was 1 x TBE. Loading buffer containing bromophenol blue was run in an unused lane as a marker of the progress of the gel. The samples were electrophoresed at 80 V (~13 V/cm) for 2.5 hours, after which time the plates were dismantled and the gel dried. The gel was exposed to autoradiograph film overnight at –70°C and the film developed the following day.

2.18.2 Assays with MelR and RNAP α subunits

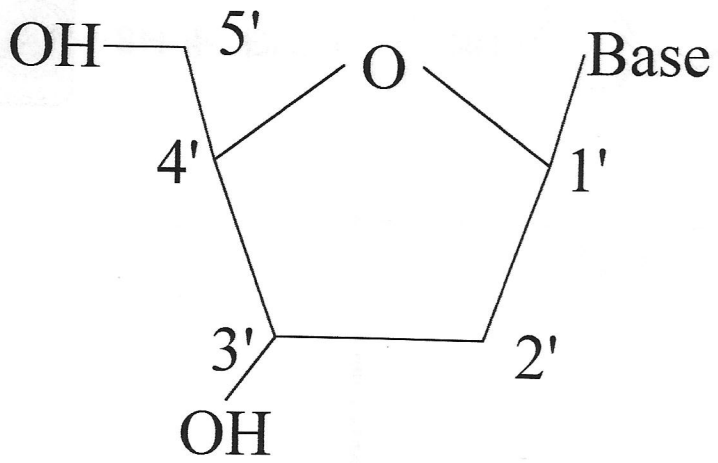
Purified RNAP α subunits, used at a concentration of 4 μM, were pre-bound to DNA fragments, for 10-15 minutes at room temperature, before the addition of MelR. When investigating the effect of RNAP α subunits on the binding of MelR to the DNA, reactions lacking α subunits contained the appropriate volume of α storage buffer.

2.19 FeBABE footprinting

The hydroxyl radical is a reactive oxygen species that mediates DNA strand breakage by the abstraction of a deoxyribose hydrogen atom from the DNA backbone. Consequently, hydroxyl radical-mediated DNA cleavage is non-sequence specific. The deoxyribose C'4-H and C'5-H bonds, which can only be accessed via the minor groove of the DNA, are the primary sites of hydroxyl radical-mediated strand breakage (Balasubramanian *et al.*, 1998; Figure 2.2). Since the hydroxyl radical can access each nucleotide of a given DNA fragment, with no bias towards particular DNA sequences, strand breaks induced by the hydroxyl radical form the basis of a variety of footprinting techniques used to study the structure of protein-DNA complexes at single nucleotide resolution.

The Fe^{2+} ion can catalyse the formation of hydroxyl radicals from H_2O_2 in a process referred to as the Fenton reaction. This reaction has been exploited to generate hydroxyl radicals in DNA footprinting experiments. The recent development of Fe^{2+} tethering reagents, such as *p*-bromoacetamidobenzyl-EDTA-Fe (FeBABE), has enabled researchers to exploit the Fenton reaction even further (Owens *et al.*, 1998; Ishihama, 2000b). FeBABE can be conjugated with proteins, via surface exposed cysteine residues, to form a stable derivative of the starting protein molecule that is capable of generating hydroxyl radicals in the presence of H_2O_2 (Figure 2.3). Crucially, as the Fe^{2+} ion is tethered to the protein, hydroxyl radical generation is localised to the site of FeBABE attachment. Thus, only a small surface of the DNA is subject to strand

A



B

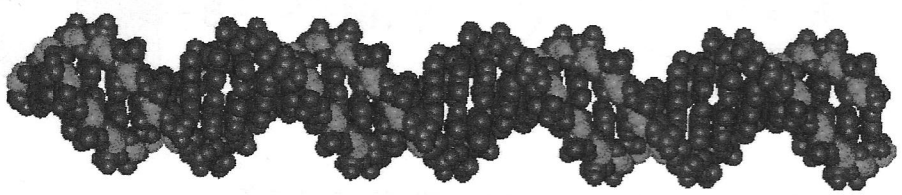


Figure 2.2 Sites of hydroxyl radical DNA strand breakage.

A) Numbering scheme for the carbon atoms of deoxyribose.

B) Location of 4' and 5' deoxyribose carbon atoms (red) in double stranded DNA.

