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Covalent Attachment of Lysine to Commercial Restriction Endonucleases

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ABSTRACT

In the present work lysine was coupled through a water-soluble carbodiimide to several restriction enzymes. The work was carried out to assess the effects on enzyme activity of attaching a small molecule to the enzyme carboxyl groups, with the intent of using carboxyl groups for subsequent immobilization of restriction enzymes on solid supports. Lysine was coupled to Eco RI, Bam HI, and Bgl I with partial to complete retention of enzyme activity. The commercial enzymes contained a large relative concentration of bovine serum albumin (BSA). Therefore, commercial Eco RI, a sample of electrophoretically pure Eco RI, and some high purity BSA each were separately labeled with ^{3}H-lysine and the products separated by dialysis and polyacrylamide gel electrophoresis. For the commercial Eco RI preparation, 0.9 μmol of lysine was attached to each μmole of the enzyme fraction; lysine was attached to the BSA and enzyme fractions in the ratio 2.3. The results agreed reasonably well with the amount coupled to the high purity Eco RI and the high purity BSA. The results suggest that carbodiimide coupling through enzyme carboxylic acid groups may be a useful approach for subsequent immobilization of restriction enzymes on solid supports.

Index Entries: Restriction endonucleases, covalent attachment of lysine to; endodeoxyribonuclease enzymes, covalent attachment of lysine to; carbodiimide coupling, of lysine to restriction endonucleases; lysine adducts, to restriction endonucleases.

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INTRODUCTION

The endodeoxyribonuclease enzymes, commonly known as restriction endonucleases, have become extremely important reagents for research in molecular biology. These enzymes have the unique property of cleaving DNA molecules at specific sites. Therefore, they are of key importance in the mapping and cloning of genes and are essential for the preparation of recombinant DNA (*1*).

The restriction endonucleases behave like many other intracellular membrane-bound (*2*) enzymes in that their stability in the isolated or cell-free state is very sensitive to (i) the specific buffer, (ii) the pH, (iii) other compounds that may be present, and (iv) temperature. Two studies have been reported in which the immobilization of restriction enzymes on solid supports was investigated as a possible method for the stabilization and reuse of the enzymes as well as for complete and rapid separation of the enzymes from the cleaved DNA fragments. Eco RI and Bam HI restriction enzymes were coupled covalently to cyanogen bromide-activated Sepharose 4B with retention of enzyme activity (*3*). The immobilized Eco RI and Bam HI were active on both linear and superhelical DNAs; and both enzymes showed marked thermal stabilization as a result of immobilization. Although a coupling efficiency of 50–90% was reported (*3*) for small-scale studies, this figure decreased very markedly on scale-up (R. Blakesley, personal communication). Eco RI, Bam HI, and Hind III also were immobilized on tritylated agarose with partial retention of enzyme activity (*4*). In this case immobilization was reported to occur through hydrophobic bonding. The coupling efficiency was 100% for all three enzymes, based on the absence of activity in the supernatant after the coupling reaction was completed. However, the trityl–agarose restriction enzymes retained their activity only for 5–12 d (P. Cashion, personal communication). Thus, neither of these immobilization procedures appears to be satisfactory for practical commercial scale use.

One approach for assessing the effects of covalently coupled molecules on restriction enzyme activity, is to attach molecules that are specific for particular functional groups on the enzyme. Thus, 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide-metho-*p*-sulfonate has been shown to attach to carboxyl groups on Eco RI subunits, but at the same time to inactivate the enzyme (*5*). The inactivation followed first-order kinetics with respect to the carbodiimide concentration, suggesting that the inactivation was caused by modification of a single carboxyl group.

In the present study, lysine was coupled to the enzyme carbodiimide conjugates as a possible site for immobilization of these enzymes through enzyme carboxylic acid groups. Lower concentrations of carbodiimide and a slightly different carbodiimide were used than was tested in the above referenced Eco RI inactivation study. The present work was done with commercially available preparations of restriction enzymes and with a highly purified sample of Eco RI. The commercial

preparations contained albumin as well as other agents for stabilization of the enzymes. However, previous literature reports of immobilization (*3,4*) and carbodiimide (*5*) studies with restriction enzymes also were done with enzyme stabilizing agents present.

