Polyoxometalates

Molecular Recognition of Basic Fibroblast Growth Factor by Polyoxometalates**

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The inhibition of angiogenesis-promoting factors such as fibroblast growth factor is considered to be a potential treatment for cancers, and has become an active area of research pursued with intense interest.[1] Two major targets of pharmacologic therapies are vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). bFGF is a globular single-chain heparin-binding polypeptide synthesized by different cell types. The 3D structure of the 18-kDa bFGF has been recently elucidated by X-ray crystallography.[2] bFGF consists entirely of β-sheet structure, which includes a threefold repeat of a four-stranded antiparallel β

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meander. It has been detected in a wide variety of normal and malignant tissues and is known to play key roles in the development, growth, and disease states in almost every organ system.[3] Ligands that recognize and bind bFGF may disrupt its interaction with endothelial cell-surface receptors such as heparan sulfate proteoglycans (HSPGs) and fibroblast growth factor receptors (FGFRs), and would represent a new class of potential therapeutic agent.

Much effort is underway in the development of drugs that inhibit bFGF-induced angiogenesis. The potential use of exogenous heparin analogues as inhibitors of angiogenesis, such as suramin,[4] pentosan polysulfate (PPS),[5] sulfonated derivatives of distamycin A,[6] and carboxylated compounds[7] have become the focus of several research groups. Suramin and PPS[8] have been evaluated in patients with various tumors, including Kaposi's sarcoma (KS), yet very large doses of these compounds are required to show activity, and their efficacy is limited by anticoagulant side effects. However, minor structural changes to suramin have resulted in a significant decrease in toxicity without loss of activity.[9]

This result was clear incentive for the design of novel compounds with high efficacy and low toxicity. Early transition metal oxygen anion clusters (polyoxometalates, or POMs) were explored as a different class of therapeutic agent. Unlike the flexible organic polysulfonated polyanionic heparin-based compounds, POMs are inorganic polyoxygen polyanions with fairly rigid cagelike structures.[10] POMs have been attractive for their promising antiviral, antitumor activities for more than a decade.[11] Based on cell-culture assays, enzymatic activities in vitro, and molecular modeling studies, POMs have regained considerable interest in recent years as a result of their remarkable interactions with HIV-1 reverse transcriptase (RT) and HIV-1 protease (P), as demonstrated by Hill, Pope, and co-workers.[12,13] Recently, it was reported that the POM $K_5SiCoW_{11}O_{39}$ could affect the mitogenic activity of bFGF,[14] yet little is known about the POM–bFGF interaction. Herein, we describe the recognition and binding of several POMs to bFGF. These POMs represent promising leads for the design of new compounds that might recognize bFGF, and which may represent potentially new avenues for the design and synthesis of new types of inhibitors of tumor angiogenesis.

Three representative POMs, the Keggin ($K_6SiNiW_{11}O_{39}$), the Wells–Dawson (α-$K_8P_2NiW_{17}O_{61}(H_2O)$), and the trivacant Keggin-derived sandwich ($K_{10}P_2Zn_4(H_2O)_2W_{18}O_{68}$) structures were selected for the present study. These ligands, whose basic anion backbones are illustrated in Figure 1, are hydrolytically stable under physiological conditions and vary in size and charge. The Keggin structure is $\approx$11.5 Å in diameter with six negative charges, the Wells–Dawson structure is $\approx 11.5 \times 15$ Å with eight negative charges, and the Keggin-derived sandwich structure is $\approx 11.5 \times 18.0$ Å with ten negative charges. The direct interaction between bFGF and POMs was first demonstrated by fluorescence, ultraviolet absorption, and circular dichroism measurements. bFGF was excited at 275 nm and its fluorescence was monitored at 303 nm in phosphate-buffered saline (PBS), pH 7.4 at 20 °C. The fluorescence intensity of bFGF was strongly quenched with an increase in the amount of POM. The titration reached a clear end point at a molar ratio of 1:1 for all compounds, which permitted a direct measurement of the interaction stoichiometry. Nonlinear least-squares fits of the data yielded apparent binding constants (K_{app}) of 3.0, 5.0, and $5.7 \times 10^6\,\text{M}^{-1}$ for the Keggin-, the Wells-Dawson-, and the sandwich-type POMs, respectively.