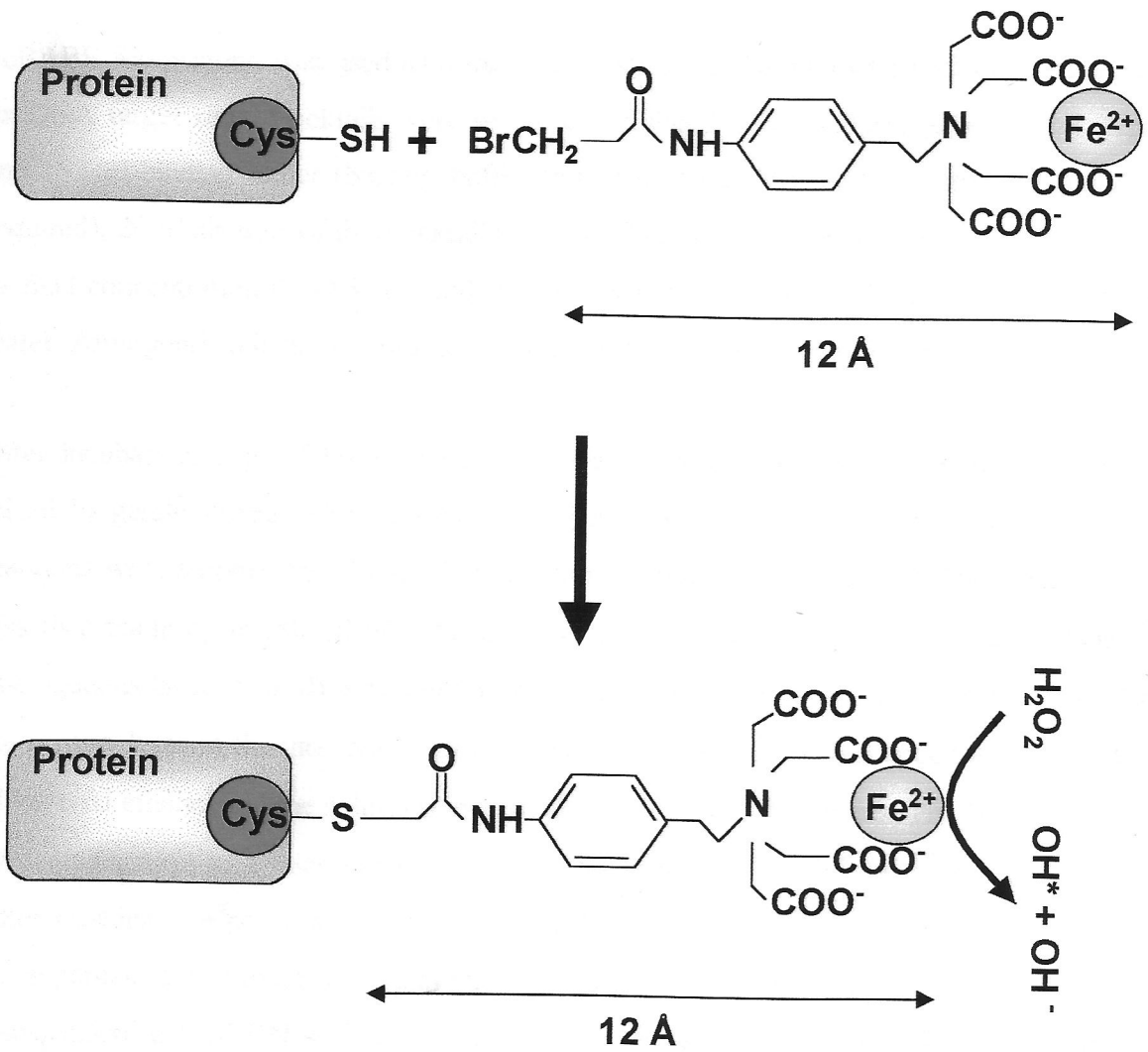


Figure 2.3 Conjugation of FeBABE with a surface exposed Cysteine residue

breakage. Consequently, FeBABE footprinting is a particularly suitable technique for mapping interactions between a discrete surface of a protein molecule and its DNA target.

