80295-58-5; complement C5b-8, 82903-91-1; complement C9, 80295-59-6; complement C5b-9, 82986-89-8.

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Production and Characterization of Monoclonal Antibodies against the Two Subunits Proteins B1 and B2 of *Escherichia coli* Ribonucleotide Reductase†

Åsa Anderson, Tamara Barlow, Elisabet Pontis, and Peter Reichard*

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

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ABSTRACT: Ribonucleotide reductase from *Escherichia coli* consists of two nonidentical subunits, named protein B1 (170 000) and protein B2 (87 000). We purified and characterized five monoclonal antibodies against B1 and three against B2 from hybridomas obtained by fusion of spleen cells from immunized mice and the myeloma cell line P3-X63Ag8. All are of the IgG_1 class with a high affinity for the antigen with dissociation constants in the nanomolar range. Four of the anti-B1 monoclonals and all three anti-B2 monoclonals neutralize reductase activity while one anti-B1 monoclonal binds tightly to B1 without affecting its activity. Fab fragments prepared from three anti-B1 monoclonals had similar dissociation constants. The anti-B1 monoclonals interacted with separate epitopes while two of the anti-B2 monoclonals appeared to react with the same epitope. In the case of B1, various allosteric states of the protein induced by binding of effectors had no apparent effect on the interaction with monoclonals, nor did their binding prevent subsequent binding of effectors. With B2, binding of monoclonals did not affect the typical electron paramagnetic resonance spectrum of the protein and thus did not involve either the tyrosyl free radical or the iron center of B2. All neutralizing antibodies interfered with the interaction between the two subunits, explaining their effect on enzyme activity, since active ribonucleotide reductase consists of a B1–B2 complex.

Ribonucleotide reductase from *Escherichia coli* catalyzes the reduction of the four common ribonucleoside diphosphates to the corresponding deoxyribonucleotides and provides the cell with the proper supply of precursors for DNA (Thelander & Reichard, 1979; Lammers & Follmann, 1983). The enzyme has been obtained in pure form, its complete nucleotide sequence was recently published (Carlsson et al., 1984), and work is in progress to determine its 3D structure by X-ray crystallography (Joelsson et al., 1984). The protein is made

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* Address correspondence to this author.

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up of two nonidentical subunits, called protein B1 (M_r 170K) and protein B2 (M_r 87K), each consisting of two identical or nearly identical polypeptide chains.

B1 contains two apparently identical binding sites for substrates (von Döbeln & Reichard, 1976) and four sites for allosteric effectors (Brown & Reichard, 1969b). From binding experiments involving equilibrium dialysis, the existence of two separate classes of effector sites was deduced: h sites or specificity sites (binding ATP, dATP, dTTP, or dGTP), directing the substrate specificity of the enzyme, and l sites or activity sites (binding only ATP or dATP), regulating the overall activity of the enzyme. Binding of the various effectors affects the dissociation constants for the various ribonucleotides (von Döbeln & Richard, 1976), presumably by an effect on the conformation of the protein.

The second subunit, protein B2, contains an iron center, consisting of two anti-ferromagnetically coupled Fe^{3+} ions linked by a μ-oxo bridge (Atkin et al., 1973; Petersson et al., 1980; Sjöberg et al., 1982) and an oxidized tyrosyl radical (Sjöberg et al., 1977, 1978; Reichard & Ehrenberg, 1983) stabilized by the iron center. This radical is derived from a tyrosyl residue of the polypeptide chain and is postulated to participate in the catalytic process (Stubbe & Ackles, 1980; Sjöberg et al., 1983; Harris et al., 1984).

Monoclonal and peptide antibodies have recently become increasingly important in studies concerning structure–function relationships of enzymes. In this paper, we describe and characterize several monoclonal antibodies to proteins B1 and B2. In an accompanying paper (Anderson et al., 1986), we report the use of some of these monoclonals to purify and study protein B1.

Experimental Procedures

Materials

Pure preparations of proteins B1 and B2 were obtained from *E. coli* overproducer strains KK546 (Eriksson et al., 1977), C600/pBS1,[1] and C600/pLSH1 (Larsson, 1984). Thioredoxin and thioredoxin reductase were provided by Dr. A. Holmgren, Karolinska Institutet. Papain was from Sigma, St. Louis, MO. Radioactive materials were obtained from Amersham, England; Sepharose 4B, Sephadex G-25, protein A, and protein A coupled to Sepharose were from Pharmacia, Sweden. Rabbit sera against mouse immunoglobulins (RAM)[2] were obtained from DAKO, Glostrup, Denmark; sera specific to mouse immunoglobulin chain subclasses were from Litton Bionetics, Kensington, MD, and alkaline phosphatase conjugated RAM was from Sigma. *Staphylococcus aureus* (Cowan 1) was a gift from Dr. Tord Holme, Department of Bacteriology, Karolinska Institutet. Cell culture media and fetal calf serum were obtained from GIBCO, Paisley, Scotland; agarose and nitrocellulose membranes were from Bio-Rad, Richmond, CA, and Freund's complete and incomplete adjuvant were purchased from Difco, Detroit, MI. All other chemicals were of reagent grade.

Methods

Coupling of Proteins to Sepharose. Proteins were coupled to Sepharose 4B by the method of Kohn & Wilchek (1982). Routinely, 150 mg of CNBr was used to activate 10 mL of packed Sepharose, and 10 mg of protein was coupled to each milliliter of activated packed Sepharose.