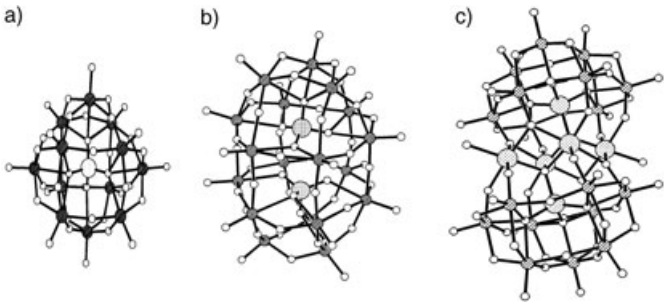

Figure 1. Basic anion backbone drawings of the three polyoxometalates: a) the Keggin structure, $[SiNiW_{11}O_{39}]^{6-}$; b) the Wells–Dawson structure, $[P_2NiW_{17}O_{61}(H_2O)]^{8-}$; and c) the trivacant Keggin-derived sandwich structure, $[P_2Zn_4(H_2O)_2W_{18}O_{68}]^{10-}$.

A number of heparin analogues that bind bFGF have various effects on the thermal stability of bFGF. Heparin and sucrose octasulfate, for example, enhance the stability of bFGF to thermal denaturation.[15] Conversely, sulfonated distamycin A derivatives destabilize the protein.[6] To determine the effect of POMs on the thermal stability of bFGF, we used temperature-dependent circular dichroism (CD) at various wavelengths to investigate the melting of the complex. Under the ionic-strength conditions described herein, bFGF alone has a thermal melting temperature (T_m) of 59 °C. Figure 2 shows that upon addition of POM at a molar ratio of 1:1, bFGF was stabilized by 9, 12, and 20 °C for the Keggin, Wells–Dawson and sandwich structures, respectively. The results of the CD experiments demonstrate that the conformation of bFGF underwent a dramatic change in the presence of POMs which manifests mainly in a decrease in the band intensity at 204 nm (Figure 3). In comparison, Wells–

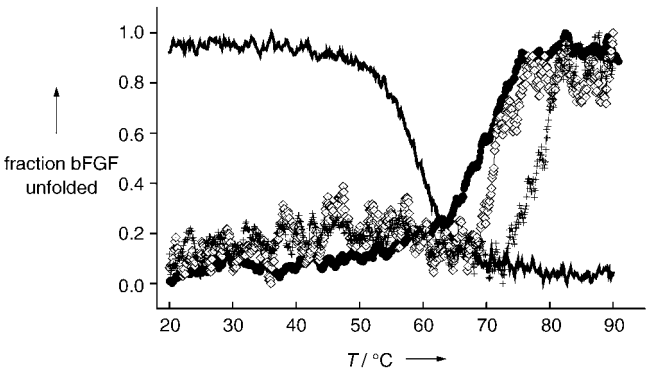

Figure 2. CD melting profiles of bFGF in the absence (——) and presence of Keggin (●), Wells–Dawson (◇), and Keggin-derived sandwich (+) compounds; POM/protein ratio = 1:1, and [bFGF] = 5 μM; solution conditions: NaCl (150 mM), PBS buffer (20 mM, pH 7.4); spectra were measured at $\lambda = 203$ or 229 nm and the temperature was raised in increments of 1 °C min^{-1} from 20 to 90 °C.

