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Osmium Tetroxide, 2,2′-Bipyridine: Electroactive Marker for Probing Accessibility of Tryptophan Residues in Proteins

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A complex of osmium tetroxide with 2,2′-bipyridine (Os,bipy) has been applied as a chemical probe of DNA structure as well as an electroactive DNA label. The Os,bipy has been known to form covalent adducts with pyrimidine DNA bases. Besides the pyrimidines, electrochemically active covalent adducts with Os,bipy are formed also by tryptophan (W) residues in peptides and proteins. In this paper we show that Os,bipy-treated proteins possessing W residues (such as avidin, streptavidin, or lysozyme) yield at the pyrolytic graphite electrode (PGE) a specific signal (peak αW) the potential of which differs from the potentials of signals produced by free Os,bipy or by Os,bipy-modified DNA. No such signal is observed with proteins lacking W (such as ribonuclease A or α-synuclein). Subpicomole amounts of W-containing proteins modified with Os,bipy can easily be detected using adsorptive transfer stripping voltammetry with the PGE. Binding of biotin to avidin interferes with Os,bipy modification of the protein, in agreement with the location of W residues within the biotin-binding site of avidin. These Ws are accessible for modification in the absence of biotin but hidden (protected from modification) in the avidin−biotin complex. The Os,bipy-modified avidin is unable to bind biotin, and its quarternary structure is disrupted. Analogous effects were observed with another biotin-binding protein, streptavidin. Our results demonstrate that modification of proteins with Os,bipy under conditions close to physiological, followed by a simple electrochemical analysis, can be applied in the microanalysis of protein structure and interactions.

Studies of structural properties and biological functions of numerous proteins belong to the most dynamically developing areas of contemporary science. Recently, instrumental techniques providing information about the overall proteomes have been developed (such as 2D electrophoresis combined with mass spectrometric techniques).[1–3] In addition to these complex proteomic approaches, techniques suitable for detailed analyses of the structure and interactions of individual proteins are needed. Site-specific chemical modifications of proteins belong to this methodological arsenal. There are a number of chemicals reacting with side chains of amino acid (AA) residues in proteins;[4–7] on the other hand, only a few of them exhibit reasonable selectivity for a particular AA residue under conditions close to physiological. Such reagents have been utilized for probing accessibility of the reactive moieties upon changes in the protein spatial structure, unfolding, denaturation, and/or intermolecular interactions. Chemical probing of AA residues in proteins has been applied in connection with various techniques of detection of the modification products (including optical methods such as absorption spectrophotometry[8–12] or fluorescence,[4] chromatographic techniques combined with site-specific proteolysis,[10,11,13] and more recently mass spectrometry.[5,14]) Literature devoted to electrochemical analysis of chemically modified peptides or proteins modified is scarce. There are several reports on the synthesis and applications of peptides bearing electrochemically active tags.[15–20] Some papers deal with derivatization of peptides or amino acids with ferrocene[17,21]

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or other "electrophores"[22–24] and their application in separation techniques coupled to electrochemical detectors. To our knowledge, no literature concerning probing of specific AA residues in native proteins with electrochemical markers has been published so far.

Among the AA residues occurring in natural proteins, tryptophan (W) has a special status in several aspects (ref 25 and references therein). This AA is relatively rare; on the other hand, it often plays crucial roles in arrangement of the protein tertiary and quarternary structures.[26–28] It is frequently found in active sites of enzymes and proteins exhibiting specific binding interactions with other molecules.[10–12,29,30] As the indole side group of W exhibits a characteristic fluorescence spectrum, the W is utilized as an intrinsic fluorophore in protein analysis.[31–33] In addition, the W residue is electrochemically oxidizable, yielding a specific signal at carbon electrodes.[34–37] Several chemical agents are available which react with the W indole group, including *N*-bromosuccinimide, 2-hydroxy-5-nitrobenzyl bromide (Hnb-Br), sulfenyl halides, or 2-nitrobenzenesulfenyl chloride.[5,7–9,30] These reagents exhibit a significant reactivity toward other AA, especially cysteine, and more or less specificity toward W can be achieved through optimization of reaction conditions. Chemical reactivity of individual W residues within a protein reflects its accessibility toward the environment, thus making it possible to distinguish between exposed and buried residues as well as to monitor shielding of residues within the protein binding sites by an interaction partner (such as avidin− or streptavidin−biotin, enzyme−substrate or −inhibitor).[9–12,29,30]

Some of us have recently reported on the utilization of the osmium tetroxide−2,2′-bipyridine complex (Os,bipy) as an electroactive marker for tryptophan residues in peptides.[38,39] Os,bipy forms a stable adduct with the W indole moiety[38–40] which is similar to adducts formed by pyrimidine residues in Os,bipy-treated nucleic acids (Figure 1).[41–43] In DNA, reaction of pyrimidines with Os,bipy and some other OsO_4 complexes (e.g., with pyridine or *N*,*N*,*N*′,*N*′-tetramethyl ethylenediamine, TEMED) is structure-selective. Modification of pyrimidines in double-stranded B-form DNA with these complexes is sterically hindered, which has been utilized in chemical probing of local open DNA structures

