdensitometer. The s values obtained were weight average sedimentation coefficients over the whole cell since components with different s values were difficult to resolve using absorbance optics. All sedimentation coefficients were recauculated to \( \beta \), the centrifuge sector of a 12-mm filled-Epon double sector cell. The densities were measured pycnometrically.

The molecular weight of the dTTP-induced complex between protein B1 and protein B2 was determined by the Archibald method as modified by LaBar (15). The liquid columns used in the Archibald run were obtained by filling 300 ml of solution over 30 ml of FC-43 in one sector and 345 ml of solvent in the other sector of a 12-mm filled-Epon double sector cell. The run was performed at 20° and 2800 rpm for 4 hours. When pictures had been taken using schlieren optics, the centrifuge was stopped and the rotor taken out. After shaking to mix the content in the cell the rotor was replaced and a sedimentation velocity run was performed. Measurements of the area under the schlieren peak gave the initial protein concentration after correction for radial dilution. Only measurements at the meniscus were used to calculate molecular weights.

Determination of Protein Concentration—On the basis of dry weight a value of 10.8 was determined for B1 and B2 was purified by affinity chromatography. Repeated dry weight analyses of protein B2 gave an E\(_{280}\) of 280 nm of 15.0 which is in good agreement with the earlier published value (7). The extinction coefficient of protein B1 in 6 M guanidine hydrochloride-0.1 M mercaptoethanol was determined by diluting a stock solution of desalted protein of known protein concentration with a guanidine hydrochloride-mercaptoethanol solution and then determining the absorbance against a proper blank. An E\(_{280}\) of 280 nm of 9.8 was obtained (cf. 16).

Partial Specific Volumes—Solutions for determining partial specific volumes were prepared by equilibrating stock solutions of proteins B1 and B2 with the appropriate buffers on columns of Sephadex G-25. Protein concentrations (2 to 4 mg per ml) were based on absorbance readings at 280 nm. In the case of guanidine hydrochloride the proteins were treated as indicated below.

Density measurements were made with the digital precision density meter DMA-02 made by Anton Paar K. G. (Gras, Austria) (17). The temperature was controlled to ±0.01° by a Hetherlyphe type 01 PT 623 cooled by a Heto ultra-cryostat 623 E-CB (Heto, Birkerød, Denmark).

Quantitative Amino Acid Analyses—Proteins B1 and B2 were desalted on columns of Sephadex G-25 equilibrated with re-distilled water, pH 8 (adjusted with 1 M NH₄OH). The protein concentration of the eluates was determined by reading the absorbance at 280 nm. Into hydrolysis tubes was pipetted 0.070 ml of protein B1 solution containing 6.67 mg per ml or 0.080 ml of protein B2 containing 2.20 mg per ml. After lyophilization 2 ml of constant boiling HCl (twice distilled, 0.66 M) were added, and the tubes were evacuated with an oil pump to 0.01 mm Hg and kept at this pressure for about 20 min before sealing. Two samples of each protein were hydrolyzed at 110° for 20-hour and two for 70-hour periods. After hydrolysis the
HCl was removed by rotary evaporation and the dry samples were dissolved in 1.0 ml of 0.2 M sodium citrate buffer, pH 2.2. Chromatography of the hydrolysates was performed according to the procedure of Moore et al. (18) with a Spinco model 120B amino acid analyzer with the high sensitivity evoutte system. The hydrolysates were analyzed using 0.159 mg of protein B1 and 0.101 mg of protein B2 on each column.

Methionine sulfoxide was determined after alkaline hydrolysis (110° for 16 hours in 1 ml of 3.75 M NaOH) of protein B1 (19). In the analyses of the chymotryptic peptides and after carboxypeptidase digestion the sensitivity of the amino acid analyzer was increased by use of a range card for the potentiometric recorder.

Dissociation of Proteins B1 and B2 in Guanidine Hydrochloride—About 5 mg of protein were dissolved in 0.5 ml of 6 M guanidine hydrochloride (51% w/w)-0.1 M mercaptoethanol, pH 7 (adjusted with NH₄OH), and incubated at room temperature overnight. The sample was then equilibrated with a fresh solution of guanidine hydrochloride-mercaptoethanol on a column of Sephadex G-25 (1 x 10 cm) calibrated with blue dextran. About 1.1 ml of eluate was collected, and the protein concentration was determined by reading the absorbance at 380 nm (protein B1) or by taking 20-μl samples for acid hydrolysis and amino acid analysis (protein B2). This amount of salt did not affect the amino acid analysis.

Most of the material eluted from the Sephadex was used directly in the determinations of apparent specific volumes (about 0.9 ml containing 2 to 4 mg of protein per ml; see above). The remainder was diluted with 6 M guanidine hydrochloride-0.1 M mercaptoethanol and the molecular weight was determined centrifugally by either the Chervenka method (12) or the Yphantis method (13) (0.3 mg per ml) or the Yphantis method (13) (0.3 mg per ml).

