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'Footprinting' proteins on DNA with peroxonitrous acid

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ABSTRACT

The peroxonitrite anion ($ONOO^-$) is a stable species in alkaline solution that quickly generates a strong oxidant at neutral pH. A convenient procedure for the preparation of ONOOK has been developed based on the procedure of Keith & Powell [(1969) *J. Chem. Soc. A*, 90], which when added to a sample of duplex DNA buffered at neutral pH rapidly generates a strong oxidant capable of nonspecifically cleaving the DNA present. We show that this solution containing ONOOK can be used to hydroxyl radical footprint the binding the *cI*-repressor (*cI*) of phage λ with the right operator, O_R. In addition, we show that the individual-site binding isotherms determined by quantitative DNase I, Fe-EDTA and ONOOK footprinting are identical within experimental error. The identical isotherms obtained with the three different reagents with greatly differing sampling times indicates that the sampling time of the footprinting probe need not be short relative to the kinetic dissociation constants that govern protein-DNA interactions.

INTRODUCTION

When ligands bind to DNA, the ribose 3′,5′-phosphodiester backbone is shielded from solvent. This limited solvent accessibility can be detected by the protection from cleavage exhibited in the presence of reagents that randomly cleave the backbone, either by enzymatic hydrolysis or chemical oxidation. When only one of the two strands in the duplex is end labeled, the ligand binding is revealed as a region of diminished intensity (a 'footprint') following separation, with single basepair resolution, by denaturing electrophoresis, and visualization of the reaction products by autoradiography. These procedures have collectively come to be accepted as 'footprinting'.

Footprinting was originally developed using the endonuclease DNase I as a probe for the occupancy of specific binding sites by proteins (1). While DNase I produces a robust experimental signal, limitations to its use include the fact that it requires divalent cations for activity (2) and shows a noticeable degree of sequence specificity (3). Binding proteins often completely block cleavage by DNase I within a binding site and 'footprints' frequently extend past the protein recognition sequences since DNase I (MW 30,400) is comparable in size to many DNA-binding proteins and must bind to the DNA in a specific configuration before cleavage can occur (4). A footprinting probe at the opposite end of the size spectrum from DNase I is the hydroxyl radical (·OH). Fe-EDTA has been successfully used to generate ·OH via the Fenton reaction for footprinting free in solution (5), tethered to an intercalator (6) or tethered to nucleic acids or proteins (7, 8). The reactivity of ·OH shows little sequence specificity for free DNA (although it is sensitive to periodic sequence induced bending, (9) and, by virtue of its small size, is able to provide details of the protein-DNA interaction within the binding site (5).

The peroxonitrite anion ($ONOO^-$) is a stable species in alkaline solution that quickly generates a strong oxidant at neutral pH. A solid solution of potassium peroxonitrite (ONOOK) in potassium nitrate (KNO_3) generated via photolysis, the 'Edwards-Plumb' reagent, was recently reported to mediate nonspecific cleavage of duplex DNA (10). This yellow solid containing 0.3% ONOOK (30 μmol ONOOK/g solid) was observed to produce nonspecific DNA strand cleavage closely analogous to that induced by an untethered Fe(II)-EDTA reagent. An alternative preparation of a stable aqueous solution of $ONOO^-$ can be easily carried out via neutralization of peroxonitrous acid (ONOOH) transiently formed during the reaction of nitrous acid (HNO_2) with hydrogen peroxide (H_2O_2) (17). Addition of the $ONOO^-$ containing solution to a sample solution buffered at pH 7 results in protonation of a significant fraction of the anion, generating peroxonitrous acid

$$ONOO^- + H^+ \rightarrow ONOOH \quad (1).$$

Peroxonitrous acid, which has a pKa of 6.8 (11) and a half-life at pH 7.4 of 1.9 seconds (12), undergoes homolytic fission to form ·OH and nitrogen dioxide (NO_2·) (13,14)

$$ONOOH \rightarrow NO_2\cdot + \cdot OH \quad (2).$$

The amount of free ·OH that may be trapped in solution is reduced by two-thirds by recombination in the solvent cage to form nitric acid (14).