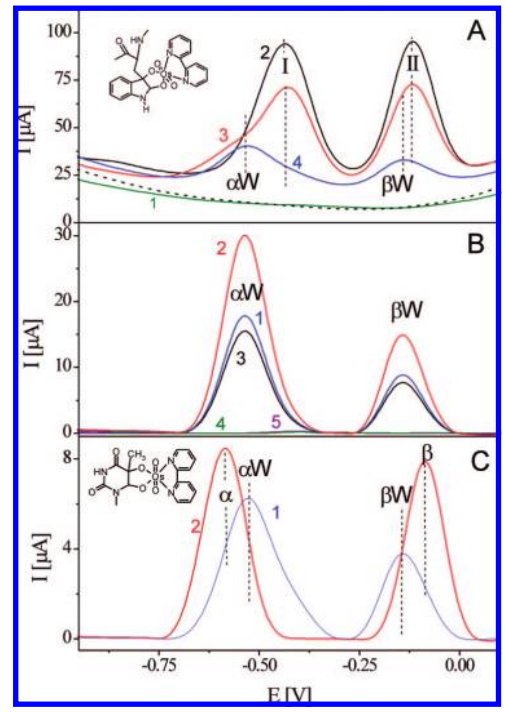

Figure 1. Square-wave voltammetric (SWV) responses of Os,bipy-treated proteins at a pyrolytic graphite electrode (PGE). (A) Voltammograms of (1) unmodified AVI, (2) Os,bipy alone, or (3 and 4), Os,bipy-treated AVI. AVI (60 μM) was treated with Os,bipy (2 mM) in 0.1 M Tris-HCl buffer, pH 7.6, at 20 °C for 3 h. Then, the solution was diluted by the same buffer three times (i.e., the resulting AVI concentration was 20 μM) and analyzed by adsorptive transfer (AdTS) SWV. The protein was adsorbed at the PGE surface from 5 μL aliquots of the diluted reaction mixture for 1 min, followed by PGE washing by deionized water and/or extraction of unreacted Os,bipy from its surface with isopropyl alcohol (60 s, whilst stirring the solvent) and the SWV measurement (see Supporting Information, Figure S1). Curves 1 and 4 were measured after the isopropyl alcohol extraction while for curves 2 and 3, this step was omitted. The dashed curve corresponds to background electrolyte (measured with bare PGE). (B) Sections of baseline-subtracted voltammograms obtained for Os,bipy-treated (1) AVI, (2) STV, (3) lysozyme, (4) ribonuclease A, or (5) α-synuclein. The isopropyl alcohol extraction procedure was used in all measurements; other conditions as in part A. (C) Sections of baseline-subtracted voltammograms of Os,bipy-treated (1), AVI; (2), oligodeoxynucleotide $(GAA)_7(T)_{20}$. Experimental conditions as in part B. Insets, Os,bipy adducts with tryptophan (in part A) or thymine (in part C).

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involving unpaired or otherwise exposed base residues.[41,44,45] The osmium adducts with pyrimidines in DNA[42,43,46–48] as well as those with tryptophan in peptides[37,38] exhibit well-pronounced electrochemical activity. Os,bipy has been used as an electroactive DNA marker detectable with carbon[46,49] as well as mercury[42,47,50] electrodes. Like Os,bipy-modified DNA, the Os,bipy-modified peptides (involving W) yield a remarkable catalytic signal at the mercury electrode which can be used for their highly sensitive determination.[38] Modification of several peptides with Os,bipy was recently studied using capillary electrophoresis and matrix-assisted laser desorption-ionization-time-of-flight mass spectrometry (MALDI-TOF MS).[39] Although several other amino acids or AA residues in proteins also exhibit reactivity toward the osmium reagents,[40] no other stable AA adduct (except that of W) bearing the osmium moiety has been identified. Oxidation products of cysteine or methionine (cysteic acid or methionine sulfone, respectively) have been observed by MALDI-TOF MS.[40] These products do not exhibit electrochemical activity characteristic for the osmium adducts; on the other hand, treatment with the osmium tetroxide reagents causes proteins to lose some electrochemical signals connected with the presence of thiol groups, such as the Brdicka catalytic hydrogen evolution.[51,52] Modification of peptides or proteins with Os,bipy followed by electrochemical analysis thus represents a convenient technique for probing of tryptophan residues.

Here we show that modification of proteins with Os,bipy can easily be analyzed by adsorptive transfer stripping (AdTS) voltammetry with pyrolytic graphite electrodes (PGE) using a specific electrochemical signal, peak αW, without preseparation of the reaction products. The technique can be utilized for monitoring of changes of accessibility of W residues upon formation of specific molecular complexes such as (strept)avidin−biotin, under conditions close to physiological.

EXPERIMENTAL SECTION

Material. Avidin, streptavidin, egg white lysozyme, biotin, osmium tetroxide, and 2,2′-bipyridine were purchased from Sigma. Other chemicals were of analytical reagent grade.