Carboxymethylated proteins B1 and B2 were prepared in the following way. After equilibration with 1 M Tris-Cl, pH 8.6-0.01 M EDTA on Sephadex G-25 columns, 0.5 ml of the protein elution containing about 7 mg of protein was pipetted into a Thunberg tube, and 50 μl of 2.7 M sodium iodoacetate, pH 9, were added. Guanidine hydrochloride (675 mg) was poured into a separate compartment of the Thunberg tube, and the tube was closed and equilibrated with argon before mixing the contents of the two compartments. This mixture was incubated at room temperature in the dark for 3 hours and was then passed through a column (1 x 9 cm) of Sephadex G-25 equilibrated with 6 M guanidine hydrochloride, pH 7. The eluted protein fractions were diluted with guanidine hydrochloride solution and analyzed ultracentrifugally as previously described.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis—The molecular weights of the polypeptide chains of proteins B1 and B2 were estimated as described by Shapiro et al. (20) using pepsin (Worthington), ovalbumin, bovine serum albumin (Sigma), and phosphorylase a (Boehringer) as markers. The proteins were denatured and reduced by incubation in 0.1 M sodium phosphate buffer, pH 7.1-1% sodium dodecyl sulfate-1% mercaptoethanol at room temperature overnight. Before electrophoresis the samples were equilibrated with 0.01 M sodium phosphate buffer, pH 7.1-0.1% sodium dodecyl sulfate-0.1% mercaptoethanol on 1-ml columns of Sephadex G-25. Electrophoresis, fixing, and staining of the gels and calculation of molecular weights were performed as in Reference 20. Protein B1 was also analyzed by the discontinuous dodecyl sulfate buffer system of Laemmli (21).

Determinations of Sulfhydryl Groups in Protein B1—Sulfhydryl determinations were performed in a Thunberg cuvette containing 1.1 ml of 0.1 M Tris-Cl, pH 7.6-8.3 M guanidine hydrochloride-0.01 M EDTA and 0.3 ml of a mixture consisting of 0.2 ml of 0.4% 5,5’-dithiobis(2-nitrobenzoic acid) in ethanol and 1.8 ml of 1 M Tris-Cl, pH 8.0. The side arm contained about 1 nmoles of protein B1 in 100 μl of 0.05 M Tris-Cl, pH 7.6-0.1 M NaCl. After the cuvette was evacuated and equilibrated with argon, the contents were mixed and the absorbance at 412 nm was recorded. The absorbance after mixing was constant for at least 20 min and the amount of sulfhydryl groups was calculated from the difference in absorbance before and after mixing using a molar extinction coefficient of 13,600 M⁻¹ cm⁻¹ (22).

COOH-Terminal End Group Analyses—Desalted and lyophilized protein B1 (2.0 mg) or protein B2 (1.3 mg) was dissolved in 0.2 ml of 0.2 M N-ethylmorpholine buffer, pH 8-1% sodium dodecyl sulfate. Carboxypeptidase was added to this solution (protein to protease ratio 90:1) and to a blank consisting of 0.5 ml of 0.2 M N-ethylmorpholine buffer, pH 8-1% sodium dodecyl sulfate. The digestion proceeded for 16 hours at 37°. The mixtures were then lyophilized and dissolved in 1.0 ml of 0.2 M sodium citrate buffer, pH 2.2. The precipitate formed was removed by centrifugation. Liberated amino acids in the supernatants were identified by amino acid analysis (see above).

NH₂-Terminal End Group Analyses—NH₂-terminal amino acids in proteins B1 and B2 were determined by three techniques: the dansyl technique of Gray and Hartley (23), the cyanate method of Stark and Smyth (24), and the fluorodinitrobenzene method of Sanger (25).

In the cyanate experiments, about 15 nmoles of proteins B1 and B2 were used. After hydrolysis the dansyl amino acids were identified by thin layer chromatography on polyamide plates as described earlier (3).

With the cyanate method, 11.5 mg of desalted protein B1 were carbamylated in 5 ml of N-ethylmorpholine-acetate buffer, pH 8-6 M guanidine hydrochloride-0.6 M KCNO at 50° for 24 hours. Protein B2 (5.2 mg) was treated in the same way. The carbamylated proteins were desalted by dialysis against 50% acetic acid. Quantitative amino acid analyses were made on aliquots of the dialysates after hydrolysis for 22 hours at 110° in 6 M HCl. Both protein concentration and extent of carbamylation were calculated from these amino acid analyses. For the cyanation step 61 nmoles of protein B1 and 57 nmoles of protein B2 were used. The remainder of the procedure including hydrolysis of pyrrolidone carboxylic acid was performed as described by Stark and Smyth (24).

In the fluorodinitrobenzene method, about 3.5 mg of protein B1 were equilibrated with 0.1 M NaHCO₃, pH 8.5-8 M urea on a column of Sephadex G-25. The volume of the protein-containing eluate was adjusted to 1.5 ml with the same buffer and 25 μl of recrystallized [PH]fluorodinitrobenzene were added. This procedure afforded approximately 100-fold excess of fluorodinitrobenzene over free amino groups in B1. The reaction was carried out for 2 hours at 37° in darkness with continuous stirring. Excess reagent was extracted three times with 4 ml of ether. After exhaustive dialysis against 0.1 M NH₄OH, a sample of the dialysate was hydrolyzed and chromatographed on the amino acid analyzer to determine the protein concentration of the dialysate.

The remainder of the dialysate (21.0 nmoles of [PH]DNP protein B1) was evaporated to dryness in a hydrolysis tube and 1.0 μmole of carrier DNP-glutamic acid plus 1.05 μmoles of carrier DNP-aspartic acid were added. Hydrolysis was performed with 2 ml of 6 M HCl at 110° for 18 hours as described earlier.

1 The abbreviations used are: dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; DNP, dinitrophenyl.
After hydrolysis the HCl solution was extracted three times with 3 ml of ether. The ether phase containing the ether-soluble DNP-amino acids was washed with 2 ml of 1 M HCl, evaporated in a stream of argon, and finally dried completely in a vacuum desiccator. This material was dissolved and chromatographed on a column of Hyflo Super Cel according to Portugal et al. (9). The fractions containing either DNP-glutamic or DNP-aspartic acid were pooled judiciously to ensure that no cross-contamination occurred and then evaporated to dryness in a vacuum desiccator. The fractions eluting before glutamic acid were also pooled and evaporated to dryness and later analyzed for the other DNP-amino acids by chromatography on polyamide plates (26). Finally, the evaporated DNP-glutamic acid pool and DNP-aspartic acid pool were dissolved in 1.0 ml of water and analyzed for tritium in a Packard scintillation counter. The concentration of DNP-amino acid was determined by absorption at 364 nm at pH 7.6 using a molar extinction coefficient of 16,000. The amount of NH₂-terminal amino acid per mole of protein Bl was calculated from the tritium determinations using the specific activity determined for the [3H]fluorodinitrobenzene (see below). The figures were corrected for the yield obtained for the added carrier DNP-amino acids. For these corrections the amounts of DNP-amino acids originating from the NH₂ termini of the protein were neglected.