2.19.1 Reactions with MelR

FeBABE footprinting was used as a method for studying the interaction of MelR with each of its DNA target sites. Cocktails were set up containing 2 µl of 400 nM radiolabelled fragment and 198 µl binding buffer (binding buffer contained 2 mM cAMP or 10 mM melibiose when required). 20 µl aliquots of this cocktail were distributed into Eppendorf tubes. MelR was added to final concentration 0.1-0.8 µM and the total reaction volume made up to 40 µl with sterile water. After gentle mixing, the reaction was incubated for 10 minutes at room temperature.

After incubation, 5 µl of H_2O_2 and 5 µl of NaAscorbate was added directly to samples and mixed by gentle stirring with the pipette tip. After an incubation time of 20 minutes at 37 °C, reactions were stopped by adding 21 µl of stop solution and mixing well. The reaction volume was then made up to 200 µl with TE and the DNA extracted with 200 µl phenol/chloroform. The aqueous layer (190 µl) was transferred to a fresh Eppendorf tube taking care not to disturb the protein layer at the interface. 1 µl of 20 mg/ml glycogen was then added together with 400 µl ice-cold ethanol and the solutions incubated at –70 °C for 15 minutes. The DNA was pelleted by centrifugation in a microcentrifuge for 15 minutes at 4 °C and the supernatant discarded. After washing, the pellet with 600 µl of cold 70 % ethanol and recentrifuging for 10 minutes, the supernatant was discarded again and the pellet dried under vacuum. Finally, the pellet was resuspended in 8 µl DNase loading buffer, often by incubating the samples for 10 minutes at 37 °C. Then samples were incubated for 2 minutes at 90 °C and loaded onto a 6 % sequencing gel which was run at 60 W. After electrophoresis, the gel plates were dismantled and the gel fixed and dried. The gel was exposed to a phosphor screen overnight at room temperature.

2.19.2 Reactions with MelR and RNAP α subunits

Purified RNAP α subunits, used at a concentration of 4 µM, were pre-bound to DNA fragments for 10-15 minutes at room temperature, before the addition of MelR. When investigating the effect of RNAP α subunits on the binding of MelR to the DNA, reactions lacking α subunits contained the appropriate volume of α storage buffer.

Stock running gel buffer: 0.75 M Tris-HCl (pH 8.3). Filtered through Whatman 47 mm diameter glass fibre filter

Stock stacking gel buffer: 1.25 M Tris-HCl (pH 6.8). Filtered through Whatman 47 mm diameter glass fibre filter

Sample buffer: 0.005 % (w/v) bromophenol blue, 125 mM Tris-HCl (pH 6.8), 2 % (w/v) SDS, 20 % (v/v) glycerol

10 x Electrode buffer: 250 mM T-HCl (pH 8.3), 1.9 M glycine, 1 % (w/v) SDS

Stain solution: 0.2 % (w/v) Coomassie brilliant blue, 50 % (v/v) methanol, 10 % (v/v) acetic acid. The solution was filtered through Whatman 47 mm diameter glass fibre filter

Destain solution: 10 % (v/v) methanol, 10 % (v/v) acetic acid

2.1.9 β-galactosidase assays

Z buffer: 0.75 g/l KCl, 0.246 g/l $MgSO_4$, 8.53 g/l Na_2HPO_4, 4.87 g/l $NaHPO_4.2H_2O$, 2.7 ml/l β-mercaptoethanol, adjusted to pH 7

2.1.10 FeBABE footprints

Binding buffer: 60 mM Hepes (pH 8), 1.25 mM Potassium glutamate, 38 µg/ml Herring sperm DNA, 0.7 % melibiose (when required)

Sodium Ascorbate: 5 mM Sodium Ascorbate made up immediately before use

Hydrogen peroxide: 5 mM Hydrogen peroxide made up immediately before use

Stop solution: 0.15 mM EDTA, 25 mM Thiourea

Gel loading buffer: 40 % (v/v) deionised formamide, 5 M urea, 5 mM NaOH, 1 mM EDTA, 0.025 % (w/v) bromophenol blue, 0.025 % xylene cyanol FF

2.1.11 FeBABE labelling of proteins

EDTA: 20 mM EDTA

FeBABE: 20 mM FeBABE

Triton-X-100: 1 % (v/v) solution of Triton-X-100 in sterile water

CPM: 1 % (v/v) solution of CPM in DMSF