Modification of Proteins with Os,bipy. The proteins (usually 3 μM) were treated with 2 mM Os,bipy in unstirred solution containing 100 mM Tris-HCl, pH 7.6, at 20 °C for 2 h, if not stated otherwise. (Note: osmium tetroxide is a volatile irritant of the eyes, respiratory tract, and skin, and it should be always handled in a hood. Millimolar solutions of Os,bipy and Os,bipy-modified DNA or proteins are relatively safe.) Prior to electrochemical analysis, the reaction mixtures were usually 3 times prediluted.

Electrochemical Analysis. Electrochemical responses of the Os,bipy-treated proteins were measured by means of ex situ adsorptive transfer stripping (AdTS) square-wave voltammetry (SWV) at a pyrolytic graphite electrode (PGE) using a CHI 440 electrochemical analyzer (CH-Instruments, Austin) connected to a three electrode system (the PGE as the working electrode, Ag/AgCl/3 M KCl as the reference electrode, and platinum wire as the counter electrode). Briefly, 5 μL aliquots of the reaction mixtures were applied at the PGE surface. After 60 s accumulation time, the electrode was washed in deionized water, followed by 30 s extraction of the unreacted Os,bipy with isopropyl alcohol (if not stated otherwise). After double rinsing with deionized water, the electrode was placed into a voltammetric cell containing blank background electrolyte (0.2 M sodium acetate, pH 5.0). The SWV measurements were performed with the following settings: initial potential −1.0 V, quiescent time 2 s, pulse amplitude 25 mV, frequency 200 Hz, potential step 5 mV, final potential +0.1 V. The PGE surface was renewed by applying a potential of +1.7 V for 60 s in the blank background electrolyte and peeling-off the graphite top layer using sticky tape.[46]

Competition Assay of (Strept)Avidin−Biotin Binding Capacity. Biotin 5′-end-labeled oligonucleotide 5′$(GAA)_7(A)_{20}$3′ probe (VBC Biotech) was incubated with AVI (STV), followed by capture at magnetic Dynabeads oligo$(dT)_{25}$ (DBT, Dynal). After magnetic separation, the beads were shaken in a solution of a streptavidin−alkaline phosphatase conjugate (SALP, Sigma) that bound to the unoccupied biotin tag of the probe. After the beads were washed, they were transferred into solution of 1-naphthyl phosphate which was enzymatically converted into electrochemically oxidizable 1-naphthol. Finally, the latter was determined by linear sweep voltammetry (initial potential −0.5 V, end potential +1.0 V, scan rate 1.0 V s^{-1}) at the PGE. For more details, see the Supporting Information.

Polyacryalmide Gel Electrophoresis in the Presence of Sodium Dodecylsulfate (SDS-PAGE). The proteins were mixed with the loading buffer containing 2% SDS, heated at 95 °C for 5 min, and loaded onto a 5% stacking/12% separation gel containing 0.1% SDS in 25 mM Tris/192 mM glycine buffer, pH 8.3. In some experiments, the heating procedure was omitted. After electrophoresis, the gels were stained with Coomassie Brilliant Blue.

RESULTS AND DISCUSSION

Electrochemical Analysis of Os,bipy-Modified Proteins with Carbon Electrodes. It has been shown previously[38] that tryptophan-containing peptides treated with Os,bipy produce at the HMDE a catalytic response similar to that of Os,bipy-modified DNAs.[48–50] When analyzing osmium-modified nucleic acids or peptides with mercury electrodes it was, however, necessary to remove carefully the unreacted Os,bipy by dialysis, making the analysis rather cumbersome. To circumvent this difficulty in the nucleic acids studies, we introduced[46] an adsorptive transfer stripping (AdTS)[53,54] voltammetric procedure with pyrolytic graphite electrodes, involving removal of the excess reagent from the PGE surface by extraction with organic solvents. Here we applied an analogous procedure to study the electrochemical

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behavior of Os,bipy-modified proteins (for details, see the Supporting Information).

We treated 60 μM avidin (AVI, a 66 kDa glycoprotein consisting of four identical subunits, each containing four W residues[11,55]) with 2 mM Os,bipy. After a 3 h incubation at 20 °C, the reaction mixture was diluted three times and a 5 μL drop of the solution was applied onto a PGE surface. The SWV responses were measured after a 60 s accumulation time using a procedure involving electroreduction of the osmium species at −1.0 V followed by an anodic scan.[46,49,56] In the potential region between −1.0 and +0.1 V, two large signals were observed, one at −0.43 V (peak I on curve 3, Figure 1A) with a shoulder at −0.59 V and the other one at −0.12 V (peak II). When the same experiment was performed with blank reaction mixture lacking AVI, both peaks I and II were obtained again but the shoulder was absent (curve 2). Unmodified AVI (in the absence of Os,bipy) yielded no voltammetric signal in the same potential region. Such behavior suggested that the two large peaks (Figure 1A) were due to consecutive redox processes of the free Os,bipy complex reduction product, while the shoulder at −0.59 V might represent a signal of the W−Os,bipy adducts within the AVI molecules. After removal of the unreacted Os,bipy by dialysis, peaks I and II were strongly decreased and the inflection turned to a separated peak at −0.54 V (we named this signal "peak αW" in analogy with the peak α due to Os,bipy-modified DNA[46]), confirming the above assumption.