The specific activity of the recrystallized [3H]fluorodinitrobenzene was determined by reacting aspartic acid and glutamic acid with [3H]fluorodinitrobenzene, isolating the [3H]DNP-aspartic acid and [3H]DNP-glutamic acid from the reaction mixture by chromatography, and analyzing for tritium and DNP-amino acids as described earlier.

Protein B2 (about 30 nmole) was reacted with [3H]fluorodinitrobenzene in the same manner as described for Bl. Before hydrolysis 1.0 μmole of carrier DNP-alanine was added to 22.2 nmole of [3H]DNP-B2. The ether-extracted DNP-amino acids were chromatographed at pH 7.4 on a column of Celite according to Matheson (10). The fractions containing DNP-amino acids were pooled and analyzed for tritium and DNP-amino acid as described earlier.

Maleylation of Protein Bl and Isolation of NH₂-terminal Peptides—After equilibration with 1 M Tris-Cl, pH 8.6-0.01 M EDTA on a Sephadex column, 2.2 ml of eluate containing 24.5 mg of protein Bl were pipetted into a Thunberg tube. Guanidine hydrochloride (2.9 g) and dithiothreitol (3.3 mg) were added after the protein solution was equilibrated with argon. The mixture was incubated 2½ hours at 37° followed by addition of 35 ml of 2.7 M sodium iodoacetate. After 45 min at room temperature, the reaction was stopped by addition of 20 ml of 5 M mercaptoethanol. The mixture was dialyzed against water at 4° and lyophilized. The residue was dissolved in 1.5 ml of 0.1 M sodium pyrophosphate buffer 6 M guanidine hydrochloride, pH 9.0. Freshly sublimed maleic anhydride (19 mg) was added to a tube containing 250 μCi of [14C]maleic anhydride in 200 μl of dry dioxane. This dioxane solution was added to the protein solution in four aliquots of 50 μl and the pH adjusted to about 9 with 2 M NaOH. After 2 hours at room temperature, the mixture was dialyzed against 0.5% ammonium bicarbonate for 3 days at 4°.

To the dialysate (13.2 ml containing 27.3 A₂₈₀ units of protein) 100 μg of chymotrypsin were added, and after 2 hours at 37° another 100 μg were added. This gave a protein to protease ratio of about 50:1. The mixture was lyophilized after an additional 3 hours and the radioactive NH₂-terminal peptides isolated by chromatography on Dowex 50 in 0.1 M acetic acid and electrophoresis at pH 6.5 as described in Reference 27. The radioactive peptides were eluted, aliquots were hydrolyzed and analyzed for amino acids, and the peptides purified further by electrophoresis at pH 3.5.

Tryptic Peptide Map of Protein Bl—Protein Bl (4.8 mg) was reduced and carboxymethylated as described for protein B1 in the preceding section and the reaction mixture dialyzed against 0.1 M ammonium bicarbonate, pH 8.6, at 4° and concentrated to 600 μl by ultradialysis. Trypsin (40 μg in 10 μl of 0.001 M HCl) was added plus 5 μl of toluene and the digestion allowed to proceed for 12 hours at 37° when another 40 μg of trypsin were added. After an additional 5 hours at 37° the sample was lyophilized and dissolved in 60 μl of 0.1 M ammonium hydroxide. Chromatography on Whatman No. 3MM paper and electrophoresis were performed as earlier described (14).

RESULTS

Characterization of Protein Bl

Purification by Affinity Chromatography—The purification scheme described earlier for protein B1 was quite involved, gave a poor yield, and the specific activities of the final material varied (7). The affinity chromatography step used here instead of chromatography on triethylamino ethyl-cellulose obviates some of these problems, resulting in a more simplified procedure with higher and more reproducible yields. Since protein Bl has a high affinity for the negative effector dATP (dissociation constant about 10⁻⁷ M (5)), dATP-Sepharose (28) was used as a specific adsorber in large scale purification. Protein Bl adsorbed strongly to dATP-substituted Sepharose, but it could be desorbed by solutions of dATP (1.6 × 10⁻⁴ M) or ATP (1 × 10⁻⁵ M). No desorption was observed with dGTP (10⁻⁴ M) or dTTP (10⁻⁵ M).

Purification of protein Bl by affinity chromatography was performed in the following way: prior to use the dATP-Sepharose column (3 × 4 cm) was equilibrated with 0.1 M Tris-Cl, pH 7.6-0.01 M MgCl₂-0.002 M dithiothreitol. The pooled fractions containing protein Bl after hydroxylapatite chromatography (about 250 μg of protein in 500 ml of approximately 0.25 M potassium phosphate buffer, pH 6.4-0.001 M dithiothreitol) was passed through the column followed by washing with 1000 ml of 0.1 M Tris-Cl, pH 7.6-0.01 M MgCl₂-0.2 M NaCl-0.002 M dithiothreitol. After an additional wash with 100 ml of 0.05 M Tris-Cl, pH 7.6-0.01 M MgCl₂-0.002 M dithiothreitol, protein B1 was eluted with 75 ml of the same buffer solution containing 0.01 M ATP and concentrated by dialysis against saturated ammonium sulfate-0.002 M dithiothreitol for 4 hours. The precipitate was dissolved and chromatographed on a column of Sephadex G-200 as described earlier (7). About 6-fold purification and 80% yield were obtained in the affinity chromatography step.

