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ECORI DNA METHYLASE ACTIVITY IS ELIMINATED UPON HISTIDINE RESIDUE MODIFICATION

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The E.coli EcoRI DNA methylase activity is completely eliminated in five minutes upon incubation with the histidine residue specific reagent diethyl pyrocarbonate. In that two moles of N-ethoxyformylimidazole per mole of methylase are detected spectroscopically upon inactivation and activity is not restored by hydroxylamine, it is likely that activity loss is due to double modification of a single histidine residue. This information is critical in determining the enzymatic mechanism, causes of the pH-activity curve, designing protein mutants and interpreting previous structure-function data.

The bell shaped pH-activity curve for the E.coli EcoRI DNA methylase exhibits a maximum at 7.8 with two pKa values at pH 7.0 and 8.4 (Mashoon, 1989). In investigating the pH profile, this work involves indicating possible participation of one or more histidine residues in S-adenosyl-L-methionine binding, DNA binding, and/or methyl group transfer. Interpreting pH profiles is difficult since they may represent singular or combinatory enzyme and substrate effects. However, such profiles can guide modifications and further activity studies which identify amino acid residues critical in binding and/or catalysis.

To date, not much structure-function information is available about DNA methylases or S-adenosyl-L-methionine binding enzymes (Razin, 1984). For DNA methylases, little is known about protein-S-adenosyl-L-methionine binding interactions or the mechanism of methyl transfer to DNA. Information about the EcoRI methylase is widely applicable since S-adenosyl-L-methionine is the methyl group donor for a large range of enzymes and DNA methylation plays a role in gene expression, DNA repair, restriction-modification systems and carcinogenesis. (Razin, 1984) The EcoRI methylase transfers the methyl group from S-adenosyl-L-methionine to the second adenine in 5'GAATTC3' DNA sequences (Rubin, 1978) thereby protecting the site from cleavage by the corresponding endonuclease.

Diethyl pyrocarbonate reacts fairly specifically with histidine residues at neutral pH as shown in Figure 1. Quantitation of modified histidines can be

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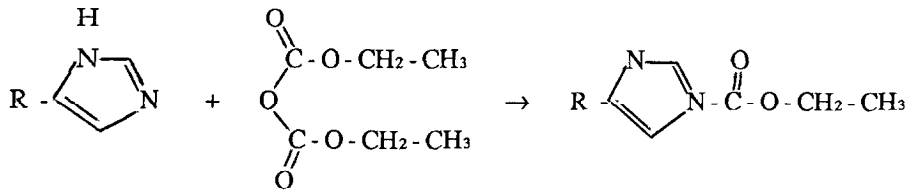

Figure 1: Reaction of diethyl pyrocarbonate with histidine residues.

achieved through an increase in absorbance at 240 nm (ε=3200 M^{-1} cm^{-1}) due to N-ethoxyformylimidazole formation. Unfortunately, diethyl pyrocarbonate is not amenable to in-depth characterization of modified residues since the adduct half-life is 55 hours at pH 7.0 and is 2 hours at pH 2.2 (Means, 1971, Eyzaguirre, 1987). Although amino groups may be modified at high diethyl pyrocarbonate concentrations or upon prolonged incubation, histidine residues are generally more reactive. The reaction can be stopped with excess histidine and reversed with excess hydroxylamine. Dicarbethoxyhistidyl derivatives which cannot be reversed by hydroxylamine result from double modification of histidine residues (Miles, 1977).

Methods

Diethyl pyrocarbonate inactivation at extended timepoints:

Diethyl pyrocarbonate was diluted from 6.79 M to 50 mM in ethanol and 2 ul was added to 3 uM methylase (Everett, 1989c) in 1 mM potassium phosphate buffer, pH 7.0, 0.7 mM 2-mercapto-ethanol, 20 mM NaCl, 1% glycerol and 0.5 mM EDTA. Ethanol (2 ul) was added to a control. Samples (5 ul) were removed, diluted 1/1000 and assayed at 5, 10, 15 and 20 minutes using the standard methylase assay (Everett, 1989c).

