Cleavage of supercoiled plasmid DNA by autoantibody Fab fragment: Application of the flow linear dichroism technique

(catalytic antibodies/nucleases/autoimmunity)

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ABSTRACT   A highly effective method consisting of two affinity chromatography steps and ion-exchange and gel-filtration chromatography steps was developed for purification of autoantibodies from human sera with DNA-hydrolyzing activity. Antibody Fab fragment, which had been purified 130-fold, was shown to catalyze plasmid DNA cleavage. The flow linear dichroism technique was used for quantitative and qualitative studying of supercoiled plasmid DNA cleavage by these autoantibodies in comparison with DNase I and EcoRI restriction endonuclease. The DNA autoantibody Fab fragment was shown to hydrolyze plasmid DNA by Mg^{2+}-dependent single-strand multiple nicking of the substrate. Kinetic properties of the DNA autoantibody Fab fragment were evaluated from the flow linear dichroism and agarose gel electrophoresis data and revealed a high affinity ($K_m^{obs} = 43$ nM) and considerable catalytic efficiency ($k_{cat}^{app}/K_m^{obs} = 0.32$ min^{-1}·nM^{-1}) of the reaction.

Antibodies able to catalyze a variety of chemical transformations were developed in the last decade, using the strategy of raising antibodies to haptens that resemble the transition state of reactions, as was suggested by Jencks (1) (for review see refs. 2 and 3). An increasing number of autoantibodies with catalytic activity toward natural substrates (4–6), as well as anti-idiotypic antibodies exhibiting a catalytic function (7), have also been described recently. To date, however, the antibody-mediated catalysis is usually characterized by relatively low rate enhancements, indicating the existence of at least two very important problems. The first one, especially important in the case of naturally occurring antibodies, is the purity of the antibody preparation used for the assay, since even a trace enzyme contaminant may mimic a high antibody turnover number (8). The second problem is the development of sensitive, continuous, and accurate methods for detection and monitoring of such low activity (9, 10). Here we describe a reproducible method of purification of DNA-hydrolyzing autoantibodies from human sera. We also demonstrate the advantages of the flow linear dichroism (FLD) technique for quantitative and qualitative characterization of the interaction of these catalytic antibodies with supercoiled (sc) plasmid DNA.

EXPERIMENTAL PROCEDURES

Chemicals. All chemicals were from Sigma and Merck. Enzymes were obtained from Boehringer Mannheim.

Plasmid DNA. Plasmid pUC19 was isolated as described elsewhere (11). More than 95% of the isolated plasmid DNA was in the sc form, judging by 1% agarose gel electrophoresis (AGE).

To estimate the FLD magnitudes for different forms of plasmid DNA, the standard samples of relaxed circular and linear forms were obtained by incubation of sc plasmid DNA with DNase I and EcoRI, as described below, and further purification of products by centrifugation in a linear CsCl

density gradient at 40,000 rpm at 4°C for 36 hr in a Beckman SW41 rotor. The purity of standard samples was checked by 1% AGE.

Glutaraldehyde-Modified DNA-Cellulose. This material was obtained as described in ref. 12. Briefly, denatured DNA solution (10 mg/ml) was heated to 96°C and AE-cellulose (Serva) was added. The mixture was stirred for 30 min at 96°C and glutaraldehyde was added to 0.5% concentration; the reaction mixture was incubated for 15 min, cooled, and washed with 1 M KCl on a glass filter until no absorption at 260 nm could be detected. The preparation was kindly provided by G. A. Nevinsky (Novosibirsk Institute of Bioorganic Chemistry, Siberian Division of the Russian Academy of Sciences).

Patient Analysis. Patients were analyzed at the Institute of Rheumatology, Russian Academy of Medical Sciences, Moscow, by T. B. Prokaeva and Z. S. Alekberova. The diagnosis was confirmed and its reliability was checked according to the criteria developed by the American Rheumatoid Association. The serum of 1 of 10 patients with systemic lupus erythematosus was selected for further investigation as the most active in DNA hydrolysis, judging by AGE (see below).