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Homolysis of peroxonitrous acid results not only in formation of the hydroxyl radical species thought to be responsible for DNA strand-scission, but also gives rise to nitrogen dioxide ($NO_2\cdot$). This radical species, while known to induce lipid peroxidation and mediate tyrosine nitration, has not been shown to inflict DNA strand cleavage (15). In aqueous solution at neutral pH, nitrogen dioxide disproportionates to yield nitrite and nitrate. This disproportionation proceeds by the two steps

$$2\ NO_2\cdot \rightarrow N_2O_4 \quad (3)$$

$$N_2O_4 + H_2O \rightarrow NO_3^- + NO_2^- + 2\ H^+ \quad (4)$$

which have rate constants $9\times10^8\ M^{-1}s^{-1}$ and $1\times10^3\ s^{-1}$, respectively (16).

The utility of the Edwards-Plumb reagent in the footprinting of protein-DNA complexes was found to be limited by the large increase in the sample solution ionic strength attendant to dissolution of this predominantly KNO_3 containing solid. To circumvent these problems, a convenient procedure for the preparation of ONOOK has been developed based on the procedure of Keith & Powell (17), which when added to a sample of duplex DNA buffered at neutral pH rapidly generates a strong oxidant capable of nonspecifically cleaving the DNA present. We show that ONOOK can be used to hydroxyl radical footprint the binding of the *c*I-repressor (*c*I) of phage λ with the right operator, O_R. In addition, we show that the individual-site binding isotherms determined by quantitative DNase I, Fe-EDTA and ONOOK footprinting are identical within experimental error. The use of ONOOK as a probe of the kinetics of site-specific protein-DNA interactions will be discussed. A preliminary account of some of this work has been presented (18).

MATERIALS AND METHODS

Peroxonitrite synthesis. All reagents were standard A.C.S. reagent grade and purchased from Aldrich or Fisher, with the exception of the KOH used which was semiconductor grade in order to minimize heavy metal contaminants. All solutions used in this synthesis were cooled to 1° ± 1°C (nominal ice bath temperature) prior to use. To 20 ml of a stirring solution of 0.6 M $NaNO_2$ – 0.9 M H_2O_2 was added 10 ml of 0.6 M HCl followed in 5–6 seconds by 10 ml of a solution containing 1.2 M KOH and 400μM diethylenetriaminepentaacetic acid (DTPA). Disproportionation of unreacted H_2O_2 was effected by immersion of a cylindrical platinum mesh electrode (addition of MnO_2 can also be used to remove the H_2O_2 present at the risk of adventitious metal ion introduction). The Pt-catalyzed disproportionation was allowed to proceed for 90 minutes at 1°C while stirring. After the H_2O_2 disproportionation, the ONOOK concentration was determined to be 80–90 mM by the absorbance at 302 nm (ϵ_{302nm} = 1670 $M^{-1}cm^{-1}$, (19). Assays of the nitrite and H_2O_2 concentrations (20, 21) were also made. The ONOOK solution was stored in 1 ml aliquots at −70°C. Such storage was found to result in a 20% decrease in ONOOK concentration over a three week period.

Proteins and DNA. Bases 37459 to 38137 of the λ genome containing O_R were cloned from pKB252 (22) into pGEM7f (Promega) to create plasmid pDS001. A Bgl II restriction site that is 35 bp upstream from O_R1 was introduced by PCR directed oligonucleotide mutagenesis (pDSFe001; D. Strahs and M. Brenowitz, in preparation) in order to provide clearer resolution of O_R upon denaturing electrophoresis. Stocks of the plasmid DNA were prepared by the alkaline lysis technique (23) followed by CsCl centrifugation (24) and stored in aliquots at −70° C until use. The 590 bp Bgl II/Hind III restriction fragment was singly end labeled at the Bgl II site with ^{32}P-dCTP as has been described except that the 'fill-in' reaction with the large fragment of DNA polymerase was conducted at 0° C.

The cI-repressor used in these studies was prepared as previously described (25). A DNA binding activity of 79% and a dimer dissociation constant of 24.4 nM (26) was used to calculate the concentration of cI dimer present in solution.