Iodination of Proteins. Proteins were labeled with ^{125}I by the method of Bolton & Hunter (1973). The protein was incubated with the reagent for 15 min; an excess of glycine in 0.2 M borate buffer, pH 8.5, was added and allowed to react for 30 min before chromatography on Sephadex G-25 to purify the labeled protein. Protein A was iodinated to a specific activity of 5×10^5 cpm/μg, anti-B1 and anti-B2 monoclonals to 3×10^5 cpm/μg, and protein B2 to 250 cpm/μg. Anti-B1 monoclonals were further purified by affinity chromatography on a B1 column. With B2, special precautions were taken to retain enzyme activity by keeping solutions on ice whenever possible. The specific enzyme activity of iodinated B2 was identical with that of the starting material. All proteins were stored at 4 °C in 50 mM Tris, pH 7.5, 0.1 M KCl, and 0.02% NaN_3 except protein B2, which was stored at −70 °C in 50 mM Tris, pH 7.6, and 20% glycerol.

Gel Electrophoresis. Samples were denatured and run on PAGE–SDS slab gels (7.5 or 10%) as described by Reiser & Stark (1982) with the Neville buffer system (Neville, 1971). The gels were stained with 0.25% Coomassie Brilliant Blue and destained as in the former reference before drying.

Immunoblotting. Proteins were transferred to nitrocellulose from slab gels by the method of Blake et al. (1984). Tween buffer (0.5% Tween 20 and 0.5 M NaCl in PBS) was used to block unoccupied sites on the nitrocellulose sheet and to make up dilutions and was used in washes when the sheet was probed with antibodies. The sheet was incubated with antibody for 3 h and then for a further 2 h with alkaline phosphatase conjugated RAM (1:1000) before developing with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium in 0.1 M ethanolamine–HCl buffer, pH 9.6.

Monoclonal Antibody Production (Fazekas et al., 1980; Galfrê & Milstein, 1981). Six week old female Balb/c mice were immunized subcutaneously either with 100 μg of a mixture of both reductase subunits containing predominantly B1 or with 80 μg of B2 alone in Freund's complete adjuvant. The animals were boosted 4 times intraperitoneally with 50 μg of the antigen at 6-week intervals. The two mice showing the highest antibody titres to B1 and B2, respectively, were boosted intravenously with 40 μg of protein and given the same dose intraperitoneally on each of the following 3 days before they were sacrificed. Half the cells from each spleen were fused with the P3-X63Ag8 (Köhler & Milstein, 1975) mouse myeloma line and the other half with the X63-Ag8.653 line (Kearney et al., 1979). The X63-Ag8.653 line is derived from the P3-X63Ag8 line, but unlike its parent does not secrete either the IgG_1 heavy chain or the κ light chain. Thus, any hybrids obtained with the former produce pure monoclonal antibodies. Supernatants from hybridomas were used for screening. Positive hybridomas were then cloned on soft agar with spleen cells in the feeder layer (Galfre & Milstein, 1981). Large amounts of monoclonal antibodies were prepared from ascites of Balb/c mice and purified by precipitation with 50% saturated ammonium sulfate followed by chromatography on protein A coupled to Sepharose 4B. The immunoglobulin fraction was in each case eluted with 0.1 M sodium citrate buffer, pH 4.0, dialyzed overnight against 50 mM Tris-HCl buffer, pH 7.6, and 100 mM KCl, and stored frozen at −20 °C. Fab fragments were obtained by digestion of IgGs with papain as described by Mage (1980). Briefly, 10 mg of protein A purified IgG was digested with 0.1 mg of papain for 60 min at 37 °C. After addition of iodoacetamide followed by dialysis, Fab and Fc fragments were separated on 2-mL Tris–acryl-DEAE columns, equilibrated with 10 mM potassium phos-

[1] B. M. Sjöberg, S. Hahne, M. Karlsson, H. Jörnvall, M. Göransson, and B. E. Uhlin, unpublished results.

[2] Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; PAGE–SDS, polyacrylamide gel electrophoresis in 1% sodium dodecyl sulfate; PBS, phosphate-buffered saline; RAM, rabbit anti-mouse serum; Tris, tris(hydroxymethyl)aminomethane; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

phate buffer, pH 7.6. Under those conditions, the Fc fragment was retained by the column while the Fab fragment was eluted directly with 10 mM phosphate buffer.

Solid-Phase Radioimmunoassay for Antibodies (Galfrê & Milstein, 1981). Poly(vinyl chloride) 96-well microtitre plates were coated for 1 h with antigen in PBS (3 μg in 30 μL per well). This and all subsequent incubations were carried out at room temperature. The plates were washed once with BSA buffer (1% BSA, 0.1% sodium azide, 5 mM EDTA in PBS) and the media incubated overnight (20 μL/well). The plates were washed twice with BSA buffer and incubated for 3 h with RAM (0.1 mg/mL in BSA buffer, 20 μL/well), washed again in the same way, and incubated for 1 further h with 20 μL of ^{125}I-labeled protein A (diluted in BSA buffer to an activity of 100.0000 cpm in 20 μL/well). Finally, the plates were washed 3 times with BSA buffer before the well bottoms were cut off and counted in a γ-counter.

Solid-Phase Competition Assay. This assay was used to determine whether two antibodies were bound to the same epitope. A fixed amount of a ^{125}I-labeled monoclonal was mixed with various amounts of other independently isolated nonlabeled monoclonals before addition to wells of a microtiter plate coated with antigen as described above. The ^{125}I-labeled anti-B1 monoclonals were purified on a B1 affinity column before use, while this was not the case with the anti-B2 monoclonals. After overnight incubation, the plates were washed 3 times with BSA buffer before the well bottoms were cut off and the amount of ^{125}I was counted.

Binding of Monoclonals in Solution. The interaction between antibodies and the reductase subunits was measured by inhibition of enzyme activity or by the formation of immunocomplexes. In the latter case, the complexes were adsorbed to protein A on *S. aureus* (Kessler, 1975) before determination of the remaining activity of free B1 or B2. The interaction between B1, B2, and IgGs was also studied by sucrose gradient centrifugation.

B1 and B2 used for the binding assays were pure proteins as judged from gel electrophoresis. Their specific activities were 400–800 (B1) and 4000–6000 (B2). Depending on minor variations in assay conditions, in particular on the quality of the complementary subunit, one and the same preparation of a subunit showed the indicated variations in specific activity.