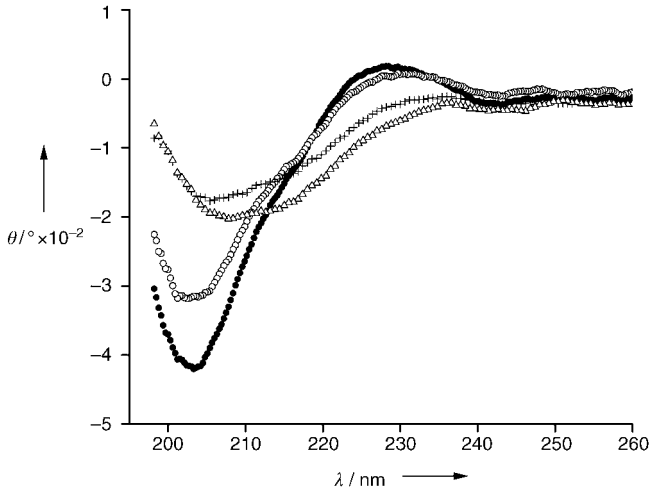

Figure 3. CD spectra of bFGF acquired at 20 °C in the absence (●) and presence of Keggin (○), Wells–Dawson (△), and Keggin-derived sandwich (+) compounds. All experimental conditions and concentrations were the same as those described for Figure 2. The spectra were recorded as an average of three scans from 195 to 260 nm.

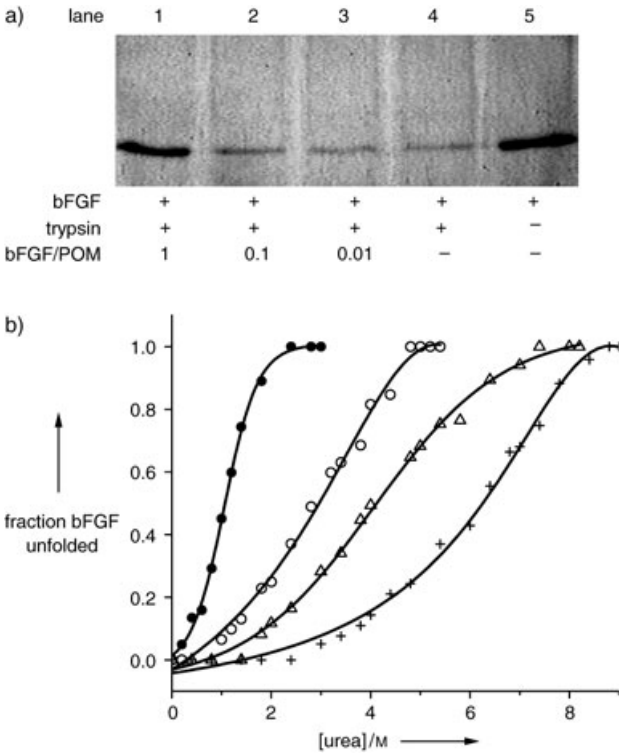

Figure 4. a) SDS-PAGE analysis of the effect of POMs on tryptic digests of bFGF: bFGF (10 μL, 4.8 μM) was incubated at 37 °C for 3 h with trypsin (1 μg in 1 μL of 0.1 N HCl) in the absence or presence of increasing concentrations of the Keggin-type POMs. At the end of the reaction, all samples were supplemented with SDS-PAGE reducing sample buffer, heated at 100 °C for 2 min, and subjected to SDS-PAGE (15%), after which the gels were stained with Coomassie blue. b) Fluorescence profiles of urea-induced bFGF denaturation in the absence (●) and presence of equimolar Keggin (○), Wells–Dawson (+), and Keggin-derived sandwich (△) compounds. All samples were incubated at 4 °C for 48–72 h prior to analysis to ensure equilibrium had been established. The fluorescence data were collected at 20 °C with a bFGF concentration of 1 μM and were presented as the fraction of unfolded bFGF. These experiments were performed in 100 mM PBS buffer, pH 7.0.