MATERIALS AND METHODS

Materials

Eco RI from *E. coli* RY13 (E.C.3.1.23.13), Bam HI from *B. amyloliquefaciens* H strain RUB 500 (E.C.3.1.23.6), and Bgl I from *B. globigii* (probably E.C.3.1.23.9) were supplied in commercial form by Bethesda Research Laboratories Inc. (Bethesda, MD). The enzymes were received in aqueous storage buffers (Table 1) and were used without further treatment. The enzymes were received at concentrations of 10, 6, and 6 activity units/μL for Eco RI, Bam HI, and Bgl I, respectively. Phage λ DNA of 33×10^6 daltons, Tris HCl, 2-mercaptoethanol, and nuclease-free bovine serum albumin (BSA) also were from Bethesda Research Laboratories. The nuclease-free BSA was listed in the Bethesda Research Laboratory product bulletin as being acetylated followed by rigorous purification. Thus, most of the BSA hydroxyl and amino groups would be present as the corresponding acetate esters and amides, respectively. L-Lysine (Sigma, St. Louis, MO), 1-ethyl-3(3-dimethyl-aminopropyl)carbodiimide hydrochloride (EDCD) (Pierce Chemical, Rockford, IL), and {4,5-^{3}H}-lysine (Amersham, Arlington Heights, IL) of specific activity 75–80 Ci/mmol also were used as received. Electrophoresis grade materials were used in preparing the polyacrylamide gel. A sample of highly purified Eco RI, free of albumin and other proteins, was very kindly provided by

TABLE 1
Commercial Enzyme Storage Buffers

	Concentration		
Component	Eco RI	Bam HI	Bgl I
Potassium phosphate buffer, m*M* (pH)	10 (7.2)	20 (7.5)	
Tris-HCl buffer, m*M* (pH)			50 (8.0)
2-Mercaptoethanol, m*M*	5.0		7.0
Dithiothreitol, m*M*		1.0	
Disodium EDTA, m*M*	0.5	0.5	1.0
BSA[a], μg/mL	500	500	500
NaCl, m*M*	300		200
Glycerol, % (vol)	50	50	50
Triton-X 100, % (vol)	0.2		

[a]Acetylated to cap amino groups.

John Rosenberg from the Department of Biological Sciences of the University of Pittsburgh. This sample was at a concentration of 1.3 mg Eco RI/mL in storage buffer consisting of 10 m*M* sodium phosphate, 7 m*M* β-mercaptoethanol, 0.1 m*M* EDTA, 0.6 m*M* NaCl, and 10% glycerol. The sample of Eco RI had been purified to electrophoretic homogeneity using procedures described elsewhere (*6*).

Determination of Activity of Soluble Enzymes

A unit of enzyme activity was defined as the minimum amount of enzyme needed to obtain complete digestion of 1 μg of λDNA after incubation for 1 h at 37°C (*3*). In a typical assay 5 μL aliquots of enzyme solution were mixed with storage buffer (Table 1) to give a series of enzyme dilutions. Five microliters of diluted enzyme were added to 45 μL of assay reagents (Table 2) containing 1 μg of λDNA. After 1.0 h at 37°C the reaction was stopped by addition of 1% SDS in aqueous glycerol containing 0.1% bromophenol blue and disodium EDTA. About 50 μL of the digested DNA mixture was loaded on a 3 mm thick slab of 1% agarose gel. Vertical gel electrophoresis was carried out at pH 7.8 and room temperature, using a Bethesda Research Laboratory Model V16 unit (*3*). After electrophoresis, the agarose gel was stained with ethidium bromide and photographed on Polaroid Type 107 ASA 3000 film under ultraviolet light, using a Polaroid-Wratten gel filter No. 16. The banding pattern was observed for the presence of partially as well as totally digested DNA.

Coupling of Lysine to Enzymes

EDCD (0.01–1.0 mg), lysine (0.01–1.0 mg), and commercial enzyme solution as received (60–100 U, 10 μL) were mixed with sufficient 0.2*M* of pH 8.0 sodium bicarbonate buffer to give a total volume of 100 μL. This mixture was incubated 20 h at 4°C and then assayed for enzyme activity.

