analytical chemistry

Article

pubs.acs.org/ac

Low-Potential Amperometric Enzyme Biosensor for Xanthine and Hypoxanthine

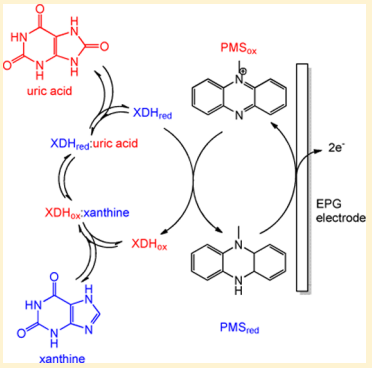
Palraj Kalimuthu,[†] Silke Leimkühler,[‡] and Paul V. Bernhardt*[,†]

[†]School of Chemistry and Molecular Biosciences, University of Queensland, Brisbane, 4072, Australia

[‡]Institut für Biochemie und Biologie, Universität Potsdam, 14476 Potsdam, Germany

Supporting Information

ABSTRACT: The bacterial xanthine dehydrogenase (XDH) from *Rhodobacter capsulatus* was immobilized on an edge-plane pyrolytic graphite (EPG) electrode to construct a hypoxanthine/xanthine biosensor that functions at physiological pH. Phenazine methosulfate (PMS) was used as a mediator which acts as an artificial electron-transfer partner for XDH. The enzyme catalyzes the oxidation of hypoxanthine to xanthine and also xanthine to uric acid by an oxidative hydroxylation mechanism. The present electrochemical biosensor was optimized in terms of applied potential and pH. The electrocatalytic oxidation response showed a linear dependence on the xanthine concentration ranging from 1.0×10^{-5} to 1.8×10^{-3} M with a correlation coefficient of 0.994. The modified electrode shows a very low detection limit for xanthine of 0.25 nM (signal-to-noise ratio = 3) using controlled potential amperometry.



Xanthine is an intermediate of the purine nucleotide and deoxynucleotide metabolism and present in most of our body tissues and fluids.[1] The normal concentration of xanthine in human plasma ranges from 0.5 to 2.5 μM (40−160 μM in urine).[2] As the metabolic precursor of uric acid, xanthine is the first indicator of an abnormal purine profile and can serve as a marker of many diseases including hyperuricemia,[3] gout,[4] xanthinuria,[5] perinatal asphyxia,[6] cerebral ischemia,[7] tumor hyperthermia,[8] and pre-eclampsia.[9] Uric acid is typically present at serum concentrations of ∼200−400 μM and is by far the dominant purine of this class in biological fluids.[10−15] Hypoxanthine (Scheme 1) is the precursor to xanthine and is typically present at plasma concentrations of ∼10−30 μM in healthy humans.[15] In xanthinuria, the concentrations of xanthine are elevated and similar to that of hypoxanthine in serum and in urine. The accumulation of xanthine is at the expense of uric acid whose concentrations are very low.[2]

In the food industry xanthine is also an important biomarker. Inosine monophosphate (present in fresh fish samples) degrades (by microbial action or the action of other endogenous enzymes) to inosine, then to hypoxanthine and xanthine. Elevated levels of hypoxanthine and xanthine are a sign of spoilage.[16] Thus, developing a stable, sensitive, and selective hypoxanthine/xanthine sensor may also have applications in food quality control.

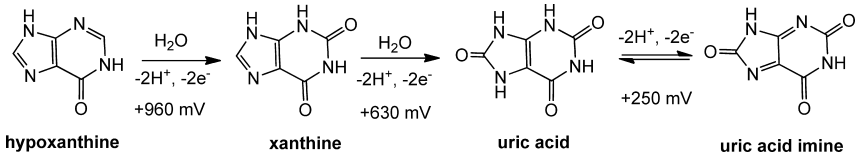
The most common methods for detecting and quantifying purines from this class are electrophoresis,[13] GC/MS,[17] HPLC,[14] chemiluminescence,[18] and UV spectrophotometry.[19] However, these methods are time-consuming, require expensive equipment, and are labor-intensive in terms of sample preparation. By comparison, electrochemical methods offer simplicity, portability, high sensitivity, and selectivity and can be applied to samples without any pretreatment. Several chemically modified electrodes have been employed for the determination of xanthine including polymer films,[20] pretreated carbon paste,[12] nanoporous carbon fiber,[21] preanodized nontronite-coated carbon,[22] and multiwall carbon nanotube composite[23] modified electrodes. Usually these chemically modified electrodes oxidize xanthine above +600 mV versus Ag/AgCl (Scheme 1), but at this potential, interference from other electroactive compounds present in biological samples limits the practicality of this approach.

Xanthine oxidoreductase, encompassing both xanthine oxidase (XO) and xanthine dehydrogenase (XDH), is a complex molybdo/iron−sulfur/flavoenzyme found in bacteria and animals.[24] The enzyme catalyzes the oxidative hydroxylation of purines, pyrimidines, pterins, and aldehyde substrates using molecular oxygen (XO) or NAD^+ (XDH) as electron acceptor.[24,25] The two principal substrates are xanthine and hypoxanthine (Scheme 1). Both XO and XDH contain a Mo active site comprising a single bidentate molybdopterin ligand, an equatorial terminal sulfido, an axial oxido, and an equatorial hydroxido/aqua ligand depending on pH.[26−28]

The most intensively studied xanthine oxidoreductase is bovine milk XO, which is commercially available. Similar to other oxidase enzymes, when dioxygen is the electron acceptor, H_2O_2 is a product of XO turnover.[29] The electroactivity of H_2O_2 enables its voltammetric detection and provides an indirect method for monitoring XO turnover. Alternatively, the enzyme horseradish peroxidase may be incorporated to