Dawson and sandwich-type POMs exerted a much more significant effect on bFGF with an approximate twofold loss in the CD signal and a marked red-shift of the minimum band. The direct interaction of POMs with bFGF was confirmed by their ability to protect bFGF from trypsin digestion; all compounds protected bFGF from protelytic cleavage. Figure 4a shows a digestion experiment with bFGF in the absence and presence of Keggin-type POMs. This protective effect was dose-dependent, and complete inhibition was observed at molar ratio of 1:1. Similar observations have been reported for the interaction of bFGF with heparin and its analogues.[16] To our knowledge, this is the first example of an enzyme or protein stabilized by a POM compound.

The binding of POMs to bFGF induced conformational changes, affected the whole structure of the protein, and had a stabilizing effect relative to free protein; all this was further verified by the effect of POMs on urea-mediated denaturation of bFGF. In the absence of POMs, the midpoint of urea-induced unfolding was observed at a urea concentration of 1.0 M (Figure 4b). Upon the addition of POMs at a molar ratio of 1:1, the unfolding transition of bFGF shifted to notably higher urea concentrations. The large shift in the transition midpoint suggests that POMs bind tightly to bFGF, which strongly supports the binding studies and thermal denaturation experiments discussed above. It should be noted that the degree of POM-based stabilization is highly dependent on the structure of the POM compound employed. The fluorescence titration, CD, and thermal unfolding studies demonstrated the varied effects of the different POM structures.

It was reported that the ability of heparin and its analogues to stabilize aFGF (acidic fibroblast growth factor) against denaturation in urea was diminished if the number of charges on these compounds was decreased.[17] In contrast to this trend, the Wells–Dawson-type POM has fewer charges than the sandwich-type POM, but exhibited the strongest stabilization of bFGF against urea-mediated unfolding. Previous studies have shown that the size, charge, and composition of POMs may all be interrelated in the inhibition of viral absorption/fusion and the inhibition of viral polymerase activity in a cell- or kinetics-based assay.[18] Modeling studies of POMs with HIV-1 P and HIV-1 RT determined that POMs exhibit reasonable steric fits in the substrate binding sites of these enzymes.[12,13]

Our results support these previous reports, and suggest that the structure of POMs might play a key role in the recognition and binding to bFGF. The POM compound $K_8ZnNiW_{11}O_{39}$ was generated through substitution of the central Si atom of the Keggin structure with Zn. It maintains the size of the smaller Keggin-type POM, but contains the same number of charges as the Wells–Dawson structure. Thermal denaturation and CD conformation studies indicate that it elicits similar effects upon the binding of bFGF as does the Keggin structure (data not shown). Whereas the exact mode of binding is not yet clear, the potential structure

relevance is unambiguous. One issue that needs to be addressed is the stability of the Keggin and Wells–Dawson anions in dilute aqueous solution. Although it is not easy to quantify the stability of these compounds experimentally, an approximate calculation can be made.[19] At the most commonly used concentration of 5 μM employed for the experiments reported herein, the Keggin anion remains intact, and the Wells–Dawson structure could experience a Ni cation loss of ≈30%. Both anionic compounds could lose a certain amount of the Ni cation upon further dilution. As a consequence, the lacunary anions might also react in a manner similar to that of the Ni derivatives. Further studies are therefore required to elucidate this point as well as the structural basis for the molecular recognition of bFGF by the compounds discussed herein, but these issues are beyond the scope of this brief communication.

