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Direct Electron Transfer of Heme- and Molybdopterin Cofactor-Containing Chicken Liver Sulfite Oxidase on Alkanethiol-Modified Gold Electrodes

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Direct heterogeneous electron transfer (ET) of sulfite oxidase (SOx), a heme- and molybdopterin cofactor-containing intermembrane enzyme, was studied on alkanethiol-modified Au electrodes both with SOx entrapped between the modified Au electrode and a permselective membrane and with SOx adsorbed at the electrode surface, in the absence of any membrane. SOx in direct electronic communication with the electrode surface gave a quasi-reversible electrochemical signal with a midpoint potential of −120 mV vs Ag | AgCl corresponding to the redox transformations of the heme domain of SOx and with a heterogeneous ET constant in the order of 15 s^{-1}. The efficiency of the bioelectrocatalytic $2e^-$ oxidation of sulfite catalyzed by SOx in direct ET exchange with the electrode was shown to depend essentially on the nature of the alkanethiol layer. Adsorption and orientation of SOx on an 11-mercapto-1-undecanol (MuD-OH) self-assembled monolayer, i.e., terminally functionalized with OH groups, provided efficient catalytic oxidation of sulfite, contrary to nonfunctionalized alkanethiols, e.g., 1-decanethiol, or alkanethiol layers terminally functionalized with NH_2 groups. Comparative studies with short-chain alkanethiols, e.g., cysteamine and 2-mercaptoethanol, revealed an evidently different mode of adsorption of SOx on these layers, onto which SOx was not catalytically active. Coadsorption of MuD-OH and 11-mercapto-1-undecanamine improved the surface properties of the SAM, resulting in a higher surface coverage with bioelectrocatalytically active SOx but not in an increased apparent catalytic rate constant, k_{cat}, ranging in the order of 18−24 s^{-1} at pH 7.4. The achieved efficiency of SOx bioelectrocatalysis in direct ET reaction between the modified electrode and the enzyme approached the rates characteristic for the catalysis mediated by cytochrome *c*, the natural redox partner of SOx, thus implying the retention of the biological function of SOx under the heterogeneous electrode reaction conditions. Results obtained enable the development of a third-generation biosensor for sulfite monitoring.

Electron transfer (ET) within multiredox cofactor-containing enzymes implies electron transport both from the biological redox partner of the enzyme and within the same enzyme molecule, between two or more of its redox sites. Mimicking the protein biological partner by a modified electrode is then of particular interest to achieve an efficient direct ET reaction from the electrode to and through the enzyme in close vicinity to the electrode surface. However, under ordinary electrochemical reaction conditions, most biological redox systems demonstrate sluggish electrochemical responses.[1−3] This results from the inaccessibility of the redox sites of the enzyme or unfavorable interactions at the electrode surface. A great amount of effort has therefore been focused on the modification of either the protein or the electrode surface in order to control the orientation at the enzyme/electrode interface to achieve a direct rapid ET between the electrode and at least one of the redox sites present in the enzyme.[1−5]

Sulfite oxidase (SOx) is an example of two redox cofactor-containing intermembrane redox enzymes,[6−7] for which direct electrochemistry has been a question for a long time.[8−11] SOx is a homodimer; each subunit contains a molybdenium complex cofactor (a molybdopterin-binding domain and a Mo ion), which is connected to a heme-containing domain (a cytochrome b_5 type, cyt b_5) by a flexible polypeptide chain region.[12] SOx catalyzes a $2e^-$ oxidation of sulfite to sulfate, the terminal reaction in the degradation of the sulfur-containing amino acids cysteine and methionine, with ferricytochrome *c* as its physiological electron acceptor:

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$$SO_3^{2-} + H_2O \xrightarrow{SOx} SO_4^{2-} + 2H^+ + 2e^- \quad (1)$$

The enzyme also plays an important role in detoxifying exogenously supplied sulfite and sulfur dioxide.[6,7] Oxidation of sulfite to sulfate occurs at the molybdenium center with concomitant reduction of Mo(VI) to Mo(IV). The reducing equivalents are then passed on from the Mo domain to the cyt b_5 domain of SOx and from there to cytochrome *c* (cyt *c*), which can, ideally, be replaced by the electrode:

$$SO_3^{2-} \rightarrow \text{Mo-cofactor} \rightarrow \text{cyt } b_5 \rightarrow \begin{matrix}\text{cyt } c \text{ or other} \\ \text{electron acceptor}\end{matrix} \quad (2)$$

Thus, SOx couples the $2e^-$ oxidation of sulfite with two $1e^-$ reductions of cyt *c*. Direct ET communication with the electrode via the heme center and the substrate conversion proceeding at the Mo−cofactor moiety enables us both to study the biological ET processes within SOx by means of electrochemical methods and to develop a third-generation biosensor, i.e., a mediatorless one,[13] for the analysis of sulfite and sulfur dioxide. However, adaptation of SOx for operation in the heterogeneous SOx | electrode system suggests very special conditions for optimal catalysis. The long ET distance between the Mo and the heme Fe, 32 Å, and the orientation of the molybdopterine ring system away from the second cofactor imply that the cyt b_5 domain should adopt a proper conformation for ET to occur, with a reduced metal-to-metal distance providing an efficient ET pathway.[12,14,15] Additionally, the conformation-dependent SOx catalytic cycle is largely affected by the composition, ionic strength, viscosity, and pH of the contacting solution.[14−18]

To electrochemically successfully follow the electrooxidation of sulfite catalyzed by SOx, the electrode surface should supposedly simulate the surface of the natural partner of SOx or the biological environment of SOx to an extent providing the necessary amount/orientation of SOx molecules capable of direct electron exchange with the electrode, as well as the conformation appropriate for efficient intramolecular ET. Our preliminary spectroelectrochemical results with thiol-modified Au electrodes demonstrated that SOx could communicate directly with the electrode through the heme-containing domain, thus offering the possibility to achieve the heterogeneous and intramolecular ET reactions between the active sites of SOx by means of electrochemical methods. However, the first reported studies of direct electrochemistry of SOx at pyrolytic graphite and thiol-modified Au electrodes showed that the bioelectrocatalytic activity of SOx was more than 25 times lower than that in homogeneous catalysis with cyt *c*.[19] The estimation demonstrated that compatibility between the electrode and the enzyme surfaces can be improved. To mimic a cyt *c* surface, self-assembled monolayers (SAMs) of synthetic terminally functionalized alkanethiols[20−22] were used in our work. Previously, the suitability of SAM surfaces for orientation of redox proteins/enzymes, e.g., cyt *c*, cellobiose dehydrogenase, and alcohol dehydrogenase, with the retention of their natural functions was successfully demonstrated.[22−26] A SAM of alkanethiols, for controlling the surface chemistry, enabled the authors to form a monolayer of conductive supports for oriented protein immobilization. The design of the alkanethiol headgroups by different functional groups can be used as well for oriented immobilization of enzymes through noncovalent coupling between the SAMs and the enzymes via electrostatic, hydrophobic, and hydrophilic interactions.[1,2,21,22] Therewith, a biomembrane-like (cellular) microenvironment predetermined by the nature of the SAM surface, may be promising for immobilization of SOx retaining its natural function, including selective sensing of specific substrates and studies of the charge transfer and the catalytic properties of the system involved.