TABLE 2
Enzyme Assay Mixture

	Concentration		
Component	Eco RI	Bam HI	Bgl I
Tris-HCl buffer, m*M* (pH)	100 (7.2)	20 (8.0)	
Glycine NaOH buffer, m*M* (pH)			20 (8.3)
$MgCl_2$, m*M*	5.0	7.0	20.
2-Mercaptoethanol, m*M*	2.0		7.0
NaCl, m*M*	50	100	50
λ DNA, μg/45 μL[a]	1.0	1.0	1.0
Enzyme solution, μL[a]	5	5	5

[a]Total assay volume of 50 μL for soluble enzyme.

This procedure was used to obtain the results with the nonradiolabeled lysine. Control runs in which the nonradiolabeled lysine was added, but the EDCD was omitted, were carried out along with each coupling reaction. The reasoning and justification for carrying out the coupling reaction at pH 8, instead of in the more usual range of pH 4–5 for carbodiimides, is given in the Discussion section.

In experiments with radiolabeled lysine 3.5 μCi (0.0080 μg) of ^{3}H-lysine plus 100 μg of nonradiolabeled lysine were mixed with 100 μg of EDCD, plus 10 μL of commercial enzyme solution (100 U of Eco RI, 60 U of Bgl I), and bicarbonate buffer and held 20 h at 4°C. The concentration used with Bam HI was reduced because of stability problems (*see* Results section for details). With the high purity Eco RI, 25.6 μCi (0.064 μg) of ^{3}H-lysine plus 100 μg of nonradiolabeled lysine was mixed with 100 μg of EDCD, 10 μg of Eco RI, and buffer and treated the same as for the commercial enzymes. Unattached EDCD, ^{3}H-lysine, and nonradiolabeled lysine were separated from the enzyme by dialysis of the entire coupling reaction mixture. This was carried out by using a Bethesda Research Laboratories Model 1200 MA microdialyzer having a 100 μL minimum dialysis volume. The Model 1202 MA membrane was permeable to molecular weights of 6000–8000 daltons or smaller. Dialysis was carried out for 48 h at 4°C at a buffer flow rate of 10–20 mL/h. The following buffers were used: 0.2*M* sodium bicarbonate, pH 8.0, for Eco RI; 20 m*M* potassium phosphate buffer, pH 7.5, plus 0.5 m*M* disodium EDTA for Bam HI; and 0.2*M* sodium bicarbonate, pH 8.0, plus 200 m*M* sodium chloride and 1 m*M* disodium EDTA for Bgl I. Aliquots of the dialyzed reaction mixture were diluted for determination of enzyme activity. Control runs for the ^{3}H-lysine experiments were done by omitting the ^{3}H-lysine and not conducting the dialysis in order to assess any loss in enzyme activity resulting from the 68-h incubation/dialysis time of the test runs. Counting was done in Aquasol-2 on a Packard Model 3320 Tri-Carb Liquid Scintillation Counter, using an external standard for quench control.

Polyacrylamide Gel Electrophoresis

Some of the ^{3}H-lysine–Eco RI and ^{3}H-lysine–BSA reaction mixtures were subjected to electrophoresis in the presence of 0.1% sodium dodecyl sulfate (*7,8*) in order to separate the labeled Eco RI from the labeled BSA that was present with the commercial Eco RI. Gels containing 7.5% acrylamide were placed in 6 mm inner diameter by 10 cm long glass tubes. The dialyzed reaction mixtures (about 120 μL) were treated as follows and then layered on top of the gels: 40 μL of dialysis mixture was mixed with 40 μL of 0.01*M* sodium phosphate buffer, pH 7.0 (containing 0.1% sodium dodecyl sulfate and 0.1% β-mercaptoethanol), 3 μL of 0.05% bromphenol blue tracking dye, 5 μL 0.1% β-mercaptoethanol, and 1 drop of glycerol. The entire dialyzed reaction mixture was electrophoresed by use of several gel tubes. After electrophoresis (*7*), some of

the gels were stained with Coomassie brilliant blue and then destained. Other gels were removed from the tubes, cut into 2 mm lengths, digested overnight at 37°C in an Econofluor liquid scintillation cocktail containing 3% Protosol, and counted. Counting was done on a Beckman Model LS 7500 Liquid Scintillation Counter, with the counting efficiency obtained from a previously determined quench curve prepared using standard ^{3}H-hexadecane.

