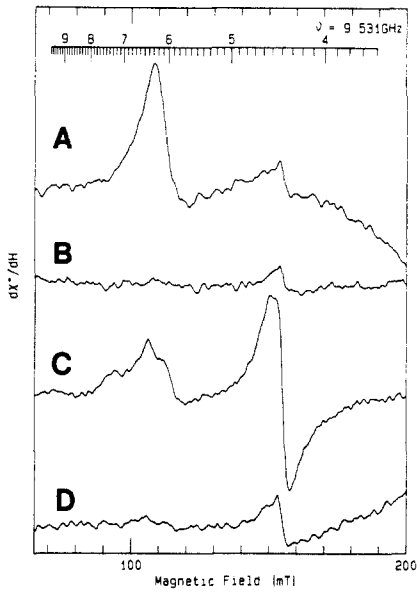

Figure 2. EPR spectra of the Mössbauer samples (Figure 1) of two product-oxidized lipoxygenases before and after treatment with substrate anaerobically: (A) oxidized P1; (B) oxidized P1 plus substrate anaerobically; (C) oxidized P4; (D) oxidized P4 plus substrate anaerobically. The spectra were taken at 40 K, with modulation amplitude = 0.63 mT and microwave power = 10 mW, and represent one 4-min scan each. The spectra all have the same vertical scaling factors.

Samples of two lipoxygenases which had been converted from the native to the iron(III) form and were subsequently treated with linoleic acid in the absence of oxygen were analyzed by Mössbauer and EPR spectroscopy.[18] The results of these experiments are presented in Figures 1 and 2. The isolation procedure we employ provides two lipoxygenases simultaneously: lipoxygenase-1 (P1) which is active at pH 9 and another isoenzyme active only near neutral pH which we operationally refer to as P4.[19] Both native lipoxygenases have Mössbauer parameters which are typical for high-spin iron(II), $\delta/Fe = 1.10$ mm s^{-1} and $\Delta Eq = 3.08$ mm s^{-1}.[20] The active enzyme was generated by treatment with 1 molar equiv of product (9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid.[21] This species does not give a well-defined Mössbauer signal under the reported conditions, but can be readily identified by the EPR spectrum of the corresponding Mössbauer sample (Figure 2). When either isoenzyme was treated with linoleic acid anaerobically, iron with Mössbauer parameters characteristic of high-spin iron(II) was obtained: P1, $\delta/Fe = 1.23$ mm s^{-1}, $\Delta Eq = 2.12$ mm s^{-1}; P4, $\delta/Fe = 1.24$ mm s^{-1}, $\Delta Eq = 2.54$ mm s^{-1} (Figure 1). The Mössbauer sample from Figure 1D was subjected to iron determination by atomic absorption spectroscopy and iron isotope ratio determination by mass spectrometry.[22] The integrated intensity of the Mössbauer spectrum in Figure 1D accounts for at least 75% of the iron-57 found to be in the sample. The data are consistent with a mechanism for catalysis in which lipoxygenase iron goes from the iron(III) to the iron(II) state as linoleic acid is oxidized presumably to a pentadienyl radical intermediate. It is interesting to note that the Mössbauer parameters of the substrate-treated enzyme were significantlly different from those of the native enzymes. This is direct evidence that oxidation and substrate binding alter the coordination sphere of the iron atom relative to the native enzyme. Also, the parameters for the two isoenzymes which had been cycled by product and substrate were significantly different from each other. This is consistent with our earlier hypothesis that the isoenzymes may act by similar yet distinguishable catalytic mechanisms.[23]

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(18) The purified isoenzymes were dialyzed against phosphate buffer (0.1 M, pH 7.0, 0.1 M NaCl). The oxidation was carried out at room temperature for 10 min using a 1.1 molar ratio of hydroperoxide to enzyme. Samples were loaded into Mössbauer cuvettes and either frozen in liquid nitrogen or transferred to a glove bag purged with argon. The sample was further treated at room temperature with an argon-purged solution of linoleic acid in methanol (<0.5% volume addition) to provide a 2.0 molar ratio of substrate to enzyme. The Mössbauer sample was then frozen immediately in liquid nitrogen.