Analytical Gel Electrophoresis—To test the homogeneity of the Bl preparation, samples were analyzed by polymeracrylamide gel electrophoresis at pH 8.74. Only one band was seen but when the gels were scanned in a densitometer this band was resolved into two very close moving components of about the same size (Fig. 1). The same result was obtained for protein Bl prepared in the presence of toluenesulfonyl fluoride which was added to inhibit proteases (cf. 29). Aside from this double band there was no evidence of inhomogeneity in the Bl preparation. This was an improvement as compared to earlier B1 preparations in which at least three different bands were observed (7).

Molecular Weight Determination—Molecular weight determi-
nations on protein B1 by the low speed sedimentation equilibriu
m technique previously did not result in linear In c versus x²
plots (7). Furthermore, such plots showed increasing upward
curvature as a function of centrifugation time indicating that
protein B1 was slowly denatured. For this reason the long
column meniscus depletion sedimentation equilibrium tech-
nique was tested, which required considerably less time before equili-
brum was reached (12). When protein B1 taken directly after
the final Sephadex G-200 chromatography was analyzed by this
technique, as described under “Experimental Procedure,” linear
plots were obtained allowing conclusive molecular weight
estimations (Fig. 2). The partial specific volume of the protein
B1 preparation was determined as 0.706 ml per g in 0.05 m
Tris-Cl, pH 7.6 - 0.01 m MgCl₂ - 0.2 m NaCl - 0.01 m dithiothreitol
at 4° and to 0.727 ml per g at 20°. Using these figures the mo-
lecular weights from three different centrifugations on three
different B1 preparations were 156,000, 160,000, and 161,000
with an average value of 160,000.

Quantitative Amino Acid Analyses—Quantitative amino acid
analyses of protein B1 were performed to permit determination
of protein concentration under various experimental conditions.
Furthermore, it was of great interest to know the number of
half-cystine residues in the protein since sulfhydryl groups were
known to be essential for enzyme activity (see below).

The results of the analyses are given in Table I. The nearest
integral numbers of amino acid residues were calculated on the
assumption of a molecular weight of 160,000 for protein B1.
From the analyses a total recovery of 95.7% by weight was
obtained. The samples were hydrolyzed for 20 and 70 hours as
described under “Experimental Procedure.” Protein B1 con-
tained 21 half-cystine residues per 160,000 g of protein.

Molecular Weight Determinations in Guanidine Hydrochloride—
The number of polypeptide chains of protein B1 was studied by
determining the molecular weight in guanidine hydrochloride
using the high speed sedimentation equilibrium technique. Ag-
egregation due to formation of disulfide bonds from exposed
sulfhydryl groups was prevented either by addition of mercapto-
ethanol or by carbamidemethylation of the protein. The apparent
specific volume of protein B1 in 6 m guanidine hydrochloride
(51% w/w)-0.1 m mercaptoethanol was determined as 0.709
ml per g at 20°.

Table II gives the molecular weights from the analyses of three
different samples, one unalkylated (71,000) and two alkylated
(77,000). All plots of ln c versus x² appeared linear, and the
same apparent specific volume was used in the calculations of all
three samples. The molecular weights were in all cases about
half of the value for the native protein, indicating the existence
of two polypeptide chains of similar size.

Analysis by Dodecyl Sulfate Polyacrylamide Gel Electrophoresis—The results from ultracentrifugation in guanidine hydro-
chloride strongly suggested that protein B1 was composed of
two polypeptide chains. This was further supported by the
electrophoresis pattern of protein B1 on sodium dodecyl sulfate
polyacrylamide gel. Only one distinct band was observed and
this had a mobility corresponding to a molecular weight of
82,000. The same result was obtained when the discontinuous
buffer system was used (21). The band gave one symmetrical
peak on scanning in a densitometer. From these results it was
concluded that protein B1 consisted of two polypeptide chains
of similar or identical size.
## Table I

**Amino acid composition of proteins B1 and B2**

The over-all accuracy was not better than \( \pm 2.5\% \).

<table>
<thead>
<tr>
<th>Amino acid residues</th>
<th>Protein B1</th>
<th>Protein B2</th>
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<tbody>
<tr>
<td>Nearest integral number of amino acid residues per 100,000 g of protein</td>
<td>Nearest integral number of amino acid residues per 75,000 g of protein</td>
<td></td>
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<tr>
<td>Aspartic acid</td>
<td>4.3</td>
<td>7.0</td>
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<td>Threonine</td>
<td>4.6</td>
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<td>Histidine</td>
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<tr>
<td>Arginine</td>
<td>10.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>12.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Total half-cystine</td>
<td>12.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Total</td>
<td>100.7%</td>
<td>100.7%</td>
</tr>
</tbody>
</table>

\( ^a \) The values were extrapolated to zero time hydrolysis.

\( ^b \) The values were those obtained after 70 hours of hydrolysis.

\( ^c \) A value of 10.2 moles per mole of protein B1 was obtained in two different experiments using a colorimetric method with p-dimethylaminobenzaldehyde (31). For this table a value of 10.0 residues per mole is chosen and this is equivalent to 1.2% of the weight of protein B1.

\( ^d \) A value of 12.4 moles per mole of protein B2 was obtained in two different experiments (31). For this table a value of 12.0 residues per mole is chosen, and this is equivalent to 2.9% of the weight of protein B2.

\( ^e \) A value of 2.9 moles per mole of protein B2 was obtained in two different experiments (31). For this table a value of 2.9 residues per mole is chosen, and this is equivalent to 1.3% of the weight of protein B2.