RESULTS

Ratio of EDCD to Units of Enzyme

A fixed ratio of mg EDCD to mg lysine of 1 : 1 was used. However, the ratio of mg EDCD to units of enzyme activity was varied by 100-fold. The results for the commercial enzymes are shown in Table 3. No loss in Eco RI activity was observed at EDCD/enzyme ratios of 0.1 mg/100 U or 0.01 mg/100 U. At the highest ratio (1.0/100), some partially digested DNA fragments were observed for the same number of enzyme units that gave complete digestion in the control run. Thus, some loss in Eco RI activity occurred during the 10-h incubation at the highest EDCD concentration.

In a preliminary test, Eco RI was diluted with 0.2*M* sodium bicarbonate, pH 8.0. No difference in enzyme activity was observed in comparison to dilution with storage buffer.

Coupling of Commercial Enzymes to ^{3}H-Lysine with EDCD

In the ^{3}H-lysine experiments, sufficient 0.2*M*, pH 8.0, sodium bicarbonate was added to give a total volume of 100 μL for the coupling reaction. Both Eco RI and Bgl I tolerated the bicarbonate buffer in place of storage buffer without any decrease in activity; however, Bam HI lost considerable activity in 0.2*M* bicarbonate and could not tolerate the presence of EDCD very well in the 0.2*M* bicarbonate buffer. Therefore, the ^{3}H-lysine experiment with 60 U of Bam HI was carried out in 0.05*M* sodium bicarbonate and with the quantities of EDCD and nonradiolabeled lysine each reduced to 0.05 mg.

The results of the ^{3}H-lysine experiments showed little loss in enzyme activity for Bgl I and for some of the Eco RI experiments. However, the control runs for Bam HI and for one of the Eco RI experiments showed partials, indicating that the enzyme activity had been influenced by the 68-h treatment time.

Coupling of Commercial and Purified Eco RI with EDCD and ^{3}H-Lysine Followed by Electrophoresis of Reaction Mixtures

Lysine (0.1 mg), EDCD (0.1 mg), and either 100 U (10 μL) of commercial Eco RI (containing BSA) or 10 μg of electrophoretically pure Eco RI were mixed with ^{3}H-lysine and bicarbonate buffer and allowed to react as in the other studies. The coupling reaction also was done using 5 μg of BSA in place of the commercial Eco RI mixture. After dialysis to remove unattached lysine, the proteins were separated by electrophoresis. The distribution of proteins along the length of the polyacrylamide gels after electrophoresis is shown in Fig. 1. Preliminary electrophoresis of nonderivatized BSA or nonderivatized electrophoretically pure Eco RI showed R_f values of 0.30 and 0.64, respectively, as determined by staining and visual observation. Electrophoresis of the ^{3}H-lysine derivatized BSA gave an R_f value of 0.31–0.33 for three gels (Fig. 1B). Thus, the peak at R_f 0.33 in Fig. 1A is clearly that of lysine-derivatized BSA. The other peak (R_f 0.57) in Fig. 1A is attributed to lysine-derivatized Eco RI, probably mixed with other similar proteins that were present in the commercial Eco RI preparation. Modrich and Zabel (*8*) reported R_f values of 0.4 for BSA and 0.7 for Eco RI after sodium dodecyl sulfate electrophoresis on 7.5% acrylamide gel. In the commercial Eco RI preparation, the BSA was acetylated and thus may have had a slightly different R_f from that of native BSA. The difference in R_f values between the commercial Eco RI-lysine (R_f 0.57) and the high purity Eco RI-lysine (R_f 0.66–0.70) may also have arisen because of different methods of enzyme preparation, purification, or storage. The methods used in the preparation and purification of the commercial enzyme were proprietory information.