To achieve efficient heterogeneous and intramolecular ET for SOx bioelectrocatalysis, we used alkanethiols of different polarity/hydrophobicity and charge to control the surface properties of the Au electrode, enabling the adsorption of SOx favorable for efficient ET reactions.

EXPERIMENTAL SECTION

Chemicals and Materials. Sulfite oxidase from chicken liver (EC 1.8.3.1) from Sigma was a 7.8 mg mL^{-1} enzyme suspension in 3.2 M ammonium sulfate containing 1.6 mM molybdic acid, pH 7.5. It was stored at +4 °C between experiments. The SOx activity assay was performed as previously described,[18] by following the increase in absorbance at 550 nm due to the reduction of cyt *c*, in 0.1 M Tris-HCl containing 0.1 mM EDTA, pH 8.5 and 7.4, where one unit of SOx is defined as the amount of SOx that oxidizes 1 μmol of sulfite to sulfate during the reduction of cyt *c* per minute at 25 °C. Cyt *c* from bovine heart was from Sigma and was used as received. Sodium sulfite (98%) and 1-decanethiol were from Fluka. 11-Mercapto-1-undecanol (97%, MuD-OH), 11-mercapto-1-undecanoic acid (95%, MuD-COOH), 2-mercaptoetanol (98%, MEt-OH), and cysteamine were from Aldrich. 11-Mercapto-1-undecanamine (MuD-NH_2) was a generous gift of Dr. Frieder Borgmeier (BASF AG, Ludwigshafen, Germany). Millipore water (18.2 MΩ) was used throughout the work.

Electrode Modification with Alkanethiols. Thiol films were prepared by 8 h of adsorption from 5 mM solutions of alkanethiols in absolute ethanol and by 2 h of adsorption from 5 mM solutions of cysteamine and MEt-OH. For mixed SAMs, 5 mM MuD-COOH

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and 5 mM MuD-OH in proportion 1:3 v/v and 5 mM MuD-OH and 5 mM MuD-NH_2 in proportion 1:1 v/v were used. The low solubility of cysteamine and MuD-NH_2 resulted in the use of saturated solutions of cysteamine and MuD-NH_2 with the real concentration essentially less than 5 mM. Modified electrode surfaces were rinsed thoroughly with water to remove weakly adsorbed molecules.

Instrumental Procedure. All measurements were performed at ambient temperature, 22 ± 1 °C. Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) experiments were done with a CV-50W voltammetric analyzer (BAS) in anaerobic solutions. SCE was the reference and a platinum plate was the auxiliary electrode. For voltammetry, gold disk electrodes (CHI, $A = 0.031$ cm^2) were abraded on 1200-grit SiC emery paper, washed with water, and successively polished to a mirror luster on Microcloth (Buehler) in aqueous alumna suspension (0.1 μm, Stuers, Denmark), rinsed with water, and finally polished electrochemically by cycling in 0.1 M H_2SO_4 between −0.3 and 1.7 V. Modification of these electrodes with alkanethiol layers was done as described above. After modification, a Teflon cap was put on the top part of the electrode, thus forming a 5-μL volume well-like microcell, with the bottom representing the electrode surface and with a well wall height of 1.6 mm. Then either a 5-μL droplet of a 72 μM SOx or 60 μM cyt *c* solution or 2.5-μL droplets of both a 72 μM SOx solution and a 60 μM cyt *c* solution were dropped into the microcell formed by the Teflon cap and the alkanethiol-modified Au surface. The system was fixed with a permselective dialysis membrane (molecular weight cutoff, 6000−8000), presoaked in water. The dialysis membrane was pressed onto the electrode Teflon cap and fitted tight to the cap surface with a rubber O-ring. In such a manner, the modified electrodes were kept for 1 h in 0.1 M Tris-HCl, pH 7.4, under intensive stirring with argon, to remove stabilizers present in the SOx sample, which interfered with the measurements. CV and DPV measurements were performed in a fresh 0.1 M Tris-HCl buffer solution, pH 7.4, either with membrane-entrapped or with physically adsorbed SOx, obtained upon stripping the membrane and removing the electrode Teflon cap. A 0.1 M aqueous solution of sulfite was prepared immediately before measurements and used within 40 min.

RESULTS AND DISCUSSION

Catalytic Activity of Sulfite Oxidase toward Cyt *c* in Homogeneous Solution. The specific catalytic activity of SOx toward cyt *c* determined spectrophotometrically[18] was 32 and 24.3 units mg^{-1}, at pH 8.5 and 7.4, respectively. Saturating concentrations of substrates, i.e., 0.4 mM sulfite (∼21K_m) and 20 μM cyt *c* (∼10K_m),[17] used in the activity assay enabled determination of the catalytic first-order rate constant k_{cat} from the rate of substrate transformation. k_{cat}, at pH 8.5 and pH 7.4, was found to be 57.6 ± 5.3 and 43.7 ± 3.4 s^{-1}, correspondingly (per cyt *c*). The relative variation of the rate constant with pH is in good agreement with previously reported data;[17] however, the absolute values are 20−35% lower.[15,17] This deviation is supposedly ascribed to the presence either of impurities in commercially available SOx or to the inhibition effect of sulfate anions being present in the sample. Sulfate anions have been shown to reduce the catalytic activity of SOx toward sulfite oxidation through competitive binding in the Mo-active site.[14,16,18] However, k_{cat} values of 50 s^{-1} at pH 8.0 have been reported as well.[27]

Bioelectrocatalysis mediated by cyt *c* was studied to estimate the catalytic activity of SOx for sulfite oxidation.[8−11] For this purpose, both redox partners, SOx and cyt *c*, were entrapped between the electrode surface and the permselective membrane. The entrapment of the enzyme (as well as a mediator) under the membrane enables study of bioelectrocatalysis using high concentrations of both reagents in a small-restricted volume. Since enzyme preparations as a rule are expensive, and in many cases sufficiently high concentrations are necessary for studies, the advantage of the membrane systems for the purpose of mediated bioelectrocatalysis is evident. Additionally, the membrane system does not require any special reconstruction of the electrochemical cell and is easy to handle.

The biological SOx electron acceptor, cyt *c*, has been shown to exhibit a well-defined direct electrochemistry on COOH-functionalized alkanethiol-modified Au electrodes, providing favorable electrostatic interactions between the positively charged lysine residues of cyt *c* and the modified electrode surface.[23,28−31] Significantly improved electrochemistry of the cyt *c* heme can be achieved at mixed MuD-OH/MuD-COOH SAMs;[32−34] thus, the modification of the Au electrode with these long-chain SAMs was used. The high ionic strength of the contacting solution, used for all the work (0.1 M Tris-HCl), prevented strong adsorption of cyt *c* at the thiol-modified Au electrodes, favorable at low ionic strength, thus suggesting optimal mobility of cyt *c* facilitating the reaction with SOx as well.

