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USE OF AMP SPECIFIC ANTIBODIES TO DIFFERENTIATE BETWEEN ADENYLYLATED AND UNADENYLYLATED E. COLI GLUTAMINE SYNTHETASE

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SUMMARY: Anti-AMP specific antibodies were purified by affinity chromatography of serum from sheep immunized with adenylylated bovine serum albumin. Results of immunotitration experiments and light scattering measurements show that these antibodies can be used to separate adenylylated from unadenylylated forms of E. coli glutamine synthetase and to detect variations in protein configurations elicited by partial adenylylation of the enzyme or by allosteric interactions with divalent cations. These results suggest that the reaction of anti-AMP antibodies with variously adenylylated forms of glutamine synthetase can be used to investigate the dependence of immunoprecipitability on the density, absolute numbers, and possibly, the spatial distribution of multiple identical antigenic sites on a given macromolecule.

INTRODUCTION: Previous investigations (see reference 1 for review) have shown that: (a) regulation of E. coli glutamine synthetase activity is mediated by cyclic adenylylation and deadenylylation of a unique tyrosyl residue in each of the enzyme's 12 identical subunits; (b) enzyme isolated from cells produced under different growth conditions can contain from zero to twelve adenylylated subunits per molecule; (c) interactions between adenylylated and unadenylylated subunits within the same enzyme molecule influence both catalytic and stability characteristics; (d) each enzyme form can exist in a *relaxed, taut* or *tightened* configuration, depending on its interactions with divalent metal cations; (e) different conformational states are elicited by allosteric interactions of the enzyme with various feedback inhibitors.

Prompted by the demonstration that enzyme-specific antibodies have been used to detect different conformational states of other allosteric enzymes (2, 3), we made several unsuccessful attempts to see if antibodies raised against adenylylated and unadenylylated forms of glutamine synthetase could distinguish between enzyme species with different states of adenylylation. In the meantime, it was reported that antibodies capable of reacting specifically with AMP moieties

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could be obtained by immunization of rabbits with AMP derivatized BSA[1] (4). The present study shows that anti-AMP antibodies prepared by immunization of sheep with BSA-AMP can discriminate between adenylylated and unadenylylated species of glutamine synthetase and also detect differences in conformational states of the enzyme due to subunit and divalent cation interactions.

METHODS AND MATERIALS: Preparation of antiserum: Adenylylated bovine serum albumin (BSA-AMP) containing 16 moles of AMP per mole of protein was prepared by the reaction of BSA with AMP in the presence of a carbodiimide as described by Halloran and Parker (5). To prepare adenylyl group specific antibodies, the sheep was injected initially at multiple sites with 0.5 mg of BSA-AMP emulsified in complete Freund's adjuvant. Booster injections of 0.5 mg of BSA-AMP in incomplete Fruend's adjuvant were made at 2 week intervals. One week after the 3rd and final injection, 600 ml of blood were collected from the sheep. The serum was separated from the clot by centrifugation and stored at -20°C.

For immunoprecipitation tests, the γ-globulin fraction was partially purified from the serum by $(NH_4)_2SO_4$ fractionation (6) and after dialysis against buffer (10 mM Imidazole, 150 mM KCl, pH 7.3) was stored at -20°C.

Immunoprecipitation Tests: Immunoprecipitation tests were carried out in a total volume of 500 µl containing buffer (10 mM Imidazole, 100 mM KCl, 1 mM $MnCl_2$, pH 7.0), a fixed amount of glutamine synthetase and decreasing amounts of paritally purified gamma globulin. Total glutamine synthetase activity and the average state of adenylylation (i.e., the average number of AMP groups per molecule) were determined by means of the γ-glutamyl transferase reaction as previously described (7). Immediately after addition of the enzyme and antibodies, glutamine synthetase activity in each reaction tube was measured and the samples were incubated for 20 min at 37°C followed by incubation at 4°C for 18 hours. Tubes were then centrifuged and residual GS activity in the supernatant solution was determined.

Purification of antibodies: AMP specific antibodies used in light scattering studies were purified by preferential absorption and elution from an affinity resin prepared by the covalent attachment of $GS_{\overline{11}}$ to AH Sepharose-4B as described by the manufacturer (Pharmacia).

Light scattering experiments: Changes in light scattering elicited by the interaction of antibodies with glutamine synthetase were observed by exposing the reaction mixture in a 1 cm cuvette to incident light at 400 nm and measuring emitted light of the same wavelength at 90° angle by means of a HPF-2A spectrofluorometer equipped with a Hewlett-Packard 700 4B X-Y recorder. Typically, millipore filtered buffer plus purified antibodies were added to the cuvette and after a baseline was established glutamine synthetase was added and the light scattering was followed with time. The initial scattering due to addition of glutamine synthetase was subtracted from all light scattering values.