Antibody Isolation. Antibodies in 5 ml of serum were precipitated twice with 50% saturated ammonium sulfate; this was followed by chromatography on a Pharmacia HR 5/5 staphylococcal protein A fast-performance liquid chromatography (FPLC) column, as described in ref. 13 (p. 310).

Antibody Purification. Samples isolated with protein A were dialyzed twice for 4 hr against 500 vol of buffer A (20 mM Tris·HCl, pH 9.0) at 4°C and applied to a Pharmacia HR 5/5 Mono Q FPLC column that was equilibrated with buffer A. The column was washed with buffer A and then developed with a linear NaCl gradient (0–1 M) in the same buffer. Samples were dialyzed twice for 4 hr against 500 vol of buffer B (20 mM Mops, pH 7.0/50 mM NaCl) at 4°C and loaded onto a glutaraldehyde-modified DNA-cellulose affinity column, which had been equilibrated with buffer B. The column was washed with the same buffer and then antibodies were eluted with a linear gradient of KCl (0–1 M) in buffer B. Collected fractions were dialyzed twice for 4 hr against 500 vol of buffer B at 4°C, and DNA-hydrolyzing activity toward sc plasmid DNA was measured by using AGE (see below). Active fractions were pooled and concentrated by ultrafiltration on Centricon-10 concentrators (Amicon), and 0.4-ml aliquots were loaded onto a Pharmacia HR 10/30 Superose-12 FPLC column that had been equilibrated with buffer C (1 M acetic acid/100 mM NaCl). Separation was performed in buffer C ("acid shock" conditions) at a flow rate of 0.5 ml/min. Fractions, corresponding by their molecular weight to IgG, were collected, immediately neutralized with 3 M Tris·HCl

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Abbreviations: AGE, agarose gel electrophoresis; FLD, flow linear dichroism; FPLC, fast-performance liquid chromatography; LD, linear dichroism; sc, supercoiled.
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buffer, pH 8.5, and dialyzed twice for 4 hr against 500 vol of buffer B at 4°C.

SDS/PAGE analysis of purified antibody preparations (14) revealed protein bands of 61 and 25 kDa (4), immunoblots of which were stained with anti-heavy (H) chain and anti-light (L) chain antibodies, respectively. Protein determinations were done according to Bradford (15).

Preparation of Antibody Fab Fragment. Protein A-isolated antibodies were digested with papain as described in ref. 13 (p. 628) and the resulting Fab and Fc fragments were separated by chromatography on a Pharmacia HR 5/5 protein A FPLC column. Further Fab fragment purification was done as described above for whole antibodies. The final Fab fragment preparation was electrophoretically pure, and its treatment with immobilized antibodies against human L chain (4) resulted in 95% reduction of the hydrolytic activity.

Activity Assays. Antibody DNA-hydrolyzing activity was determined in a standard assay mixture containing 20 mM Tris·HCl at pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 0.1 μg of sc pUC19 plasmid DNA, and 2–10 μg of antibody preparations in a total volume of 20 μl. One unit of specific activity was defined as the amount of antibody required to convert 0.1 μg of sc plasmid DNA into its circular form in 1 hr at 37°C. The hydrolysis was assessed by 1% AGE of the reaction products; the gel was stained with ethidium bromide.

In the kinetic studies, increasing concentrations of sc plasmid DNA were incubated with antibodies. Aliquots were taken out and reaction products were separated by 1% AGE. Ethidium bromide-stained gels were photographed and the films were scanned with a Molecular Dynamics 300A computer densitometer. Molar ratios of reaction products were determined from the scanning data.

FLD. This technique was applied for continuous monitoring of sc plasmid DNA degradation. The method is based on the fact that oriented molecules are generally characterized by different absorption of the light with different linear polarization—i.e., they demonstrate linear dichroism (LD) (for review see ref. 16). The magnitude of LD is defined as $LD = \Delta A = A_{\parallel} - A_{\perp}$, where $A_{\parallel}$ and $A_{\perp}$ are the absorbancies of polarized light parallel or perpendicular to the orientation axis, respectively (17). LD is usually characterized by a dimensionless parameter, so-called reduced linear dichroism LD_r, defined as $LD_r = \Delta A/A_{iso}$, where A_{iso} is the isotropic absorption of an unoriented sample. Long polymers, such as DNA, can be partially oriented by a shear flow (16, 18, 19), and any changes in the molecule length, conformation, or stiffness would affect the LD signal.