Footprinting protocols. A binding buffer containing 25 mM Na_2HPO_4, 2 mM $MgCl_2$, 75 mM KCl, 2 μg/ml calf-thymus DNA (CT-DNA), 50 μg/ml bovine serum albumin (BSA), pH 7.0 at 20° C was employed for DNase I, Fe-EDTA and ONOOK mediated footprinting. The DNase I footprint titration experiments were conducted essentially as described (25,27). A series of dilutions of *c*I-repressor were incubated for ~30 min with less than 1 pM of the ^{32}P-labeled DNA in binding buffer in a regulated water bath at 20° ± 0.1° C. The final volume of all samples was 200 μl. A sufficient concentration of DNase I was added for 2 min to cleave approximately 50% of the DNA. The reactions were quenched and the ^{32}P-DNA precipitated, denatured and the components separated by electrophoresis on 12% denaturing polyacrylamide gels as described in the published protocols. Densitometric analysis of the footprint titration autoradiograms was accomplished with a microcomputer based video densitometer (28) implementing the computer software originally described in (25).

For hydroxyl radical footprinting with Fe-EDTA, mixtures of *c*I and ^{32}P-labeled DNA were prepared as described for the DNase I titrations. The reactions were conducted essentially as described by Tullius and coworkers (29). Solutions of 2 μl each containing 1 mM $Fe(NH_4)_2(SO_4)_2$ and 2 mM EDTA, 100 mM sodium ascorbate and 3% H_2O_2 (made up in the binding buffer without the CT-DNA and BSA) were pipetted to the sides of the microfuge tubes as separate drops, not allowing contact with each other or the protein-DNA containing sample solution. The tubes were then immediately placed upon a vortexer and gently mixed to initiate the cleavage reaction. The reaction was allowed to proceed for 5 min and quenched with 20 μl of a solution containing 100 mM thiourea and 200 mM EDTA. The ^{32}P-DNA was precipitated, denatured and separated by electrophoresis as described above for DNase I.

Cleavage reactions with ONOOK were carried out by addition of the peroxonitrite solution to sample solution buffered at pH 6.8. A frozen aliquot was thawed in an ice bath 20 minutes prior to use. The ONOOK solution (pH 12.5) was then pipetted in a 2–10 μl aliquot directly into a vortexing 200 μl sample containing 25 mM sodium phosphate, 2 mM $MgCl_2$ and 75 mM KCl. Due to the rapid decomposition of the oxidant formed, addition of a thiolic or alcoholic quench solution was found to be unnecessary. Additions of ONOOK solution, made as detailed above, resulted in a negligible increase in sample solution pH (~0.05). The ^{32}P-DNA was precipitated and separated by electrophoresis as described above.

RESULTS

Characterization of peroxonitrite. The reaction of ·OH with dimethylsulfoxide (DMSO) results in methyl radical formation which, in the presence of O_2, gives rise to one-half mole formaldehyde (HCHO) per mole ·OH scavenged (30,31). This production of HCHO was used to assay the fraction of trappable

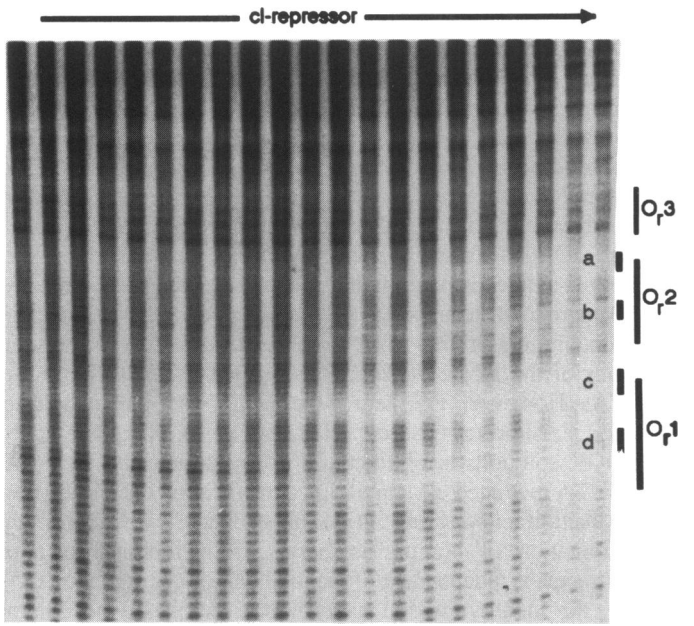

Figure 1. Peroxonitrite anion footprint titration autoradiogram for the binding of cI-repressor to the right operator (O_R) of phage λ. The far left lane has no added protein. The concentration of cI increases over the range 0.038 to 381.0 nM (total *c*I-repressor concentration in units of dimer) from left to right. The vertical bars at the far right indicate the positions of the three 17 bp operators. The smaller vertical bars labeled a–d represent the bases that are protected from ONOOK mediated hydroxyl radical cleavage. (Protection of the weakly associating O_R3 could not be clearly discerned due to the phosphate present in the sample buffer.) Seven μl of 92 mM ONOOK solution was added to each sample to yield a final concentration of 3.1 mM.