A CV of cyt *c* at the (MuD-OH/MuD-COOH)-modified Au electrode, in 0.1 M Tris-HCl, pH 7.4, exhibits a couple of redox peaks, which correspond to a quasi-reversible redox transformation of cyt *c* (Figure 1, inset). Close peak separations of 63−78 mV at 2−10 mV s^{-1} were detected both in the absence and in the presence of SOx under the membrane, the smaller peak areas in the presence of SOx being connected with the lower concentration of cyt *c* under the membrane (see Experimental Section). The peak current varied linearly with the square of the scan rate[35] (slope of 16.5 ± 1.1 nA (mV $s^{-1})^{1/2}$, $r^2 = 0.999$), designating soluble protein (not immobilized one) electrochemistry, which was expected at this ionic strength. The formal redox potential, $E^{0\prime}$, estimated as the mean value of the voltammetric peaks was 89.5 ± 1.5 mV at pH 7.4. This value correlates well with 68 and 59 mV versus Ag | AgCl, at pH 7.0, reported for horse heart and bovine heart cyt *c*, respectively;[23,28−34] the deviations due to different pH, ionic strength, and solution composition should be taken into account. The rate constant, $k^{0\prime}$, for the heterogeneous ET between the alkanethiol-modified Au electrode and cyt *c*, calculated with the Nicholson approximation,[36,37] was (1.1 ± 0.2) × 10^{-3} cm s^{-1}

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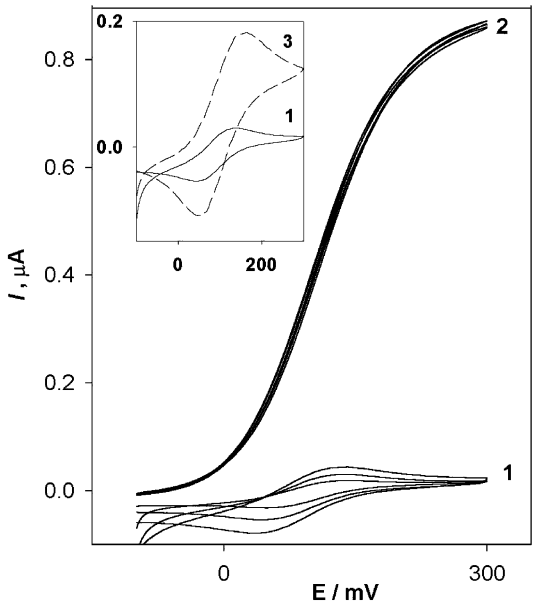

Figure 1. Bioelectrocatalytic CVs of SOx (2) entrapped between the MuD-OH/MuD-COOH-modified Au electrode and the membrane, with cyt *c* as mediator (1), in 0.1 M Tris-HCl, pH 7.4, at scan rate *v* 2, 5, and 10 mV s^{-1}. [SOx] = 36 μM, [cyt *c*] = 30 μM, and [SO_3^{2-}] = 3.3 mM. Inset: CVs for cyt *c* under the membrane, in the presence (1) and in the absence (3) of SOx; [cyt *c*] is 30 and 60 μM, respectively.

at 4−10 mV s^{-1} and increased to (2.1 ± 0.2) × 10^{-3} cm s^{-1} when the scan rate decreased to 2 mV s^{-1} (see Supporting Information). A cyt *c* diffusion coefficient, $D_{\text{cyt } c}$, of (3.3 ± 0.4) × 10^{-7} cm^2 s^{-1} estimated from the dependence of the oxidation−reduction peak currents on the square root of scan rate (in the presence of SOx under the membrane)[35] was used for the calculations. The obtained $k^{0/}$ value suites well the 10^{-4}−10^{-3} cm s^{-1} range obtained for soluble cyt *c* at modified Au electrodes,[4,23] approaching the upper limit and thus reflecting optimized electrochemistry of cyt *c*.

In the absence of cyt *c*, SOx exhibits no electrochemical activity on the MuD-OH/MuD-COOH layer, evidently due to noncompatibility of the negatively charged SAM surface and the negatively charged surface moieties in the vicinity of the heme domain.[27] No sulfite oxidation current was observed upon addition of sulfite within the potential range studied (from −300 to +300 mV) as well. The formation of the SAM inhibits the direct oxidation of sulfite at the modified Au electrode, shifting the starting oxidation potential from 170−200 mV, for bare Au surface, to 300−350 mV, for alkanethiol-modified surfaces (data not shown). In the presence of both SOx and cyt *c*, upon the addition of a saturating excess of sulfite, the original redox wave of cyt *c* transforms into a characteristic catalytic wave, with a steady-state current plateau, independent of potential scan rate or solution stirring (Figure 1, curves 2), in the same manner as was demonstrated by Murray and co-workers with cyt *c* and cobalt/ruthenium complexes, acting as mediators, at pyrolytic graphite electrodes.[8−11] The limiting current, corresponding to the plateau, is independent of further additions of sulfite, indicating a kinetically fast step of SOx reaction with sulfite, much faster than the rates of electrooxidation of cyt *c* by the electrode or the ET interaction of cyt *c* with the reduced enzyme. Under these conditions, the rate of the reaction between cyt *c* and SOx was estimated using two approaches. First, the CV data were processed in accordance with the theory of Nicholson and Shain,[37] when a pseudo-first-order rate constant, k_f, for the reaction between cyt *c* and SOx, can be derived from the ratio of the catalytic (the plateau current value, I_{cat}, in Figure 1) and diffusion-controlled (the cyt *c* oxidation peak current, I_{dif}) currents plotted versus the reciprocal scan rate *v*:

$$(I_{cat}/I_{dif})^2 = \lambda = k_f(RT/nF)/v \qquad (3)$$

Here, *n* is the number of electrons exchanged, and *R*, *T*, and *F* are the universal gas constant, the Faraday constant, and the temperature, respectively. The cyt *c* oxidation/reduction is a reversible $1e^-$ process, i.e., $n = 1$; thus, a k_f value of 91.6 ± 8.2 s^{-1} (per cyt *c*) was obtained. For calculating the second-order rate constant, *k*, for the reaction between SOx and cyt *c* at pH 7.4, the value of k_f was divided by the enzyme concentration C_{SOx} under the membrane, which resulted in a *k* equal to (2.53 ± 0.24) × 10^6 M^{-1} s^{-1}.

With the second approach, *k* is calculated with the methodology proposed first by Savéant and Vianello[38] and developed later by Murray and co-workers,[8−11] from the value of the sulfite-saturated limiting current I_{cat}:

$$I_{cat} = nFAC_{\text{cyt } c}(D_{\text{cyt } c}C_{SOx}k)^{1/2} \qquad (4)$$

Here, $C_{\text{cyt } c}$ is the concentration of cyt *c* under the membrane and *A* is the electrode surface area (using a roughness factor of 1.2). Calculations with eq 4 give *k* equal to (2.93 ± 0.08) × 10^6 M^{-1} s^{-1} at pH 7.4, close to the value 2.5 × 10^6 M^{-1} s^{-1} obtained with eq 3. Both values exceed by 4−5-fold the value obtained under similar (but not the same) conditions of catalysis 0.63 × 10^6 M^{-1} s^{-1} (20 mM Tris, 0.1 M KCl, pH 7.5);[9] however, the difference in the original catalytic activity of the SOx sample should be taken into account as well. The parameters, characterizing the catalytic activity of SOx in the reaction with cyt *c* and determined by the different methods, were compared. The catalytic first-order rate constant k_{cat} (per cyt *c*) determined spectrophotometrically from the rate of substrate transformation at pH 7.4 (43.7 ± 3.4 s^{-1}) was twice lower than the pseudo-first-order rate constant k_f for the reaction between cyt *c* and SOx (91.6 ± 8.2 s^{-1}), determined electrochemically, under similar conditions and at saturation levels of substrates. This raises a question of the reasons for the discrepancy and mutual validity of the results. However, assuming that both modes of catalysis have the same limiting step, i.e., the reaction between cyt *c* and SOx, the deviation in turnover numbers might be ascribed to the higher activity of SOx entrapped (and stored during the deaeration of the working solution) under the membrane. Thus, removal of inhibitors, e.g., sulfate, or impurities from cyt *c* and SOx samples through the membrane out into the bulk solution should enhance the efficiency of catalysis.