The FLD signal was measured as a function of time by using a JASCO J500C spectropolarimeter equipped with an achromatic quarter-wavelength prism at 260 nm. Plasmid DNA was oriented by the flow gradient provided by pumping the solution through the flow cell, designed and constructed by V. L. Makarov (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences). The cell volume was 1.6 ml; optical path length, 2 mm; motor rate, 2 rpm; and average flow gradient in the cell, 6000 s^{-1}. Experiments were performed in 200 μl of the reaction mixture containing 20 mM Tris·HCl at pH 8.0, 5 mM $MgCl_2$, 10% (vol/vol) glycerol, and 1 μg of pUC19 plasmid DNA. Reactions were initiated by addition of DNase I (0.01 ng), *Eco*RI (50 unit), or antibody Fab fragment (30 unit), and the FLD signal was recorded continuously as a function of time.

Kinetic constants were determined by FLD curve fitting using the integral form of the rate equation (20).

RESULTS AND DISCUSSION

Purification of DNA-Hydrolyzing Antibodies. In catalytic antibody studies it is critical to ensure that the catalytic activity observed does belong to the antibodies and is not due to minor quantities of highly active enzyme contaminants. We developed a scheme for purification of DNA-hydrolyzing autoantibody (Fig. 1) that is based on two main features of the newly described biocatalyst—i.e., its antibody nature and DNA-specific character. The use of protein A or G affinity columns (step 2) is effective, yet reliable evidence that catalysis occurs at the antibody binding site should be provided. Further, the ion-exchange chromatography step allows us to eliminate minor enzyme contaminants. To enrich DNA-specific hydrolytic antibodies, total IgG fractions were affinity purified on a glutaraldehyde-modified DNA cellulose column (step 4), in the absence of metal ions to prevent DNA hydrolysis. The specific activity of the antibodies retained by the column was 75-fold greater than that of the preparation before the affinity chromatography. Gel-filtration chromatography under the acid shock conditions (step 5) made it possible both to avoid minor enzyme contaminants if such existed in the noncovalent complexes with antibodies and to prove the analytical purity of the final catalytically active IgG preparation.

The protocol developed was routinely used for several DNA-hydrolyzing antibody preparations and proved its reproducibility and efficiency. The described multistep functional approach to the DNA antibody purification may be recommended to minimize the probability of contamination of the final preparation with enzymes. A specific cleavage of the antibody molecule, intended to produce the Fab fragment, did not reveal any significant loss in the specific DNA-hydrolyzing activity, thus indicating that the hydrolytic activity was peculiar to the antibody Fab fragment and raising the possibility of using this minimal biocatalyst in our further investigations. For the DNA autoantibody Fab fragment under investigation a 130-fold increase of the specific activity during purification was achieved. The specific activity of the final preparation was 25,000 units/mg of protein.

Characterization of Plasmid DNA States During the Antibody-Mediated Hydrolysis. For the broad group of artificial catalytic antibodies, a marked end product of the catalyzed reaction can be detected (21). The multiple-site hydrolysis of biopolymers requires a convenient assay allowing determination of the reaction parameters. A variety of different methods can be applied to assay the DNA cleavage (22). Some of them
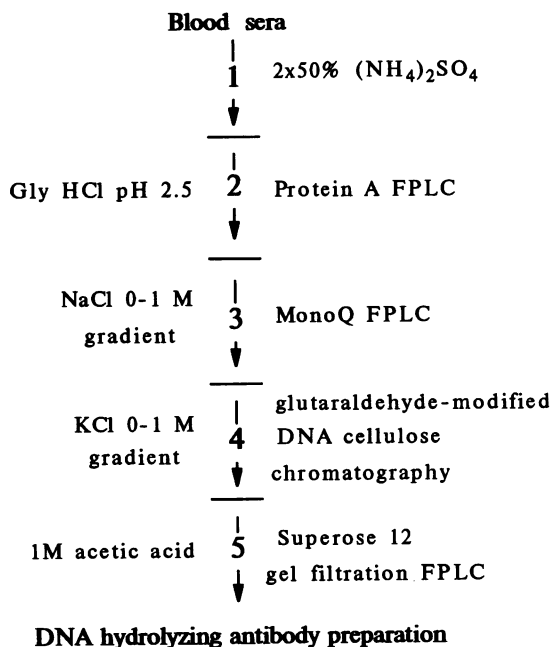
Blood sera