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General Fluorescence Assay for Enzyme-Catalyzed Polyanion Hydrolysis Based on Template-Directed Excimer Formation. Application to Heparin and Polyglutamate

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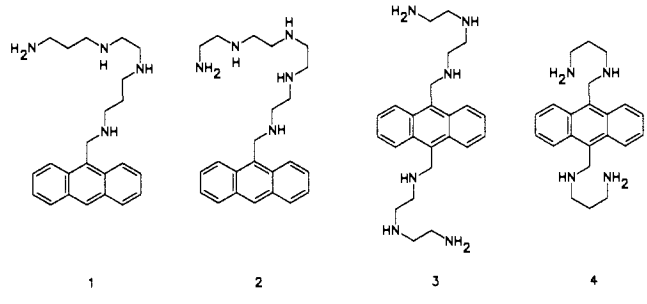
While the hydrolyses of DNA and RNA can be followed by monitoring changes in their UV absorption spectra, the hydrolyses of biological polyanions lacking a chromophore must be accomplished indirectly. Both radiolabeling techniques and coupled enzyme systems are used frequently as indirect methods for enzyme assay. We now report that the activity of hydrolytic enzymes acting on the polyanions heparin and polyglutamate can be monitored by fluorescence using a template directed excimer formation effect obtained with anthrylpolyamine conjugate probes.[1]

Anthrylpolyamines **1–4** were prepared via simple substitution reactions using conditions similar to those reported previously.[2,3] The full emission spectra of anthrylpolyamines **1–4** (all 1 μM) were collected during titration with ds DNA, ss DNA, heparin,



1 2 3 4

and poly-L-glutamate;[4] representative titration data from the monitoring of compound **3** at 422 nm are shown in Figure 1.[5]

(1) Presented at the 23rd Reaction Mechanisms Symposium, Boulder, CO, June 1990.

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(3) The products were isolated as their HCl salts via a series of aqueous and organic extractions and purified on a column of neutral alumina. Characterizations of **1–4** using ^{1}H NMR, ^{13}C NMR, and high-resolution FAB mass spectrometry were supportive of the structural assignments.

(4) DNAs (calf thymus), heparin (bovine mucosa), and poly-L-glutamate were purchased from Sigma Chemical Company and used as received. All titrations were performed at pH 5.0 in 0.1 M NaOAc buffer with 0.05 mM EDTA.

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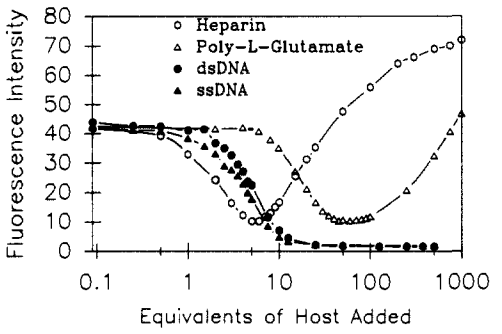

Figure 1. Effects on the fluorescence of anthrylpolyamine **3** upon titration by four biological polyanions.

Both disubstituted anthracene probes (**3** and **4**) exhibit a 6-nm red shift in λ_{max} when bound either to ds DNA or to ss DNA; likewise, both monosubstituted anthracene probes (**1** and **2**) show a 14-nm red shift in their emission spectra in the presence of either ds DNA or ss DNA. Interaction of the nucleotide bases with the anthracene is a likely source of the bathochromic shift and the observed chelation enhanced quenching (CHEQ) effect. Such π-stacking with ss DNA, seldom observed with intercalating compounds, may result from the favorable entropy effect of electrostatic preassociation.[6]

The heparin and poly-L-glutamate titrations show a markedly different behavior than do the DNA titrations. As polyanion is added, the fluorescence of the anthrylpolyamine solution decreases until a well-defined minimum is reached. A new emission at 510 nm, which we assign to the anthracene excimer of **3**, increases and decreases coincidently with the titrated fluorescence minimum. Likewise, the UV spectrum of 10 μM **3** with added heparin shows hypochromism that occurs and disappears coincidently with the fluorescence minimum and a 2-nm red shift. We propose *template-directed excimer formation* as the physical basis for these observations. In the absence of heparin, fluorescence of the unassociated probe is observed. As heparin is added, the fluorescence decreases as a result of heparin-directed interaction between probe molecules. Additional heparin permits the fluorophore population to diffuse over the length of the polyanion, thus avoiding excimer formation.

The anthrylpolyamine most effective in binding to heparin (**3**) was used to follow the activity of heparinase[7] at pH 5. Samples were prepared containing 1 μM probe **3** and 5 μM heparin in 0.1 M pH 5 NaOAc buffer with 0.05 mM EDTA. Under these conditions, the fluorescence of probe **3** is at its minimum as a result of template-directed excimer formation. Addition of heparinase, an enzyme that hydrolyzes heparin to oligosaccharide units,[8] results in a fluorescence enhancement as shown in Figure 2. One of the most effective polyglutamate binders (**2**) was used to test the activity of pronase[7] at pH 5. Samples were prepared containing 1 μM probe **1** and 90 μM poly-L-glutamate in 0.1 M pH 5 NaOAc buffer with 0.05 mM EDTA. Under these conditions, the fluorescence of probe **2** is also at its minimum. Addition of pronase, a nonselective proteolytic enzyme that hydrolyzes polyglutamate to glutamic acid,[9] results in a fluorescence enhancement analogous to that shown in Figure 2.