Further, in control experiments, cyt *c* was covalently attached to the studied SAMs by means of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide, which restricted the mobility of the protein and

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Table 1. Apparent Catalytic Rate Constants k_{cat} Estimated from the Bioelectrocatalytic Currents of Sulfite Oxidation Catalyzed by SOx Immobilized on Different Alkanethiol-Modified Au Electrodes

modifier	$E^{0\prime}$, V	ΔE_n, V	k_s,[a] s^{-1}	Q, n C	Γ, pmol cm^{-2}	k_{cat}, s^{-1}
cysteamine[b]	-119 ± 5	45 ± 5	13.8 ± 1.5	1.4 ± 0.1	0.45 ± 0.5	no catalysis
MuD−OH[b]	-120 ± 2	52 ± 3	12.0 ± 1.0	1.8 ± 0.1	0.59 ± 0.03	24.2 ± 0.2
MuD−OH	-121 ± 4	45 ± 3	13.9 ± 1.0	0.8 ± 0.1	0.26 ± 0.04	23.8 ± 3.4
MuD−OH/MuD−NH_2[b]	-120 ± 6	50 ± 5	12.6 ± 1.5	4.1 ± 0.4	1.30 ± 0.10	18.3 ± 1.5
MuD−OH/MuD−NH_2	-119 ± 3	43 ± 1	15.2 ± 0.3	2.7 ± 0.4	0.90 ± 0.10	18.3 ± 1.6

[a] k_s values were calculated at 500 mV s^{-1}. [b] Experiments were performed with SOx entrapped under the membrane.

fixed cyt *c* with its heme region exposed to the electrode surface, for feasible exchange of electrons with the electrode.[33] Despite the pronounced electrochemistry of covalently attached cyt *c* at these layers, SOx had no access to the e^- acceptor site of cyt *c*; thus, intramolecular ET was suppressed and no SOx catalysis was observed with these electrodes upon the addition of SOx and sulfite.

Direct Electrochemistry of Sulfite Oxidase on Alkanethiol-Modified Au Electrodes. The membrane used in the experiments on SOx bioelectrocatalysis mediated by cyt *c* enabled measurements in the restricted volume using essentially high concentrations of the enzyme and the protein. For experiments on direct nonmediated electrochemistry of SOx, the same approach was used as well to achieve the possibly higher concentration of SOx in contact with the electrode surface.

Initial experiments with SOx entrapped between bare Au electrodes and the permselective membrane failed to reveal any redox activity of SOx in the SOx|Au electrode system. We suggest that specific macromolecular interactions, similar to those prevailing in the course of natural biocatalysis, are necessary for the enzyme to approach the electrode and to adsorb productively for an efficient direct ET reaction. To simulate the natural conditions of biocatalysis, the Au surface was modified with various alkanethiol SAMs bearing NH_2, COOH, and OH headgroups or without them (1-decanethiol). Variations in the charge and nature of the SAM were expected to provide a specific direct ET orientation of SOx on the SAM-modified Au electrode favorable for efficient heterogeneous ET.

No direct electrochemistry of SOx on the MuD-COOH or MuD-OH/MuD-COOH layers was obtained. It is an anticipated result, since intermolecular ET between cyt *c* and the cyt b_5 domain of SOx implies interactions between the negatively charged deprotonated residues of the SOx cyt b_5 domain and the positively charged lysine residues around the exposed heme of cyt *c*.[27] Thus, positively charged SAM surfaces, specifically, containing (NH_2) headgroups, were expected to provide adsorption of SOx that would favor the direct ET reaction. Nevertheless, no electrochemical activity of the SOx heme domain was attained using only MuD-NH_2. Modification of the Au electrode with a short-length alkanethiol, i.e., cysteamine, however, resulted in pronounced direct electrochemistry of SOx (see Table 1 and Supporting Information), with a value of $E^{0\prime}$ corresponding to a redox transformation of the SOx heme domain.[39,40] Similar results were obtained with MEt-OH and with cysteamine layers "diluted" with MEt-OH. However, as will be discussed below, despite the direct ET exchange between the SOx heme and the electrode surface, no catalysis was observed in these cases. Additionally, the ET process degraded rapidly when the potential was cycled. When changing for a hydrophobic neutral 1-decanethiol SAM, no signs of an ET reaction were obtained. Thus, both electrostatic and hydrophilic surface interactions were supposed to play an important role for electronic communication between SOx and the electrode.

With SOx entrapped between the permselective membrane and a (MuD-OH)-modified Au electrode, a pair of stable well-defined oxidation−reduction peaks was detected within −90 to −150 mV and the peaks were very reproducible upon repetitive cycling. Modifying the Au surface with a mixed MuD-OH/MuD-NH_2 SAM resulted in a significantly higher intensity of the oxidation−reduction process compared with using the MuD-OH SAM (Figure 2A). A linear dependence of the peak current intensity, I_p, on scan rate v supported a surface-confined[41] direct electrochemistry of SOx entrapped under the membrane (Figure 2A, inset). The $E^{0\prime}$ calculated as the average value of the oxidation and reduction peaks corresponded to −120 mV versus Ag | AgCl (KCl_{sat}), correlating fairly well with the previously reported $1e^-$ redox potential for the heme Fe(III/II) couple in SOx obtained through optical redox titration, −146 mV at pH 9.0[40] and −113 mV at pH 7.0,[39] if the data are corrected for the corresponding pH. Upon stripping the membrane SOx still remained in the adsorbed state (Figure 2A). However, the intensity of the redox process decreased, evidently resulting from a partial desorption of the adsorbed enzyme (see the variation in the peak area, Figure 2A inset). The electrochemically active amount of adsorbed SOx M_{SOx} estimated from the slopes b_1 of the $I_p - v$ dependencies[41] ($b_1 = n^2F^2M_{SOx}/4RT$, $n = 1$) therewith changed from 3.87×10^{-14} mol for the membrane entrapped adsorbed SOx to 2.4×10^{-14} mol of adsorbed SOx after membrane stripping.