B1 Activity by Isotope Assay (Brown et al., 1969). Various amounts of B1 and antibody werre first incubated on ice for 1–4 h in 50 mM Tris-HCl, pH 8.5, 0.5 mM DTT, and BSA (1 mg/mL) in a final volume of 10 μL. In the direct enzyme neutralization assay, 5 μL of a concentrated assay mixture was added to give final concentrations of 1.5 mM ATP, 12 mM $MgCl_2$, 10 mM DTT, 10 μM thioredoxin, 40 mM HEPES buffer, pH 8.0, and 0.5 mM [^{3}H]CDP (30 cpm/pmol) supplemented with an at least 5-fold molar excess of the B2 subunit. After 30-min incubation at 30 °C, 0.5 mL of 1 M $HClO_4$ was added, the solution was heated for 10 min in boiling water, and the amount of [^{3}H]dCMP formed was determined after separation from CMP on Dowex 50W-X8 (Reichard, 1958).

B2 Activity by Isotope Assay (Brown et al., 1969). The first incubation of B2 with antibody was made in 50 mM Tris-HCl, pH 8.5, and 20% glycerol. Subsequent steps were as described for B1 except that an excess of B1 was used as the complementary subunit of the reductase during the final incubation.

B1 Activity by Spectrophotometric Assay (Brown et al., 1969). This assay was used for the titration of B1 with antibodies, e.g., in the experiments described in Figure 4. Antibody was added directly to an assay mixture (final volume 0.15 mL) in a quartz cuvette containing an excess of B2 and ATP, $MgCl_2$, thioredoxin, and buffer (but *not* DTT or CDP) at the concentrations given above for the isotope assay. In addition, the mixture contained 0.4 mM NADPH and 0.3 μM thioredoxin reductase. The solution was incubated for 10 min at room temperature in a PMQ3 Zeiss spectrophotometer that automatically recorded the small decrease in the absorption at 340 nm (background slope). The reaction started by addition of CDP (final concentration 0.5 mM). B1 activity was obtained from the decrease in the absorbance at 340 nm.

Immune Complexes. When the formation of immune complexes was assayed, 4 μL of RAM (10 mg/mL) was added after the first 10-μL incubation of enzyme with antibody, and after an additional 1 h on ice, the immune complexes were adsorbed to *S. aureus* (3 μL of a 20% suspension) and removed by centrifugation. Enzyme activity remaining in the supernant solution was determined after addition of 5 μL of the concentrated assay mixture as described above.

Calculations. In Figures 3–5, the amount of unbound B1 or B2 was calculated from the difference in enzyme activity between the sample containing no antibody and that containing a given amount of antibody. From this value and from the known molecular weights of B1 (170 000) and B2 (87 000), the molar concentrations of free antigen (L) were calculated for the Scatchard plots. The difference between total antigen and free antigen gave the value for bound antigen as a basis for the calculation of the moles of antigen bound by 1 mol of antibody ($\bar{v}$). In this latter calculation, we assumed molecular weights of 150 000 for IgGs and 50 000 for Fab fragments.

Sucrose Gradient Centrifugations. These experiments measured the effects of IgGs on the formation of a complex between ^{125}I-labeled B2 and nonlabeled B1. Enzymes (25 μg of B2 and 75 μg of B1) and IgG (250 μg of anti-B2 or 375 μg of anti-B1) were preincubated on ice for 1 h in 50 mM Tris-HCl, pH 7.6, with 15 mM $MgCl_2$, 10 mM DTT, and 0.1 mM dTTP, the complementary subunit was added where relevant, and the sample (0.1 mL) was layered onto a 4.6-mL 5–20% sucrose gradient in the same buffer. Centrifugation was made in a SW 50.1 rotor in a Beckmann L2-65B centrifuge at 20 °C and 28 000 rpm for 14 h. The tubes were punctured at the bottom, 10-drop fractions were collected at room temperature, and the samples were counted directly in a γ-counter.

EPR Spectra. To measure the effect of anti-B2 monoclonals on the tyrosyl free radical of B2, we preincubated 26 μg of B2 with 440 μg of the various anti-B2 monoclonals in 50 mM Tris HCl, pH 7.6, 0.1 M KCl, and 20% glycerol for 1 h at 0 °C in a final volume of 300 μL. The amplitude and the shape of the signal for the tyrosyl radical was then monitored at 77 K in a Bruker 100 EPR spectrometer (Barlow et al., 1983) after 0, 5, 15, 30, and 60 min. In addition, aliquots were taken at each time point to measure B2 activity by the isotope method described above.

RESULTS

Anti-B1 Monoclonals. Spleen cells from one mouse yielding a high-titer anti-B1 serum were hybridized with either P3-X63Ag8 (P3) or X63-Ag8.653 myeloma cells. The resulting hybridomas were analyzed with a solid-phase radioimmunoassay for their ability to secrete B1 binding antibodies. Ten hybridomas, designated anti-B1-a to anti-B1-k, gave high values in this assay and were used for our further experiments. All were derived from fusions with P3 cells.