Inactivation at different diethyl pyrocarbonate concentrations:

Methylase (3 uM) was incubated with 0 (ethanol), 0.0003, 0.003, 0.03, 0.30 and 3.0 mM diethyl pyrocarbonate for six minutes. Samples (5 ul) were diluted 1/20 into 30 mM L-histidine or 20 mM hydroxylamine and diluted further for assay.

Inactivation at short timepoints:

Methylase was inactivated with 0.3 mM diethyl pyrocarbonate and diluted 1/20 into 30 mM L-histidine at 15 seconds, 1, 2, 3, 4 and 5 minutes and diluted further for assay.

Quantitation of modified histidine residues:

The absorbance of 0.3 mM diethyl pyrocarbonate in water was scanned from 200-300 nm. The absorbance of 7.5 uM methylase in 800 ul water was scanned and subtracted from scans after the addition of 0.3 mM (3.5 ul) diethyl pyrocarbonate. Absorbance scans of the reaction were taken at 30 seconds, 3, 6, 9 and 12 minutes. The absorbance of diethyl pyrocarbonate at 240 nm was subtracted from the increase in absorbance at 240 nm for the reaction mixture before the number of N-ethoxyformylimidazole residues was calculated.

All experiments were conducted at 25°C.

Results

As shown in Figure 2a, the diethyl pyrocarbonate treated methylase has lost 100% activity by the five minute timepoint. Consistent with findings in other studies (Everett, 1989a), the methylase looses about 20% activity due to room temperature incubation.

Activity is completely lost at diethyl pyrocarbonate concentrations between 0.03 and 0.30 mM. Reactivation cannot be achieved by hydroxylamine. (Data not shown.)