Received: August 30, 2012
Accepted: November 7, 2012
Published: November 7, 2012

Scheme 1. Redox Reactions and Potentials (mV vs Ag/AgCl, pH 8) of Purines in This Work



catalytically reduce the product H_2O_2 to water (mediated by a coimmobilized electron-transfer relay).[30] Several XO-based electrochemical biosensors have been reported where XO is immobilized within various supporting matrixes including polypyrrole,[31] graphite (mediated by ferricyanide),[32] osmium poly(vinylpyridine) gel polymer,[30] silk fibroin/cellulose acetate membranes,[33] gold nanoparticles,[34] multiwall carbon nanotubes,[35] calcium carbonate nanoparticles,[36] poly(mercapto-*p*-benzoquinone),[37] layered double hydroxides,[38] and Nafion[39] modified electrodes. However, these sensors operate at high overpotentials (>+0.20 V vs Ag/AgCl). In the detection of xanthine, this is particularly important as the product of xanthine oxidation, uric acid, is also oxidized at this potential (Scheme 1). Other species such as catecholamines and ascorbic acid are also electroactive in this region.

On the other hand, XDH from *Rhodobacter capsulatus* has very low reactivity with O_2 and preferentially uses NAD^+ as an electron acceptor.[40] In a recent paper we showed that *N*-methyl phenazinium methanesulfonate (phenazine methosulfate, PMS) is an effective substitute for NAD^+ and can mediate electrons between XDH and a thiol-modified Au working electrode.[41] Moreover, its redox potential (ca. −160 mV vs Ag/AgCl, pH 8) is very low, so interference from the oxidation of other compounds at the electrode is avoided. In the presence of either xanthine or hypoxanthine, pronounced catalytic currents were observed at the redox potential of PMS.[41]

In earlier papers,[42,43] we observed that XDH adsorbed on an edge-plane pyrolytic graphite (EPG) electrode shows pronounced electrocatalytic activity (for the oxidation of xanthine to uric acid) even in the absence of an added mediator. It is now understood[43] that uric acid (the product of this catalytic reaction) acts as a mediator of catalysis, being oxidized at ca. +250 mV versus Ag/AgCl (pH 8, Scheme 1) in an unusual enzyme electroautocatalytic mechanism. In terms of a sensing application, the simultaneous oxidation of both xanthine (enzymatically) and uric acid at the electrode in this system makes unique determination of xanthine impractical as the observed current is a composite of both processes. Indeed similar behavior was seen for the XDH-catalyzed oxidation of hypoxanthine to xanthine, so the catalytic current in that case was due to the oxidation of three molecules concurrently: hypoxanthine and xanthine by XDH and uric acid at the electrode. On this point, uric acid is present in biological fluids at concentrations ∼10−100 times higher than the xanthine. Therefore, interference from uric acid oxidation is a chronic issue in any practical electrochemical hypoxanthine/xanthine sensor unless the operating potential can be lower than that of the uric acid redox couple.

In the present study, we have developed an electrochemical hypoxanthine/xanthine biosensor based on *R. capsulatus* XDH immobilized on an EPG electrode. To avoid uric acid interference, which is oxidized at +250 mV versus Ag/AgCl, we have employed the mediator PMS. The present electrochemical biosensor has been optimized in terms of pH and applied potential.

EXPERIMENTAL SECTION

Materials. Xanthine dehydrogenase was purified from a heterogeneous expression system in *E. coli* as previously described.[28] Xanthine, hypoxanthine, uric acid, and phenazine methosulfate were purchased from Aldrich and were used as received. All other reagents used were of analytical grade purity and used without any further purification. All solutions were prepared in purified water (Millipore, resistivity 18.2 MΩ·cm). Phosphate buffer solution (PBS) was prepared using 100 mM Na_2HPO_4 and NaH_2PO_4, and the desired pH was obtained with dilute H_3PO_4 or NaOH.

Electrochemical Measurements and Electrode Cleaning. Cyclic voltammetry (CV) and controlled potential amperometry were carried out with a BAS 100B/W electrochemical workstation using a three-electrode system consisting of an EPG working electrode, a platinum wire counter electrode, and a Ag/AgCl reference electrode. Unless otherwise stated, electrochemical solutions were purged with nitrogen for at least 30 min prior to the series of experiments and all experiments were performed under a blanket of nitrogen. The EPG electrode surface was cleaned by cleaving several 1 μm layers from the face of the electrode with a microtome followed by sonication in Milli-Q water for 15 min. No abrasives were used. No other electrode surface conditioning was necessary, and no promoters were used. All CV measurements were made with a cell that was protected from light to avoid photochemical degradation of the PMS mediator.

Enzyme Electrode Preparation. A 6 μL droplet of XDH (66 μM) was transferred to a freshly prepared, inverted EPG electrode, and this was allowed to dry to a film at 4 °C. To prevent protein loss the electrode surface was carefully covered with a perm-selective dialysis membrane presoaked in water (MW cutoff 12 kDa). The dialysis membrane was pressed onto the electrode using a Teflon cap and fastened to the electrode with a rubber O-ring to prevent leakage of the internal membrane solution. The resulting modified electrode was stored at 4 °C in 100 mM phosphate buffer solution (pH 7.0) when not in use.

