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Antibodies specific for NADPH-binding region of enzymes possessing dehydrogenase activities

(site-specific antibodies/antigenic determinants in NADPH-binding region/antigenic crossreactions)

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ABSTRACT **The results reported in this paper show the presence of a population of antibodies in rabbit polyclonal antiserum that recognize an antigenic site at the NADPH-binding region of enzymes possessing dehydrogenase activities. Antisera from rabbits immunized with glucose-6-phosphate dehydrogenase or fatty acid synthetase were found to inactivate the enzyme dihydrofolate reductase. The inhibitory effect of this site-specific antibody is a time- and concentration-dependent reaction. This immunoinactivation is prevented by preincubation of the enzyme with NADPH.**

Recent experiments with monoclonal antibodies have shown the existence of crossreacting sites on protein molecules that do not have homology as detected by conventional serological methods (1–3). For example, some monoclonal antibodies against large tumor (T) antigen of simian virus (SV40) have been demonstrated to crossreact with host proteins (4). Similarly, a monoclonal antibody to herpesvirus, which was raised by immunizing mice with the virus, binds a glycoprotein specific to the virus and virus-infected cells. This antibody was also found to crossreact with neutral glycolipids present in normal uninfected cells (5). Some other antigenic crossreactivities have been detected in various other proteins that are related to one another (6–12).

The implications of this crossreactivity of related protein molecules could be very significant. It is possible that a monoclonal antibody crossreacting with two related proteins may be detecting a conserved recognition site on the protein molecule. Thus such antibody may be used as a tool for investigating molecular identity.

We have discovered in our studies that conventional antiserum raised in a rabbit against an enzyme containing a nucleotide-binding site (e.g., NADPH) crossreacts with an unrelated enzyme that also possesses a structural domain for the binding of NADPH. Apparently the polyclonal antiserum contains a population of antibodies that recognize an antigenic site located in or around the NADPH-binding region of the protein. It is these site-specific antibodies that crossreact with unrelated enzyme proteins. In this paper we present evidence for antibodies specific for the NADPH-binding region of dehydrogenases from various sources.

MATERIALS AND METHODS

Materials. The enzymes glucose-6-phosphate dehydrogenase (yeast; D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase, EC 1.1.1.49) and dihydrofolate reductase (bovine liver; 5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) were obtained from Boehringer Mannheim and Sigma, respectively. Fatty acid synthetase was purified by the procedure of Muesing and Porter (13). Glucose 6-phosphate, dihydrofolic acid, NADP, and NADPH were obtained from Sigma; acetyl- and malonyl-CoA were from P-L Biochemicals; and complete and incomplete Freund's adjuvant were from Difco Laboratories. All other chemicals used were of analytical grade.

Immunization Protocol and Production of Antibodies. New Zealand White rabbits (5 to 6 months old) were immunized with purified preparations of fatty acid synthetase and glucose-6-phosphate dehydrogenase. Initially, 1 mg of enzyme emulsified with 2 ml of complete Freund's adjuvant was injected into each rabbit subcutaneously at 10 different sites. The animals were given booster injections at 2 and 4 weeks after the first injection at intramuscular and subcutaneous sites, and blood was withdrawn 10 days thereafter. In all cases blood was allowed to clot at 37°C for 1 hr and then stored at 0°C overnight. After removal of the clot, the rabbit serum IgG fraction was purified by precipitation with 50% saturated ammonium sulfate and chromatography on DEAE-cellulose (14).

Enzyme Assays. Glucose-6-phosphate dehydrogenase activity was assayed spectrophotometrically by measuring the rate of reduction of NADP at 340 nm in the presence of glucose 6-phosphate (15). The activity of pigeon liver fatty acid synthetase was determined by following the oxidation of NADPH in the presence of the substrates acetyl- and malonyl-CoA (16). Dihydrofolate reductase activity was measured by following the oxidation of NADPH spectrophotometrically in the presence of dihydrofolate (17).

Immunotitration of Enzyme Activities. The antigen–antibody reaction was followed by an immunotitration procedure that we reported earlier (14, 18, 19). Immunotitrations were performed by incubating enzyme with various amounts of DEAE-cellulose-purified (or ammonium sulfate-purified) antibody to glucose-6-phosphate dehydrogenase or pigeon liver fatty acid synthetase. Incubations were carried out in a buffer, pH 7.0, in the presence of 1 mM EDTA and bovine serum albumin at 1 mg/ml in a final volume of 100 μl for various time intervals at 30°C. In a typical titration increasing amounts of anti-pigeon liver fatty acid synthetase IgG (0–500 μg) were added to 3.44 μg of dihydrofolate reductase in the presence of 100 μg of bovine serum albumin in 0.1 M potassium phosphate buffer, pH 7.0/1 mM EDTA in a final volume of 100 μl. After a fixed incubation time, aliquots (10 μl) were assayed for the remaining dihydrofolate reductase activity. A similar protocol was used for immunotitrations involving other enzymes.