The hybridomas were injected intraperitoneally into mice; antibodies were precipitated from the ascites with ammonium

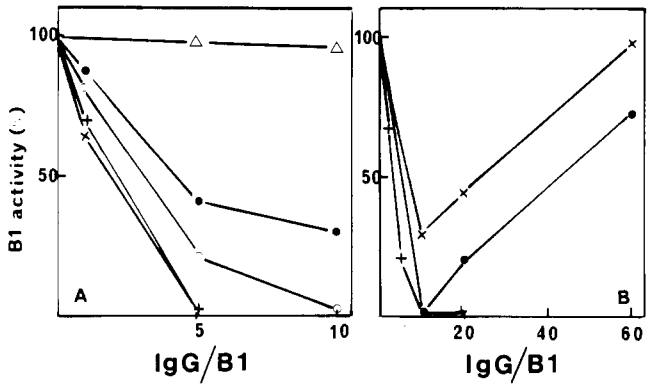

FIGURE 1: Inhibition of protein B1 by monoclonal antibodies. (Panel A) B1 (0.7 μg) was incubated at 0 °C for 4 h with various amounts of anti-B1-a (●), anti-B1-c (+), anti-B1-g (×), anti-B1-i (○), and anti-B1-k (△) in a final volume of 20 μL; 5 μL were then analyzed for B1 activity with the isotope assay. (Panel B) B1 (0.3 μg) was incubated with anti-B1-k as described for panel A. After 4 h at 0 °C, rabbit antimouse IgG [(×) 10 μg; (●) 20 μg; (+) 40 μg] was added during 1 additional h incubation at 0 °C. The immune complexes were adsorbed to *S. aureus* (3 μL of a 20% suspension) during 30 min at 0 °C, and the suspension was then centrifuged. B1 activity was determined with the isotope assay on 10 μL of supernatant solution. The abscissa gives the excess of IgG on a weight basis.

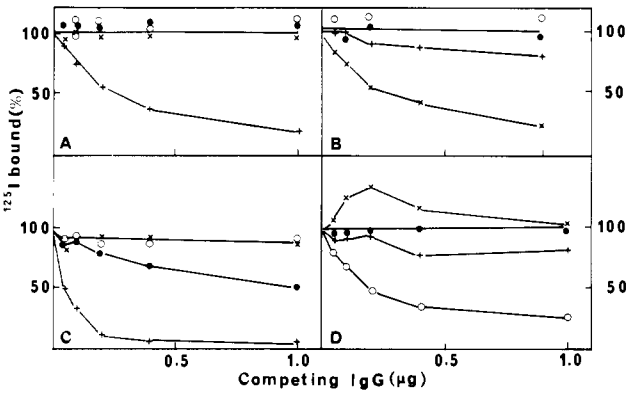

FIGURE 2: Competition between IgGs for separate epitopes. Each panel demonstrates the competition between 40 ng of ^{125}I-labeled IgG (purified by affinity chromatography on protein B1 columns) and increasing amounts of unlabeled IgGs (purified by chromatography on protein A columns) for binding to B1 in solid-phase radioimmunoassays. Panel A describes the binding of labeled anti-B1-c; panel B, labeled anti-B1-g; panel C, labeled anti-B1-i; panel D, labeled anti-B1-k. Symbols for nonlabeled IgGs: (+) anti-B1-c; (×) anti-B1-g; (●) anti-B1-i; (○) anti-B1-k.

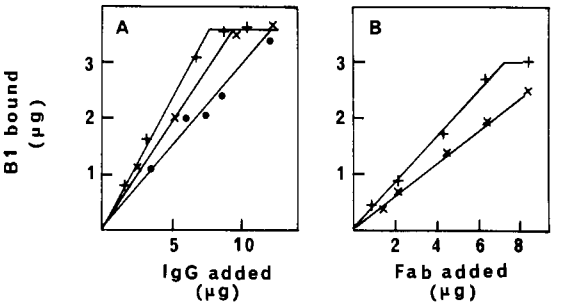

FIGURE 3: Titration of B1 activity with IgGs (panel A) or Fab fragments (panel B). B1 [3.6 μg in (A); 3.0 μg in (B)] was incubated for 10 min at room temperature in microcuvettes with the amounts of IgG indicated and under standard conditions for the spectrophotometric B1 assay, except for the absence of substrate (CDP). During this time period, the background decrease in the absorbance at 340 nm was recorded. The reaction was then started by addition of CDP. For each sample, the amount of active B1 could be calculated from the slopes of the decrease in the absorbance at 340 nm. The amount of B1 bound by the IgG or Fab fragment could then be calculated from the differences between a given sample and the sample containing no antibody. Symbols were as follows: (+) anti-B1-c; (×) anti-B1-g; (●) anti-B1-i.

sulfate and, after dialysis, assayed for their ability to neutralize B1 activity. Results from five positive assays are depicted in Figure 1. Four of the antibodies showed neutralizing activity while the fifth, anti-B1-k, did not inhibit the activity of the enzyme (Figure 1A). However, anti-B1-k bound to the enzyme, and after addition of RAM, an immune complex could be adsorbed to protein A present on formalinized *S. aureus* and removed from solution by centrifugation of the bacterial suspension. In these experiments it was of crucial importance for RAM to be present in excess over the mouse monoclonal (Figure 1B).

The five antibodies described in Figure 1 were purified by chromatography on protein A. Their chromatographic behavior suggested that all belonged to the IgG_1 class. This was confirmed by gel diffusion analysis (data not shown). From three of the antibodies (anti-B1-c, anti-B1-g, and anti-B1-k) as well as from the parent P3 line the corresponding Fab fragments were prepared. During denaturing gel electrophoresis of the IgGs and their Fab fragments (data not shown), each IgG contained one heavy and one light chain whose mobilities corresponded to those of the parent P3 IgG. Each monoclonal gave an additional light chain, and anti-B1-c also showed a second heavy chain. The pattern for the Fab fragments was more complex, but here again each fragment gave rise to two bands corresponding to those found for the P3 fragment as well as an additional band(s). These results demonstrated that the monoclonal antibodies consist of a mixture of IgG molecules containing heavy and light chains from both the parent P3 myeloma and from the mouse lymphocyte responsible for the specific anti-B1 activity.