RESULTS: The ability of the antibodies to react with adenylylated glutamine

[1] Abbreviations: $GS_{\bar{n}}$, refers to glutamine synthetase preparation containing on the average $\bar{n}$ adenylylated subunits per dodecameric molecule. The value of $\bar{n}$ can vary from 0 to 12 and is sometimes referred to as the state of adenylylation. BSA, bovine serum albumin, BSA-AMP, bovine serum containing covalently bound AMP moieties.

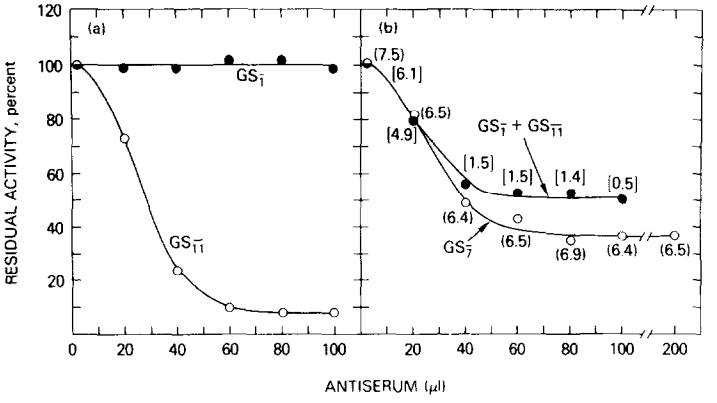

Figure 1. Immunoprecipitation curves of glutamine synthetase with anti-AMP antiserum. Experiments were performed as described in Methods.

$$\text{Percentage residual activity} = \frac{\text{non-precipitated enzyme activity}}{\text{total enzyme activity per reaction tube}} \times 100$$

For each point, reaction mixtures contained: (a) either 46 μg/ml $GS_{\overline{1}}$ (•) or 61 μg/ml $GS_{\overline{11}}$ (o); (b) either 46 μg/ml $GS_{\overline{1}}$ plus 46 μg/ml $GS_{\overline{11}}$ (•); or 50 μg/ml of natural $GS_{\overline{7.5}}$ (o). The numbers in brackets indicate the $\bar{n}$ value of the non-precipitated fraction.

synthetase could be demonstrated by light scattering measurements and by immunoprecipitation tests as described in Methods. Measurements by either method (Fig. 1a and 2a, 2b) show that antibodies react with adenylylated but not with unadenylylated enzyme.

Figure 1b shows that antibody titration of an artificial $GS_{\overline{6}}$ preparation (obtained by mixing equal amounts of pure $GS_{\overline{1}}$ and $GS_{\overline{11}}$) led to quantitative precipitation of the $GS_{\overline{11}}$ fraction but none of the $GS_{\overline{1}}$. In contrast, titration of a native $GS_{\overline{7.5}}$ preparation led to precipitation of only 60% of the partially adenylylated enzyme preparation; moreover, the nonprecipitable fraction contained about the same number of adenylylated subunits ([6.5] *vs* [7.5]) as the untreated enzyme.

Figure 2b shows that when compared at a fixed concentration of antibodies and enzyme the rate of light scattering increases as the state of adenylylation is increased. This is not due solely to a progressive increase in the total number of antigenic sites with increased state of adenylylation since qualitatively

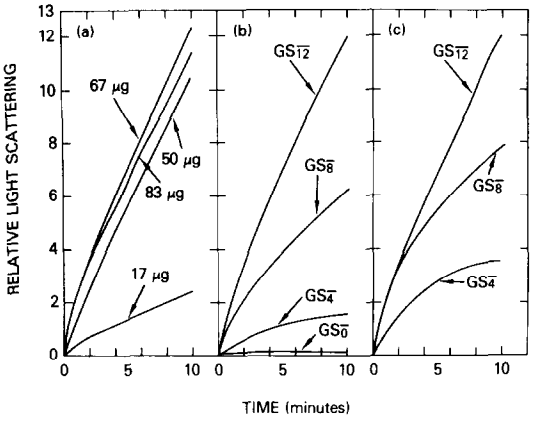

Figure 2. Increase in light scattering as a function of time, enzyme concentration and state of adenylylation. 200 µg of anti-AMP antibodies purified by affinity chromatograph (see Methods) were used for each time course. Buffer for all experiments was 10 mM Imidazole, 100 mM KCl, 1 mM $MnCl_2$, pH 7.0. In addition, reaction mixtures contained: (a) 17, 50, 67, or 83 µg of GS_{12} as indicated; (b) 67 µg of either GS_0, GS_4, or GS_8, or GS_{12}, as indicated; (c) 70 µg GS_{12}, 105 µg GS_8, or 210 µg GS_4, as indicated.

similar results are obtained when the concentrations of GS_4 , GS_8 and GS_{12} are adjusted so that each reaction mixture contains the same number of AMP groups (Fig. 2C).