Electrochemical Measurements and Data Processing. The variation of the observed limiting catalytic current (i_{lim}) as a function of substrate concentration ([S]) followed Michaelis−Menten kinetics, and the data were fit to the eq 1 which is applicable to steady-state enzyme kinetics and where the concentration of substrate [S] is constant. The presence of a membrane covering the electrode inhibits diffusion of the substrate to the enzyme, so steady-state conditions are not strictly met, but the catalytic current does in fact saturate at high substrate concentrations (vide infra Figures 3B and 4B). For these reasons though, the apparent Michaelis constant ($K_{M,app}$) will reflect the diffusion-limited mass transport of substrate to the enzyme:

$$i_{lim} = \frac{i_{max}[S]}{[S] + K_{M,app}} \quad (1)$$

dx.doi.org/10.1021/ac3025027 | *Anal. Chem.* 2012, 84, 10359−10365

where i_{max} is the saturation limiting current and $K_{M,app}$ is the apparent Michaelis constant.

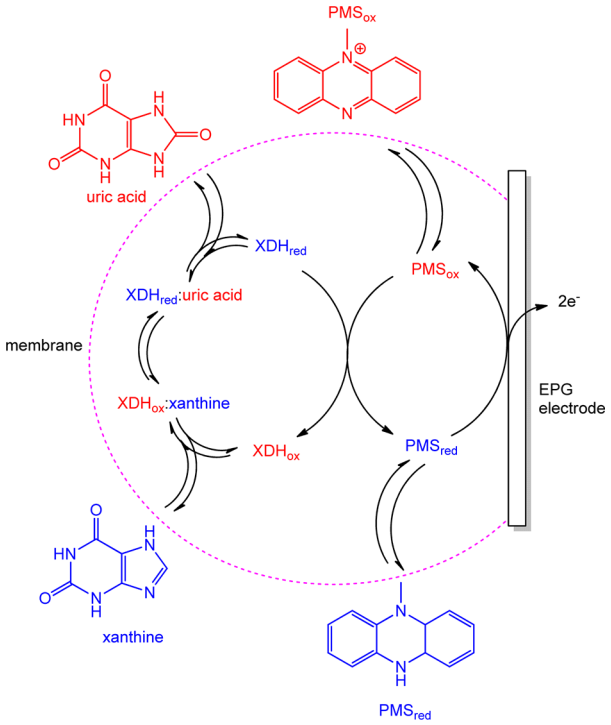
The pH dependence of the catalytic current was modeled by eq 2 which is applicable for an active form of the enzyme that is reversibly deactivated by either deprotonation (at pK_{a1}) or protonation (at pK_{a2}).

$$i_{lim}(pH) = \frac{i_{opt}}{1 + 10^{(pH-pK_{a1})} + 10^{(pK_{a2}-pH)}} \quad (2)$$

RESULTS AND DISCUSSION

Mechanism. The catalytic electrochemical reaction is a ternary system comprising XDH, substrate (xanthine or hypoxanthine), and mediator (PMS). In the absence of any of these three components no catalysis can occur. Scheme 2 summarizes the overall coupled electrochemical and chemical reactions that are involved in this process. The membrane shown in Scheme 2 traps the enzyme XDH but permits diffusion of xanthine and PMS from the bulk solution to the electrode and also allows uric acid to diffuse away from the enzyme. The kinetics of these various steps have been discussed in detail in a recent publication.[41]

Scheme 2



Catalytic Voltammetry. The response of bare EPG and XDH-modified EPG electrodes toward 800 μM xanthine in the presence of 5 μM PMS was investigated, and the results are displayed in Figure 1. In the control experiment, at a bare EPG electrode, PMS (5 μM, pH 7) shows a reversible redox wave at −139 mV with a peak-to-peak separation of 47 mV along with a weaker response at −268 mV (Figure 1A, red curve a). Two redox waves at −139 and −268 mV were attributed to the diffusional and adsorbed PMS responses, respectively, at the EPG electrode.[44] This was established by the sweep rate dependence of these responses where the lower potential peak currents increased linearly with sweep rate while the higher potential peaks increased with the square root of sweep rate.[45] No change was found upon addition of 800 μM xanthine to the same solution (Figure 1A, blue curve b), illustrating that xanthine is electroinactive within this range and does not react with PMS$_{ox}$. The EPG/XDH electrode also shows a similar response for 5 μM PMS in the absence of xanthine (Figure 1B, red curve a; note different vertical scale). In this case the capacitive current is somewhat smaller.

On addition of xanthine (800 μM) to the cell the EPG/XDH electrode shows a pronounced anodic current and loss of the corresponding cathodic peak (Figure 1B, blue curve b). The enhancement of anodic current and asymmetry of the waveform are indicative of a catalytic (EC_{cat}) mechanism. The potential coincides with that of the PMS$_{ox/red}$ couple as this compound is the only electroactive species present within this potential window. Briefly, the anodic sweep switches on catalysis as the mediator PMS$_{red}$ is converted to its active PMS$_{ox}$ form which may accept electrons from XDH$_{red}$ after turnover (Scheme 2) regenerating PMS$_{red}$ for further electrochemical oxidation. The XDH chemical reaction comprises hydroxylation of xanthine to uric acid. The XDH$_{red}$/PMS$_{ox}$ reaction is an outer-sphere electron-transfer reaction.