The identification of the specific bFGF binding site of POMs is essential for the understanding of their biological function. Unlike heparin and its analogues, POMs are fairly rigid, cagelike structures coated with oxygen atoms bearing partial negative charges; the binding modes of POMs may therefore be different from the organic polyanions. For example, the Hill and co-workers recently demonstrated that POMs function not by binding to the active site of HIV-1 P, but by binding to a cationic pocket of lysine residues on the outer surface of the flaps that cover the active site.[13] This led us to explore the binding site of POMs on bFGF; the cationic heparin-binding cleft of bFGF was decided as a good candidate target site.[20] Competition assays were first used to verify that the putative POM binding site on bFGF is at or near the cationic pocket of the previously described heparin-binding site. The fluorescent polyanion suramin, a polysulfonated naphthylurea compound, is a common chaotropic agent. It is known to inhibit a large number of important enzymes[21] and to block the activity of several growth factors, one of which is bFGF.[22] A comprehensive analysis of the interaction of suramin with the growth factor has shown that suramin caused nonspecific and irreversible protein aggregation with a stoichiometry that depends on drug concentration. Furthermore, suramin was found to bind at or near the heparin binding site of bFGF, and the fluorescence intensity was strongly enhanced upon binding bFGF.[23] The addition of POMs to the bFGF–suramin complex resulted in a progressive decrease of suramin fluorescence intensity (Supporting Information), which suggests that POMs can displace the drug from the bFGF. This observation implies that both suramin and POMs compete for the same binding site.

We subsequently constructed the C78S–C96S double mutant of bFGF and studied its interaction with POMs. Fluorescence titration studies showed that POMs also form a 1:1 stoichiometric complex with the mutant bFGF with similar affinities. The apparent binding constants of the POM–mutant bFGF complexes were found to be three to fivefold less than the corresponding complexes with wild-type bFGF. At a 1:1 molar ratio, the POMs showed no effect on the thermal denaturation profiles of the mutant bFGF, except for a slight increase in T_m (2 °C) for the sandwich-type complex. Although the change of local microenvironment could not be ruled out with the substitution of cysteine by serine residues, the overall conformation of the mutant bFGF was identical to that of the wild-type as monitored by CD studies and X-ray crystallography.[6] CD data showed that the Wells–Dawson and sandwich structures caused a compatible conformational change of mutant bFGF, whereas the Keggin-type showed no effect (data not shown). These results indicate that the replacement of Cys 78 and Cys 96 considerably affects the interaction of the protein with POMs. Among the four cysteine residues in bFGF, Cys 34 is completely buried and Cys 101 is partly buried within the folded peptide chain. Only Cys 78 and Cys 96 can be modified by thiolation or carboxymethylation.[13,24] Under nondenaturing conditions with the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) method,[25] direct thiol titration studies revealed two free SH groups in the absence of POMs, and one free SH group in the presence of POMs, which indicates that POMs bind near one of the two, and subsequently prevent the protein from the thiol-mediated disulfide exchange reaction with the DTNB reagent. Cysteines 78 and 96 are located on opposing surfaces of the protein, and Cys 96 is known to be in the vicinity of the cationic pocket of the heparin binding site.[6,20] The competition study, the cysteine mutation data, and the thiol titration studies all strongly suggest that POMs bind in the vicinity of the heparin binding region of bFGF, whereas the exact binding site awaits high-resolution structural analysis of bFGF–POM complexes.

The design of synthetic molecules that can bind to a protein and block biologically important protein–protein interactions remains a major challenge. The results of the experiments described herein have identified a new structural family of bFGF-binding ligands. The unique structure of POMs may play a key role in the recognition and binding to the protein. The development of POMs as selective FGF binders may be limited, as many derivatives have little or no hydrolytic stability at physiologically relevant pH values and some POMs exhibit toxicity. However, the versatility of the POM compounds render them attractive, because many of the properties that dictate their utility, including elemental composition, structure, charge density, redox potential, acidity, and solubility can be controlled synthetically to varying degrees. Furthermore, a number of new POMs with hydrolytic stability at physiologically relevant pH values have been synthesized recently, and the toxicity problems exhibited by some POMs are considerably smaller or nonexistent in the second generation of POM-based chemotherapeutic agents. The data reported herein show promise for the development of new types of selective binders of fibroblast growth factors. Although we have just begun to address the issue of antiangiogenic activity, preliminary studies of POMs have shown an inhibition effect similar to that caused by heparin; further investigations of these points are currently underway.

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