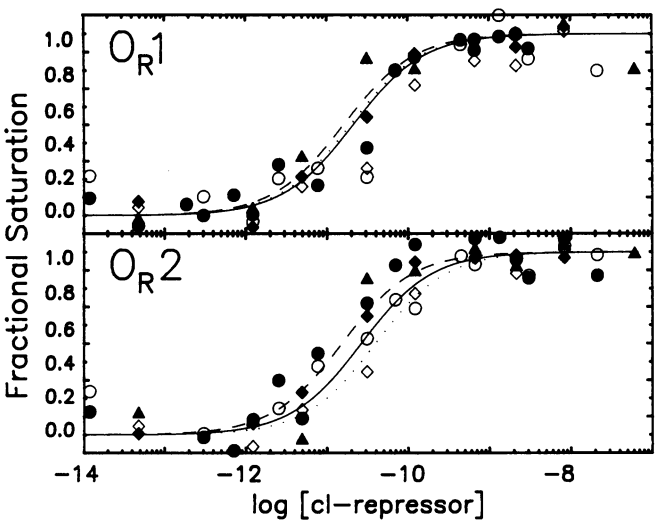

Figure 2. The individual-site titration curves determined for the binding of cI-repressor to O_R1 and O_R2. ONOOK, circles, DNase I, triangles, Fe-EDTA, diamonds. The solid symbols denote quantitation of regions a and c for the ONOOK and Fe-EDTA titrations, the open symbols regions b and d (Fig. 1). The Gibbs free energies of loading each site (the individual-site 'loading' free energy, 38) calculated for each of the individual-site titrations are $\Delta G_1^L = -14.5 \pm 0.3$, -14.3 ± 0.4, -14.4 ± 0.3 and $\Delta G_2^L = -14.4 \pm 0.3$, -13.8 ± 0.4, -14.3 ± 0.4 for the DNase I, ONOOK and Fe-EDTA titrations, respectively. The loading energies include all energetic contributions that affect binding of ligand at the specified site and are evaluated in a model-independent fashion by $\Delta G_i^L = RT \ln \bar{X}i = RT \int_0^{\ln XdYi} \ln XdYi$ where $\bar{X}i$ is the median ligand activity (38, 40).

oxidant formed upon addition of ONOOK to 1 M DMSO in 100 mM phosphate buffer (pH 6.8). The amount of ·OH observed to be produced by this assay in terms of amount of HCHO formed per ONOOK added varied from 17–23%. These values are consistent with those obtained previously by Beckman and co-workers (11).

The concentrations of nitrite anion and H_2O_2 were determined as these species are themselves ·OH scavengers. The nitrite anion is a particularly efficient ·OH scavenger and every effort was made to keep the concentration of this anion to a minimum. The nitrite anion was assayed to be present in the undiluted ONOOK solution at concentrations of 250–500 μM. The second order rate constant for reaction of H_2O_2 with ·OH is 120 times smaller than that for nitrite, making it a much less effective trap of ·OH. Its concentration following disproportionation was found to be 10–30 mM. Controls utilizing peroxonitrite samples, in which the ONOOK was allowed to decompose (yielding nitrate and nitrite), as well as standard additions of H_2O_2 demonstrated that DNA cleavage did not arise from either nitrite or H_2O_2 alone (data not shown).

The pH of the ONOOK solution following the Pt-catalyzed disproportionation was between 12.3–12.6. It was found that preparations whose pH was less than 12.3 were insufficiently stable once thawed to be useful. Increasing the pH beyond pH 12.6, while resulting in more stable ONOOK solutions, makes the use of more highly buffered sample solutions necessary to counter increases in sample pH subsequent to addition of the more alkaline ONOOK reagent.