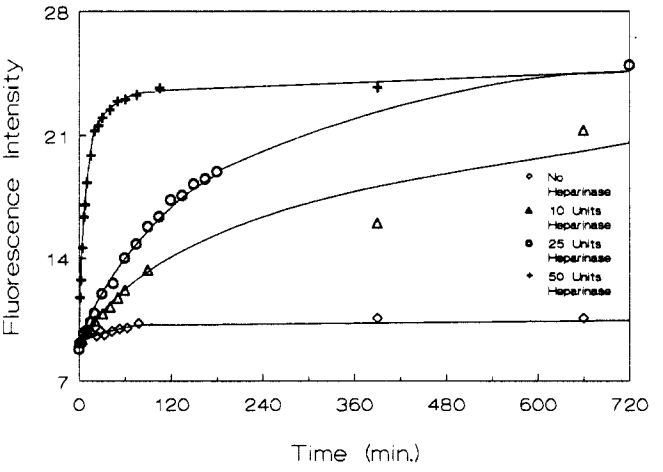

Figure 2. Effect of heparinase on the CHEQ of probe **1** with 5 equiv of heparin.

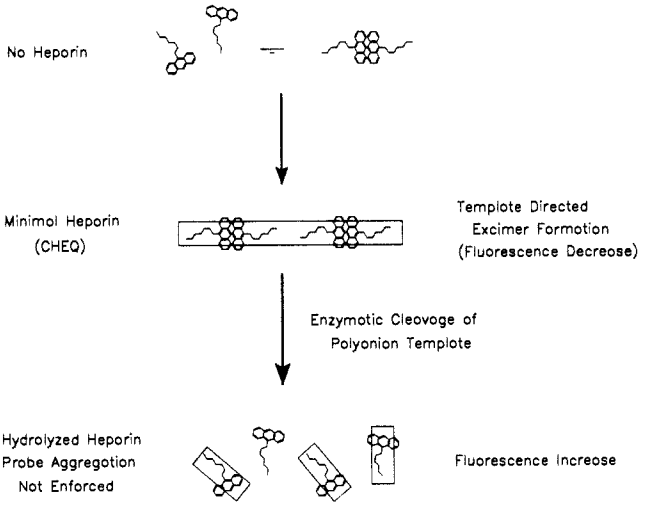

Figure 3.

Our rationale for the physical basis of these assays is shown in Figure 3. The cationic fluorophores, which do not associate appreciably in dilute solution, are brought into proximity upon binding to the polyanion; excimer formation is thus enforced, and the fluorescence of the probe is quenched. Addition of hydrolytic enzyme causes cleavage of the substrate into fragments that no longer enhance probe aggregation. Consequently, quenching of the fluorescence decreases with time until hydrolysis of the template is complete. Because the binding interaction is principally electrostatic, it should be possible to follow the hydrolyses of other polyanions by this technique as well. Furthermore, it is not even necessary to know the structure of the polyanion for the assay to be useful. For this reason, we believe that the template directed excimer formation method may prove useful for the assay of other enzymes involved in the anabolism or catabolism of anionic biopolymers.

Acknowledgment. We gratefully acknowledge support for this work from The Ohio State University. Shared resources, including the use of a fluorometer, were made available by Prof. M. Platz of this department. We thank Dr. Kent Stewart for his collaboration, which was both intellectual and instrumental. FT-NMR spectra were obtained with equipment funded in part by NIH Grant 1 S10 RR01458-01A1. A.W.C. thanks the A. P. Sloan and Dreyfus Foundations for support in the form of fellowships and Eli Lilly and Co. and Merck for support in the form of granteeships.

(5) For ds DNA and ss DNA, the number of equivalents of host is expressed in terms of the nucleotide base; the titration results obtained were virtually identical when either purchased or freshly heat denatured ss DNA was used. For heparin, an equivalent of host is expressed in terms of a repeating six hexose oligosaccharide unit. For poly-L-glutamate, an equivalent of host is expressed in terms of monopeptide.

(6) Previous work on polyamine DNA probes has been reported by Gabbay and by Ware: (a) Gabbay, E. J. *J. Am. Chem. Soc.* **1969**, *91*, 5136. (b) Ware, E. R.; Klein, J. W.; Zero, K. *Langmuir* **1988**, *4*, 458.

(7) Heparin, heparinase, poly-L-glutamate, and pronase were purchased from Sigma Chemical Company and used as received. Stock solutions of the enzymes were prepared in 0.1 M pH 5 NaOAc buffer with 0.05 mM EDTA for delivery to the fluorophore/substrate sample.

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