For the studied SOx | modified Au systems (Table 1), the half-width of the reduction and oxidation peaks of SOx varied from 91 to 125 mV (Figure 2A, inset), which can be correlated with a quasi-reversible $1e^-$ transfer process.[41,42] The areas under the peaks gave the surface coverage of SOx on the studied SAMs, calculated for $n = 1$, where n is the number of electrons transferred (Table 1). As can be seen from Table 1, the nature of the SAM predetermines the amount of adsorbed SOx in direct ET contact with the modified electrode surface, the mixed MuD-

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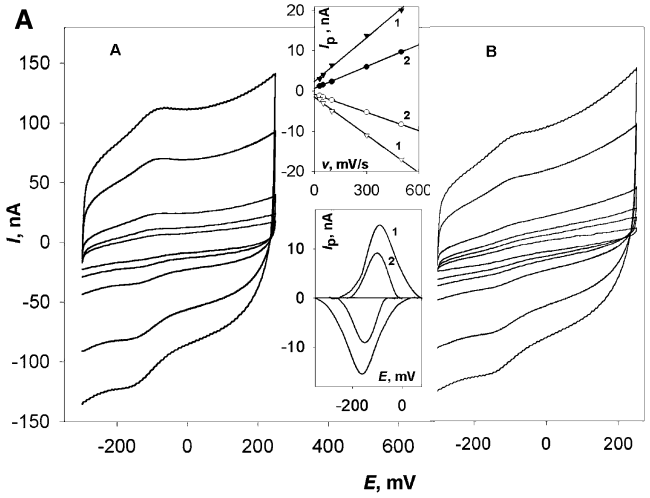

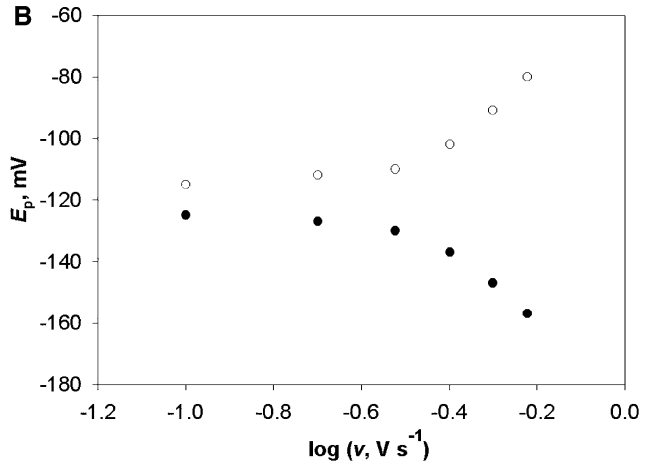

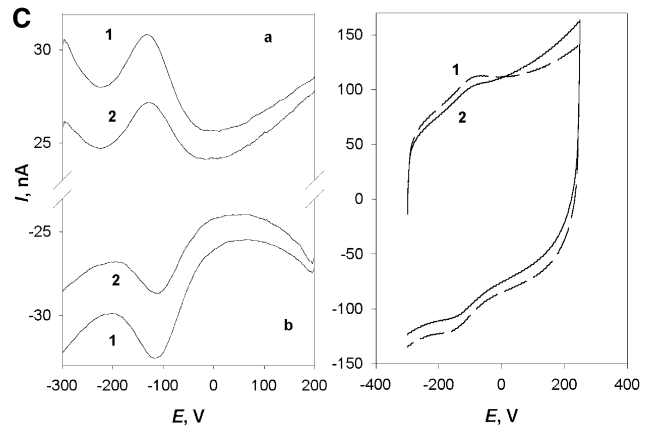

Figure 2. (A) CVs of SOx (A) entrapped between a MuD-OH/MuD-NH_2-modified Au electrode and the membrane and (B) in the absence of the membrane, 0.1 M Tris-HCl, pH 7.4, at scan rate v 500, 300, 100, 50, and 30 mV s^{-1}. Insets: scan rate dependencies of the cathodic and anodic peak intensities and baseline-subtracted CV at 500 mV s^{-1} (1) and the same data upon stripping the membrane (2). (B) Variations of the peak potentials E_p with the logarithmic scan rate (log v) for SOx redox transformations at the MuD-OH/MuD-NH_2-modified Au electrode. (C) DPVs and CVs of SOx on MuD-OH/MuD-NH_2-modified Au electrode (1) under the membrane and (2) without the membrane in 0.1 M Tris-HCl, pH 7.4. DPV: (a) anodic and (b) cathodic direction of scanning, pulse amplitude 25 mV, and pulse width 50 ms, Δt 20 ms, pulse period 200 ms, scan rate v 10 mV s^{-1}. CV: scan rate v = 500 mV s^{-1}.

Table 2. Surface Coverage with SOx Estimated from the Maximal[a] DPV Peak Currents $(i_p)_m$ Corresponding to SOx Redox Transformations on Alkanethiol-Modified Electrodes

modifier	$E^{0\prime}$, V	$(I_p)_m$, nA	Γ, pmol cm^{-2}	k_{cat},[c] s^{-1}
MuD-OH[b]	−124 ± 1	3.09 ± 0.01	0.57 ± 0.03	24.6 ± 0.2
MuD-OH	−125 ± 1	1.34 ± 0.03	0.25 ± 0.01	24.7 ± 3.4[c]
MuD-OH/-MuD-NH_2[b]	−119 ± 1	6.12 ± 0.23	1.15 ± 0.05	20.7 ± 1.7
MuD-OH/-MuD-NH_2	−117 ± 1	4.55 ± 0.10	0.83 ± 0.02	19.9 ± 1.7 [c]

[a] Data were obtained with v = 10 mV s^{-1}, ΔE = 25 mV, τ = 50 ms, sample width 20 ms, and pulse period 200 ms. [b] Experiments were performed with SOx entrapped under the membrane. [c] Calculated with eq 5 and Γ from DPV.

OH/MuD-NH_2 layer significantly increasing the amount of SOx capable of direct ET reaction. Approximately a 2−3-fold increase in the surface coverage with electrochemically active SOx is attained with the mixed SAM compared with the MuD-OH SAM. The peak potentials for direct redox transformation of SOx on the alkanethiol layers varied linearly with a logarithmic scan rate,[42] with a slope of 115 ± 21 and −110 ± 18 mV/decade for the anodic and cathodic processes, respectively (Figure 2B). This corresponds to a kinetically limited heterogeneous ET reaction, with $(1 - \alpha)n$ and αn close to 0.5 (and n = 1). The values of the heterogeneous ET constant, k_s, calculated according to the Laviron theory[42] from the separation of the peak potentials (ΔE_p) at 200−600 mV s^{-1} were in the order of 12−15 s^{-1} for the studied systems and does not principally vary when changing from one alkanethiol system to another (Table 1).