The $ONOO^-$ solution was capable of efficiently cleaving the DNA in the 25 mM phosphate buffer (Fig. 1, lane 1). The addition of $ONOO^-$ was adjusted so that ~50% of the input DNA was nicked at least once using the experimental protocol described in Methods and Materials (data not shown). For a fixed concentration of ONOOK, a limitation to the maximum degree of cleavage that can be obtained is the need for sufficient buffering capacity to maintain the solution at neutral pH following addition of the reagent. Increasing the phosphate buffer beyond 25 mM resulted in a significant loss in electrophoretic resolution. Although samples can be de-salted prior to electrophoresis by G-25 'spin column' techniques, such a protocol is not desirable in a titration experiment. Substitution of 25 mM Tris buffer resulted in little to no detectable $ONOO^-$ cleavage of the radio-labeled DNA due to the efficient scavenging of ·OH by this primary alcohol containing buffer (data not shown).

Footprinting with ONOO⁻. A titration of O_R with *c*I probed with $ONOO^-$ is shown in Fig. 1. Two protected regions (designated a and c) within O_R1 and O_R2 become clearly visible with increasing concentrations of *c*I. Two other regions are protected (b and d) although to a lesser extent. Protection within O_R3 could not be unequivocally discerned due, in part, to the poor resolution of the upper part of the gel. This loss of resolution is a result of the phosphate buffer employed in these studies. The individual-site binding isotherms determined by densitometric analysis of bands within regions a–d are shown in Fig. 2 (solid and open circles). It is clear that even the poorly protected regions B and D, clearly define the titration of *c*I with O_R1 and O_R2. The triangles in Fig. ?? show the binding isotherms determined from a DNase I titration conducted under identical experimental conditions (data not shown). It is clear that both $ONOO^-$ and DNase I identically report the occupancy of O_R1 and O_R2 by *c*I.

Comparison with Fe-EDTA mediated ·OH cleavage. A direct

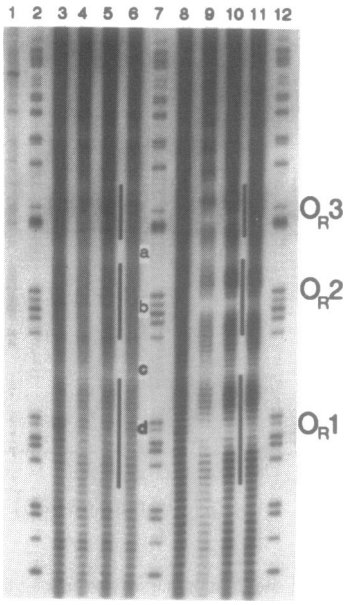

Figure 3. Comparison of ONOOK and Fe-EDTA footprinting of *c*I binding to O_R. Lane 1 is the ^{32}P-DNA untreated by reagent. Lanes 2, 7 and 12 show guanines detected by DMS methylation (41). Lanes 3 and 8 show ·OH cleavage mediated by $ONOO^-$ and Fe-EDTA, respectively, in the absence of *c*I-repressor. Lanes 4 and 9 contain 190.5 nM *c*I. Lanes 5 and 10 contain 47.7 nM *c*I. Lanes 6 and 11 contain 3.82 nM *c*I (total *c*I-repressor concentration in units of dimer). Seven μl of 92 mM ONOOK solution was added to the samples shown in lanes 3–6 to yield a final concentration of 3.1 mM. The concentration of Fe-EDTA in lanes 7–9 is 10 μM.

comparison of Fe-EDTA and $ONOO^-$ ·OH-mediated footprinting is shown in Fig. 3. Lanes 8–11 show Fe-EDTA mediated cleavage in the absence and presence of *c*I. The protection pattern observed for O_R1 on the three-site wild-type O_R is identical to that observed by Tullius and Dombroski (5) on DNA containing only O_R1. The protection patterns for O_R2 and O_R3 are consistent with *c*I interacting with these sites in a comparable manner.

$ONOO^-$ mediated footprinting results in protection of the same regions although the degree of protection is reduced compared with Fe-EDTA. That the reduced protection is not due to a reduction in the occupancy of the binding sites by *c*I (perhaps due to ·OH mediated degradation of the protein) is shown by the identity of the resolved binding isotherms (Fig. 2). In addition, there is an asymmetry to the degree of protection within each site. Within each site, the upstream region is less well protected (Fig. 1).