RESULTS AND DISCUSSION

The presence of an antibody population that is specific for binding at a NADPH-binding region of an enzyme suggested that

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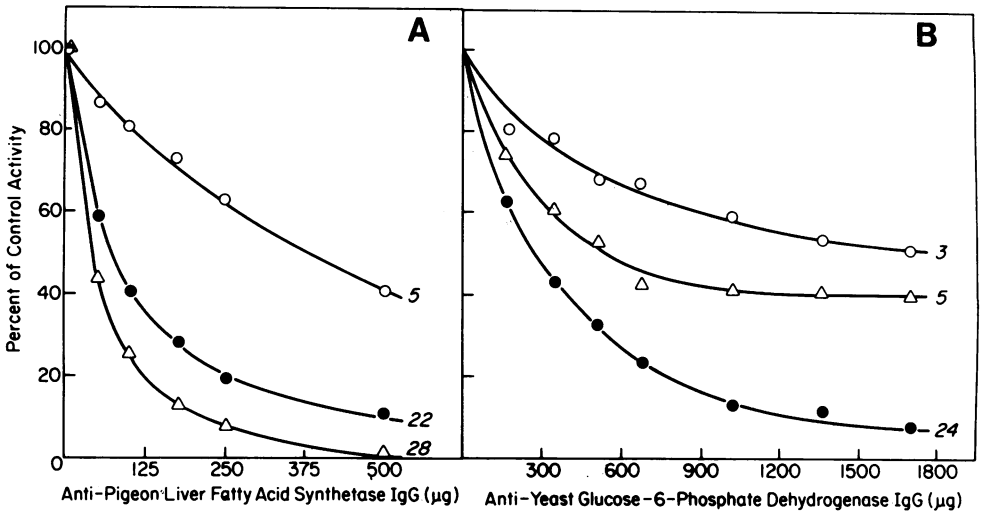

FIG. 1. Inhibition of dihydrofolate reductase activity by anti-glucose-6-phosphate dehydrogenase IgG (*A*) and anti-fatty acid synthetase IgG (*B*). Dihydrofolate reductase, 3.4 μg of protein, in 0.1 M potassium phosphate/1 mM EDTA buffer, pH 7.0, containing 100 μg of bovine serum albumin, was incubated at 30°C with increasing concentrations of IgG in a final volume of 0.09 ml. Aliquots (0.01 ml) were assayed for dihydrofolate reductase activity after a fixed interval of time; numbers on the curves are time in hr. Controls were run in duplicate in the absence of antisera. No loss of dihydrofolate reductase activity was observed in the controls during the periods of incubation reported in this figure.

the polyclonal antiserum against pigeon liver fatty acid synthetase might inhibit dehydrogenases that have a requirement for NADPH. Fig. 1 shows the immunoinactivation of dihydrofolate reductase with increasing concentrations of anti-pigeon liver fatty acid synthetase IgG and anti-yeast glucose-6-phosphate dehydrogenase IgG. It is of interest to note that both antibodies showed a remarkable inhibition of the activity of dihydrofolate reductase, a totally unrelated enzyme.

The inhibition of dihydrofolate reductase by anti-pigeon liver fatty acid synthetase IgG was time dependent (Fig. 2). More than 50% of the enzyme activity was lost within 60 min, and over 90% inhibition of enzyme activity was observed in about 3 hr. A similar experiment (data not shown) with pigeon liver fatty acid synthetase and anti-yeast glucose-6-phosphate dehydrogenase IgG showed a greater immunoinactivation. Fifty percent of the fatty acid synthetase activity was lost within 27 min. The above results lead us to the conclusion that this crossreactivity

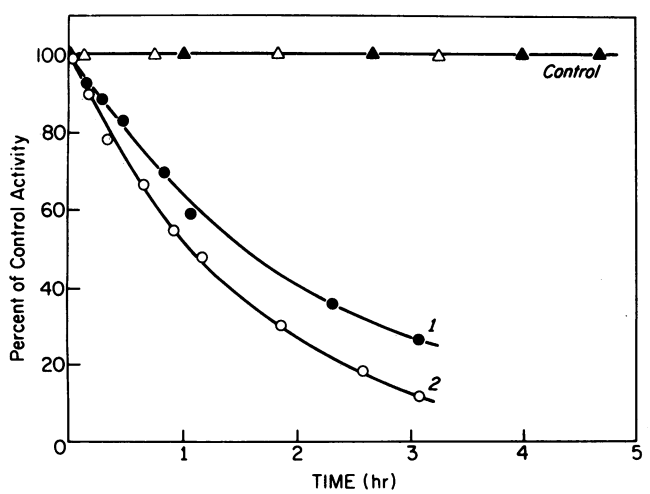

FIG. 2. Time course of inhibition of dihydrofolate reductase by anti-pigeon liver fatty acid synthetase IgG. Dihydrofolate reductase, 3.4 μg of protein, was incubated with 1,500 μg of IgG (curve 1) and 2,000 μg of IgG (curve 2) in the presence of bovine serum albumin at 1 mg/ml in 0.10 ml of 0.1 M potassium phosphate/1 mM EDTA buffer, pH 7.0, at 30°C. Aliquots (10 μl) were assayed for dihydrofolate reductase activity at the indicated times.

is due to the binding of antibodies to an antigenic site that is present on the unrelated enzyme proteins dihydrofolate reductase, pigeon liver fatty acid synthetase, and yeast glucose-6-phosphate dehydrogenase. Because all these enzymes possess dehydrogenase activities, they would be expected to have a common structural domain that binds NADPH.