Direct ET of SOx on alkanethiol-modified Au was also studied with DPV to obtain a deeper insight.[43−45] Some registered CVs and DPVs of SOx adsorbed at the alkanethiol-modified Au electrodes, under the membrane and without it, are compared in Figure 2C. The significant increase in the sensitivity of DPV enabled us to monitor small quantities of SOx adsorbed more precisely as well as values of $E^{0\prime}$. The values of $E^{0\prime}$ evaluated as midpoint potentials from DPV agree quite well with values from optical titration[39,40] and CV measurements of SOx (compare Table 1 and Table 2), with a confidence interval of ±1 mV. As can be seen from the DPV data (Table 2), the $E^{0\prime}$ of SOx at the MuD-OH/MuD-NH_2 layer is slightly shifted to more positive values compared with the MuD-OH SAM (5 mV), which can be expected from the more positive character of the first one. The surface coverage of SOx was calculated using the approach proposed by Brown and Anson,[44] but assuming the absence of destabilizing interactions between the oxidizing/reducing species in the adsorbed layer, according to

$$\Gamma = 4RT(I_p)_{max}\tau e/(\Delta E n^2 F^2) \qquad (5)$$

where $(I_p)_{max}$ is the maximal detected DPV peak current at a fixed sampling time, the experimental conditions for $(I_p)_{max}$ being

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optimized by selecting the scan rate and the potential pulse amplitude, τ is the pulse width, ΔE is the pulse amplitude, and e equals 2.718. The estimated surface concentrations of SOx adsorbed on the different alkanethiols, with and without membrane, using eq 5, are presented in Table. 2. As seen when the amounts of SOx on the different surfaces were compared, the DPV data at the mixed SAM confirmed a higher amount of adsorbed enzyme in favorable orientation for direct ET. Additionally, the entrapment under the membrane increased the amount of the surface-"bound" SOx molecules, likely providing a special microenvironment by the constant excess of SOx in the vicinity of the electrode. The data obtained from integrating the area under the peak in the CVs obtained with the same electrode correlate well with the data of DPV processed with eq 5. However, a significant dependence of the CV data on the form of the background subtracted, which should be properly chosen, leads in some cases to overestimated values of Γ.

Thus, the appropriate choice of the alkanethiol | Au electrode system enables an efficient direct ET reaction between the electrode and the heme active site of SOx, at potentials corresponding to the redox transformation of the heme domain, i.e., −120 mV versus Ag | AgCl. The nature of the alkanethiol layer predetermines the amount of properly adsorbed SOx for direct electronic communication with the modified electrode surface, which was found to vary from 0.26 to 1.3 pmol cm^{-2} (minimal and maximal detected values). However, the ET rate constant k_s for the ET reaction between the heme of SOx and the electrode does not principally differ for the studied alkanethiol systems, ranging from 12 to 15 s^{-1}.

Oxidation of Sulfite Catalyzed by Sulfite Oxidase Adsorbed on Alkanethiol-Modified Au Electrodes. When a saturation concentration of sulfite (174K_m, K_m of 19.1 ± 1.6 μM at pH 8.0[17]) was added, the CVs of the SOx adsorbed on (MuD-OH/MuD-NH_2)- and (MuD-OH)-modified Au electrodes transformed into oxidative catalytic waves (seen in Figure 3A,B) at potentials similar to the redox transformation of SOx in the absence of the substrate. In the absence of SOx, the studied SAMs totally suppress sulfite oxidation in the potential range involved. At low scan rates (in the order of 30 mV s^{-1} and lower) in the presence of 3.3 mM sulfite, the catalytic signal converts to a sigmoid wave with a limiting current independent of stirring the solution (Figure 3A). With a further increase of the concentration of sulfite, no increase in the response was observed. A more efficient catalysis, characterized by a higher catalytic current plateau, was achieved with SOx entrapped under the membrane (Figure 3A,B), as well as with SOx immobilized on the mixed SAM, evidently correlated with higher surface concentrations of the enzyme (Figure 3C). As the coverage was low, and the concentration of the substrate was more than 100 times higher than the K_m value (thus presenting saturation conditions with substrate), the catalytic properties of the enzyme were expected to determine the waveform of the CVs (Figure 4). Under these conditions, the limiting current i_{lim} may be related directly to the turnover rate constant k_{cat}:

$$k_{cat} = i_{lim}/(\Gamma_{SOx} AnF) \qquad (6)$$

The values of k_{cat} were calculated from the values of the catalytic currents at 250 mV (Figure 4), taking into account the correspondingly different surface concentrations of electrochemically active SOx on the different SAMs (Tables 1 and 2). k_{cat} was in the order of 18−20 and 24−25 s^{-1} for SOx immobilized on mixed MuD-OH/MuD-NH_2 and MuD-OH layers, respectively (data presented in Tables 1 and 2), thus revealing a similar efficiency of bioelectrocatalytic oxidation of sulfite on the studied surfaces, despite the different surface coverage with electrochemically active SOx. Still it was interesting to compare these values with the catalytic activity of SOx with its natural redox partner cyt *c*, obtained with the same SOx sample in the batch experiments. k_{cat}, characterizing direct ET-based bioelectrocatalysis, are 2-fold less than that obtained spectrophotometrically (44 s^{-1} at pH 7.4) and 4-fold less than the pseudo-first-order rate constant k_f for the reaction between cyt *c* and SOx (91.6 ± 8.2 s^{-1}). Thus, the rates of sulfite oxidation catalyzed by SOx in direct ET reaction with the modified Au electrode were lower but comparable with that achieved with cyt *c* as a redox partner, thus indicating a good mimicking of the biological SOx redox partner by the surface of the modified electrodes. We were looking for reasons for the lower efficiency of direct ET-based bioelectrocatalysis compared to SOx catalysis with cyt *c*. It could be connected both with the rate-limiting heterogeneous ET reaction and with a lower efficiency of intramolecular ET in SOx adsorbed at the electrode surface; the mutual dependence of these ETs and catalysis should be kept in mind as well.

Comparable values for the heterogeneous ET constant k_s (close to 15 s^{-1}) and the values of k_{cat} (on the order of 18−25 s^{-1}) allowed considering the catalysis with a rate-determining heterogeneous ET step. Considering SOx catalysis with soluble mediators, the intramolecular ET was shown to be fast and to depend essentially on the composition of the solution.[14−16] Rate constants for the intramolecular ET in SOx were reported to change from 2400 to 60 s^{-1} (20 mM Tris, pH 7.0 and 9.0, respectively).[16] The effect of reversible anion inhibition (with Cl^-, SO_4^{2-}, and phosphates) revealed a decrease in the intramolecular ET rate from 1500 to less than 100 s^{-1}, at pH 6.0 and 7.0.[16] The dependence of intramolecular ET on the ionic strength was clearly shown in Tris buffer solutions, pH 7.7,[14] and under these conditions the ET rates were still well above 1000 s^{-1}. We studied the bioelectrocatalysis under optimal solution composition when the intramolecular ET rates should be incomparably high with respect to the observed heterogeneous ET (2400−1000 vs 15 s^{-1}). Apparently, the enzyme adsorption might affect the intramolecular ET rate; however, we suppose that the oxidation of SO_3^{2-} catalyzed by the adsorbed SOx was limited by the slow heterogeneous ET reaction. This was verified by experiments on the inhibition of intramolecular ET in the presence of sulfate and phosphate anions (solutions were adjusted to the same pH). If the intramolecular ET rates were limiting, a decrease in the observed catalytic currents could be observed. However, the catalytic responses due to sulfite oxidation in 0.1 M Tris, pH 7.4, remained the same upon addition of the inhibitors, supporting the suggestion of a kinetically slow heterogeneous ET reaction of SOx at alkanethiol-modified Au electrodes.