DISCUSSION

The protocol for the synthesis of ONOOK described in this paper has the inherent advantage of simplicity, and generates final ONOOK concentrations of 80–90 mM. This concentration of ONOOK affords optimal DNA cleavage via additions of 2–10 μl. A quench flow reactor designed for the synthesis of unstable intermediate species, stabilized by deprotonation with strong base, has also been used to generate solutions of ONOOK (32). Such an apparatus can be used to increase the final yield of ONOOK by 50–60%. While beneficial in some instances, higher concentrations of the stock solution of ONOOK would require that either very small additions be made or that the solution be appropriately diluted.

It has recently been proposed that the actual oxidant formed upon protonation of $ONOO^-$ is an activated isomer of peroxonitrous acid rather than ·OH arising form homolysis (33). For the purpose of footprinting protein-DNA interactions, exact knowledge of the nature of the oxidant is not critical. The oxidant formed, whether it be hydroxyl radical or an activated isomer of ONOOH, is a small, neutral molecule whose reaction with dissolved solutes is random and limited by solvent accessibility.

The peroxonitrite anion has been used to quantitatively footprint the binding of a protein to DNA. cI-repressor protects the same regions of the DNA from ·OH generated by $ONOO^-$ or Fe-EDTA. However, the degree to which the phosphodiester backbone is protected from cleavage by ·OH generated by ONOOK solutions is significantly less than that observed with Fe-EDTA mediated cleavage. Since the titration data are treated as transition curves and fit to upper and lower limits, the determination of binding isotherms from footprint titration experiments is independent of the total degree of protection (25). Thus, the identity, within experimental error, of the binding curves resolved utilizing DNase I and ·OH (Fig. 2) is evidence that the reduced protection is not the result of dissociation of the *c*I-O_R complex or degradation of the protein. Also, since the half-life of the *c*I-O_R complex greatly exceeds the time of the $ONOO^-$mediated ·OH cleavage under these experimental conditions (34), any modification introduced during the $ONOO^-$ cleavage reaction would have to have a drastic effect on the stability of the *c*I-O_R complex in order to be detected. Thus, it is unlikely that $ONOO^-$ induced modification or degradation of *c*I would have a discernible effect on the footprints.

The question of concomitant damage to DNA binding proteins during cleavage of DNA by ·OH is a real concern. However, this concern it is not limited to the peroxynitrous acid mediated cleavage, but applies to all footprinting techniques conducted with Fe-EDTA. Recent studies have demonstrated that Fe-chelate complexes can cleave proteins (35,36,37). In fact, the close proximity of protein ligands to the coupled Fe-chelate may localize and intensify the relative damage to the protein when footprinting is performed. Studies have also demonstrated that backbone cleavage is a relatively less frequent result of protein oxidation by hydroxyl radical, with side chain oxidation being the predominant event (10). Preferential modification of proteins differs from DNA where the solvent accessibility of the backbone leads to cleavage being a common consequence of reaction with hydroxyl radical. However, an argument that mitigates against significant protein damage during the footprinting studies is that reaction conditions are adjusted so that less than 50% of the DNA molecules interact with ·OH. Because of their relative sizes, the rate constant for DNA reacting with ·OH is greater than that for the protein ligand and one may estimate that less than 10% of the bound protein will have encountered a single ·OH molecule during the oxidation reaction.

The possibility that the differences in the degree of protection observed with Fe-EDTA and $ONOO^-$ footprinting are due to an interaction of $ONOO^-$ with the protein-DNA complex is unlikely, but cannot be excluded at this time. Alternatively, incomplete mixing of the reagent with the sample solution would result in only a fraction of the radio-labeled DNA being cleaved, and decrease the observed degree of protection. However, control experiments in which the rate of sample addition and the speed

with which the sample solution was vortexed was varied, suggest that complete mixing was obtained during the ~2 sec lifetime of the reagent. Further experiments will be required to resolve this issue.