Further, we applied the AdTS procedure involving extraction of the interfering free Os,bipy from the PGE surface[46,56] to the analysis of the modified protein directly from the (undialyzed) reaction mixture. The procedure (namely, the organic solvent choice) was optimized for protein analysis (see the Supporting Information). Curve 4 in Figure 1A corresponds to the AdTS SWV response of 20 μM Os,bipy-modified AVI (the 3-fold prediluted reaction mixture) after extraction of unreacted Os,bipy from the PGE surface by isopropyl alcohol. The same voltammogram, after baseline subtraction, is shown in Figure 1B as curve 1. Besides the above-mentioned peak αW, the voltammogram displayed another signal at −0.14 V (peak βW). The potential of this signal was close to that of peak II due to the free Os,bipy (potential difference −20 mV, Figure 1A), in contrast to a considerable separation of the peak αW from the peak I (difference −110 mV) allowing distinction of both species. Both peak αW and peak βW differed in their potentials from analogous signals of Os,bipy-modified DNA (peak α and peak β, Figure 1C).

In addition to AVI, we treated with Os,bipy two other W-containing proteins (streptavidin (STV)[10,13,33] and egg-white lysozyme[28,29]) and two proteins lacking W (ribonuclease A and α-synuclein[57–59]) all at a concentration of 60 μM (the measurements were performed after 3-fold dilution again). Both modified proteins yielded peaks αW and βW at the same respective potentials, as did AVI (Figure 1B), but intensities of signals obtained with various proteins differed. The peak αW of Os,bipy-modified STV (60 kDa protein consisting of four subunits, each

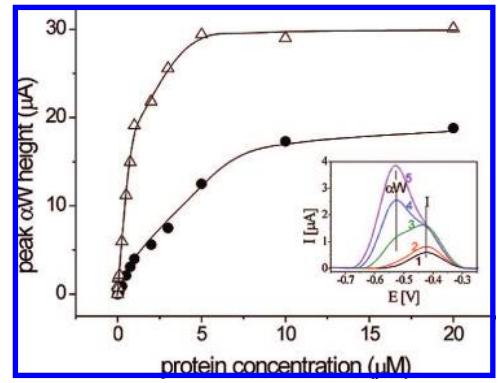

Figure 2. Dependence of the peak αW height on AVI (●) or STV (△) concentration. Both proteins were modified at a concentration of 60 μM with 2 mM Os,bipy, and concentrations given in the graph were achieved by dilution of this reaction mixture. Inset, sections of voltammograms showing peak αW obtained for AVI concentrations of (1) 0, (2) 0.03, (3) 0.1, (4) 0.17, and (5) 0.25 μM. In this case, all samples contained 2 mM Os,bipy and varying concentrations of AVI; prior to measurements, the samples were 3 times diluted. For other details, see Figure 1B.

containing six tryptophans) was about 1.5 times more intense, compared to peak αW of AVI modified under the same conditions (Figure 1B, curve 2). Lysozyme, a 14.6 kDa protein consisting of a single polypeptide chain with six W residues,[28] yielded a peak αW which was by about 20% lower than the analogous signal of AVI (Figure 1B, curve 3). On the contrary, neither of the W-lacking proteins yielded peak αW or peak βW (Figure 1B, curves 4 and 5). Similarly, small W-containing peptides (such as human or salmon luteinizing hormone-releasing hormones) yielded the peak αW upon modification with Os,bipy while peptides lacking W (such as the "substance-P" neuropeptide[60]) did not (data not shown), confirming that presence of W residues in the protein molecule was a prerequisite for appearance of this signal.

Effects of the AVI or STV concentrations on the peak αW intensities are presented in Figure 2. The proteins, both 60 μM, were modified with 2 mM Os,bipy as above, and the desired protein concentration was achieved by dilution of these reaction mixtures by the buffer solution. Both AVI and STV exhibited a linear dependence of the peak αW on concentration up to approximately 1 μM, followed by a region of less steeply increasing peak αW intensity. Leveling the peak αW height off (around 5 μM for STV or 10 μM for AVI) suggested saturation of the electrode surface. Under these conditions, STV or AVI were easily detectable down to 10 or 50 nM, respectively, after a 60 s accumulation time. In the following experiment, the Os,bipy concentration was kept constant (2 mM) and the concentration of AVI in the reaction mixture varied. Prior to the measurements, the samples were 3-fold prediluted (the Os,bipy was thus diluted to 0.67 mM). Under such conditions, the peak αW was measurable down to AVI concentrations of 0.1 μM (inset in Figure 2). Considering the sample volume used per measurement (5−7 μL), these data suggest that subpicomole amounts of the osmium-modified proteins can easily be analyzed using the simple voltammetric method.