We next investigated whether the anti-B1 activity of the IgGs was directed against separate antigenic determinants of B1. Competition experiments were made with four monoclonals labeled with ^{125}I. Each was purified by affinity chromatography on B1 columns since it seemed possible that a fraction of the antibodies had been denatured by the labeling procedure. The four corresponding nonlabeled monoclonals were then allowed to compete with each ^{125}I-labeled anti-B1 preparation in solid-phase radioimmunoassays (Figure 2). For three of the labeled antibodies (anti-B1-c, anti-B1-g, and anti-B1-k), the results were clear-cut and indicated that each was directed against a separate epitope. The results with the fourth, anti-B1-i, were more complicated. Here (Figure 2C), the strongest "competition" occurred with nonlabeled anti-B1-c rather than with anti-B1-i. At first sight, this might suggest that both antibodies bind to the same epitope, which has a higher affinity for anti-B1-c. However, we will show below that both monoclonals in solution have about the same affinity for B1. Even though the two tests measure slightly different parameters, we believe that the combined results suggest that the two antibodies bind to separate epitopes and that the epitope responsible for the binding of anti-B1-i is masked when anti-B1-c is attached to B1. The reverse is not true since nonlabeled anti-B1-i did not inhibit the binding of labeled anti-B1-c (Figure 2A).

Four of the monoclonal antibodies neutralized the activity of B1. In the following experiments with protein A purified IgGs and their corresponding Fab fragments, we investigated in more detail the interactions between B1 and antibodies. Figure 3 depicts titrations of B1 activity by IgGs (Figure 3A) or Fab fragments (Figure 3B). In these experiments, a spectrophotometric assay (Brown et al., 1969) was used to measure enzyme activity. In this assay, the reduction of a ribonucleotide is coupled via the thioredoxin system to the

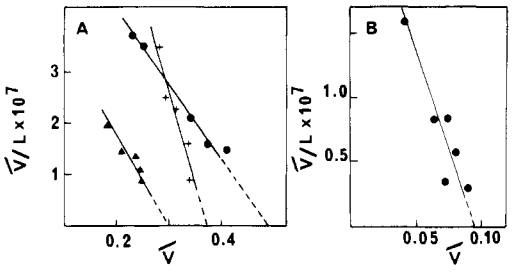

FIGURE 4: Scatchard plots for binding of IgGs (panel A) and Fab fragment of anti-B1-c (panel B). Binding was measured by neutralization of B1 activity with IgGs after adsorption of immune complexes to *S. aureus*. Between 20 and 120 ng of B1 was preincubated with 100–300 ng of IgG or Fab fragment and then treated with RAM and staphylococci as described in the legend to panel B of Figure 1 before assay for B1 activity. For a given sample, the amount of B1 bound was calculated from the difference between the remaining B1 activity and the B1 activity of the noninhibited sample. Enzyme activities were then transformed into values for L and $\bar{v}$ as described under Methods. From the slopes of the Scatchard plots, we determine in panel A K_D values of 7 nM for anti-B1-c (●), 6 nM for anti-B1-i (▲), and 3 nM for anti-B1-k (+) and in panel B 4 nM for the Fab fragment of anti-B1-c.

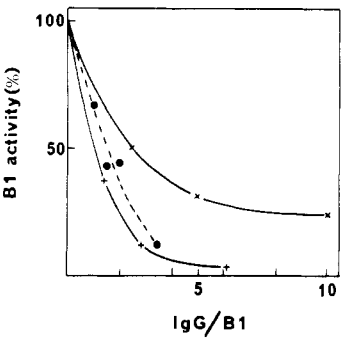

FIGURE 5: Neutralization of B1 activity in extracts of *E. coli*. A crude extract from wild-type *E. coli* containing 96 μg of protein (×) or from KK546 containing 9.6 μg of protein (+) or pure protein B1 (●) was first incubated at 0 °C for 1 h with various amounts of anti-B1-c and then assayed for B1 activity by the isotope assay. The abscissa gives the molar excess of anti-B1-c over protein B1. For the crude extracts, the concentration of B1 was computed from the known specific activity of pure B1 and the determined specific activity in the extract.

oxidation of NADPH and is monitored by the decrease in the absorbance at 340 nm. IgGs or Fab fragments were added directly to cuvettes containing all the components required for the assay of B1 activity except for the substrate, CDP. Reaction mixtures were first incubated for 10 min in the absence of substrate to allow the antibody and B1 to interact and to obtain a value for the background oxidation of NADPH. The assay proper was then started by addition of CDP, and the reaction rate was determined from the linear decrease in the absorbance at 340 nm.

In these experiments, we determined the effect of the addition of increasing amounts of IgGs or Fab fragments to a series of reaction mixtures containing a constant amount of B1. From the inhibition of the oxidation of NADPH, we could then calculate the amount of B1 bound by the antibody. Figure 3 demonstrates that at these concentrations of reactants binding of antibody to B1 was stoichiometric and that between 2 and 3 mg of antibody or Fab fragment was required to neutralize 1 mg of B1.

CDP was the substrate in these experiments, with ATP as the positive allosteric effector. Similar experiments were carried out with GDP or ADP as substrates using dTTP and dGTP, respectively, as effectors. In all cases, IgGs and Fab fragments neutralized B1 activity (data not shown). Whenever detailed quantitative analyses were made, the results were indistinguishable from those described for CDP, demonstrating that binding of antibodies did not depend on any specific allosteric conformations of B1.

The results described so far demonstrate a high affinity of all antibodies for B1. The precise measurements of binding constants required much lower concentrations of the reactants. To this purpose, we used a more sensitive isotope assay (Brown et al., 1969). Figure 4 shows data for three IgGs and one Fab fragment. The affinities of the fourth IgG, anti-B1-g, and its Fab fragment were too high to be measured even with this assay. The Scatchard plots of Figure 4A are derived from experiments in which various concentrations of IgGs and B1 were preincubated on ice for 1 h before the immune complexes thus formed were adsorbed to protein A on *S. aureus* in the presence of an excess of RAM. After centrifugation, the remaining B1 activity was determined. In this way we could also measure the affinity of anti-B1-k, a nonneutralizing IgG. The values for L and $\bar{v}$ were calculated as described under Methods. Dissociation constants ranging from 3 to 7 nM can be calculated from the curves. The binding of the Fab fragment of anti-B1-c (Figure 4B) was determined in a direct enzyme neutralization test. In this case, a dissociation constant of 4 nM was found.

