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†

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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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and were directed against separate epitopes. Three monoclonals neutralized enzyme activity and interfered with the binding of B1 to the second subunit B2 but had little effect on binding of substrates or effectors to B1.

In the experiments described here, we investigate the use of affinity chromatography on anti-B1 columns for the purification of B1 from crude extracts of *E. coli*. The monoclonal antibodies are also used to investigate conformational changes of B1 induced by effectors.

Experimental Procedures

Materials

Sepharose 4B used for affinity columns was from Pharmacia Sweden, nitrocellulose sheets for immunoblotting were from Bio-Rad, Richmond, CA, subtilisin was from Dako, Glostrup, Denmark, chymotrypsin was from Merck, Darmstadt, West Germany, and trypsin and alkaline phosphatase conjugated rabbit anti-mouse serum were from Sigma, St. Louis, MO.

Protein B1 was prepared from the overproducer strain *E. coli* C600/pLSH1 (Larsson, 1984). The preparation of anti-B1 monoclonal antibodies is described in the accompanying paper (Anderson et al., 1986).

Crude bacterial extracts containing B1 were prepared from pelleted bacteria by treatment with lysozyme (Fuller et al., 1981), followed by centrifugation at 45 000 rpm for 45 min. *E. coli* C600 was used as the wild-type strain. Ribonucleotide reductase overproducers were strains KK546 (Eriksson et al., 1977) and C600/pPS2 (Platz & Sjöberg, 1980). Buffer A used for washing of affinity columns was 50 mM Tris-HCl,[1] pH 7.6–0.1 M KCl–0.5 mM DTT.

Methods

Affinity Chromatography of B1 on Antibody Columns. All affinity gels were prepared from IgGs obtained by ammonium sulfate precipitation of ascites. Except where stated otherwise, 10 mg of protein was coupled to 1 mL of wet, packed Sepharose 4B activated with 15 mg of CNBr as described by Kohn & Wilchek (1982). Eighty to ninety-five percent of the protein was usually bound by the gel. The gel was washed extensively with 50 mM Tris-HCl, pH 7.6–0.1 M KCl and stored in the same buffer, containing 0.02% azide. Analytical experiments were done with columns of 0.25 or 0.5 mL of gel in Pasteur pipets; for preparative use, correspondingly larger columns were employed. All experiments were made in a cold room at 4 °C. As far as possible, air was excluded from the buffers and gels during chromatography. All buffers were extensively flushed with argon before and during use, and a stream of this gas was directed over the top of the column throughout the whole procedure. The column was first washed with approximately 10 volumes of buffer A before adsorption of the bacterial extract. After proteins had been introduced, the gel was washed with 10–20 volumes of buffer A to remove nonadsorbed proteins. B1 was then eluted with a 0.1 M glycine buffer, usually of pH 11. The drops emerging from the column were immediately neutralized by collecting each fraction (0.5 mL) in 0.125 mL of 2 M Tris-HCl, pH 7.0, containing 40 mM DTT. Finally, the gels were usually washed rapidly with 0.75 mL of 0.1 M glycine buffer, pH 12, immediately followed by 50 mM Tris-HCl, pH 7.6. Affinity gels should be exposed to DTT or alkali (no DTT) as briefly as possible since these treatments destroy the binding capacity of the gels. Fractions eluted from the gel by buffer A or glycine buffers were collected for protein determination, enzyme assay (Brown et al., 1969), and analytical PAGE–SDS.