	1	2x50% $(NH_4)_2SO_4$
Gly HCl pH 2.5	**2**	Protein A FPLC
NaCl 0–1 M gradient	**3**	MonoQ FPLC
KCl 0–1 M gradient	**4**	glutaraldehyde-modified DNA cellulose chromatography
1M acetic acid	**5**	Superose 12 gel filtration FPLC

DNA hydrolyzing antibody preparation

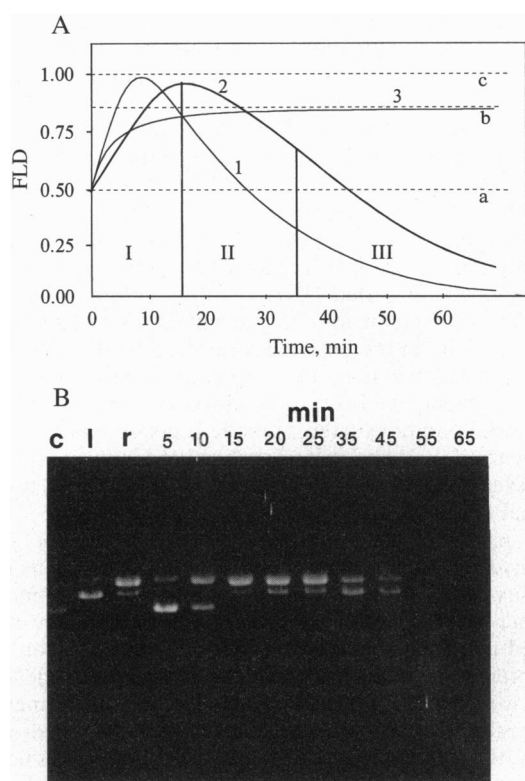FIG. 1. Purification of DNA-hydrolyzing autoantibodies. See text for details.

A

B

FIG. 2. Monitoring of the sc pUC19 plasmid DNA cleavage. (*A*) Analysis of cleavage by the FLD method. One microgram of sc plasmid DNA was incubated in 200 μl of 20 mM Tris·HCl, pH 8.0/5 mM MgCl₂/10% glycerol at 37°C with 50 units of *Eco*RI restriction endonuclease (curve 2), 0.01 ng of DNase I (curve 3), or 30 units of DNA autoantibody Fab fragment (curve 1) in the 1.6-ml flow cell. FLD signal was measured with a JASCO J-500C spectropolarimeter at 260 nm and is presented in relative units. FLD signals of sc (a), linear (b), and relaxed (c) forms of DNA are indicated by dashed lines. See text for details. (*B*) Analysis of plasmid DNA states during cleavage mediated by the autoantibody Fab fragment. Reaction conditions were as in *A*; 20-μl aliquots were taken from the reaction mixture at the indicated time points, and digested products were analyzed by electrophoresis in 1% agarose slab gel, using ethidium bromide as staining agent (23). Control lanes: c, untreated control; 1, linear form standard; r, relaxed form standard.