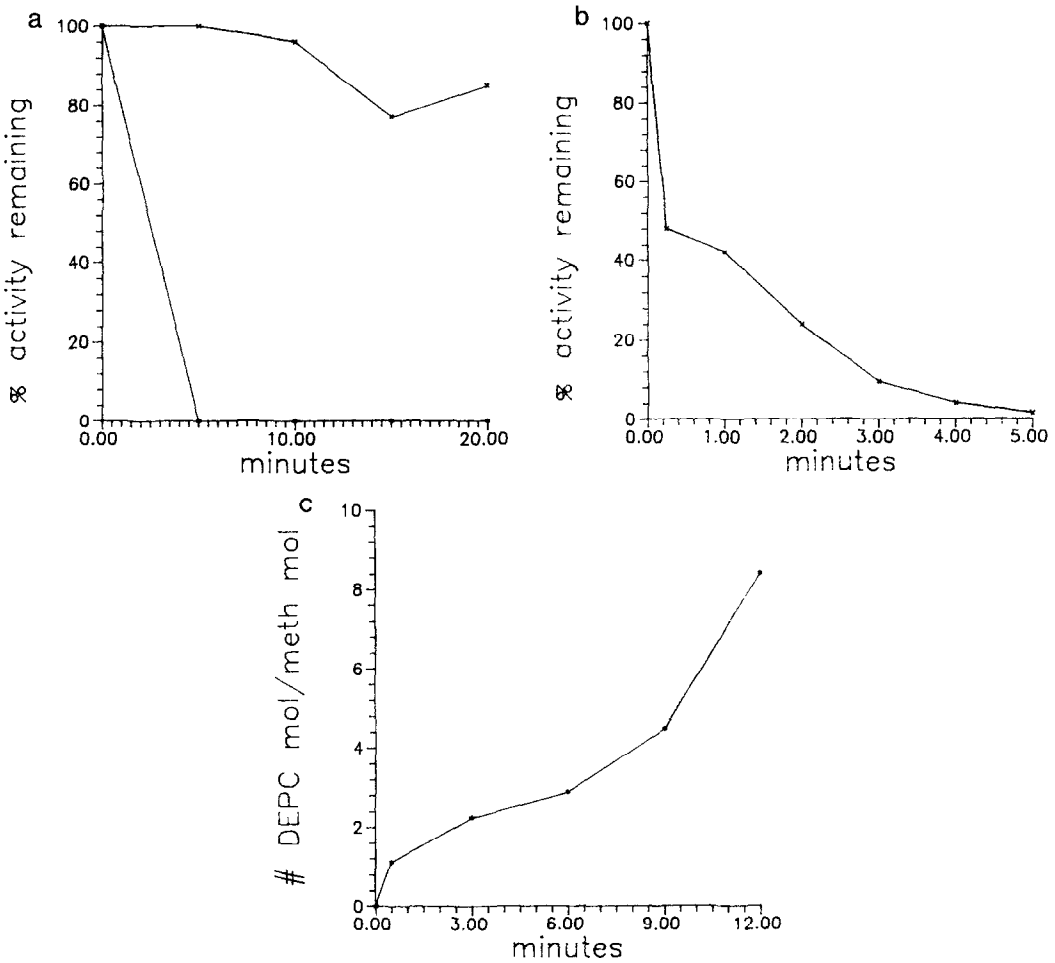

Figure 2a: Inactivation of methylase (3 uM) with diethyl pyrocarbonate (50 mM) at 25°C. Samples were removed at 5, 10, 15 and 20 minutes for assay. The control is methylase with ethanol added (2 ul) to mimic the ethanol added with the diethyl pyrocarbonate. The upper line (x) is methylase and ethanol, the lower line (*) is methylase and diethyl pyrocarbonate.

Figure 2b: Inactivation of methylase (3 uM) with 0.3 mM diethyl pyrocarbonate. Samples were assayed at 15 seconds, 1, 2, 3, 4 and 5 minutes.

Figure 2c: The number of histidine residues modified by diethyl pyrocarbonate per molecule of methylase as determined from the absorbance at 240 nm. The absorbance of 7.5 uM methylase and 0.3 mM diethyl pyrocarbonate was scanned at 0.5, 3, 6, 9 and 12 minutes and the absorbance of 0.3 mM diethyl pyrocarbonate subtracted.

Figure 2b gives the results of inactivation at short timepoints. Rapid inactivation occurs as only 48% activity remains after 15 seconds of incubation with diethyl pyrocarbonate. The activity rapidly declines until only 1.5% remains at 5 minutes.

One histidine has been modified at 30 seconds (Figure 2c) and approximately two upon full inactivation. The number of modified residues continued increasing through the final timepoint to 8.4 per methylase molecule. This indicates probable double modification of histidine residues since the methylase contains only six histidine residues and hydroxylamine does not restore activity. Considering that the methylase is extremely labile, it is possible that long-range modification effects and/or hydroxylamine itself irreversibly inactivate the protein.

Discussion

The EcoRI methylase has six histidine residues and His-235 has been shown to be part of the S-adenosyl-L-methionine binding site (Everett, 1989a). Although the two pKa values might reflect protonation or deprotonation of the S-adenosyl-L-methionine or DNA or a methylase amino acid with an environmentally altered pKa, one most likely candidate is a histidine residue. The 2-amino, 6-amino and carboxyl group of S-adenosyl-L-methionine are probably important in enzyme binding interactions and have pKa values of approximately 10.8 and 1.7 respectively (Zappia, 1969, Oliva, 1980). The pKa's of DNA groups are probably not a factor although DNA binding interactions are taking place and might be affected by changes in pH. Hydrogen bond participants on the enzyme for S-adenosyl-L-methionine and DNA might include histidine, lysine, tyrosine, arginine, aspartic acid, glutamic acid or cysteine. Since cysteine residue modification destroys activity it is possible that the pH effect partially reflects a pKa for this residue (Everett, 1989b).

Modification of the EcoRI methylase with diethyl pyrocarbonate resulted in complete loss of activity after a five minute incubation. This correlated with formation of approximately two N-ethoxyformylimidazole groups. Activity could not be restored with hydroxylamine, although the methylase lability precludes interpretation of the data (Everett, 1989a). Inactivation and quantitation in the presence of S-adenosyl-L-methionine and DNA will be useful in determining how many histidine residues are actually involved in binding or catalysis. Mass spectrometric analysis of peptide maps, already used to determine the critical cysteine residue (Everett, 1989b), could be used to identify the modified residue(s) and confirm histidine (versus amino group) modification.

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