The radiolabeling results showed that 0.011 μg of labeled plus nonlabeled lysine was coupled to the 10 μg of high purity Eco RI. This amounted to 0.21 μmol coupled lysine/μmol of Eco RI subunit, based on an Eco RI subunit molecular weight of 29,000 (*9*). The radiolabel-electrophoretic results with 100 U of commercial Eco RI showed that 7.1×10^{-4} μg of labeled plus nonlabeled lysine was attached to the Eco RI fraction (R_f 0.57) and 1.6×10^{-3} μg to the BSA fraction (R_f 0.33) (Fig. 1A). In the case of the purified Eco RI, 1.06×10^{-2} μg of total lysine was attached to 10 μg of enzyme (Fig. 1C). When 5 μg of BSA was derivatized, 5.9×10^{-4} μg of total lysine was attached (Fig. 1B). The relative quantities of lysine coupled to the Eco RI and BSA are discussed in the next section.

DISCUSSION

The results showed that 7.1×10^{-4} μg of lysine was attached per 100 units of commercial preparation of Eco RI. The specific activity of the

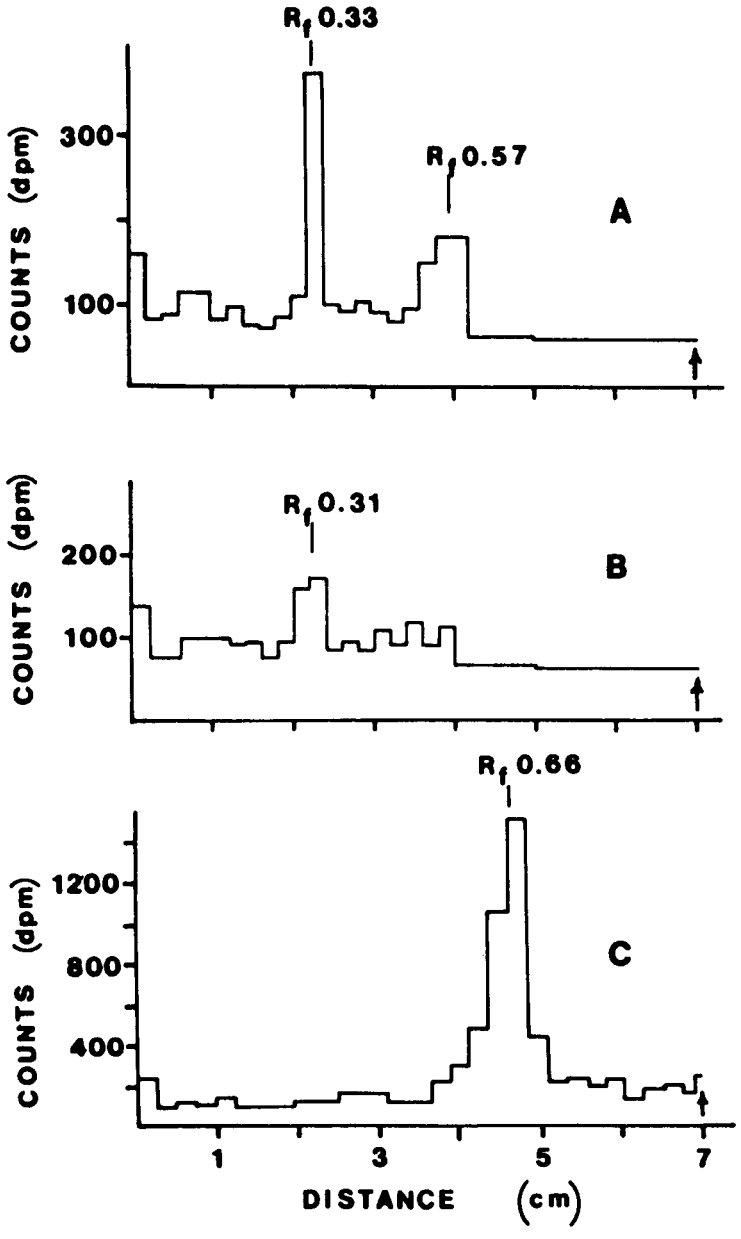

Fig. 1. Distribution of radioactivity along length of polyacrylamide gel tubes after sodium dodecyl suflate electrophoresis of ^{3}H-lysine derivatized preparations of the following proteins: (A) commercial Eco RI-BSA mixture, (B) high purity BSA, and (C) electrophoretically pure Eco RI. Derivatization reaction mixtures were dialyzed to remove unattached ^{3}H-lysine. After electrophoresis the 7.5% acrylamide gels were cut into 2-mm long segments and counted. The results shown are for single acrylamide gel tubes. Several gel tubes were needed in order that the total volume of the ^{3}H-lysine derivation protein could be electrophoresed and counted. The arrows represent the positions of the tracking dye at the end of the electrophoresis.

Eco RI could not be provided by Bethesda Research Laboratories; however, an approximate value was estimated from the literature. Eco RI from the same microbial source as used in the present study was found to have a specific activity of 880,000 U/mg protein (5). In the referenced system a unit of activity was defined as the amount of enzyme giving complete digestion of 0.5 μg of ColE1 DNA during 15 min at 37°C; whereas the unit definition we used was for complete digestion of 1.0 μg of λDNA incubated 60 min at 37°C. It takes 2.5 ColE1 based units to equal 1.0 λ based unit (R. Blakesley, personal communication). Therefore, the 880,000 ColEl based specific activity was equivalent to 352,000 U/mg for