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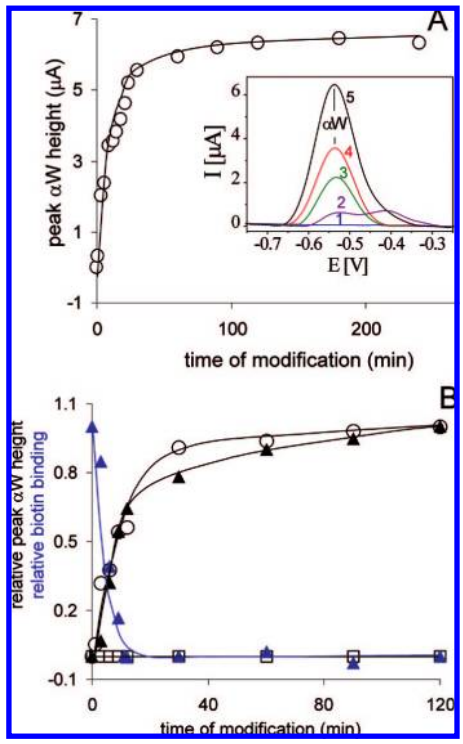

Figure 3. (A) Dependence of the peak αW height on time of AVI modification. AVI (3 μM) was treated with 2 mM Os,bipy at 20 °C and aliquots of the reaction mixture were analyzed by the AdTS SWV procedure at time intervals indicated in the graph. Inset, sections of voltammograms showing peak αW obtained with (1) unmodified AVI, (2−5) AVI treated with 2 mM Os,bipy for the following reaction times: (2) immediately after mixing, (3) after 3 min, (4) 9 min, or (5) 30 min. (B) Effects of biotin on AVI modification with Os,bipy and effects of AVI modification with Os,bipy on biotin binding capacity of the proteins. The experiment was performed as in part A, but the AVI was treated with Os,bipy in the absence (○) or in the presence (□) of 24 μM biotin. In another experiment (black ▲), biotin was added to the reaction mixture (to reach a concentration of 24 μM) at time intervals indicated in the graph and the samples were further incubated up to 120 min, followed by the measurements. Blue ▲ represent the dependence of relative biotin binding capacity of the AVI on the modification time. AVI was incubated with Os,bipy, and at time intervals indicated in the graph, aliquots of the reaction mixture were withdrawn and added to biotinylated oligonucleotide probe (0.4 μM) solution to reach an AVI concentration of 0.2 μM. The biotin binding competition assay is described in the Supporting Information.

Intensity of the peak αW was further measured as a function of the time of AVI modification (Figure 3A). Reaction mixture containing 3 μM AVI and 2 mM Os,bipy was incubated at 20 °C and at the given time intervals, aliquots were withdrawn and analyzed by means of the AdTS SWV as above. The peak αW increased steeply with reaction time up to 30 min (slope 1.9 μA min^{-1}). Between 30 and 120 min, a very slow increase of the peak αW height was detected (0.06 μA min^{-1}) followed by leveling off for longer modification times. Inset in Figure 3A shows that a distinct peak αW was detected even for a reaction time of "1 min" which corresponds to AVI modification only during adsorption at the electrode surface (in 3 times diluted reaction mixture withdrawn immediately after mixing the protein with the reagent). Formation of the electrochemically detectable W−Os,bipy adducts was accompanied by diminution of the characteristic W fluorescence[25,31,33] (for more details, see the Supporting Information).

Effects of Biotin on Reactivity of AVI or STV toward Os,bipy. In AVI, three (W70, W97, and W110) of the four (per subunit) W residues are involved in biotin binding, and two of these (W70 and W110) were reported to be readily modified with Hnb-Br.[11] Similarly, experiments with fluorescence quenchers such as acrylamide or iodide revealed two Ws of AVI (in the absence of biotin) exposed and two buried.[32] In STV, four Ws (79, 92, 108, and 120) are located within the biotin binding site and three of them (except 79) exhibit a remarkable reactivity toward Hnb-Br[10] and accessibility toward fluorescence quenchers.[33] Formation of the (strept)avidin−biotin complexes resulted in shielding of W residues in these proteins from modification by chemical reagents such as Hnb-Br[10,11] as well as from the fluorescence quenchers.[32,33]

Here, we were interested in whether the specific binding of biotin to these proteins affects modification of the W residues with the Os,bipy, measured using the SWV peak αW. We first prepared a solution containing 3 μM AVI and 24 μM biotin (i.e., a ratio of two biotin molecules per binding site in the AVI tetramer) and examined time dependence of the protein modification as above. Figure 3B shows that no peak αW was observed for any modification time up to 120 min (and even after 6 h incubation with the reagent, data not shown).

In the next experiment, a reaction mixture of AVI with Os,bipy (but without biotin) was prepared. At chosen time intervals (Figure 3B), aliquots of this mixture were mixed with biotin to reach the 2:1 ratio of biotin per binding site, and the incubation of the samples was continued to at least 120 min (measured from mixing AVI with Os,bipy). Under such conditions, the height of peak αW increased with time elapsed from mixing Os,bipy with the biotin-free AVI to adding the biotin (following a similar course as the time dependence of the peak αW intensity obtained for biotin-free AVI, see Figure 3). Such behavior suggests that upon adding biotin to the reaction mixture, the modification reaction was stopped and further incubation of the AVI-biotin complex with Os,bipy did not result in formation of a considerable amount of additional W−Os,bipy adducts.