\( ^f \) After performic acid oxidation (32) of protein B1, 19.5 cysteic acid residues were obtained per mole of protein B1. If this value is corrected for the reported recovery of cysteic acid of about 94%, the result will be 21 residues per mole. This is equivalent to 1.4% of the weight of protein B1.

\( ^g \) After performic acid oxidation (32) of protein B2, 9.3 cystic acid residues were obtained per mole of protein B2. If this value is corrected for the reported recovery of cysteic acid of about 94%, the result will be 10 residues per mole. This is equivalent to 1.3% of the weight of protein B2.

## Table II

**Molecular weight of protein B1 in guanidine hydrochloride solutions**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Method</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein B1 in 6 M guanidine hydrochloride—0.1 mM mercaptoethanol</td>
<td>Chervenka (12)</td>
<td>71,000</td>
</tr>
<tr>
<td>Carboxymethylated protein B1 in 6 M guanidine hydrochloride</td>
<td>Chervenka (12)</td>
<td>77,000</td>
</tr>
<tr>
<td>Carboxymethylated protein B1 in 6 M guanidine hydrochloride</td>
<td>Yphantis (13)</td>
<td>77,000</td>
</tr>
</tbody>
</table>

## Table III

**NH\(_2\)-terminal groups of protein B1 determined by the cyanate method**

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Protein B1</th>
<th>Control</th>
<th>Corrected values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>1.62</td>
<td>1.12</td>
<td>0.50</td>
</tr>
<tr>
<td>Serine</td>
<td>0.11</td>
<td>0.14</td>
<td>0.57</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1.33</td>
<td>0.47</td>
<td>0.76</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.28</td>
<td>0.32</td>
<td>0.32</td>
</tr>
</tbody>
</table>

result suggested that dissociation of the B1 molecule occurred in the absence of magnesium ions.

Oxidation of protein B1 by air or by diluted hydrogen peroxide (about 0.1 mM) also led to a dissociation of the molecule as seen from a gradual decrease in \( s_{20, w} \) down to 5.7 S. At the same time the enzymatic activity decreased to about 20% of that of the starting material. Both effects seemed to be caused by a time-dependent oxidation of cysteine residues in the B1 molecule to cystines since during inactivation the number of sulfhydryl groups in protein B1 decreased to about 50% of that in the fully active protein and increasing amounts of cystines appeared in hydrolylates of the protein. The hydrolylates contained no methionine sulfoxide, methionine sulfone, or cysteic acid.

Both the dissociation and inactivation of protein B1 could be reversed to a great extent by incubation of the inactivated protein in dithiothreitol (40 mM). Reactivation was pronounced when the protein had not been kept in the inactivated, dissociated state for a long time (some hours).

**COOH-terminal End Groups**—Analyses of the COOH-terminal amino acids were performed by quantitative amino acid analyses after digestion with carboxypeptidase in 1% sodium dodecyl sulfate. This treatment released 1.5 moles of isoleucine per mole of protein B1 after subtraction of the blank values. No significant amount of other amino acids was detected. Increasing the ratio of carboxypeptidase to protein B1 four times only resulted in higher blank values.

**NH\(_2\)-terminal End Groups**—Quantitative NH\(_2\)-terminal amino acid analysis by the cyanate method (24) showed that protein B1 contained two different NH\(_2\)-terminal amino acids: glutamic acid and aspartic acid. Results of the analysis are shown in Table III. After subtraction of the amount of amino acids found in noncarbamylated protein B1, aspartic acid and glutamic acid in the amounts of 0.5 and 0.8 mole per mole of protein B1, respectively, were obtained. Analyses of protein B1
TABLE IV

<p>| Ammonium groups of proteins B1 and B2 determined by the fluorodinitrobenzene method |
|---------------------------------|-------------------------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>Recovery of added carrier*</th>
<th>Amount of [3H]DNP-amino acid corrected for recovery of carrier*</th>
<th>Amount of [3H]DNP-amino acid per mole of protein**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein Bl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>33.2</td>
<td>3.81</td>
<td>11.4</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>34.7</td>
<td>5.92</td>
<td>17.1</td>
</tr>
<tr>
<td>Protein B2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>43.0</td>
<td>15.1</td>
<td>35.0</td>
</tr>
</tbody>
</table>

* Calculated from the absorbance at 364 nm of the [3H]DNP-amino acid pool after chromatography using a molar extinction coefficient of 16,000.

** Calculated from the total amount of radioactivity in the [3H]DNP-amino acid pool after chromatography using a specific activity of 548 cpm per nmole of [3H]DNP-amino acid (see “Experimental Procedure”).

+ Obtained by combining the two first columns of the table.

++ In the experiments 21.0 nmols of protein B1 and 22.2 nmols of protein B2 were used.

Selective Isolation of NH2-terminal Peptides of Protein B1—The data presented thus far indicate that protein B1 consists of two polypeptide chains of the same size but with different NH2-terminal amino acids. The number of lysine plus arginine residues in B1 was too high to permit separation of the tryptic peptides, therefore I isolated the NH2-terminal peptides of protein B1 by the technique of Bruton and Hartley (27) to determine whether more amino acids than the NH2-terminal differed.

The free amino groups of carboxymethylated protein B1 were reacted with [14C]maleic anhydride and after digestion with chymotrypsin the labeled NH2-terminal peptides were isolated by chromatography on Dowex 50 equilibrated with 0.1 M acetic acid. All peptides containing free amino groups will be retarded by such a column while peptides with maleylated amino groups—except for those containing arginine—will pass through (27). In Fig. 3 electrophoresis at pH 6.5 of the Dowex 50 eluate is shown. The radioactive peptides were eluted and samples were hydrolyzed and analyzed for amino acids. Peptides 3 and 4 did not contain any amino acids and from their mobility it was highly probable that they represented maleamic acid and maleic acid (27).