If the heterogeneous ET between SOx and the modified electrode is the rate-limiting step in the catalytic oxidation of

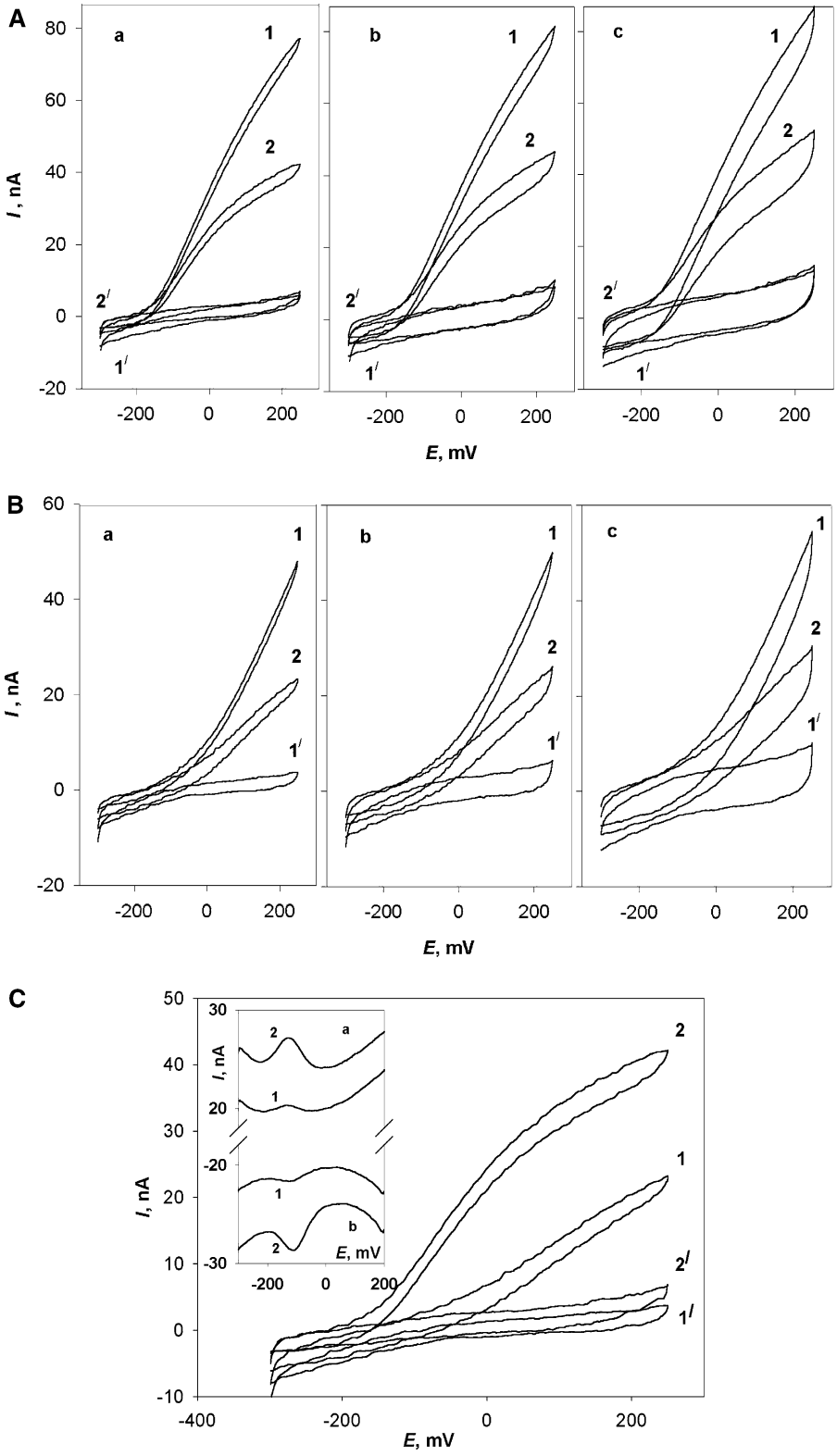

Figure 3. (A) Catalytic CVs of SOx adsorbed at MuD-OH/MuD-NH_2-modified Au (1) under the membrane and (2) without the membrane, in the presence of 3.3 mM Na_2SO_3 in 0.1 M Tris-HCl, pH 7.4; (1′), (2′) corresponding noncatalytic CVs in the absence of sulfite; scan rates v (a) 2, (b) 5, and (c) 10 mV s^{-1}. (B) Catalytic CVs of SOx adsorbed at MuD-OH-modified Au (1) under the membrane and (2) without the membrane, in the presence of 3.3 mM Na_2SO_3 in 0.1 M Tris-HCl, pH 7.4; (1′) corresponding noncatalytic CVs in the absence of sulfite; scan rates v (a) 2, (b) 5, and (c) 10 mV s^{-1}. (C) Catalytic CV of SOx adsorbed (1) at MuD-OH-modified Au and (2) at a mixed-thiol layer (MuD-OH/MuD-NH_2)-modified Au electrode in the presence of 3.3 mM Na_2SO_3; (1′), (2′) corresponding noncatalytic CVs in the absence of sulfite; 0.1 M Tris-HCl, pH 7.4, and $v = 2$ mV s^{-1}. Inset: differential pulse voltammetry in the absence of sulfite, (a) anodic and (b) cathodic direction of scanning, pulse amplitude 25 mV, pulse width 50 ms, Δt 20 ms, pulse period 200 ms, and scan rate 10 mV s^{-1}. All the rest conditions and notations as in the main figure.

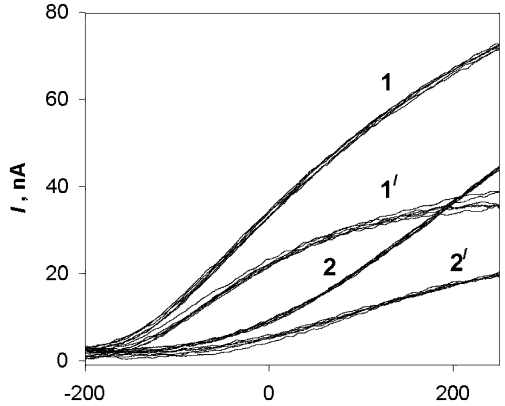

Figure 4. Background-subtracted catalytic CVs of SOx adsorbed (1, 1′) at the (MuD-OH/MuD-NH_2)- modified Au electrodes and (2, 2′) at the MuD-OH-modified Au electrodes in the presence of 3.3 and 6.6 mM Na_2SO_3; (1, 2) in the presence and (1′, 2′) in the absence of membrane; 0.1 M Tris-HCl, pH 7.4, and scan rates 2 and 10 mV s^{-1}.

sulfite, then there is a question of why there was no catalysis observed with the short-length alkanethiol layers, despite the pronounced direct ET communication between the electrode and the enzyme. Another question is why, despite the direct electrochemistry of SOx on cysteamine-, MuD-OH-, and MuD-OH/MuD-NH_2-modified electrodes, no direct electrochemistry of SOx was observed on MuD-NH_2 layers. Reductive desorption[21] of the studied alkanethiol layers at −1.1 to −1.4 V (in 0.5 M KOH) gave virtually the same surface coverage for MuD-OH, MuD-NH_2, and mixed MuD-OH/MuD-NH_2 layers ($9.30 \pm 0.5 \times 10^{-10}$ mol cm^{-2}, the coefficient for a surface roughness of 1.2 being used). In accordance with previously reported data,[21] this value corresponds to a closely packed monolayer of alkanethiol on polycrystalline Au. Thus, modification of Au with MuD-OH or mixed MuD-OH/MuD-NH_2 was supposed to optimize the charge distribution/hydrophobic−hydrophilic interactions between SOx and the SAM, providing the necessary conditions for an efficient direct ET reaction, i.e., adsorption and orientation of the SOx molecules. However, that does not clarify the absence of direct ET reaction of SOx on the positively charged MuD-NH_2 SAM and, as a result, no bioelectrocatalytic activity of SOx on this SAM. Additionally, no catalytic activity was attained with SOx on cysteamine- and MEt-OH-modified Au electrodes, despite the observed direct ET reaction between the electrode and the SOx heme domain and this ET process degraded rapidly (within 20−40 cycles), contrary to direct electrochemistry of SOx on long-chained alkanethiol layers.