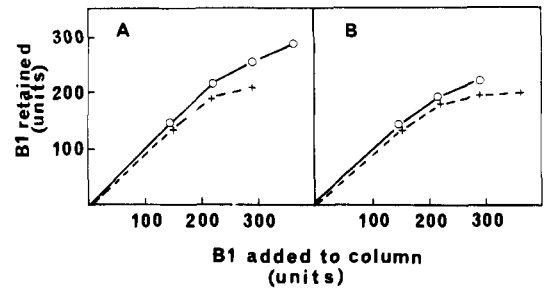

FIGURE 1: Binding of protein B1 by affinity gels prepared from anti-B1-k. B1 present in crude extracts of KK546 (6 mg of protein and 144 units of B1 per mL) was adsorbed stepwise to four separate 0.25-mL columns of anti-B1-k gel. Between each step, the columns were washed with buffer A, and a portion of the eluate was analyzed for breakthrough of B1 activity. The amount of B1 retained on the column was calculated from the difference between the amount of B1 added to the column and the amount present in the breakthrough. In panel A, the gels were prepared by first activating 1 mL of wet Sepharose with 15 mg of CNBr followed by coupling to 12 (+) or 24 (O) mg of anti-B1-k. In panel B, activation was with 30 mg of CNBr followed by coupling to 12 (+) or 24 (O) mg of anti-B1-k.

Proteolytic Digestion of B1. Protein B1 (80–110 μg) was digested at 0 °C with 3 μg of protease in 20 μL of 50 mM Tris-HCl, pH 7.6–20% glycerol–10 mM DTT, with 0.1 mM dATP included where indicated. In the experiment depicted in Figure 9, digestion was instead made with 1 μg of subtilisin at room temperature. At the indicated time intervals, samples (3 or 5 μL) were removed, added to an excess (10 or 25 μL) of 1 mM PMSF to stop proteolysis, and stored overnight in the cold room. Portions from each sample were then used for the determination of remaining B1 activity and the capacity to bind anti-B1 antibodies in a solid-phase radioimmune assay and for PAGE–SDS, followed in some cases by immunoblotting with anti-B1 antibodies.

PAGE–SDS Electrophoresis and Immunoblots. Most separations were made by conventional PAGE–SDS on 10% slab gels (Renart et al., 1979). For immunoblotting after proteolysis of B1, separation was instead on a 10–16% gradient gel (Hashimoto et al., 1983). Immunoblots were made as described in the preceding paper (Anderson et al., 1986). There, we also give details for other methods used in this paper.

Results

Affinity Chromatography on Anti-B1 Columns. In an attempt to optimize conditions for the preparation of affinity gels, we first activated Sepharose with either 15 or 30 mg of CNBr/mL of gel and then coupled each preparation to either 12 or 24 mg of anti-B1-k. Columns from the resulting four affinity gels were then used to adsorb B1 from a crude extract of KK546, an *E. coli* strain overproducing ribonucleotide reductase (Platz & Sjöberg, 1980). Increasing amounts of extracts were added in a stepwise fashion, and the run-through fraction from each step was analyzed for its content of B1 activity. Knowing the amount of B1 activity loaded onto the columns, we could calculate the amount of enzyme retained in each step (Figure 1). Two main conclusions can be drawn from this experiment: (i) the gels prepared from Sepharose activated with more cyanogen bromide (Figure 1B) retained less enzyme, in spite of the fact that they had coupled more IgG; (ii) doubling the amount of IgG during the coupling step (from 12 to 24 mg/mL of Sepharose) resulted only in a minor increase in binding capacity. Furthermore, when in a second

[1] Abbreviations: PAGE–SDS, polyacrylamide gel electrophoresis in 1% sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol.

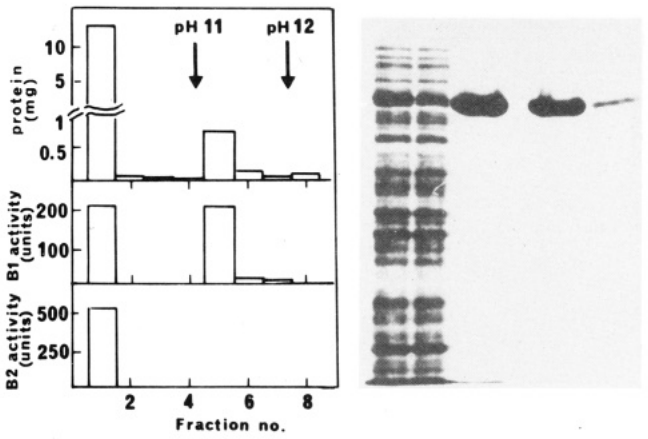

FIGURE 2: Chromatography of an extract from KK546 on an anti-B1-k column. An extract containing 18 mg of protein, 570 units of B1, and 540 units of B2 was chromatographed on a 0.25-mL column of anti-B1-k. Each fraction (2 mL in fraction 1, 0.5 mL thereafter) was analyzed for total protein and B1 and B2 activity (left part of figure). The right part of the figure shows the following gel patterns (from left to right): crude extract and fractions 1, 5, 6, and 8.