Table I: Effect of Anti-B1 Monoclonals on Binding of dATP and dTTP to Protein B1[a]

additions	dATP		dTTP	
	μM	pmol bound	μM	pmol bound
none	0.06	24	0.08	12
	4.5	86		
anti-B1-c	0.06	20	0.08	4
	4.5	80		
anti-B1-g	0.06	33	0.08	9
	4.5	104		
anti-B1-i	0.06	20		
	4.5	68		
anti-B1-k	0.06	30	0.08	9
	4.5	91		
P3	0.06	20	0.08	7
	4.5	76		

[a] The ^{32}P-labeled nucleotides at the indicated concentrations were mixed with 10 μg of protein B1 in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 2 mM DTT in a final volume of 1.0 mL and filtered through 0.22-μm millipore filters.[3] When indicated, protein B1 was preincubated at 0 °C with 40 μg of monoclonal for 30 min.

To what extent is B1 activity in crude bacterial extracts neutralized by addition of IgGs? To answer this question, extracts from either wild-type *E. coli* C600 or from the lysogenic strain KK546 (Eriksson et al., 1977), an overproducer or ribonucleotide reductase, were preincubated with various amounts of anti-B1-c and then assayed for B1 activity (Figure 5). The extract from the wild-type bacterium contained 2 μg of B1/mg of total protein; that of KK546, 50 μg, as calculated from the determined B1 activity of the extract and the known specific activity of the pure protein. The activity of both extracts was strongly inhibited. For KK546, the degree of inhibition was identical with that observed for pure B1, while the activity of the wild-type extract was inhibited less effectively.

Can we localize the inhibition of B1 to specific functions of the protein? The B1 subunit of ribonucleotide reductase is responsible for the allosteric properties of the enzyme and can bind various effector nucleoside triphosphates. It also binds the ribonucleoside diphosphates acting as substrates. Finally, B1 must bind to the second subunit of the reductase, protein B2, in order to form an active enzyme.

The effect of the monoclonals on binding of effectors to B1 was investigated with a newly developed assay that measures

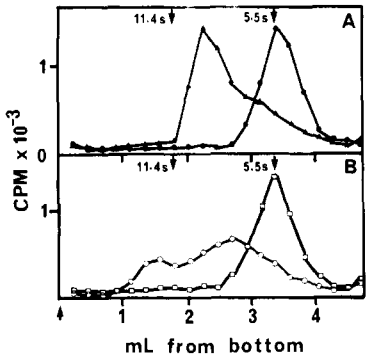

FIGURE 6: Effect of the monoclonal IgGs on the sedimentation of the B1–B2 complex in 5–20% neutral sucrose gradients. Sample preparation and conditions for centrifugation are described under Methods. The arrows at 5.5 and 11.4 S show the positions of ^{125}I-B2 and catalase, respectively. (Panel A) (●) ^{125}I-B2; (▲) ^{125}I-B2 + B1. (Panel B) (□) B1 + B2 + anti-B1-c; (○) B1 + B2 + anti-B1-k. Anti-B1-g and anti-B1-i gave the same distribution as anti-B1-c.

the retention of radioactive nucleotides by nitrocellulose filters in the presence of B1.[3] The results of Table I demonstrate that, with one exception, binding of either dATP or dTTP to B1 was not affected more by the specific anti-B1 IgGs than by IgG from the parent P3 line. The exception was the effect of anti-B1-c on binding of dTTP. In this case, more extensive data from binding at different nucleotide concentrations revealed that the K_D value of the B1–IgG complex for dTTP was increased to 0.9 μM from a value of 0.4 μM for free B1 (data not shown). At high concentrations of dTTP, both free B1 and B1–IgG complex bound approximately 1.5 mol of dTTP/mol of B1. Thus, these data do not suggest that monoclonals bind to allosteric sites of B1.

Instead, we found that all those monoclonals that inhibit the activity of B1 also inhibited the interaction between B1 and B2, the second subunit of the reductase. In these experiments, B2 had been labeled with ^{125}I, and its interaction with B1 was studied by neutral sucrose gradient centrifugation. Earlier experiments (Brown & Reichard, 1969a) had shown that the separate B1 and B2 proteins sediment at 7.8 and 5.5 S, respectively, in 5–20% sucrose gradients containing 10 mM $MgCl_2$ and 5 mM dithiothreitol. Together, the two subunits formed a relatively loose 1:1 complex with a sedimentation coefficient approaching a value of 9.7 S. The complex dissociated easily during centrifugation. Its tightness was favored by high protein concentration and by some effectors, such as dTTP.

In the present experiments, complex formation was monitored by the shift of ^{125}I-labeled B2 from the 5.5S position to a heavier one in the gradient, induced by addition of protein B1. Panel A of Figure 6 demonstrates such a partial shift of B2 from 5.5 S to approximately 9.7 S in the presence of a 1.5 molar excess of B1. Addition of the anti-B1 IgGs that neutralize B1 activity inhibited this shift completely (Figure 6B). Addition of anti-B1-k, a monoclonal that binds to but does not inhibit B1, gave rise instead to the formation of an additional heavy peak, sedimenting at approximately 12.8 S (Figure 6B). This peak probably represents a complex beween B2, B1, and anti-B1-k.

Anti-B2 Monoclonals. To obtain anti-B2 monoclonals, we used the strategy described earlier for anti-B1 monoclonals. Twelve hybridomas giving positive results in the solid-phase radioimmunoassay were cloned in soft agar and injected intraperitoneally into mice. Of these, only three, designated anti-B2-a, anti-B2-c, and anti-B2-g, produced IgGs that bound protein B2. All three had been obtained by fusion of spleen cells from a mouse, immunized with B2, with P3 myeloma cells, and all belonged to the IgG_1 class. Anti-B2-a and anti-B2-c showed a banding pattern on PAGE–SDS gels identical with that of P3 while anti-B2-g had an additional light chain (data not shown). We assume, however, that all three anti-B2 monoclonals contained mixtures of IgGs with heavy and light chains from both parent lines, which happened to have (near) identical mobilities on the gels. Only those IgGs with both chains from the spleen cell are expected to bind B2.