Reactivity of the AVI W residues toward Os,bipy was further studied for different biotin/AVI ratios (Figure 4). AVI (3 μM) was first mixed with biotin at concentrations given in the graph (Figure 4), followed by addition of Os,bipy and 120 min incubation at 20 °C. Except for the slight increase of the peak αW observed at 1 μM biotin, the dependence of peak αW height was monotonously decreasing, following an approximately linear course. For 6 μM biotin (corresponding to semisaturated AVI), the peak αW height was only 33%, compared to the signal measured with free AVI. From a concentration that starts at 9 μM biotin (corresponding to three biotin molecules per an AVI tetramer), no peak αW was detected suggesting that occupation of three of the four binding pockets of the AVI tetramer by biotin was sufficient for practically complete loss of the apparent reactivity of its W residues toward the reagent.

Binding of biotin to STV also inhibited modification of the protein with Os,bipy but in this case, the decreasing part of the dependence of peak αW height on biotin concentration was less

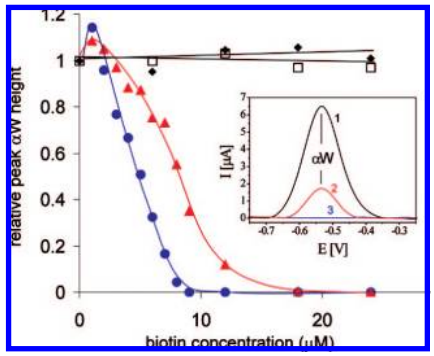

Figure 4. Effect of biotin on modification of AVI, STV, lysozyme, or the $(GAA)_7(T)_{20}$ oligonucleotide with 2 mM Os,bipy. Dependence of the relative heights of the peak αW: (blue ●) AVI; (red ▲) STV; (□) lysozyme; or (black ◆) peak α yielded by the oligonucleotide treated in the same way. Signal intensity obtained in the absence of biotin was taken as 1.0 for each set of data. Inset, section of voltammograms obtained with AVI for (1) 0, (2) 6, or (3) 12 μM biotin.

steep than observed with AVI (Figure 4). For STV semisaturated with biotin, the relative peak αW height decreased to about 75% of the value corresponding to free STV. At 9 μM biotin ($^3/_4$ sites occupied), the peak αW due to Os,bipy-modified STV was still 35%, and a small (about 10%) signal was obtained even for 12 μM biotin (one biotin per one binding site). For higher biotin concentrations, no modification of the protein was detected. Differences between the biotin titration curves obtained for AVI or STV were not due to mixing errors, as confirmed by tests of the residuary biotin binding capacity of proteins subsaturated or saturated with the ligand (not shown). Fluorescence experiments confirmed protection of W residues in AVI or STV complexes with biotin from modification with the osmium reagent (see Supporting Information for more details).

Analogous titration experiments with lysozyme or the 41-mer oligonucleotide showed no significant effects of biotin on reactivity of these substances toward Os,bipy (Figure 4). Neither of these species can form specific complexes with the vitamin. Hence, the lack of AVI or STV modification in the presence of biotin was not due to nonspecific effects but reflected formation of the specific (strept)avidin−biotin complexes.

Effects of Modification with Os,bipy on the Biotin Binding Activity of (Strept)Avidin. Previous studies of the role of W residues within biotin binding sites of AVI or STV revealed that modification of these residues with Hnb-Br (in the absence of biotin) caused the proteins to lose their biotin-binding activities.[10,11] To test the influence of AVI modification with Os,bipy on its ability to bind the vitamin, we introduced a magnetic beads-based electrochemical enzyme-linked competition assay[61] employing a biotinylated oligonucleotide probe (for details, see the Supporting Information). AVI fully modified with Os,bipy was unable to bind the probe (exhibiting, in contrast to unmodified AVI, no competition for any AVI concentration tested, see Figure S5 in the Supporting Information). Analogous results were obtained with unmodified and Os,bipy-modified STV (not shown). Further, we examined the effects of the time of modification with Os,bipy on the AVI biotin-binding capacity. AVI was incubated with Os,bipy and at chosen time intervals, aliquots of the reaction mixture were added to the probe. The subsequent competition assay showed that the AVI biotin-binding capacity strongly decreased with the modification time. After 15 min of modification, the biotin binding was completely lost. When compared with dependence of the peak αW on the modification time (Figure 3B), the time interval in which the biotin binding capacity of AVI was decreasing coincided with the interval of steep increase of the peak αW intensity. Hence, increasing the degree of AVI modification with Os,bipy was accompanied by gradual inhibition of the AVI-biotin binding.