experiment B1 was eluted at pH 11 from two columns such as those described in Figure 1A, identical yields of active enzyme were recovered (data not shown). With guidance from these results, all further affinity gels were synthesized by coupling 10 mg of IgG to 1 mL of packed Sepharose, activated with 15 mg of CNBr.

The B1 activity adsorbed to the affinity gels could be eluted with buffers of alkaline pH. The enzyme is highly unstable under these conditions, in particular since it was not possible to protect its redox-active SH groups with DTT during elution. However, most of the activity was recovered on elution at pH 11 provided oxygen-free buffers were used during chromatography, the enzyme solution was neutralized, and DTT was added after elution as described under Experimental Procedures. An elution profile of a crude extract of KK546 from an affinity column of anti-B1-k is shown in Figure 2. In this experiment, 95% of the total protein, all B2 activity, and approximately half of the B1 activity emerged in the buffer A wash, while the remaining B1 activity was eluted at pH 11. On gel electrophoresis under denaturing conditions, the latter material gave a single band at the position of B1 (inset in Figure 2). The specific B1 activity of this fraction was 380. Absolute values for the specific activity of B1 depend on the amount and quality of protein B2, thioredoxin, and thioredoxin reductase used in the assays. While values as high as 1000 may be obtained, B1 of the highest purity prepared by conventional means during our assay conditions routinely had a specific activity of 4–500. The specific activity of B1 prepared by chromatography on anti-B1-k columns of an extract of KK546 thus was approximately 80% of the theoretical value.

Of the various IgGs, anti-B1-k proved to be best for the purification of B1 by affinity chromatography. Table I compares the chromatographic behavior of B1 present in a crude extract of KK546 on affinity gels prepared from five different IgGs. In each case, 12 mg of protein was chromatographed on a 0.25-mL column. Table I gives the percentage of B1 units in the buffer A wash and in the pH 11 and 12 fractions. The fraction of B1 activity eluted at pH 11 varied considerably between the various gels, but in each case this fraction contained one main band at the position of B1 when analyzed by gel electrophoresis (cf. Figure 4). However, the specific activity of B1 eluted at pH 11 varied considerably in the five experiments, suggesting various degrees of denaturation of the enzyme. The gel prepared from anti-B1-a bound the enzyme

Table I: Recovery of B1 Activity after Chromatography of an Extract from *E. coli* KK545 on Affinity Gels Prepared from Various Anti-B1 Monoclonal Antibodies[a]

	recovery of B1 activity (% of that added to column)				sp act. of B1 (units/mg)[b]
antibody	total	in buffer A wash	in glycine, pH 11	in glycine, pH 12	
anti-B1-a	82	75	7	0	200
anti-B1-c	56	46	12	1	160
anti-B1-g	53	40	13	2	470
anti-B1-i	78	49	27	2	220
anti-B1-k[c]	72–81	22–35	43–52	1–2	360–390

[a] Extracts containing 13 mg of total protein and 450 units of B1 were chromatographed on 0.25-ml gel columns as described under Experimental Procedures. [b] Refers to the pH 11 fraction. [c] Includes results from three experiments.