All three monoclonals had neutralizing activity and bound tightly to B2. From experiments similar to those described for B1 in Figure 4, we could obtain Scatchard plots (data not shown) from which we could calculate K_D values of 5.8 nM for anti-B2-a, 3.3 nM for anti-B2-c, and 2.5 nM for anti-B2-g.

Competition experiments similar to those described for anti-B1 monoclonals in Figure 3 demonstrated that nonlabeled anti-B2-g competed with all three ^{125}I-labeled anti-B2 IgGs, while anti-B2-a and anti-B2-c competed only with each other but not with anti-B2-g (data not shown). From this, we tentatively conclude that anti-B2-a and anti-B2-c are directed against the same epitope while anti-B2-g is directed against a separate epitope but, when bound to the latter, prevents binding of the other two monoclonals.

What specific B2 function is affected by antibody binding? Protein B2 harbors an iron center and a tyrosyl radical. The catalytic activity of the reductase depends on an intact radical for which, in turn, the iron center is a prerequisite (Atkin et al., 1973; Sjöberg et al., 1977). The presence of the radical can be monitored by its characteristic EPR signal. We now investigated whether addition of an excess of either antibody to B2 changed or destroyed the EPR signal but found no such effect even though the enzyme was shown to be completely inactivated (data not shown). Thus, the monoclonals do not inhibit the activity of B2 by interaction with the tyrosyl radical or the iron center.

Instead, all three monoclonals interfered with the binding of B1 to B2. In experiments similar to those shown in Figure 6 for anti-B1 monoclonals, we analyzed by neutral sucrose gradient centrifugation the effects of anti-B2 monoclonals on complex formation between ^{125}I-labeled and a 1.5-fold excess of nonradioactive B1. The interpretation of these experiments was complicated by the fact that the antibodies themselves form a heavy complex with B2, sedimenting at 11 S. The 11S complex was also observed when a mixture of B1, B2, and antibody was centrifuged, suggesting that B1 did not bind to B2 in the presence of the antibody (data not shown). Such binding should have resulted in the formation of a heavier ternary B1–B2–antibody complex, as was found in Figure 6B for the anti-B1-k monoclonal.

The three anti-B2 monoclonals did not neutralize B1 activity and formed no immune complexes with the native B1 protein. Also, the reaction in the solid-phase radioimmunoassay was specific for B2. It was therefore surprising to find that in immunoblots of crude extracts of KK546 a prominent band was also found in the position of B1 (Figure 7). The identity of this band with B1 was established from immunoblots of pure B1 stained with anti-B2-g (Figure 7). Apparently under these conditions B1 also reacted strongly with the antibody. We will return to this point under Discussion.

DISCUSSION

The aim of this investigation was to provide us with a battery of monoclonal antibodies directed against the two subunits of ribonucleotide reductase. Such antibodies should be useful

[3] K. Söderman and P. Reichard, unpublished results.

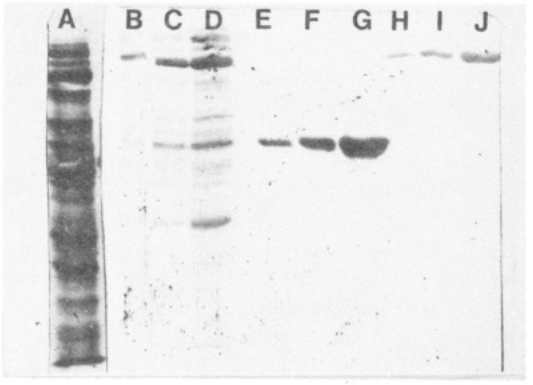

FIGURE 7: Immunoblotting experiments with anti-B2 monoclonals. The results shown here are for anti-B2-g; those for anti-B2-a and anti-B2-c are similar. Proteins were first run on a 10% PAGE–SDS slab gel and then transferred to a nitrocellulose sheet. Lane A was stained with 0.25% Coomassie brilliant blue, and lanes B–J were incubated with anti-B2-g (5 μg/mL). (Lane A) *E. coli* KK546 lysate, 10 μg; (lanes B–D) *E. coli* KK546 lysate, 1, 3, and 10 μg; (lanes E–G) B2 protein, 0.1, 0.3, and 1.0 μg; (lanes H–J) B1 protein, 0.1, 0.3, and 1.0 μg.

in the purification of the enzyme as well as in investigations of structure–function relationships. In this paper we describe and characterize eight monoclonals, five directed against B1 and three against B2. All were obtained by conventional techniques involving the fusion of spleen cells from hyperimmunized mice with cells from an established mouse myeloma line. Selection of the particular hybridomas described here was made with a solid-phase radioimmunoassay. Only hybridomas giving a signal over 20 times the background were selected, cloned in soft agar, and used for the large-scale production of antibodies. Other hybridomas, with lower activity in the assay, were stored in liquid nitrogen.

Anti-B1 Antibodies. Of 10 anti-B1 hybridomas selected originally as described above, only five produced B1-binding antibodies after induction of ascites tumors. Four of these neutralized the enzymatic activity of B1, while the fifth bound to B1, as demonstrated by formation of an immune complex, without neutralizing its activity. All antibodies belonged to the IgG_1 class and were purified by chromatography on protein A. Since they had been selected from their high scores in a solid-phase binding assay, it was not surprising to find also that they bound tightly to B1 in solution with K_D values below 10 nM. In one case (anti-B1-g), the sensitivity of our enzyme assay was not high enought to quantitate the very high affinity. When investigated, Fab fragments and the corresponding IgGs had the same affinity for B1. Since the experiments with monovalent Fab fragments measure true dissociation constants, we believe that the low K_D values found for IgGs do indeed measure the binding of the monoclonal antibody.