Effects of Os,bipy Modification on the AVI or STV Quaternary Structures. Both AVI and STV form extremely stable tetramers that can resist even conditions of SDS-PAGE electrophoresis.[62–64] The tetramers are composed as "dimers of dimers", and their stabilization involves contacts mediated by a W residue from one subunit involved in the biotin binding site in the neighboring subunit.[65] Accordingly, saturation with biotin was shown to considerably increase the tetramer stability.[62–64] Here we utilized SDS PAGE to test the effects of Os,bipy treatment on the stability of the AVI or STV quarternary structures.

We first studied behavior of unmodified or Os,bipy-modified (in the absence of biotin) proteins in SDS-PAGE (Figure 5A). In this experiment, the protein samples were not thermally denatured prior to loading on the gel. In agreement with previous observations,[63,64] unmodified STV migrated in the gel as a tetramer while unmodified AVI formed in the presence of the anionic detergent SDS a heavy aggregate that failed to pass through the 5% stacking gel. For Os,bipy-modified AVI or STV, a major part of the proteins migrated in the gel as monomers and only minor fractions were observed as dimers, trimers, tetramers, or (in the case of AVI) aggregates (Figure 5A). After thermal treatment of the (both unmodified or modified) biotin-free proteins in the presence of 2% SDS (lanes 1 and 3 in Figure 5B), all oligomers (aggregates) disappeared, with the exception of a small amount of species migrating as a STV dimer after the Os,bipy treatment (see below).

Addition of biotin to the unmodified proteins resulted in stabilization of the AVI aggregates or STV tetramers that resisted the thermal treatment (Figure 5B, lanes 2). Modification of the biotin-saturated AVI or STV with Os,bipy had no effects on the SDS PAGE results (lanes 4 in Figure 5B) i.e., no breakdown of the tetramers or aggregates was observed (even after the thermal treatment). Such observation was in agreement with the lack of reactivity of the fully liganded proteins toward the reagent (see above). When biotin-free AVI was mixed with Os,bipy and at different time intervals, the modification was stopped by adding biotin (as in Figure 5B), distribution of the species in the gel was changing in accord with different degrees of the protein modification (for supplementary data see Supporting Information, Figure S5).

Treatment with Os,bipy Does Not Result in Significant Protein Cross-Linking. The osmium(VIII) reagents are known to induce protein cleavage or cross-linking under certain

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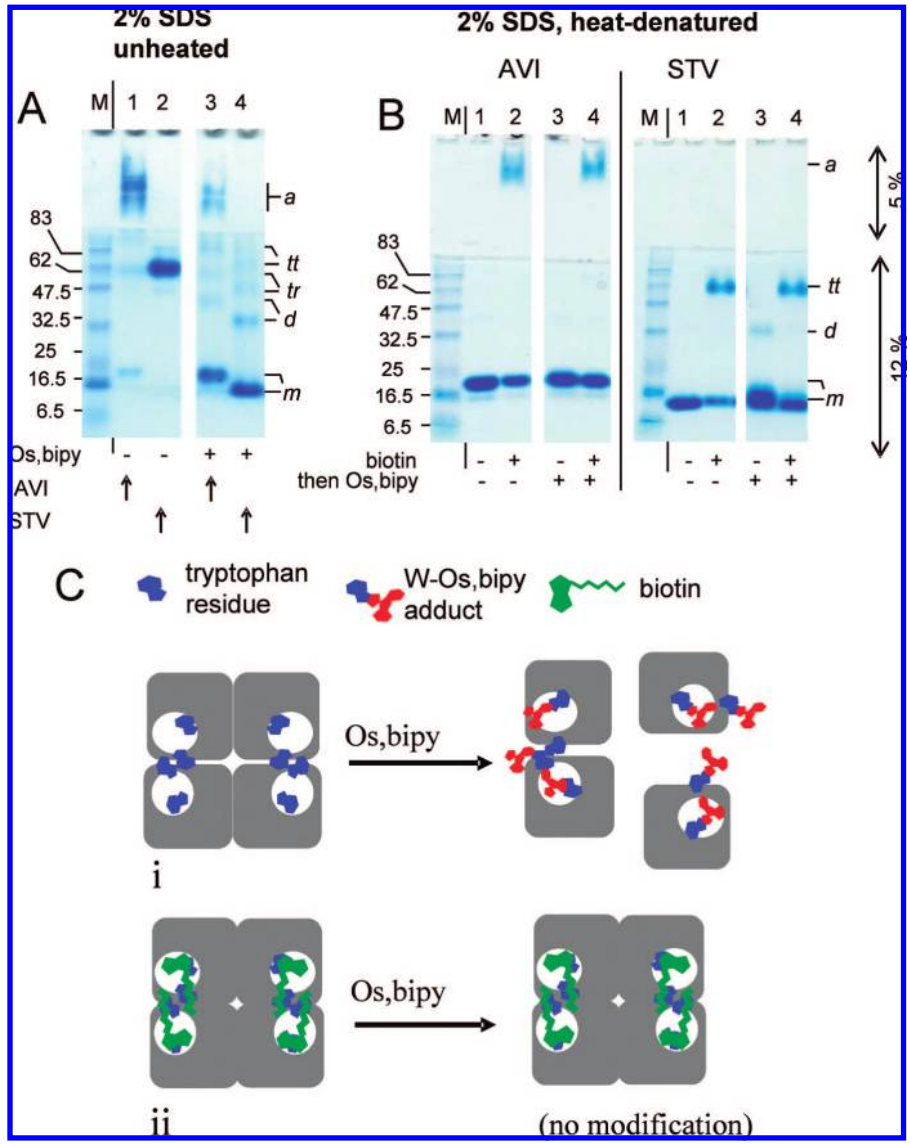