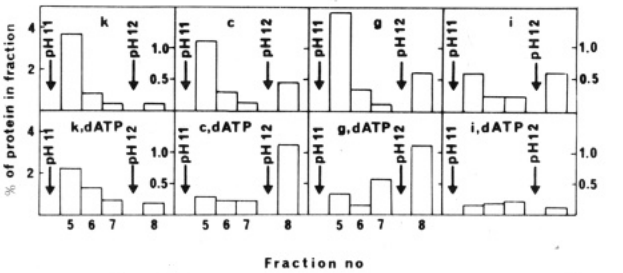

FIGURE 3: Effect of dATP on the chromatographic behavior of B1. An extract from KK546 (15 mg of protein) was adsorbed to 0.25-mL anti-B1 columns (anti-B1-k, anti-B1-c, anti-B1-g, and anti-B1-i) in the absence (top row) or presence of 0.1 mM dATP (bottom row) and chromatographed as described under Experimental Procedures. The ordinate shows the percentage of total protein eluted in the pH 11 and 12 fractions. An analysis of the proteins by PAGE–SDS electrophoresis is shown in Figure 4.

poorly, with most of the activity appearing in the buffer A wash. Anti-B1-c and anti-B1-g gels bound more B1, but only little active enzyme was recovered at pH 11, and the total recovery of B1 activity was low. These results suggest tight binding of B1 to the gels, making elution of active enzyme a difficult task since enzyme denaturation increased considerably at pH values above 11.

Ribonucleotide reductase is an allosteric enzyme, with the B1 subunit containing binding sites for nucleoside triphosphates acting as effectors (Brown & Reichard, 1969b). Binding of, e.g., dATP or dTTP is correlated with specific functional and conformational states of B1. It was of interest to investigate whether addition of nucleotides to B1 would affect the behavior of the enzyme during chromatography on affinity gels.

The experiments depicted in Figure 3 demonstrate that dATP indeed had an effect on the retention of B1. No such effect was found with dTTP (data not shown). In eight chromatograms, 15 mg of protein from crude extracts of KK546 was added to columns containing 0.25 mL of four different gels and, after being washed at pH 7.6, eluted stepwise at pH 11 and 12. With each affinity gel, chromatography was carried out with and without dATP present throughout the whole experiment. The data of Figure 3 give the percentage of the total protein eluted in the chromatographic fractions eluted at pH 11 and 12. In the absence of dATP, protein was easily eluted at pH 11 from the anti-B1-k gel, but was more strongly retained on the other gels. Note the difference in scales between anti-B1-k and other IgGs. In the presence of dATP, the affinity for B1 is increased in all cases. This was most pronounced with the anti-B1-c and anti-B1-g gels, where about half of the protein required elution at pH 12. From the anti-B1-i gel, little protein was recovered at any pH in the presence of dATP.

Table II: Purification on Affinity Gels Prepared from Anti-B1-k of Extracts from Various Strains of *E. coli*[a]

source	extract added to column			buffer A wash			pH 11 fraction		
	mg	units	sp act.	mg	units	sp act.	mg	units	sp act.
C600	69	90	1.5	62	20	0.3	0.2	32	160
C600/pPS2	21	300	14	19	32	1.7	0.5	175	350
KK546	12	450	38	10	100	10	0.7	260	370

[a] Chromatography was made on 0.25-ml columns of anti-B1-k as described under Experimental Procedures. The wild-type C600 extract was diluted to a final volume of 9 mL and the extracts from the two overproducers to 3 mL before the solutions were applied to the affinity columns.

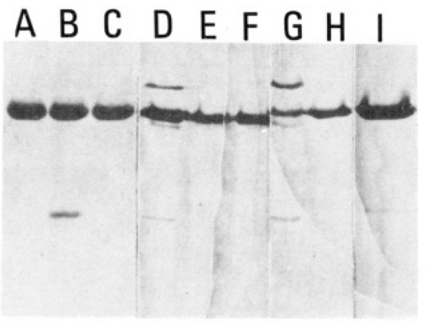

FIGURE 4: Analysis of PAGE–SDS of protein fractions from Figure 3. Rows A–C represent fractions from the two anti-B1-k columns eluted at pH 11. Row A is fraction 5 eluted without dATP; rows B and C are fractions 5 and 6, respectively, eluted in the presence of dATP. Rows D–F show fractions 5, 6 and 8, respectively, from the anti-B1-c without dATP; rows G–I show the corresponding fractions eluted from anti-B1-c with dATP.