The composition and yields of peptides 1 and 2 are given in Table V. It was clear that the peptides were not identical and further that one could not have been formed from the other by partial proteolysis. Hydrolysis and amino acid analyses of peptides 1 and 2 purified further by electrophoresis at pH 6.5 and the peptides were localized by autoradiography.

Characterization of Protein B2

Molecular Weight Determinations—The molecular weight of a homogeneous preparation of protein B2 was determined earlier as 78,000 by low speed sedimentation equilibrium centrifugation.
plots of In c versus x² were linear. The molecular weight of VI. All centrifugation studies were performed at 20°C and all molecular weights obtained ranged from 32,000 to 42,000 (Table VI). The samples analyzed in the presence of mercaptoethanol to prevent aggregation by disulfide formation. The apparent specific volume was 0.685 ml per g at 20°C, and this value was also used to calculate the molecular weights of the alkylated samples. The samples were hydrolyzed for 20 and 70 hours on the assumption of a molecular weight of 78,000. From these figures the molecular weights of protein B2 from three different long column meniscus depletion runs, two at 4°C and one at 20°C, were 78,000, 79,000, and 78,000, giving an average value of 78,000.

Quantitative Amino Acid Analyses—The results of quantitative amino acid analyses of protein B2 are given in Table I. The nearest integral numbers of amino acid residues were calculated on the assumption of a molecular weight of 78,000. From the analyses a total recovery of 102.7% by weight was obtained for protein B2. The samples were hydrolyzed for 90 and 70 hours as described under “Experimental Procedure.” Protein B2 contained 10 half-cystine residues per 78,000 g of protein.

Molecular Weight Determination in Guanidine Hydrochloride—Protein B2 contains 2 atoms of non-heme iron bound per molecule, suggesting the existence of two polypeptide chains. To investigate this, protein B2 in guanidine hydrochloride solutions was analyzed in the ultracentrifuge by the high speed sedimentation equilibrium method. Two samples were carboxymethylated without prior reduction before centrifugation and two samples analyzed in the presence of mercaptoethanol to prevent aggregation by disulfide formation. The apparent specific volume was 0.685 ml per g at 20°C, and this value was also used to calculate the molecular weights of the alkylated samples. The molecular weights obtained ranged from 32,000 to 42,000 (Table VI). All centrifugation studies were performed at 20°C and all plots of In c versus x² were linear. The molecular weight of protein B2 in guanidine hydrochloride was about half of the value of the native protein, indicating the presence of two polypeptide chains. These were not held together by disulfide bonds, since the same results were obtained with protein carboxymethylated and analyzed in the absence of a reducing agent.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis—Protein B2 showed only one band when analyzed by dodecyl sulfate gel electrophoresis and this had a mobility corresponding to a molecular weight of 43,000. This together with the results from the centrifugations in guanidine hydrochloride indicated that protein B2 contained two polypeptide chains of identical or very similar size.

COOH-terminal End Groups—Carboxypeptidase digestion of protein B2 in 1% dodecyl sulfate released 1.6 moles of leucine per mole of protein after subtraction of the blank values. In addition about 0.4 mole of phenylalanine per mole of protein B2 was detected.

NH₂-terminal End Groups—Determination of NH₂-terminal amino acids by the “dansyl” method (20) showed alanine to be the only NH₂-terminal amino acid in protein B2. This was confirmed by a quantitative NH₂-terminal amino acid analysis by the cyanate method. For some reason the carboxymethylation was incomplete since only 32% of the lysine residues were recovered as homocitrulline after acid hydrolysis of carboxamylated protein B2 (21). No control with non-carboxamylated protein was analyzed but the uncorrected figure was 1.0 mole of alanine per mole of protein B2. When the analysis was repeated by the fluorodinitrobenzene method (25) in an attempt to get a more conclusive value 1.6 moles of NH₂-terminal alanine per mole of protein B2 were obtained (Table IV).

Analysis of Tryptic Peptides of Protein B2—The results from ultracentrifugational analyses and the NH₂-terminal and COOH-terminal end group determinations indicated that protein B2 was composed of two identical polypeptide chains. In a further attempt to determine whether the polypeptide chains of protein B2 were identical, the tryptic peptides derived from the protein were analyzed. Amino acid analyses showed that protein B2 contained 64 lysine plus arginine residues and therefore 64 potential sites of cleavage by trypsin. However, after tryptic digestion of reduced and carboxymethylated protein B2 only about 25 peptides were detected (Figs. 4 and 5). This is a little less than half the number of peptides one would expect if the

**Table V**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Peptide 1</th>
<th>Peptide 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mole/mole peptide</td>
<td>mole/mole peptide</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>1.85 (2)</td>
<td>1.00 (1)</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.16 (0)</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>1.42 (1-2)</td>
<td>4.60 (4-5)</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1.00 (0)</td>
<td>0.30 (0)</td>
</tr>
<tr>
<td>Proline</td>
<td>1.01 (1)</td>
<td>0.56 (0-1)</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.79 (0-1)</td>
<td>0.74 (0-1)</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.19 (0)</td>
<td>1.74 (1-2)</td>
</tr>
<tr>
<td>Valine</td>
<td>0.61 (1)</td>
<td>0.20 (0-1)</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.00 (1)</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.25 (2)</td>
<td>2.04 (2)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.47 (0-1)</td>
<td>0.96 (1)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.55 (1-2)</td>
<td></td>
</tr>
</tbody>
</table>

*Values less than 0.15 mole of amino acid per mole of peptide are not included. In the analyses of peptide 1 about 11 moles of peptide were put on the chromatography column of the amino acid analyzer. The corresponding value for peptide 2 was about 5 moles of peptide.*

**Table VI**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Protein B2 in 6 M guanidine hydrochloride—0.1 M mercaptoethanol</td>
<td>Chervenka (12) 33,000</td>
</tr>
<tr>
<td>2. Carboxymethylated protein B2 in 6 M guanidine hydrochloride</td>
<td>Chervenka (12) 42,000</td>
</tr>
<tr>
<td>3. Protein B2 in 6 M guanidine hydrochloride—0.1 M mercaptoethanol</td>
<td>Yphantis (13) 32,000</td>
</tr>
<tr>
<td>4. Carboxymethylated protein B2 in 6 M guanidine hydrochloride</td>
<td>Yphantis (13) 36,000</td>
</tr>
</tbody>
</table>
two polypeptide chains of protein B2 had different amino acid sequence and trypsin split every lysine and arginine residue.