action on λDNA. Since our assay required cleaving twice as much DNA, the activity would be half of that based on 0.5 μg DNA. However, the activity based on a 60 min incubation would be four times that based on a 15 min incubation. Applying these factors gave an estimated specific activity for our commercial Eco RI of 704,000 U/mg protein, based on our unit of activity and our assay procedure. There was some error inherent in this calculation because the activity during each of the four 15-min periods at 37°C probably was not the same. Based on this calculated specific activity, 100 U of Eco RI contained 0.14 μg or 4.9×10^{-6} μmol of enzyme protein (subunit molecular weight 29,000) (9).

The amount of lysine coupled to the 10 μg of purified Eco RI compared reasonably well with that coupled to the Eco RI fraction (0.14 μg) of 100 U of the commercial enzyme preparation. The commercial Eco RI should have had 1.5×10^{-4} μg of lysine coupled to the 0.14 μg of enzyme, if the loading had been the same as with the purified enzyme. This compares favorably with the actual loading of 7.1×10^{-4} μg of lysine coupled to the enzyme (0.9 μmol lysine/μmol enzyme). Since the commercial Eco RI appeared to contain other proteins besides Eco RI (based on modified R_f value for enzyme peak), a greater loading would be expected than if only pure Eco RI was present. Therefore, it was concluded that the loading results were consistent between the commercial Eco RI and the purified Eco RI.

A similar comparison between the lysine loading of 5 μg of purified acetylated BSA and the lysine loading of the BSA fraction of the commercial enzyme preparation also showed reasonably good agreement. The purified BSA had 5.9×10^{-4} μg of lysine attached, as compared to 1.6×10^{-3} μg of lysine for the commercial enzyme BSA. This difference by a factor of 3 was attributed to differences in the concentrations of BSA. Both the purified BSA and enzyme BSA were supplied by Bethesda Research Laboratories as 100 μL solutions; so the accuracy of the BSA concentrations could not be determined.

For the commercial Eco RI preparation the ratio of the lysine attached to BSA to that attached to Eco RI was 2.3. If the lysine loading on the commercial Eco RI had been based on the loading of 1.5×10^{-4} μg (equivalent to that for the purified Eco RI) instead of 7.1×10^{-4} μg, then the attached lysine ratio for BSA/Eco RI would have been 11. The maxi-