When the different fractions were analyzed by denaturing gel electrophoresis, the presence of dATP during chromatography induced an interesting effect (Figure 4). The first chromatographic fraction eluted at pH 11 (fraction 5) from an anti-B1-k column, in addition to the B1 band, contained a second band at the position of protein B2, the other subunit of ribonucleotide reductase. No B2 band was found in fraction 6, the second fraction eluted at pH 11. An identical result was obtained with anti-B1-g and anti-B1-i gels (data not shown). This result suggests that in the presence of dATP a substoichiometric amount of B2 was attached to B1. Furthermore, the affinity of B1 for the gel was apparently stronger than its affinity for B2, which explains the absence of B2 from fraction 6. The results with the anti-B1-c gel were still more complicated (Figure 4). In this case gel electrophoresis of the first fraction eluted at pH 11 demonstrated the presence of two additional protein bands, besides B1 and B2. These bands were also found in the absence of dATP, but their relative abundance increased considerably in the presence of the nucleotide. Again, the bands were absent in later fractions. It seems possible that the protein(s) corresponding to these bands bind specifically to B1 in the presence of dATP and therefore are retained on the column. It would be interesting to establish their nature since they might be functionally related to ribonucleotide reductase. We can, however, not exclude that the protein(s), per se, has (have) a high affinity for the gel, which is increased in the presence of dATP.

Affinity chromatography on anti-B1-k gels was useful for rapid purification of B1 from various kinds of bacterial extracts. Table II summarizes three experiments with extracts from wild-type *E. coli* C600 and two *E. coli* strains overproducing B1 and B2 (KK546 (Eriksson et al., 1977) and *E. coli* carrying the plasmid pPS2 (Platz & Sjöberg, 1980). From the extracts of the two overproducers, B1 was obtained in 58% yield, with a specific activity approaching that of the enzyme prepared in the conventional way. From the extracts of wild-type *E. coli*, the yield of B1 was only 35%, and the specific activity of the enzyme was low. Nevertheless, analysis by denaturing gel electrophoresis gave one major band in the

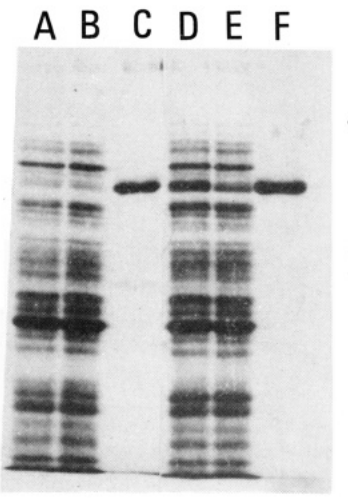

FIGURE 5: PAGE–SDS of fractions obtained by gel chromatography of extracts from *E. coli* C600 or from strain C600/pPS2. Rows A–C show gel patterns of crude extract, buffer A wash, and pH 11 fraction from C600; rows D–F show the corresponding fractions from the overproducer C600/pPS2.

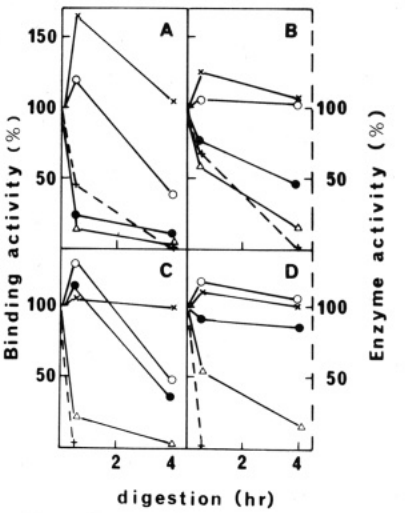

FIGURE 6: Binding of antibodies to proteolytic fragments of B1. Protein B1 (80 μg) was digested with 3 μg of trypsin (panel A), chymotrypsin (panel B), subtilisin (panel C), or subtilisin + 0.1 mM dATP (panel D). At the indicated time intervals, digestion was stopped by addition of 1 mM PMSF. One portion from each sample was used to determine remaining B1 activity (+); a second portion (0.1 μg of fragmented B1) was used to determine the binding of 0.1 or 0.3 μg of anti-B1-c (×), anti-B1-i (○), anti-B1-g (●), or anti-B1-k (△) in a solid-phase radioimmune assay; a third portion was analyzed by PAGE–SDS electrophoresis with results shown in Figure 7.