It is attractive to speculate that these enzymes have similarities in the nucleotide-binding region, because each has a requirement for a pyridine nucleotide cofactor. A striking similarity in the sequence of *Escherichia coli* dihydrofolate reductase and the sequences of other pyridine nucleotide-requiring enzymes has been pointed out by Bennett (20). In addition, Rossmann *et al.* (21) have discussed the evolutionary aspects of a nucleotide-binding protein and have compared the similarity of nucleotide-binding structural domains in several dehydrogenases. Thus the presence of similarities in the amino acid sequence of several dehydrogenases and the suggestion of the chemical and biological evolution of a nucleotide-binding structural domain is in agreement with our proposal of the existence of a specific antigenic site in the nucleotide-binding region of the enzymes under investigation in this paper.

The binding of the site-specific antibodies present in anti-yeast glucose-6-phosphate dehydrogenase IgG to the enzyme dihydrofolate reductase was studied by the competitive binding of NADPH. Amounts of dihydrofolate reductase and anti-glucose-6-phosphate dehydrogenase IgG were chosen so that the inhibition of dihydrofolate reductase was approximately 75%. Various amounts of NADPH were incubated with the enzyme for about 5 min at 30°C, and then anti-glucose-6-phosphate dehydrogenase IgG was added to the incubation mixture. After an incubation of 12 or 18 hr the remaining enzyme activity was measured. It may be seen from the data of Fig. 3 that enzyme activity increased with increasing concentrations of NADPH. This indicates that NADPH is competing for the same binding site as the site-specific antibody. Up to 94% of the enzyme activity was recovered at 250 μM NADPH. These results strongly support our proposal that the inhibitory effect of anti-glucose-6-phosphate dehydrogenase or anti-pigeon liver fatty acid synthetase IgG on dihydrofolate reductase activity is due to the binding of the site-specific antibody population to the nucleotide-binding domain of the enzyme.

The magnitude of inhibition and the rate of inhibition vary

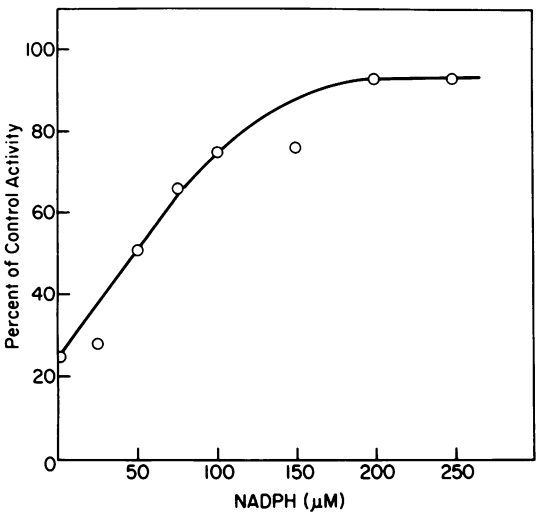

FIG. 3. Protection by NADPH against the inhibition of dihydrofolate reductase activity by anti-glucose-6-phosphate dehydrogenase IgG. Potassium phosphate buffer, pH 7.0, 0.1 M, containing 3.4 μg of dihydrofolate reductase, 100 μg of bovine serum albumin, and 1 mM EDTA, was incubated with various amounts of NADPH for 1 min; anti-glucose-6-phosphate dehydrogenase IgG, 680 μg of protein, was added and the incubation was continued for 24 hr. Residual dihydrofolate reductase activity in the incubation mixture was then determined.

significantly for the crossreaction of antibody with different antigens that possess the NADPH-binding domain. It may be pointed out, too, that antisera to glucose-6-phosphate dehydrogenase did not show immunoprecipitability with either fatty acid synthetase or dihydrofolate reductase. This is not unexpected or unusual, because it is known that antibodies raised to a specific site by immunizing a rabbit with a hapten conjugated to a protein may not be able to precipitate another protein that possesses the same specific site. For example, antibodies raised to biotin-conjugated carrier protein failed to precipitate biotin-containing enzymes such as transcarboxylase and pyruvate carboxylase although the antisera rapidly inactivated these enzymes (22). It is obvious from the above that further work on the production of site-specific antibody, presumably by the conventional adsorption techniques, is needed. We believe that antibody to the NADPH-binding site, and other site-specific antibodies, can be produced by conventional and hybridoma techniques. Site-specific antibodies should have great potential in medicine and in the study of the physical properties and biological activity of an antigen.

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