are suitable for measuring the initial stages of the enzyme attack (23–26), whereas other techniques allow us to assay the extensive attack of endonucleases (27–30). In our previous report (4) we have described an application of the coupled DNA-polymerase reaction and gel electrophoresis for detection of the hydrolysis mediated by DNA autoantibodies. Although extensively used, none of these methods are without drawbacks; most are time consuming and only approximately quantitative. Here we used the FLD technique (18) for monitoring of the sc plasmid DNA cleavage. Continuity, high sensitivity and accuracy of measurements, and the relatively low amount of biocatalyst used are among the main advantages of this method (16). Depending on whether single- or double-

strand breaks were induced by the enzyme, relaxed circular or linear forms of plasmid DNA could be produced. We have demonstrated (see *Experimental Procedures*) that different pUC19 plasmid DNA forms revealed quite different FLD signals; the ratio of FLD amplitudes was found to be as follows: supercoiled:linear:relaxed = 1:1.75:2.0. These variations in FLD magnitude can be used to follow the interconversions of different DNA forms and to discriminate between single-strand and double-strand DNA cleavage mechanisms.

Time dependencies of the FLD signal during sc DNA hydrolysis by both the autoantibody Fab fragment and two nucleases, DNase I and *Eco*RI restriction nuclease, are presented in Fig. 2*A*. For the *Eco*RI-mediated plasmid DNA hydrolysis, a simple kinetic curve may be obtained by the FLD method, reflecting a single-step conversion of sc DNA into a linear form as the result of double-stranded DNA cutting by the enzyme (Fig. 2*B*). All kinetic parameters could be easily determined in this case. Quite different FLD curves were observed when sc DNA was treated with DNase I or the Fab fragment of DNA autoantibodies. The whole curves in these cases may be divided into three parts, as indicated in Fig. 2*A* for the Fab fragment-mediated hydrolysis. At the first part (I), a pseudo-first-order increase of the FLD signal was observed. The maximal values of the FLD signal at this step were close to those measured for the standard sample of the relaxed plasmid form. Data from AGE (Fig. 2*B*) also indicate that a single-strand-nick-mediated relaxation of sc DNA has occurred at this time. At the next part of the curve (II), a quasilinear decrease of the FLD signal was detected. Nondenaturing AGE did not show any alterations except partial linearization of the relaxed plasmid form at this stage, but denaturing PAGE revealed that a further single-strand DNA cleavage occurs at this step (not shown). We attribute the observed decrease of the FLD signal to a decrease in the plasmid DNA orientation, caused by an increased DNA flexibility upon accumulation of single-strand nicks. The subsequent plasmid DNA hydrolysis (III) led to plasmid DNA degradation and formation of low molecular weight products that could be monitored by native AGE. Further FLD signal reduction at this stage may be naturally explained by a decrease of the contour length of the DNA molecule (19). The FLD experiments provided conclusive evidence that DNA-hydrolyzing autoantibodies cleave DNA with the step-by-step formation of a number of single-strand nicks without a pronounced site specificity.

Kinetic Investigation. Since the FLD method allows us to single out the plasmid relaxation stage of plasmid degradation reaction (I in Fig. 2*A*), we applied the Michaelis–Menten equation to this part of the FLD time curve and calculated the kinetic parameters by using the integral form of the equation. To check the applicability of such an approach, the kinetic constants for nucleases with known parameters, such as DNase I and *Eco*RI, were evaluated by using the FLD data; they were found to be in good agreement with the published values (Table 1). On the other hand, the results of AGE quantitative analysis showed that the DNA-autoantibody-mediated plasmid relaxation could be fitted to the Michaelis–Menten equation (Fig. 3).

Table 1. Kinetic parameters of DNA cleavage by DNase I, *Eco*RI, and human Fab fragment

	FLD method			Electrophoresis method			Published value*	
Enzyme	K_m^{obs}, nM	k_{cat}^{app}, min^{-1}	k_{cat}^{app}/K_m^{obs}, min^{-1}·nM^{-1}	K_m^{obs}, nM	k_{cat}^{app}, min^{-1}	k_{cat}^{app}/K_m^{obs}, min^{-1}·nM^{-1}	K_m^{obs}, nM	k_{cat}^{app}, min^{-1}
DNase I	58,000 ± 3200	320,000 ± 18,000	5.0	46,000 ± 8600	250,000 ± 47,000	5.4	25,000	100,000
*Eco*RI	3.9 ± 0.24	10 ± 0.6	2.6	1.7 ± 0.30	2.3 ± 0.50	1.3	4.0	6.5
Fab	43 ± 5.8	14 ± 1.8	0.32	92 ± 32	40 ± 14	0.43	—	—