position of B1 (Figure 5). In this case, the whole procedure required more time, and B1 was more dilute during elution from the column. This may explain why the protein was partially inactivated.

Binding of IgGs to Proteolytic Cleavage Products of B1. Fragments of B1 obtained by proteolytic cleavage retained some capacity to bind the monoclonal antibodies. Figure 6

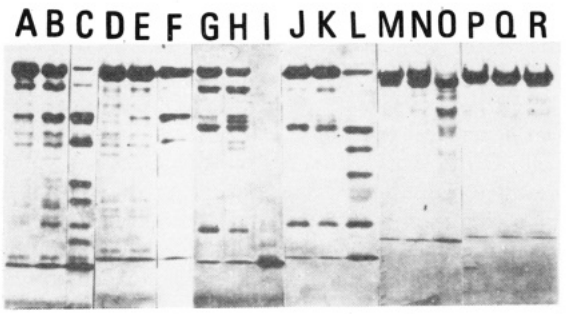

FIGURE 7: Effect of dATP on the digestion of B1 by proteolytic enzymes. B1 was digested as described in the legend to Figure 6 with trypsin (lanes A–F), subtilisin (G–L), or chymotrypsin (M–R) with or without dATP. In each case, the first three lanes (A–C, G–I, and M–O) represent samples taken at 0, 30, and 240 min from digestions without dATP, while the following three lanes (D–F, J–L, and P–R) are the corresponding samples obtained by digestion with dATP. All samples were analyzed by 10% PAGE–SDS.

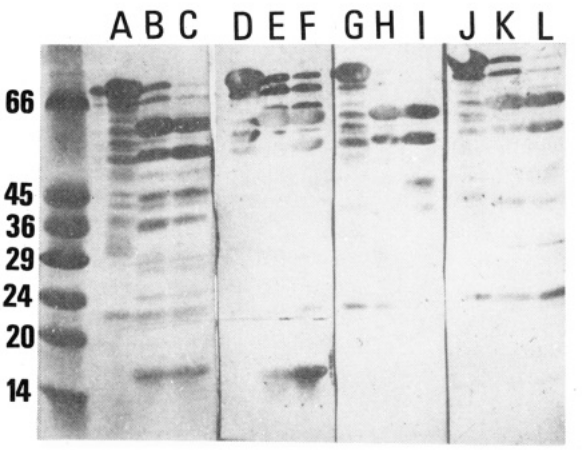

FIGURE 8: Immunoblots of proteolytic fragments of B1 obtained by digestion with trypsin. Digestion with trypsin and immunoblotting was made as described under Experimental Procedures. Lanes A–C were stained with Coomassie blue; lanes D–F were treated with anti-B1-c, lanes G–I with anti-B1-g, and lanes J–L with anti-B1-i. Within each group, the material in the first lane was digested for 0 min, in the second lane for 120 min, and in the third lane for 240 min. The lane to the far left shows the positions of molecular weight standards.