mum expected ratio for lysine coupling to BSA/Eco RI is about 26. This arises from the relative number of carboxylic acid groups on the BSA and Eco RI present in the reaction mixture. Carbodiimides react primarily with carboxylic acids to form active intermediates which in turn react readily with amines. Previous studies with Eco RI and a carbodiimide suggested that coupling occurred to a single carboxyl group; however, the number of available carboxylic acid groups per Eco RI subunit was not listed (*5*). Ten μL of enzyme solution contained 5 μg or 7.7×10^{-5} μmol of BSA (MW 65,000). If we assume 62 carboxyl groups per Eco RI subunit (*8*) and 100 carboxyl groups per BSA molecule (*10*), then the total available carbonyl groups were 7.7×10^{-3} on BSA and 3.0×10^{-4} on Eco RI for a ratio of 26 :1. Our results suggest that all of the carboxylic acid groups did not have the same availability for reaction with the carbodiimide or lysine. This availability also may have been influenced by the choice of pH used for the coupling reaction.

In the present study, the carbodiimide coupling reaction was carried out at pH 8 since the restriction enzymes rapidly lose activity at pH values below about 6.8. The overall coupling reaction worked at pH 8, as evidenced by the results showing 0.21 μmol lysine coupled/μmol (subunits) of purified Eco RI. Other studies also have reported the successful reaction of carbodiimides with proteins at pH 7–8. The previous Eco RI carbodiimide study was done successfully at pH 7.0 (*5*); and Mosbach and coworkers coupled enzymes via carbodiimide linkages at pH 3.5 and also in 0.2*M* $NaHCO_3$ at pH 8 (*11*). The reaction between a carboxylic acid and a carbodiimide proceeds most rapidly at acid pH. The carbodiimide undergoes protonation followed by coupling to the carboxylic acid anion to form an *O*-acyl intermediate. The intermediate can undergo rearrangement to an acyl urea or couple with a nucleophile, i.e., a primary amine. Under basic conditions the formation of the O-acyl intermediate would be expected to proceed very slowly. However, the more basic pK_a of the epsilon-amino group of lysine would tend to push the reaction between the amine and the intermediate to completion.

Rate constants for carbodiimide inactivation of Eco RI at 20°C were reported previously as a function of carbodiimide concentration over the range of 10–50 m*M* carbodiimide (*5*). In our studies no loss in Eco RI activity was observed after 20 h incubation with either 0.1 mg (5.2 m*M*) or 0.01 mg (0.52 m*M*) EDCD (Table 3) at 4°C. These concentrations are well below those used in the previous study (*5*). Extrapolation of the first-order deactivation rate constant data to 0.52 and 0.052 m*M* carbodiimide gave values of 4.7×10^{-4} min^{-1} and 5.5×10^{-5} min^{-1}, respectively. These results would predict 43 and 6% loss of enzyme activity, respectively, after 20 h at 20°C. Since our couplings were carried out at 4°C, the inactivation data are not directly comparable. However, even at 20°C and 0.52 m*M* carbodiimide, little loss in Eco RI activity would be expected. Thus our results at 4°C, where no inactivation was observed, are consistent with the previous work.

TABLE 3
Eco RI Activity after Incubation with EDCD and Nonradiolabeled Lysine

EDCD:lysine:units enzyme mg:mg:units	Eco RI activity, % of control[a]
1.0:1.0:100	<100
0.1:0.1:100	100
0.01:0.01:100	100

[a]Controls were identical to test mixtures, but with EDCD omitted.

The results of this study showed that commercial preparations of Eco RI, Bam HI, and Bgl I could be reacted with low concentrations of a carbodiimide and lysine without significant loss of enzyme activity. The amount of lysine attached to 100 U of commercial Eco RI suggested strongly that useful immobilized Eco RI preparations could result if roughly the same enzyme/support loading as enzyme/lysine loading could be obtained. In addition, the enzyme would need to be positioned some distance away from the solid support in order to allow DNA molecules of 10^6 daltons molecular weight to come in contact with the immobilized enzyme. One method for controlling the orientation of the enzyme, relative to the support, during immobilization would be to carry out the enzyme–carbodiimide-support coupling reaction in the presence of a small molecular weight piece of DNA. This substrate would need to have the proper recognition sequence so as to protect the enzyme active site. However, the diffiult task of preparing such a segment of DNA is made more demanding since it would also need to be free of functional groups that could couple during the enzyme immobilization procedure.

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