In addition to providing qualitative information regarding the binding of proteins to DNA, quantitative DNase I footprinting has been shown to yield thermodynamically valid, individual-site binding isotherms that describe the site-specific binding of proteins to one or more sites on the DNA. It has been concluded that DNase I 'samples', without perturbing, the equilibrium distribution of the binding protein with DNA rather than 'taking a snapshot' of the complex at a rate faster than the dissociation kinetics (25,38,39).Because of the manipulations required for DNase I addition and quenching of the reaction, sampling times of less than 30 sec can not be reproducibly conducted and quantitative titration assays are routinely conducted with reaction times equal to or exceeding 2 min (Brenowitz et al., 1993). Similar, or longer, reaction times are employed for Fe-EDTA footprinting (29). Fe-EDTA experiments utilizing the Fenton reaction are typically conducted with reaction times of 2–10 minutes (29). Shorter reaction times can be achieved, in principle, by increasing the concentration of Fe-EDTA. However, the need to separately add the Fe-EDTA, ascorbate and H_2O_2 solutions as drops on the side of a microfuge tube prior to initiating the reaction and the requirement for a separate solution to quench the reaction makes achieving *reproducible* reaction times of 2-5 sec technically very demanding, if not impossible. In contrast, the rapid decomposition of the oxidant formed via protonation of ONOOK, resulting in a ~2 second half-life of ONOOH, makes it possible to examine cleavage events that occur in a much shorter time frame. The important feature of the peroxonitrous acid cleavage is that it can be initiated at a precise time by mixing a single reagent with the DNA-protein equilibration mixture and that it does not need to be subsequently quenched. Thus, $ONOO^-$ offers significant advantages with proteins that might be sensitive to the oxidation conditions required for Fenton chemistry as well as the potential for use in time resolved footprinting experiments. An additional benefit of ONOOK is that the amount of oxidant added can be quantitated directly by its UV absorbance, making it possible to add the reagent in reproducible amounts with a single volumetric addition.

The fact that DNase I, Fe-EDTA and $ONOO^-$ yield individual-site isotherms that are identical within experimental error, strengthens the argument that it is not necessary to probe protein-DNA interactions at a rate that exceeds the kinetic dissociation constant(s) of the binding reaction(s). In addition, the fact that ·OH can be used to generate quantitative titrations suggests that determination of thermodynamically valid binding isotherms can be combined with detailed structural analysis of protein-DNA interactions. Such analysis may yield a better understanding of the role of the DNA in cooperative interactions among site-specific binding proteins.

The half-lives of site-specific protein-DNA complexes are highly sensitive to solution conditions, varying from seconds to tens of minutes. The ability to assay dissociation reactions is dependent upon the ability to sample the relative concentration of protein-DNA complex at a rate significantly faster than the rate of the dissociation reaction. The ~2 sec half-life of ONOOH clearly has advantages in the analysis of systems having faster rates of dissociation. The reagent should also be useful in the analysis of association reactions that cannot be conducted under sufficiently dilute conditions to allow convenient analysis by other techniques. In addition, 'time-resolved footprinting' conducted with ONOOK will allow the separate analysis of individual proteins in multiple protein complexes.