We have also investigated the response of EPG/XDH toward uric acid in the presence of 5 μM PMS (Supporting Information Figure S1). This is relevant because uric acid is the product of XDH turnover and also a potential electroactive

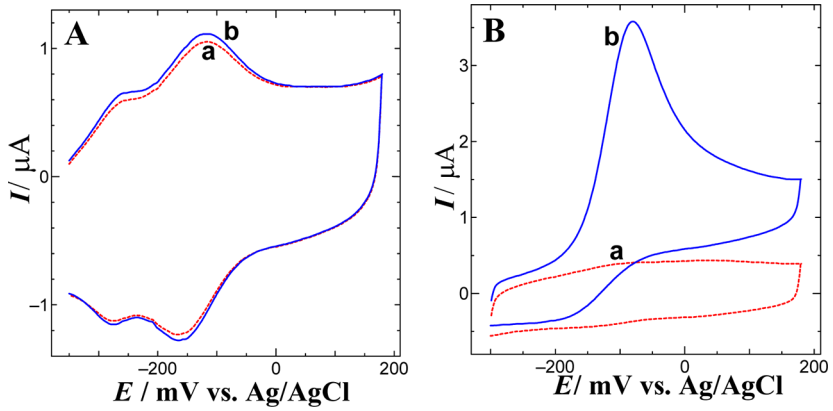


Figure 1. CVs obtained for 5 μM PMS in the (a) absence and (b) presence of 800 μM xanthine at (A) bare EPG (no XDH) and (B) the EPG/XDH electrode [100 mM phosphate buffer (pH 7), sweep rate of 5 mV s^{-1}].

dx.doi.org/10.1021/ac3025027 | Anal. Chem. 2012, 84, 10359−10365

interfering species due to its high concentrations in biological fluids and low redox potential. However, the EPG/XDH electrode does not show any electrochemical response toward uric acid within the potential window of −350 to +180 mV versus Ag/AgCl, so the observed catalytic current is exclusively due to the PMS-mediated XDH/xanthine reaction.

PMS Concentration Dependence. Figure 2 illustrates the CVs obtained for various PMS concentrations in the presence

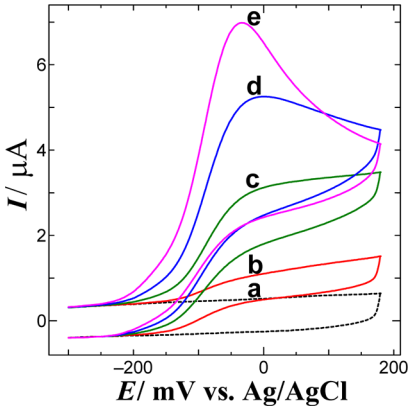


Figure 2. CVs obtained for the EPG/XDH electrode with 1 mM xanthine at varying PMS concentrations of (a) 0, (b) 2, (c) 4, (d) 6, and (e) 8 μM in 100 mM phosphate buffer solution at a scan rate of 5 mV s^{-1}.

of 1 mM xanthine at the EPG/XDH electrode. In the absence of PMS, no faradaic response is seen within the potential window of −300 to +180 mV (Figure 2, broken curve a). As PMS is added a pronounced sigmoidal oxidation wave emerges (Figure 2, curves b and c) which is indicative of an electrochemical steady state where PMS_{ox} is consumed by XDH_{red} at the same rate as it is replenished at the electrode (Scheme 2). At higher bulk concentrations of PMS (Figure 2, curves d and e) the waveform becomes peak-shaped (transient voltammetry) because the XDH_{red}/PMS_{ox} reaction overruns the XDH_{ox}/xanthine reaction and the concentration of PMS_{ox} at the electrode surface accumulates thus establishing a time-dependent diffusion layer.

Xanthine Concentration Dependence. The xanthine concentration dependence of the voltammetry in the presence of 5 μM PMS is shown in Figure 3. The main catalytic anodic peak current at −70 mV versus Ag/AgCl increases linearly with xanthine concentration within the range of 25 μM to 1 mM and then nonlinearly at higher concentrations before saturating at around 7.3 mM as predicted by Michaelis−Menten kinetics ($K_{M,app}$ 1.0 ± 0.1 mM, Figure 3B). This value is much higher than the previously reported value (K_M = 64.5 μM) for an XDH solution assay at pH 7.8 in 50 mM Tris buffer solution.[46] This increase is most likely a consequence of inhibited mass transport of xanthine from the bulk solution across the membrane to the reaction layer and restricted access to the enzyme active site within a protein film at the working electrode surface.

Hypoxanthine Concentration Dependence. We also investigated the electrocatalytic response of the EPG/XDH electrode toward hypoxanthine under identical experimental conditions as those in Figure 3, in fact using the exact same EPG/XDH electrode to eliminate any variations in XDH concentration or electrode surface area, and the results are shown in Figure 4A. After the xanthine data were acquired (see

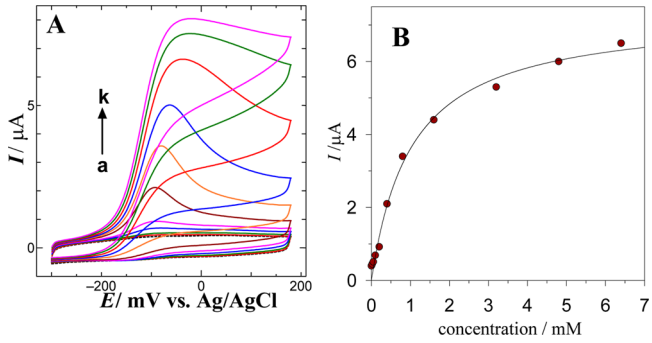


Figure 3. (A) CVs obtained for 5 μM PMS at the EPG/XDH electrode at varying xanthine concentrations of (a) 0, (b) 20, (c) 50, (d) 100, (e) 200, (f) 400, (g) 800, (h) 1600, (i) 3200, (j) 4800, and (k) 6400 μM in 100 mM phosphate buffer solution at a scan rate of 5 mV s^{-1}. (B) Plot of the electrocatalytic anodic current at −90 mV vs Ag/AgCl as a function of xanthine concentration in panel A. The curve is from data fit to eq 1 [$K_{M,app}$ (xanthine) 1.0 ± 0.1 mM].