Interaction between Proteins B1 and B2

Molecular Weight Determinations of Ribonucleoside Diphosphate Reductase—Data from sucrose gradient centrifugations showed that under certain conditions a complex containing equimolar amounts of proteins B1 and B2 was formed. This complex which had an s value of 9.7 S was thought to represent the enzymatically active form of ribonucleotide reductase (4). It was also reported earlier that the presence of the negative effector dATP in the sucrose gradient led to the formation of a complex with an s value of 15.5 S, which was thought to represent an inactive, inhibited form of ribonucleotide reductase (4). However, it was reported in the literature that sucrose can influence protein-protein interaction (30). Therefore, it seemed desirable to repeat these experiments in the absence of sucrose and if possible find conditions where the complexes were stable enough to permit molecular weight determinations.

When an equimolar solution of proteins B1 and B2 in 0.05 M Tris-Cl, pH 7.6-0.015 M MgCl₂-0.004 M dithiothreitol-10⁻⁴ M dTTP was analyzed in the ultracentrifuge the schlieren pattern seen in Fig. 6A was obtained. The protein complex moved as a single peak with an s value of 10.1 S. However, the peak was not symmetrical but showed a slight tailing towards the meniscus. The molecular weight of this dTTP complex was 245,000 as determined by the Archibald method using a partial specific

![Fig. 4. Tryptic peptide pattern of carboxymethylated protein B2. The electrophoresis was performed at pH 6.5. Spots encircled by a broken line were ninhydrin negative. The details of the experiment are described under “Experimental Procedure.”](image)

![Fig. 5. Tryptic peptide pattern of carboxymethylated protein B2. The electrophoresis was performed at pH 3.5. The peptides were labeled as described in Fig. 4. The details of the experiment are described under “Experimental Procedure.”](image)

![Fig. 6. Sedimentation of the dTTP- and the dATP-induced complexes between proteins B1 and B2. A, an equimolar solution of proteins B1 and B2 containing 7.7 mg of protein per ml of 0.05 M Tris-Cl, pH 7.6-0.015 M MgCl₂-0.004 M dithiothreitol-10⁻⁴ M dTTP was centrifuged at 59,780 rpm and 20°. The exposure was made with a bar angle of 70° at 12 min after top speed was reached. B, an equimolar solution of proteins B1 and B2 containing 7.7 mg of protein per ml of 0.05 M Tris-Cl, pH 7.6-0.015 M MgCl₂-0.01 M dithiothreitol-10⁻⁴ M dATP was centrifuged at 42,040 rpm and 20°. The exposure was made with a bar angle of 70° at 16 min after top speed was reached.](image)
volume of 0.715 ml per g, which is the weight average of the partial specific volumes of proteins B1 and B2 assuming a 1:1 complex.

When an equimolar solution of proteins B1 and B2 was analyzed under the same conditions as above except that dATP was used instead of dTTP the pattern seen in Fig. 6B was obtained. The fastest moving peak containing about 60% of the total material had an s value of 22.1 S. In addition two slower peaks with s values of 16.6 S and 8.0 S were seen. No meaningful molecular weight determination could be made on this material due to the pronounced heterogeneity.

When the solution containing the dATP complex was made 10% with respect to sucrose a completely changed sedimentation pattern was obtained. Now only one peak with an s2,0, of about 13.0 S appeared instead of the three peaks seen in the absence of sucrose. I will return to this point in the discussion.

**DISCUSSION**

Protein B1 had a high affinity for dATP-substituted Sepharose and was specifically eluted by dATP or ATP. The concentration needed for elution was about 1000-fold higher than the dissociation constant in the case of dATP and about 100-fold higher than the dissociation constant in the case of ATP. No elution was observed with dTTP or dGTP. This fits the model of protein B1 having two types of binding sites for effectors, the h sites binding all four effectors (dATP, ATP, dTTP, dGTP) and the l sites binding only dATP and ATP (6). No elution of protein B1 bound via the l sites would be expected to occur with dTTP or dGTP.

Affinity chromatography on dATP-Sepharose resulted in a protein B1 preparation which, in the presence of dithiothreitol, appeared homogeneous by ultracentrifugational criteria. When the partial specific volume of this B1 preparation was redetermined, significantly lower values were obtained than the ones published earlier (7). However, the old values were determined by the density gradient technique, and this way of measuring density is more liable to systematic errors, e.g. transfer of water from drops to surrounding solvent (33), than density measurements performed in the precision density meter used in this work (17). Therefore, the new partial specific volume of protein B1 is believed to be correct.

Molecular weight determinations by ultracentrifugation in guanidine hydrochloride and dodecyl sulfate polyacrylamide gel electrophoresis showed that protein B1 was composed of two polypeptide chains of similar size. The two chains seemed to have the same COOH-terminal amino acid (isoleucine) but different NH2-terminal amino acids (glutamic acid and aspartic acid). The latter was shown by two independent methods and the same result was obtained when thiol sulfonyl fluoride was added during the preparation of protein B1 to reduce the possibility of proteolysis (cf. 20).