The structural significance of the binding data is unclear since the exact stoichiometry between IgGs and B1 is unknown. Unfortunately, all hybridomas producing high-affinity monoclonal antibodies are the result of fusions of spleen cells with the P3 myeloma line and therefore contain light and heavy chains from both parents (Köhler & Milstein, 1975). Only IgGs constructed from chains derived from spleen lymphocytes are expected to bind to B1. A further complication arises from the fact that IgGs as well as protein B1 are divalent. B1 consists of two identical polypeptide chains, each presumably with one epitope. We considered it to be beyond the purpose of the present investigation to try to unravel this situation.

We tried instead to relate the interactions between monoclonals and B1 to functional aspects of the protein. To begin with, competition experiments between IgGs suggested that each monoclonal reacted with a separate epitope on B1. This conclusion remains tentative for anti-B1-c and anti-B1-i, since anti-B1-c strongly suppressed binding of anti-B1-i. However, there was not competition in reverse in spite of our finding that both antibodies bound equally well to B1 in solution. In the accompanying paper, we demonstrate that the two monoclonals bind differently to proteolytic fragments of B1. Taken together, our results suggest that the monoclonals bind to separate epitopes and that binding of anti-B1-c masks the anti-B1-i site.

None of the monoclonals showed specificity for a particular allosteric conformation of B1 elicited by binding of effectors. Thus, the stoichiometries between IgGs and B1 in enzyme neutralization tests were not affected by the nature of the substrate or effector. These experiments were carried out at quite high concentrations of IgGs, and therefore do not exclude minor differences in K_D values. Furthermore the monoclonals did not bind to effector binding sites since they did not keep dATP or dTTP from being bound to B1. In one case (anti-B1-c), the monoclonal decreased the affinity of B1 for dTTP probably by inducing a minor conformational change in the protein.

Instead, results from sucrose gradient centrifugation, detailed in Figure 7, clearly establish that four of the five monoclonals prevented the formation of a B1–B2 complex. Active ribonucleotide reductase is made up by a B1–B2 complex, and the inhibitory effect of these four monoclonals on B1 activity is therefore probably connected with their effect on complex formation. Further support for this comes from the finding that the fifth monoclonal, anti-B1-k, that permits the complex formation does not affect the activity of B1.

Anti-B2 Antibodies. Our efforts to obtain monoclonals against B2 resulted in the final production of three IgG_1s, all neutralizing B2 activity and binding tightly to the subunit with K_D values in the nanomolar range. As was the case with the anti-B1 monoclonals, our primary assay had selected for high-affinity monoclonals. Two of them were apparently directed against the same epitope, while anti-B2-g probably reacted with a separate epitope but interfered with the binding of the other two monoclonals.

The monoclonals did not affect the EPR spectrum of B2 and thus did not interfere with either the free radical or the iron center of the molecule. This is not surprising, since these centers are buried inside the protein structure. Instead, all monoclonals perturbed the interaction between B1 and B2. It is interesting to note that the neutralizing anti-B1 monoclonals had a similar effect. At first sight these results might suggest that these monoclonals are directed against the protein structures involved in subunit interaction, maybe with the corollary that these structures are highly immunogenic. However, a different explanation could be that the B1–B2 interaction is easily disturbed and that the attachment of a monoclonal to areas of one subunit not involved in subunit interaction causes a disturbance through a change in tertiary structures.

One final point concerns the specificity of the anti-B2 monoclonals. The results of the immunoblotting experiments depicted in Figure 7 demonstrate that anti-B2-g bound equally well to B1 as to B2 when the two proteins were present on nitrocellulose, and presumably in a denatured state. Similar results were obtained with the other anti-B2 monoclonals. Several other proteins present in an *E. coli* extract behaved similarly, but from a comparison with the Coomassie blue staining pattern it is clear that the reaction with the monoclonal was far from nonspecific. These antibodies do not bind at all

to protein B1 in the native state. It seems that denaturation of some proteins on nitrocellulose reveals binding sites for anti-B2 monoclonals that are not apparent in the native configuration.

Registry No. Ribonucleotide reductase, 9047-64-7.

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Affinity Chromatography on Anti-B1 Monoclonal Gels for Purification and Characterization of Protein B1 from *Escherichia coli* Ribonucleotide Reductase†

Åsa Anderson, Lars Höglund, Elisabet Pontis, and Peter Reichard*

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

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ABSTRACT: Affinity gels were prepared from four monoclonal antibodies against the B1 protein of ribonucleotide reductase of *Escherichia coli*. The gels were used to purify protein B1 and also to study some of its properties. Gels from the nonneutralizing monoclonal anti-B1-k bound as much as 2 mg of B1/mL and were employed to prepare essentially pure B1 protein in a single step from extracts of wild-type *E. coli* and strains overproducing the subunit. However, B1 prepared from wild-type extracts had a lowered specific activity, suggesting some denaturation during elution of the protein from the column. Addition of the allosteric effector dATP during affinity chromatography changed the chromatographic pattern. Some protein B2, the second subunit of the reductase, remained in all cases bound to the gels together with B1. The gel prepared from anti-B1-c retained two additional proteins. In other experiments involving binding of proteolytic fragments of B1 to various antibodies, we also found a striking effect of dATP, suggesting that dATP made protein B1 less accessible to proteolysis. In these experiments fragments around 15K still had the ability to bind monoclonals, making possible more detailed investigations of the structural contacts between B1 and the monoclonals.

The preceding paper (Anderson et al., 1986) describes the production of monoclonal antibodies against the two subunits of ribonucleotide reductase from *Escherichia coli* (proteins B1 and B2). B1 is the subunit of the reductase that contains binding sites for substrates and allosteric effectors (Brown & Reichard, 1969b). Four anti-B1 monoclonals belonging to the IgG_1 class were characterized in some detail. All four had a high affinity for the protein with K_D values below 10 nM

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* Address correspondence to this author.

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