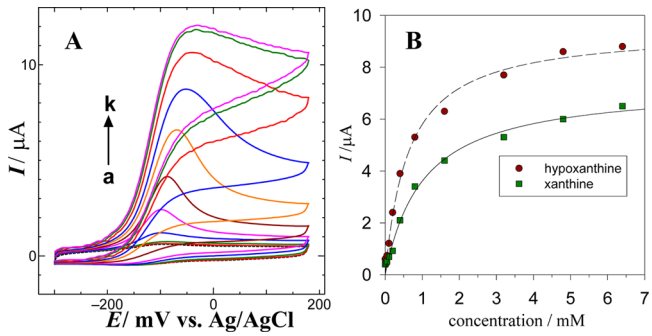


Figure 4. (A) CVs obtained at the EPG/XDH electrode for 5 μM PMS at varying hypoxanthine concentrations of (a) 0, (b) 20, (c) 50, (d) 100, (e) 200, (f) 400, (g) 800, (h) 1600, (i) 3200, (j) 4800, and (k) 6400 μM in 100 mM phosphate buffer solution at a scan rate of 5 mV s^{-1}. (B) Plot of the electrocatalytic anodic currents at −90 mV vs Ag/AgCl as a function of both hypoxanthine and xanthine concentration. The curves are from data fit to eq 1 [$K_{M,app}$ (hypoxanthine) 630 ± 70 μM and $K_{M,app}$ (xanthine) 1.0 ± 0.1 mM]. Note: CV data were acquired for both substrates using the same EPG/XDH electrode after washing all previous substrate out with fresh buffer.

Figure 3) the same electrode was removed from the solution and placed in fresh buffer containing only PMS (5 μM). The original (noncatalytic) PMS redox response was again observed; then additional amounts of hypoxanthine were added to produce the responses in Figure 4. The EPG/XDH electrode shows very similar catalytic voltammetry in the presence of hypoxanthine, but the current response to hypoxanthine is ∼50% greater than that from xanthine (Figure 4B). This higher current may be attributed to a contribution from the consecutive hypoxanthine → xanthine → uric acid (four-electron) process in addition to the simple hypoxanthine → xanthine (two-electron) process. We have noted similar behavior previously where the consecutive oxidation processes hypoxanthine → xanthine and xanthine → uric acid were observed.[41,43] The response to hypoxanthine does not simply double relative to xanthine as evidently not all of the xanthine intermediate is recaptured by the enzyme in competition with hypoxanthine for the active site.

pH Dependence. To optimize the electrochemical biosensor, the catalytic response of the EPG/XDH electrode

dx.doi.org/10.1021/ac3025027 | Anal. Chem. 2012, 84, 10359−10365

toward xanthine was studied at different pH values. Figure 5 shows the pH dependence of the EPG/XDH electrode maximum anodic current over the pH range of 6−10 in 100 mM phosphate buffer solution containing 1 mM xanthine and 5 μM PMS.

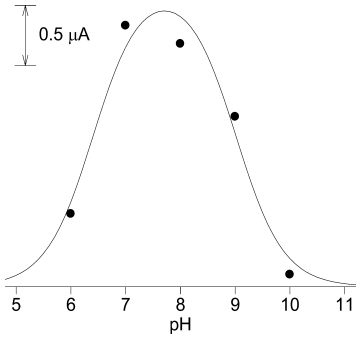


Figure 5. pH dependence of the maximum catalytic current for 1 mM xanthine in the presence of 5 μM PMS at the EPG/XDH electrode in 100 mM phosphate buffer at a scan rate of 5 mV s^{-1}. The curve is a fit to a dibasic acid model (eq 2) (ref 47) where the intermediate protonated form is active (pK_{a1} = 9.0 and pK_{a2} = 6.4).

A classic "bell-shaped" profile was obtained which is consistent with a dibasic acid model (eq 2) where the intermediate (monoprotonated) form is active.[47] This model gave approximate pK_a values of 9.0 and 6.4. On the basis of previous studies,[28,46] xanthine deprotonation (pK_a 7.7)[48] inhibits catalysis. Similarly, protonation of a catalytically essential glutamate residue (E730),[46] which acts as a base to accept a proton from the oxidized substrate, switches off catalysis. Optimal catalytic current was found at pH 7. These results are comparable with the known activity profile of XDH in solution assays[28] and reflect the pH dependence of the enzyme and substrate but not the mediator PMS.

Amperometric Determination of Xanthine. Constant potential amperometry was performed to quantify the sensitivity of the EPG/XDH electrode toward xanthine. Figure 6A shows the amperometric *i*−*t* curve for the catalytic xanthine oxidation at the EPG/XDH electrode in a constantly stirred 100 mM phosphate buffer solution (pH 7) at an applied potential of +50 mV versus Ag/AgCl. An initial steady-state current response (Figure 6B) was observed in the presence of 10 μM xanthine, and further increments of 10 μM xanthine at intervals of 100 s led to a step in the current, with a steady-state current (plateau) being reached within 3 s. The current increased linearly with each xanthine addition from 10 to 120 μM, and the detection limit calculated from the standard deviation of the baseline current[49] was found to be 0.25 nM [signal-to-noise ratio (S/N) = 3] (Figure 6). In separate experiments, we found that the amperometric current was linear from 1.0 × 10^{-5} to 1.8 × 10^{-3} M xanthine with a regression coefficient of 0.994. The sensitivity of the EPG/XDH electrode for xanthine was found to be 0.31 nA/μM.

Analytical Performance. The analytical performance of the xanthine biosensor was compared with other reported xanthine oxidase modified electrodes based on oxidation of enzymatically generated H_2O_2, as summarized in Table 1. Evidently the EPG/XDH electrode exhibits an excellent lower detection limit and very wide linear range compared to the bovine XO immobilized on different substrates. To the best of our knowledge, the present electrochemical biosensor showed the lowest detection limit for xanthine [0.25 nM (S/N = 3)] compared with XO-based modified electrodes. Most importantly, the present biosensor operates at very low potential (ca. +50 mV vs Ag/AgCl) and does not suffer from interferences from other electroactive biologically important molecules such as uric acid, ascorbic acid, and catecholamines which are oxidized around +300 mV.