It was not possible to analyze the tryptic pattern of protein B1 to learn if more than the NH2-terminal amino acids differed since the number of arginine plus lysine residues was too high. Instead the method of Bruton and Hartley (27) was used to isolate specifically peptides containing the NH2-terminal sequences of the two polypeptide chains of protein B1. This resulted in the isolation of two peptides with different amino acid composition. One peptide contained NH2-terminal glutamic acid just like one of the polypeptide chains of protein B1, thereby confirming its origin. The NH2-terminal residue of the other peptide could not be identified because of technical difficulties. However, from the composition of this peptide, one aspartic acid and no glutamic acid, it was likely that it originated from that polypeptide chain of protein B1 which contained NH2-terminal aspartic acid.

Together all of these results suggest that the two polypeptide chains of protein B1 are nonidentical, at least in the NH2-terminal ends. An additional observation that might fit this "non-identical chain" model is the two-component pattern observed when protein B1 was analyzed by polyacrylamide gel electrophoresis. The two components might represent the two not completely separated polypeptide chains of protein B1 which dissociated during electrophoresis. The B1 molecule readily dissociates on oxidation (see below), and this may occur during electrophoresis. Hopefully further studies will reveal whether there really exist two different genes for the polypeptide chains of protein B1 or whether the observed dissimilarities are only a consequence of a preparation artefact. The identity or non-identity of the two polypeptide chains of protein B1 will be of great importance in the study of the binding sites for substrates and effectors to the B1 molecule.

Magnesium ions seemed to be required to keep the polypeptide chains of protein B1 together much in the same way as Mg2+ is required to form a complex between proteins B1 and B2 (4). However, in the first case the magnesium requirement was observed only after treatment with EDTA, which suggested a stronger binding of the Mg2+ than in the second case.

During preparation of protein B1 it was necessary to include thiol reagents such as dithiothreitol in the buffers to get good yields of B1 activity (7). This is explained by the finding in this paper that oxidation of protein B1 led to dissociation and inactivation of the molecule. The ability of protein B1 to dissociate on oxidation and associate on reduction is not unique but has been observed for many proteins (34). The reason for the inactivation and dissociation of the B1 molecule seems to be an oxidation of cysteine residues to cystines. Sulfenic acid formation as in the case of glyceraldehyde 3-phosphate dehydrogenase (35) did not occur, since arsenite could not reactivate protein B1 (not shown here). Furthermore, no formation of methionine sulfoxide was observed on oxidative inactivation of protein B1. This has been reported to be the reason for loss of activity in the case of ribonuclease and adrenocorticotropic hormone (19). Cystines can be reduced by thiol and this explains the reactivating effect of dithiothreitol. On the other hand the failure to reactivate completely protein B1 which had been kept in the inactivated form for long times (some hours) could not be explained by the formation of cystic acid or methionine sulfoxide since these were absent in hydrolysates of inactivated protein B1.

The binding of protein B2 to protein B1 was too weak to allow the purification of both proteins on dATP-Sepharose. Instead protein B2 used in these studies was a homogeneous preparation purified by the procedure given in Reference 7. The final step was Sephadex G-200 chromatography which replaced the gel electrophoresis used earlier, since the latter procedure was shown to partly inactivate protein B2 (6). The partial specific volume of this B2 preparation was much lower than the earlier published value, as in the case of protein B1. However, the molecular weight as determined by sedimentation equilibrium centrifugations was the same as that published earlier, 78,000. One reason for these discrepancies might be that the B2 preparation used in the earlier studies was produced by gel electrophoresis which did not result in a completely native preparation (6).

Ultracentrifugation in guanidine hydrochloride and dodecyl sulfate gel electrophoresis showed that protein B2 contained...
two polypeptide chains of similar or identical size. These and the following data strongly indicate that protein B2 consists of two identical polypeptide chains. (a) Only one type of NH$_2$-terminal amino acid ( = alanine) was found with three different methods. The low value of less than 1 mole of alanine per mole of protein B2 obtained by the cyanate method most probably was a result of incomplete carbamylation. The fluorodinitrobenzene method gave 1.6 moles of alanine per mole of B2, which is in good agreement with the proposed model. (b) Carboxypeptidase digestion in dodecyl sulfate released 1.6 moles of leucine per mole of protein B2. (c) The number of tryptic peptides of protein B2 was in agreement with that which would be expected if the molecule consisted of two identical polypeptide chains.

When equimolar amounts of proteins B1 and B2 were incubated with dTTP in the presence of magnesium ions and dithiothreitol a complex sedimenting with an $s_{20,w}$ of 10.1 S and with a molecular weight of 245,000 was observed in the analytical ultracentrifuge. The molecular weight is close to what would be expected of a 1:1 complex between proteins B1 and B2, indicating that the active form of ribonucleotide reductase consists of one B1 molecule bound to one B2 molecule. This is in agreement with data from sucrose gradient centrifugations (4). However, both this and earlier experiments were performed in the absence of substrate and that might influence the result.

Under conditions where the ribonucleotide reductase was inhibited by binding the negative effector dATP the active 9.7 S complex was shifted to an inactive 15.5 S complex as determined by sucrose gradient centrifugation (4). However, both this and earlier experiments were performed in the absence of substrate and that might influence the result. When sucrose was added to the dATP-induced complex between B1 and B2 in the analytical centrifuge, the sedimentation pattern changed completely. This time only one peak with an $s_{20,w}$ of about 13.0 S was obtained, showing that the interaction between B1 and B2 in the presence of dATP was strongly influenced by sucrose (cf. 30). Definition of the nature of the inhibitory complex of ribonucleotide reductase must await further studies.

REFERENCES


Physicochemical Characterization of Ribonucleoside Diphosphate Reductase from *Escherichia coli*
Lars Thelander


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