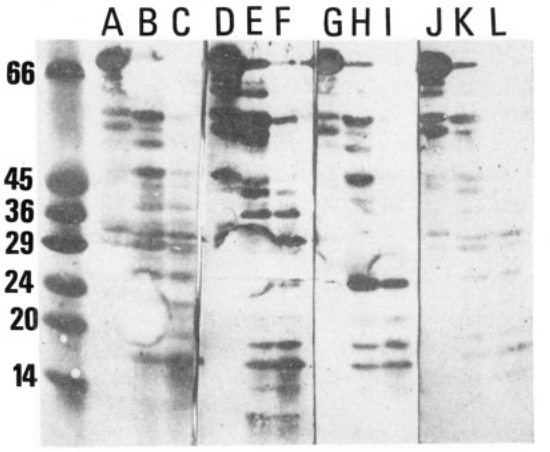

FIGURE 9: Immunoblots of proteolytic fragments of B1 obtained by digestion with subtilisin in the presence of dATP. The designations in this figure are identical with those in Figure 8.

describes the results from a series of experiments in which B1 was first digested for 0.5 or 4 h with trypsin (Figure 6A), subtilisin (Figure 6B,C), or chymotrypsin (Figure 6D) and the products of digestion then were used to bind various IgGs in the solid-phase radioimmune assay. In each panel, the solid lines show the remaining binding capacity while the broken lines represent enzyme activity. Subtilisin rapidly destroyed this activity, while digestion with the two other proteases decreased it to 50% during the first 30 min but had destroyed it completely after 4 h. The fragments showed great variations in their ability to bind different monoclonals. Anti-B1-c was bound strongly even after 4-h digestion with any protease; 30-min digestion actually appeared to result in increased binding of this monoclonal. At the other extreme, binding of anti-B1-k declined rapidly. The two remaining IgGs, anti-B1-g and anti-B1-i, showed an intermediate behavior.

Panel D of Figure 6 demonstrates an experiment with dATP present during treatment of B1 with subtilisin. In this case, as well as in similar experiments with trypsin and chymotrypsin (data not shown), the binding ability of fragments for all four IgGs was preserved for a longer time, suggesting a decreased rate of digestion in the presence of dATP. Such a protective effect on dATP was clearly apparent when the proteolytic fragments were analyzed by denaturing gel electrophoresis (Figure 7). With each enzyme, the B1 band disappeared more slowly when dATP was present during digestion, indicating that binding of dATP to B1 resulted in a tighter conformation of the protein, less accessible to proteolytic attack. A similar protective effect was found with ATP, but not with two other nucleotides that bind to B1, namely, dTTP or CDP (data not shown).

Figure 7 also shows that well-defined protein fragments accumulate transiently during digestion with subtilisin or trypsin, as witnessed by the appearance of distinct bands during gel electrophoresis. We next investigated by immunoblotting whether such fragments still had the ability to bind monoclonals. Figure 8 shows results from an experiment with trypsin; Figure 9, those from a digestion with subtilisin carried out in the presence of dATP. The fragments obtained after 2- or 4-h digestion were first separated electrophoretically on polyacrylamide gradients (10–16%) and then transferred to nitrocellulose sheets and either stained with Coomassie Blue (lanes A–C) or tested for their ability to bind anti-B1-c (lanes D–F), anti-B1-g (lanes G–I), or anti-B1-i (lanes K–M).

At all time points, we attempted to stop proteolysis by addition of PMSF and then left the sample on ice overnight before gel electrophoresis. This treatment apparently did not completely inactivate the proteases as is apparent from the bands moving faster than protein B1 at the zero time points. Nevertheless, one notices a clear progression of digestion with time for both enzymes. With trypsin (Figure 8), little intact B1 remained after 4-h digestion. Coomassie Blue stained two bands located at 65K and 55K most heavily, but several minor bands could also be distinguished (43K, 36K, 22K, and 16K). The two major bands, as well as the 22K band, reacted with all three antibodies. The 16K band reacted exclusively with anti-B1-c, while the 43K and 36K bands reacted both with anti-B1-g and with anti-B1-i. The latter, in addition, reacted with a 28K band that was just barely visible with the Coomassie stain. Digestion with subtilisin (Figure 9) was more extensive than with trypsin. Again, several major bands of higher molecular weight reacted with all three antibodies, but clear differences were apparent for the smaller fragments. Thus, anti-B1-c reacted with certain fragments (43K, 35K, 30K, 13K, and 12K) that did not show up after treatment with anti-B1-g. Other bands (24K, 16K, and 15K) showed up with all three IgGs. Taken together, the results from the digestion experiments clearly indicate that the three IgGs bound to both similar and dissimilar fragments, again indicating that binding occurs to separate epitopes on B1.