As noted in the Introduction, glutamine synthetase can exist in different configurations in response to divalent cation interactions. Data in Table 1 show that when compared at identical enzyme and antibody concentrations, the rate of light scattering is different for each configuration. The relative rates of reaction are as follows: Mn^{2+}-taut > Mg^{2+}-tightened > Mg^{2+}-taut >> relaxed (divalent cation free) enzyme.

DISCUSSION: The highly specific anti-AMP antibodies described here should facilitate studies on the participation of adenylylation in the regulation of glutamine synthetase, and other enzymes, in various organisms. They have already been useful in demonstrating that the glutamine synthetase of Azotobacter vinelandii is regulated by adenylylation (J. Siedel, Proc. Am. Soc. Biol. Chem., 1978, in press). The studies used with *relaxed, taut* and *tightened* forms of glutamine synthetase indicate that the antibodies can also detect conformational

TABLE 1

Reactivity of antibodies with $GS_{\overline{12}}$

as a function of divalent cation environment

Enzyme Form	Initial Rate $\Delta A_{400\ nm}$ /min
Mn^{2+} taut	2.34
Mg^{2+} taut	1.45
Mg^{2+} tightened	1.69
Relaxed	0.62

Light scattering experiments performed as described in text. 200 µg anti-AMP antibodies and 75 µg $GS_{\overline{12}}$ used in each experiment. Values given in table represent the initial slope of the light scattering reaction. $GS_{\overline{12}}$ had been dialyzed against 10 mM Imidazole, 100 mM KCl, pH 7.0 plus either 1 mM $MgCl_2$, 1 mM $MnCl_2$ or no metal. Buffer used in the experiments was the final dialysate. Relaxed enzyme was dialyzed against 5 mM EDTA followed by 10 mM Imidazole, 100 mM KCl, pH 7.0. The enzyme was tightened by incubating relaxed enzyme with 10 nM $MgCl_2$ at 37°C for 20 min before running the experiment.

changes elicited by the binding of allosteric effectors to the enzyme. Particularly noteworthy is the fact that anti-AMP antibodies will precipitate all of the adenylylated and none of the unadenylylated enzyme from a mixture of $GS_{\overline{1}}$ and $GS_{\overline{11}}$, whereas they precipitate only 60% of the paritally adenylylated enzyme in native $GS_{\overline{7.5}}$ preparations. In agreement with other lines of evidence (1), this shows that naturally occuring enzymes at intermediate states of adenylylation are not mixtures of $GS_{\overline{0}}$ and $GS_{\overline{12}}$ but are composed of hybrid molecules containing both adenylylated and unadenylylated subunits. Incomplete precipitation of the natural $GS_{\overline{7.5}}$ might reflect conformational variations attributable to differences in the distribution of adenylylated subunits in hybrid variants containing the same number of adenylylated subunits, or be due to differences in the rates of intra- *vs* inter- molecular reactions of the enzyme with bivalent antibodies. (Intra-molecular bivalent reaction of an antibody with proximally positioned adenylylated subunits would preclude its involvement in lattice formation).

Similar considerations might explain the rate dependence of the antibody reaction on the state of adenylylation of glutamine synthetase as measured by the light scattering technique.

Since many previous efforts to separate the adenylylated from unadenylylated enzyme by a variety of physical-chemical techniques have failed, it is noteworthy that a quantitative separation of $GS_{\overline{1}}$ and $GS_{\overline{11}}$ could be achieved by immunoprecipitation with anti-AMP antibodies. Hopefully, the observed variations in reactivity of antibodies as a function of the state of adenylylation can be exploited to develop procedures for the separation of glutamine synthetase species at different intermediate states of adenylylation.

Because of its unique multimeric subunit structure and the extraordinary clarity of its molecular imagery as viewed in the electron microscope, E. coli glutamine synthetase is well designed for fundamental studies at the immunochemical level. Among other problems the relationship between immunoprecipitability and the density, absolute number, and spatial distribution of identical antigentic sites on a single macromolecule can be studied using the anti-AMP antibodies.

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