Reproducibility and Stability. The stability of the EPG/XDH electrode toward xanthine was assessed in two ways, first for repeated use and second with regards to long-term storage. The CVs for 500 μM xanthine in the presence of 5 μM PMS in 100 mM phosphate buffer solution were recorded every 3 min to evaluate the stability of the biosensor. It was found that the anodic peak current was constant with a relative standard deviation of <2% for 10 repeat measurements. To check the long-term storage stability of the biosensor, the EPG/XDH electrode was kept in 100 mM phosphate buffer solution in a refrigerator (4 °C). No apparent decrease in the catalytic current response of xanthine was observed over the first 4 days while the current decreased by 12% after 2 weeks and 22% after 4 weeks. The lowering in electrocatalytic activity could be due

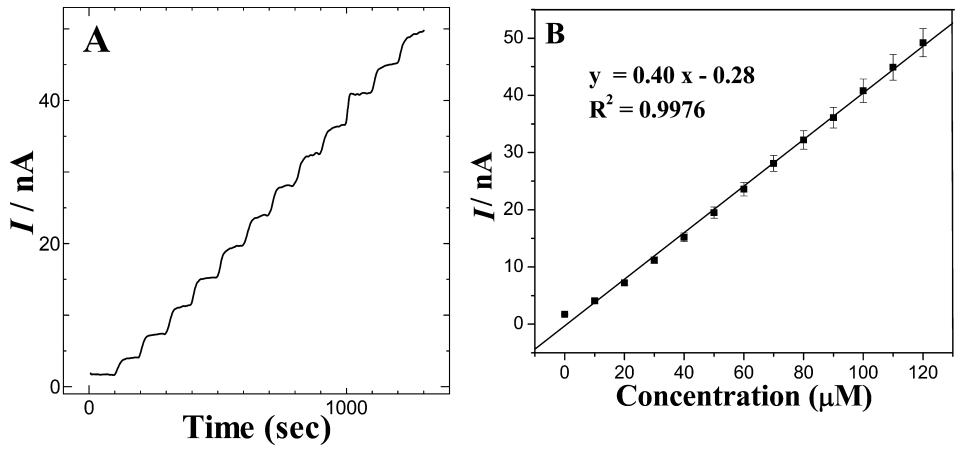


Figure 6. (A) Amperometric *i*−*t* curve obtained for the determination of xanthine at an EPG/XDH electrode in stirred 100 mM phosphate buffer solution. Each step comprises a 10 μM increment in added xanthine concentration at an applied potential of +50 mV vs Ag/AgCl. (B) Linear response range for xanthine.

dx.doi.org/10.1021/ac3025027 | *Anal. Chem.* 2012, 84, 10359−10365

Table 1. Comparison of Analytical Performance of Reported Xanthine-Based Biosensors

electrode	E_{app} (mV vs Ag/AgCl)	pH	linear range (M)	detection limit (M)
XO/calcium carbonate nanoparticles[a]	+590	7.5	2.0×10^{-6}–2.5×10^{-4}	2.0×10^{-6}
XO/layered double hydroxide[b]	+550	7.5	1.0×10^{-6}–2.0×10^{-4}	1.0×10^{-7}
XO/laponite nanoparticles[c]	+390	7.5	3.9×10^{-8}–2.1×10^{-5}	1.0×10^{-8}
XO/ZnOx/polypyrrole[d]	+380	7.0	8.0×10^{-7}–4.0×10^{-5}	8.0×10^{-7}
XO/carbon nanotubes[e]	+400	7.0	5.0×10^{-6}–6.0×10^{-4}	2.0×10^{-6}
XO/poly(terthiophine-3-carboxylic acid)[f]	+350	7.5	5.0×10^{-6}–1.0×10^{-4}	1.0×10^{-6}
XO/glassy carbon paste[g]	+900	7.0	5.0×10^{-7}–4.0×10^{-5}	1.0×10^{-7}
EPG/XDH[h]	+50	7.0	1.0×10^{-5}–1.8×10^{-3}	2.5×10^{-10}

[a]Ref 36. [b]Ref 38. [c]Ref 50. [d]Ref 51. [e]Ref 52. [f]Ref 53. [g]Ref 54. [h]This work.

to degradation of XDH or leakage of XDH from the inner membrane solution.

To ascertain the reproducibility of the results further, three different EPG electrodes were modified with XDH and their response toward the oxidation of 500 μM xanthine in the presence of 5 μM PMS was tested by 10 repeat measurements. The peak current obtained in the 10 repeat measurements of three independent electrodes showed a relative standard deviation (RSD) of 2.3%.

Limitations of the Biosensor. The present electrochemical xanthine biosensor is capable of accurately determining either xanthine or hypoxanthine across a wide range of concentrations in buffered solution. However, as XDH oxidizes both hypoxanthine and xanthine, this method cannot discriminate between the two in a mixture of both purines, which would require separation (HPLC) techniques to be included. In biological fluids (plasma, urine) of healthy humans the concentration of hypoxanthine greatly exceeds that of xanthine (by at least a factor of 5).[2,10,13] So under normal circumstances the major current response will be due to hypoxanthine, but the proportion of hypoxanthine and xanthine cannot, as such, be determined. This limitation has been overlooked in previous studies that have employed xanthine oxidase biosensors for hypoxanthine or xanthine determination in fish samples[36,55−59] or human fluids[53] where both will be present in different concentrations and the enzyme will give a cumulative response rather than being selective for one or the other. In some samples it may be known that only hypoxanthine or xanthine is present, and in this case an enzymatic method is entirely appropriate. A significant advance of the present biosensor is that it functions at a very low potential and avoids any interference from uric acid (product) oxidation which has been an issue in previous xanthine oxidase based electrochemical sensors.