Discussion

Monoclonal antibodies have been used in several instances with great success for the purification of proteins. With the appropriate antibody it may be possible to isolate in a single step an essentially pure protein from a crude cellular extract. Aside from the inherent convenience of such a method, rapid purification also minimizes degradation of the desired product by proteolysis, which is often a considerable problem during more extended procedures. Of the five anti-B1 IgGs studied here, the nonneutralizing anti-B1-k proved to be best for the preparation of affinity gels for the purification of B1. It may be of more general interest that the best performing gels were obtained by moderate derivatization of Sepharose. Higher amounts of CNBr increased the amount of IgG attached to Sepharose, but such gels bound less protein B1 (Figure 1).

Columns prepared from anti-B1-k had a high capacity for B1 and per 1 mL of gel bound 2 mg of B1 present in extracts from overproducing *E. coli* strains. However, B1 present at much lower concentration in wild-type extracts was not bound equally well. The protein was eluted from the column at pH values above 10.5. Since under these conditions B1 is highly unstable, it was of utmost importance to neutralize the eluate as soon as it emerged from the column and also to add DTT to reduce and protect functionally important SH groups. Best results were obtained when the gel was completely saturated with B1 before elution was started, since inactivation was less pronounced at high protein concentrations.

While B1 of good specific activity could be prepared in a one-step procedure from extracts of overproducing bacteria, loss of activity occurred on purification of the protein from wild-type extracts. However, in both instances few other proteins were present in the final product as judged by gel electrophoresis. It should now be possible to investigate also with B1 from wild-type *E. coli* whether the earlier found inhomogeneity of its NH_2 terminus (Thelander, 1973) was an artifact of the then prolonged purification procedure or represents intracellular processing of the protein.

Aside from its use for the purification of B1, affinity chromatography provided additional insights into functional aspects of the protein. B1 contains two classes of allosteric sites (Brown & Reichard, 1969b): substrate specificity sites (h sites), where binding of ATP, dATP, dTTP, or dGTP affects the substrate specificity of the enzyme; activity sites (l sites), where binding of ATP or dATP (but *not* other nucleotides) affects the overall activity of the enzyme. We now found that dATP, but not dTTP, influenced the chromatographic behavior of B1. With dATP, the protein was more strongly retained by all affinity gels, and still more interesting, the columns now retained other proteins in addition to B1. These data indicate that binding of dATP to l sites resulted in a change of the conformation of B1 large enough to influence its binding both to the column and to other proteins present in the extract. One of these proteins was B2, the other subunit of the reductase. Since we know from other experiments (Brown & Reichard, 1969a) that B1 has a high affinity for B2, it is indeed probable that B2 was bound to the column via B1. Other explanations are possible for the two proteins represented by the additional bands eluted from the anti-B1-c column (Figure 4). However, since also in this case dATP increased the relative amounts of these proteins, it seems likely that they also were bound via B1. In that case, chromatography on anti-B1-c gels might provide a tool to identify proteins that are functionally related to B1.

The idea of a major conformational change of B1 induced by binding of effectors to l sites is also borne out by our experiments involving limited proteolysis. In this case, dATP and ATP, both nucleotides that bind to l sites, protected B1 from cleavage by several proteases. dTTP or CDP had no such effect. Protection could be demonstrated by the fact that the cleavage products retained the ability to bind to anti-B1 monoclonals and also by their gel electrophoretic patterns. Many of the separated cleavage products retained the capacity to bind three of the four IgGs. The pattern of binding varied between the monoclonals, again demonstrating their specificity for different epitopes. In all three cases, also quite small fragments around 15K showed affinity for IgGs on immunoblots. Further work involving limited proteolysis may make it possible to arrive at a circumscript description of the areas of contact between B1 and IgGs.

Registry No. dATP, 1927-31-7; ribonucleotide reductase, 9047-64-7.

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