Figure 5. Effects of Os,bipy treatment and/or presence of biotin on the behavior of AVI or STV in SDS polyacrylamide gel electrophoresis. (A) Biotin-free, Os,bipy-treated or untreated proteins were loaded on the gel without thermal pretreatment (for sample identification see marking on the bottom). (B) Prior to loading on the gel, biotin-free or biotin-saturated samples were subjected to thermal denaturation procedure. Os,bipy modification of the proteins as in Figure 3; M, markers (molecular weights indicated in kilodaltons on the left); legends on right side of the panels: m, protein monomers; d, dimers; tr, trimers; tt, tetramers; a, high molecular mass aggregates; 5%, 12%, concentrations of stacking and separation gels, respectively. (C) Scheme of modification of the (strept)avidin with Os,bipy and protection of tryptophan residues in the (strept)avidin−biotin complexes. In the absence of biotin (i), tryptophan residues within the binding site are accessible for modification. Modification with Os,bipy results in destabilization and disintegration of the protein quarternary structure in accordance with involvement of one of the accessible tryptophan residues (W110 in AVI or W120 in STV) in the mutual contact between two neighboring subunits.[65] When the binding pockets are occupied by biotin (ii), the W residues are shielded and no modification takes place (W residues which are buried within the protein spatial structures are omitted).

conditions.[40,66] It has been shown that the cleavage reaction induced by osmium tetroxide alone can be suppressed by addition of nitrogenous ligands stabilizing the resulting osmate esters.[66] On the other hand, the cross-linking reaction was promoted by additions of TEMED, and it was observed also in the presence of 2,2′-bipyridine.[66] Formation of considerable amounts covalently cross-linked protein aggregates might affect reactivity of particular W residues toward Os,bipy as well as accessibility of the W−Os,bipy adducts for the electrochemical process giving rise to the peak αW. Results of our SDS PAGE experiments (Figures 5A,B and 5S in the Supporting Information) have shown that under conditions used in this work, neither protein cleavage nor cross-linking took place to a significant extent. In particular, no detectable products of Os,bipy-induced covalent cross-linking of the AVI monomers appeared in the gels. With STV, a distinct band corresponding to molecular mass of the protein dimer, attributable to two covalently cross-linked STV monomers, was detected after the Os,bipy treatment in the absence of biotin (Figure 5B). Nevertheless, densitometric tracing of the gel revealed that only

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minor portion (≤4%) of the protein had undergone the assumed cross-linking.

CONCLUSIONS

Osmium tetroxide complexes proved useful as electroactive nucleic acids markers[42,43,46–50,56,67] as well as probes of the DNA structure.[41,44,45] Reaction of pyrimidine bases with Os,L takes place in aqueous solutions under physiological conditions, convenient for DNA structure analysis and show an ability to penetrate easily in bacterial[44] and eukaryotic[68] cells. Most of these complexes, such as Os,bipy recognize single-stranded and distorted regions in DNA where the pyrimidines are accessible for chemical modification. Similar electrochemically active adducts are formed also upon reaction of the Os,bipy with tryptophan. We demonstrate that modification of W residues in proteins (such as AVI or STV) with Os,bipy is sensitive to accessibility of the Ws in the protein structure, suggesting applicability of this reagent as an electrochemically active structural probe for proteins. Subpicomole amounts of Os,bipy-modified proteins can easily be detected using a simple voltammetric procedure with the PGE. Similarly as the Os,bipy modified DNA, the modified proteins can also be detected with other types of carbon electrodes (e.g., screen-printed or carbon paste, data not shown). Alternatively, mercury or amalgam electrodes can be used for the measurements of the catalytic osmium signal.[38,69–71]

There is a growing amount of data documenting that simple voltammetric or chronopotentiometric techniques can be used for fast and sensitive determination of specific peptides and proteins in biological specimens[72,73] and for studies of protein denaturation,[74] aggregation,[58] as well as for protein interactions with low-molecular-mass ligands,[75] with nucleic acids,[76] etc. These emerging properties of electrochemical methods and their combinations with chemical modification and magnetic separation[61,77,78] make these methods good candidates for their application in biomedical research.

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SUPPORTING INFORMATION AVAILABLE

Optimization of the AdTS SWV procedure for the analysis of protein reaction mixtures with Os,bipy, effects of Os,bipy treatment on the tryptophan fluorescence, competition assay of the (strept)avidin−biotin binding activity, and supplementary results. This material is available free of charge via the Internet at http://pubs.acs.org.

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