CONCLUSIONS

This paper demonstrates the determination of xanthine using a bacterial xanthine dehydrogenase enzyme adsorbed at an EPG electrode with the mediator PMS. The enzyme-modified electrode shows an enhanced anodic current response at pH 7 in the potential range of −100 to +50 mV versus Ag/AgCl. The amperometric oxidation current was linearly dependent on xanthine concentration within the range from 1.0×10^{-5} to 1.8×10^{-3} M. The wide dynamic range is attributed to an enhanced apparent Michaelis constant for the enzyme (1.0 mM). Further, the modified electrode showed the excellent sensitivity and a low detection limit of 0.25 nM (S/N = 3). The sensor provides a cumulative response to the presence of hypoxanthine and xanthine with an ∼50% enhanced response to hypoxanthine due to a subsequent oxidation of the product xanthine by XDH to uric acid.

ASSOCIATED CONTENT

Supporting Information

Voltammetry of PMS in the presence of uric acid. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: p.bernhardt@uq.edu.au.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We gratefully acknowledge financial support from the Australian Research Council Discovery Grants DP0880288 and DP12014665 (to P.V.B.) and from the Deutsche Forschungsgemeinschaft Cluster of Excellence “Unifying concepts in catalysis” (to S.L.).

REFERENCES

(1) Luong, J. H. T.; Male, K. B.; Nguyen, A. L. *Enzyme Microb. Technol.* **1989**, *11*, 277−282.
(2) Boulieu, R.; Bory, C.; Baltassat, P.; Gonnet, C. *Anal. Biochem.* **1983**, *129*, 398−404.
(3) Campion, E. W.; Glynn, R. J.; Delabry, L. O. *Am. J. Med.* **1987**, *82*, 421−426.
(4) Kim, K. Y.; Ralph Schumacher, H.; Hunsche, E.; Wertheimer, A. I.; Kong, S. X. *Clin. Ther.* **2003**, *25*, 1593−1617.
(5) Kawachi, M.; Kono, N.; Mineo, I.; Yamada, Y.; Tarui, S. *Clin. Chim. Acta* **1990**, *188*, 137−146.
(6) Banupriya, C.; Ratnakar; Doureradjou, P.; Mondal, N.; Vishnu, B.; Koner, B. C. *Clin. Biochem.* **2008**, *41*, 968−973.
(7) Ono, T.; Tsuruta, R.; Fujita, M.; Aki, H. S.; Kutsuna, S.; Kawamura, Y.; Wakatsuki, J.; Aoki, T.; Kobayashi, C.; Kasaoka, S.; Maruyama, I.; Yuasa, M.; Maekawa, T. *Brain Res.* **2009**, *1305*, 158−167.
(8) Frank, J.; Kelleher, D. K.; Pompella, A.; Thews, O.; Vaupel, P.; Biesalski, H. K. *Free Radical Biol. Med.* **1998**, *25* (Supplement1), S116.
(9) McMaster-Fay, R. A. *Biosci. Hypotheses* **2008**, *1*, 35−43.
(10) Boulieu, R.; Bory, C.; Baltassat, P.; Divry, P. *Clin. Chim. Acta* **1984**, *142*, 83−89.
(11) Kojima, T.; Nishina, T.; Kitamura, M.; Kamatani, N.; Nishioka, K. *Clin. Chem.* **1986**, *32*, 287−290.
(12) Cai, X.; Kalcher, K.; Neuhold, C. *Fresenius' J. Anal. Chem.* **1994**, *348*, 660−665.
(13) Bory, C.; Chanting, C.; Boulieu, R. *J. Chromatogr., A* **1996**, *730*, 329−331.
(14) Yamamoto, T.; Moriwaki, Y.; Takahashi, S.; Tsutsumi, Z.; Yamakita, J.-i.; Nasako, Y.; Hiroishi, K.; Higashino, K. *J. Chromatogr., B: Biomed. Sci. Appl.* **1996**, *681*, 395−400.