Evidently, two factors play a role in the observed phenomena, the ET distance and the effect of the metal electrode surface, pronounced in the case of short alkanethiols (they form relatively less ordered SAM at the electrode surface compared to long-chain ones, and thus the underlying metal is not well shielded). Ideally, an exponential decay of the ET rate constant with the number of methylenes in the alkyl chain should be observed.[21,46−48] For example, an increase in the ET distance for ferrocenes tethered at the ends of alkanethiols from five C to nine C decreases the ET rate constant from 1.6×10^6 to 1.1×10^4 s^{-1}; therewith the ET rates for seven C and less are so high that they cannot be adequately measured by chronoamperometry.[48] However, the heterogeneous rate constants for SOx ET calculated for short- and long-chain alkanethiols have close values, demonstrating no evident dependence on the ET distance through the alkanethiol SAMs. Thus, it may be supposed that the adsorption of SOx on the cysteamine, MuD-NH_2, and MEt-OH provides a longer ET pathway within the enzyme structure and that this orientation of SOx at the electrode surface excludes the possibility for catalysis. To estimate the variation in ET distance within the enzyme for a supposedly different orientation of SOx on different SAMs, we used the approach previously developed for ET processes in cyt *c* and cyt *c* adsorbed on alkanethiols.[49−53] Approximate calculations of ET distances in SOx adsorbed on cysteamine- and MuD-OH/MuD-NH_2-modified electrodes (for both cases, an ET rate constant of 15 s^{-1} was used) gave an additional ET distance equivalent to 11 carbons (11 C) within the SOx structure to the 2 C present in the cysteamine layer and an additional 1 C for the 11 C of the MuD-OH or MuD-OH/MuD-NH_2 SAMs. That means that the amine headgroup as well as the vicinity of the electrode (inefficient shielding from the metal surface achieved with the MEt-OH is anticipated) provides an orientation and a conformation of SOx different from that observed with the long-chained MuD-OH or MuD-OH/MuD-NH_2 layers. In the case of a long-chained alkaneamine, it is logic to suppose the same SOx orientation as with cysteamine, which implies an ET distance of the 11 C of the aminealkanethiol and additionally further a distance equivalent to 11 C within the SOx structure. In this case, the ET should be sluggish and difficult to detect by the techniques used. Thus, the highly developed specificity of the interactions between SOx and its redox partner implies that the ET reactions of SOx are kinetically sluggish in the case when the electrode surface does not resemble the natural redox partner: specific molecular interactions between the electrode and the enzyme surfaces are necessary for the protein to approach the electrode productively in a correct orientation for direct ET. Of the investigated SAMs, only the slightly polar/positive character of the MuD-OH and the mixed MuD-OH/MuD-NH_2 SAMs provides specific interactions between the modified Au electrode and SOx, including electrostatic compatibility of the surfaces and a proper orientation for fast ET and for an efficient bioelectrocatalysis of sulfite oxidation with SOx.

Analytical Application for the Analysis of Sulfite. The potential analytical application of direct ET-based bioelectrocatalytic oxidation of of SO_3^{2-} by SOx was studied with CV (Figure 5). The variation of the catalytic current with the concentration of SO_3^{2-} is presented in the inset of Figure 5, at 100 and 200 mV. The variation of the signal is more pronounced at 200 mV; however, at both potentials, the catalytic currents gave a substrate

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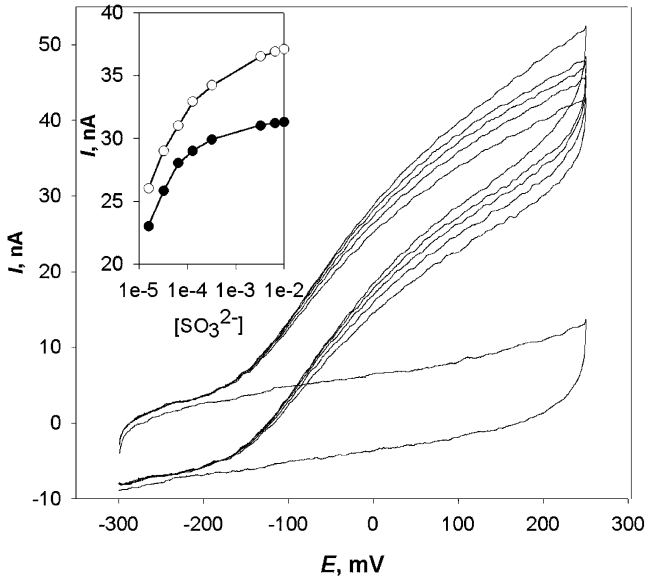

Figure 5. Catalytic CVs of SOx adsorbed at MuD-OH/MuD-NH_2-modified Au, in the absence and upon the addition of 16, 33, and 66 μM and 0.132, 0.33, and 3.3 mM Na_2SO_3 in 0.1 M Tris-HCl, pH 7.4; scan rate v 10 mV s^{-1}. Inset: the concentration dependence of the current intensity upon subtraction of the background, at 100 (●) and 200 mV (○).

concentration dependence with a linear calibration curve between 10 and 100 μM SO_3^{2-}. For concentrations of sulfite higher than 3 mM, the current leveled off indicating a substrate-saturated regime. Thus, the studied system points to the development of an amperometric biosensor for SO_3^{2-} based on direct ET in contrast to the current SO_3^{2-} biosensors all based on a mediated approach.[8−11,54]

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CONCLUSIONS

Direct ET coupling between Au electrodes and the heme domain of SOx was achieved with alkanethiol-modified Au electrodes, at potentials corresponding to the redox transformations of the heme of SOx, −120 mV versus Ag | AgCl, with a heterogeneous ET rate constant k_s in the order of 15 s^{-1}. The nature of the alkanethiol headgroups was shown to drastically affect both the direct ET reaction of the SOx heme and the conformation-dependent catalytic activity of SOx. Modification of the electrode surface with either MuD-OH or mixed MuD-OH/MuD-NH_2 SAMs provided the necessary molecular interactions between the surface of the electrode and SOx, which resulted in an efficient intramolecular ET from the Mo active site, reduced in the presence of sulfite, to the heme. The efficiency of adsorbed SOx to bioelectrocatalytically oxidize sulfite (k_{cat} ∼20 s^{-1} at pH 7.4) was comparable with the efficiency of the enzymatic oxidation in solution with cyt *c* as a second substrate (k_{cat} of 44 s^{-1} at pH 7.4). In general, the above-described methodology may be applicable to other multicofactor-containing membrane-bound and intermembrane enzymes and proteins as well as for the development of a third-generation biosensor for SO_3^{2-}.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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