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REFERENCES

1. Galas, D. & Schmitz, A. (1978) *Nuc. Acids. Res. 5,* 3157–3170.
2. Price, P. (1975) *J. Biol. Chem. 250,* 1981–1986.
3. Lomonossoff, G.P., Butler, P.J.G. & Klug, A. (1981) *J. Mol. Biol. 149,* 745–760.
4. Oefner, C. & Suck, D. (1986) J. Mol. Biol. 192, 605–632.
5. Tullius, T.D. & Dombroski, B.A. (1986) *Proc. Natl. Acad. Sci. USA 83,* 5469–5473.
6. Hertzberg, R.P. & Dervan, P.B. (1982) *J. Am. Chem. Soc. 104,* 313–315.
7. Dreyer, G.B. & Dervan, P.B. (1985) *Proc. Natl. Acad. Sci. USA 82,* 968–972.
8. Sluka, J.P., Horvath, S.J., Bruist, M.F., Simon, M.I. & Dervan, P.B. (1987) *Science 238,* 1129–1132.
9. Burkhoff, A.M. & Tullius, T.D. (1987) *Cell 48,* 935.
10. King, P.A., Anderson, V.E., Edwards, J.O., Gustafson, G., Plumb, R.C. & Suggs, J.W. (1992) *J. Am. Chem. Soc. 114,* 5430–5432.
11. Radi, R., Beckman, J.S., Bush, K.M. & Freeman, B.A. (1991) *J. Biol. Chem. 266,* 4244–4250.
12. Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A. & Freeman, B.A. (1990) *Proc. Natl. Acad. Sci. USA 87,* 1620–1624.
13. Halfpenny, E. & Robinson, P.L. (1952) *J. Chem. Soc. A.*, 928–938.
14. Mahoney, L.R. (1970) *J. Am. Chem. Soc. 92,* 5262–5263.
15. Prütz, W. A., Mönig, H., Butler, J. and Edward, J. L. (1985) *Arch. Biochem. Bioph. 243,* 125–134.
16. Forni, L. G., Mora-Arellano, V. O., Packer, J. E. and Willson, R. L. (1986) *J. Chem. Soc., Perkin Trans. 2,* 1–6.
17. Keith, W.G. & Powell, R.E. (1969) *J. Chem. Soc. A,* 90.
18. King, P.A., Jamison, E., Strahs, D., Anderson, V.E. & Brenowitz, M. (1993) *Biophysical J. 64,* A179.
19. Hughes, M.N. & Nicklin, H.G. (1968) *J. Chem. Soc. A,* 450 –452.
20. Standard Methods for the Examination of Water and Wastewater (1985) 16th ed., Am. Pub. Health Assoc., 404–406.
21. Frew, J.E., Jones, P. & Scholes, G. (1983) *Anal. Chim. Acta. 155,* 139–150.
22. Backman, K., Ptashne, M. & Gilbert, W. (1975) *Proc. Natl. Acad. Sci. USA 73.* 4175–4178.
23. Birnboim, H. & Doly, J. (1979) *Nuc. Acids. Res. 7,* 1513–1523.
24. Maniatis, T., Fritsch, E.F. & Sambrook, J. (1982) *Molecular Cloning–A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
25. Brenowitz, M., Senear, D.F., Shea, M.A. & Ackers, G.K. (1986) *Methods Enzymol. 130,* 132–181.
26. Koblan, K.A. & Ackers, G.K. (1991) *Biochemistry 30,* 7817–7821.
27. Brenowitz, M. & Senear, D.F. (1989) 'DNase I Footprint Analysis of Protein-DNA Binding.' in *Current Protocols in Molecular Biology,* Unit 12.4, F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl, eds., John Wiley and Sons, New York.
28. Reiner, P. & Brenowitz, M. (1991) *Computer Applications in the Biosciences* 7, 337–340.
29. Tullius, T.D., Dombroski, B.A., Churchill, M.E.A. & Kam, L. (1987) *Meth. Enzymol. 155,* 537–558.
30. Klein, S.M., Cohen, G. & Cederbaum, A.K. (1981) *Biochemistry 20,* 6006–6012.
31. Johansson, L.H. & Hakan Borg, L.A. (1988) *Anal. Biochem. 174,* 331–336.
32. Reed, J. W., Ho, H. H. and Jolly, W. L. (1973) *J. Am. Chem. Soc. 96,* 1248-1249.

33. Koppenol, W.H., Moreno, J.J., Pryor, W.A., Ischiropoulos, H. & Beckman, J.S. (1992) *Chem. Res. Toxicol.*, in press.
34. Johnson, A.D., Pabo, C.O. & Sauer, R.T. (1980) *Meth. Enz. 65*, 839–856.
35. Ermacora, M. R., Delfino, J. M., Cuenod, B., Schepartz, A.& Fox, R. O. (1993) *Proc. Natl. Acad. Sci. USA*, in press.
36. Rana, T. M. and Meares, C.F. (1991) *J. Am. Chem. Soc.* 113, 1859–1861.
37. Kim, K., Rhee, S. G., and Stadtman, E. R. (1985) *J. Biol. Chem.* 260, 15394–15397.
38. Ackers, G.K., Johnson, A.D. & Shea, M.A. (1982) *Proc. Natl. Acad. Sci. USA 79*, 1129–1133.
39. Ackers, G.K., Shea, M.A. & Smith, F.R. (1983) *J. Mol. Biol. 170*, 223–242.
40. Wyman, J. (1964) *Adv. Protein Chem. 19*, 224–394.
41. Maxam, A.M. & Gilbert, W. (1980) *Meth. Enzymol. 74*, 560–539.