(15) Caussé, E.; Pradelles, A.; Dirat, B.; Negre-Salvayre, A.; Salvayre, R.; Couderc, F. *Electrophoresis* **2007**, *28*, 381−387.
(16) Carsol, M. A.; Volpe, G.; Mascini, M. *Talanta* **1997**, *44*, 2151−2159.
(17) Chabard, J. L.; Lartigue-Mattei, C.; Vedrine, F.; Petit, J.; Berger, J. A. *J. Chromatogra., B: Biomed. Sci. Appl.* **1980**, *221*, 9−17.
(18) Hlavay, J.; Haemmerli, S. D.; Guilbault, G. G. *Biosens. Bioelectron.* **1994**, *9*, 189−195.
(19) Kito, M.; Tawa, R.; Takeshima, S.; Hirose, S. *J. Chromatogr., B: Biomed. Sci. Appl.* **1983**, *278*, 35−42.
(20) Kalimuthu, P.; John, S. A. *Talanta* **2009**, *80*, 1686−1691.
(21) Kathiwala, M.; Affum, A. O.; Perry, J.; Brajter-Toth, A. *Analyst* **2008**, *133*, 810−816.
(22) Zen, J. M.; Lai, Y. Y.; Yang, H. H.; Senthil Kumar, A. *Sens. Actuators, B* **2002**, *84*, 237−244.
(23) Wang, Y. *Colloids Surf., B* **2011**, *88*, 614−621.
(24) Hille, R. *Chem. Rev.* **1996**, *96*, 2757−2816.
(25) Enroth, C.; Eger, B. T.; Okamoto, K.; Nishino, T.; Nishino, T.; Pai, E. F. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 10723−10728.
(26) Leimkühler, S.; Kern, M.; Solomon, P. S.; McEwan, A. G.; Schwarz, G.; Mendel, R. R.; Klipp, W. *Mol. Microbiol.* **1998**, *27*, 853−869.
(27) Truglio, J. J.; Theis, K.; Leimkühler, S.; Rappa, R.; Rajagopalan, K. V.; Kisker, C. *Structure* **2002**, *10*, 115−125.
(28) Leimkühler, S.; Hodson, R.; George, G. N.; Rajagopalan, K. V. *J. Biol. Chem.* **2003**, *278*, 20802−20811.
(29) Mao, L.; Xu, F.; Xu, Q.; Jin, L. *Anal. Biochem.* **2001**, *292*, 94−101.
(30) Mao, L.; Yamamoto, K. *Anal. Chim. Acta* **2000**, *415*, 143−150.
(31) Xue, H.; Mu, S. *J. Electroanal. Chem.* **1995**, *397*, 241−247.
(32) Ianniello, R. M.; Lindsay, T. J.; Yacynych, A. M. *Anal. Chem.* **1982**, *54*, 1980−1984.
(33) Qiong, C.; Tuzhi, P.; Liju, Y. *Anal. Chim. Acta* **1998**, *369*, 245−251.
(34) Zhao, J.; O'Daly, J. P.; Henkens, R. W.; Stonehuerner, J.; Crumbliss, A. L. *Biosens. Bioelectron.* **1996**, *11*, 493−502.
(35) Devi, R.; Yadav, S.; Pundir, C. S. *Biochem. Eng. J.* **2011**, *58*−*59*, 148−153.
(36) Shan, D.; Wang, Y.; Xue, H.; Cosnier, S. *Sens. Actuators, B* **2009**, *136*, 510−515.
(37) Arai, G.; Takahashi, S.; Yasumori, I. *J. Electroanal. Chem.* **1996**, *410*, 173−179.
(38) Shan, D.; Wang, Y.; Zhu, M.; Xue, H.; Cosnier, S.; Wang, C. *Biosens. Bioelectron.* **2009**, *24*, 1171−1176.
(39) Shi, G.; Liu, M.; Zhu, M.; Zhou, T.; Chen, J.; Jin, L.; Jin, J.-Y. *Analyst* **2002**, *127*, 396−400.
(40) Nishino, T.; Nishino, T.; Schopfer, L. M.; Massey, V. *J. Biol. Chem.* **1989**, *264*, 2518−2527.
(41) Kalimuthu, P.; Leimkühler, S.; Bernhardt, P. V. *J. Phys. Chem. B* **2012**, *116*, 11600−11607.
(42) Aguey-Zinsou, K. F.; Bernhardt, P. V.; Leimkühler, S. *J. Am. Chem. Soc.* **2003**, *125*, 15352−15358.
(43) Kalimuthu, P.; Leimkühler, S.; Bernhardt, P. V. *J. Phys. Chem. B* **2011**, *115*, 2655−2662.
(44) Miyawaki, O.; Yano, T. *Enzyme Microb. Technol.* **1992**, *14*, 474−478.
(45) Bard, A. J.; Faulkner, L. R. *Electrochemical Methods: Fundamentals and Applications*; Wiley: New York, 2001.
(46) Leimkühler, S.; Stockert, A. L.; Igarashi, K.; Nishino, T.; Hille, R. *J. Biol. Chem.* **2004**, *279*, 40437−40444.
(47) Brody, M. S.; Hille, R. *Biochemistry* **1999**, *38*, 6668−6677.
(48) Stoychev, G.; Kierdaszuk, B.; Shugar, D. *Eur. J. Biochem.* **2002**, *269*, 4048−4057.
(49) Zare, H. R.; Memarzadeh, F.; Ardakani, M. M.; Namazian, M.; Golabi, S. M. *Electrochim. Acta* **2005**, *50*, 3495−3502.
(50) Shan, D.; Wang, Y.-N.; Xue, H.-G.; Cosnier, S.; Ding, S.-N. *Biosens. Bioelectron.* **2009**, *24*, 3556−3561.
(51) Liu, Y.; Nie, L.; Tao, W.; Yao, S. *Electroanalysis* **2004**, *16*, 1271−1278.
(52) Villalonga, R.; Diez, P.; Gamella, M.; Reviejo, J.; Pingarrón, J. M. *Electroanalysis* **2011**, *23*, 1790−1796.
(53) Rahman, M. A.; Won, M.-S.; Shim, Y.-B. *Electroanalysis* **2007**, *19*, 631−637.
(54) Kirgöz, Ü. A.; Timur, S.; Wang, J.; Telefoncu, A. *Electrochem. Commun.* **2004**, *6*, 913−916.
(55) Hu, S.; Liu, C.-C. *Electroanalysis* **1997**, *9*, 372−377.
(56) Zhang, J.; Lei, J.; Pan, R.; Xue, Y.; Ju, H. *Biosens. Bioelectron.* **2010**, *26*, 371−376.
(57) Lawal, A. T.; Adeloju, S. B. *Food Chem.* **2012**, *135*, 2982−2987.
(58) Pundir, C. S.; Devi, R.; Narang, J.; Singh, S.; Nehra, J.; Chaudhry, S. *J. Food Biochem.* **2012**, *36*, 21−27.
(59) Devi, R.; Narang, J.; Yadav, S.; Pundir, C. S. *J. Anal. Chem.* **2012**, *67*, 273−277.