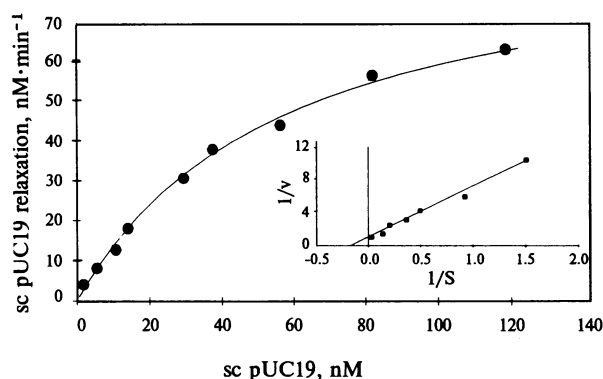± indicates rms deviation values.
*Refs. 31 and 32.

FIG. 3. Saturable hydrolysis of sc pUC19 plasmid DNA by the DNA autoantibody Fab fragment. Indicated concentrations of sc pUC19 plasmid DNA were incubated with 30 units of DNA autoantibody Fab fragment in 20 mM Tris·HCl, pH 8.0/50 mM NaCl/5 mM MgCl$_2$ buffer. Aliquots were taken out at 5-min intervals, the reaction products were separated by 1% AGE, and the gel was stained with ethidium bromide at 5 μg/ml and then photographed. The films were scanned with a Molecular Dynamics 300A computer densitometer and molar ratios of reaction products were determined. (*Inset*) Lineweaver-Burk plot of the same data.

The kinetic parameters of antibody-mediated plasmid DNA hydrolysis are presented in Table 1. The data obtained by both FLD and AGE techniques were used to simulate the kinetic curve of sc DNA relaxation. As can be seen from Fig. 4, the FLD experimental data fit well to the Michaelis–Menten hyperbolic equation.

The K_m^{obs} value obtained for DNA-hydrolyzing autoantibody is close to that for *Eco*RI and nearly three orders of magnitude less than that for DNase I. The hydrolysis efficiency (k_{cat}^{app}/K_m^{obs}, min^{-1}·M^{-1}) for antibody-mediated hydrolysis appeared to be just one order of magnitude less than that for DNase I and *Eco*RI. It should be noted that this activity is underestimated because we used a polyclonal antibody preparation that contained DNA-binding antibodies both with and without DNA-hydrolyzing activity; thus the real efficiency of antibody-mediated DNA hydrolysis would be even higher. To date there are no reports of such a high efficiency of antibody-mediated catalysis. However, in earlier reports (2, 3) antibodies have been raised generally against transition state analogs, and
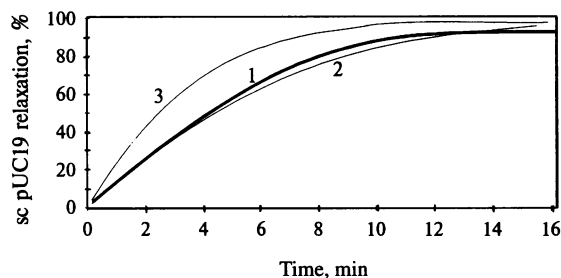

FIG. 4. Simulation of kinetic data of sc DNA cleavage mediated by the DNA autoantibody Fab fragment. Experimental FLD time curve (1) and Michaelis–Menten curves fitted from the FLD (2) and AGE (3) data are presented.

unusually high K_m values for these antibody-catalyzed reactions are caused by the artificial nature of these catalysts. In our case, natural antibodies with high affinity for the substrate have been studied, and their kinetic parameters appear to be rather close to those of related DNA-metabolizing enzymes. The possibility of such effective catalysis by antibodies promises broad perspectives in the biomedical application of antibody-based catalysts.

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