Eur. J. Biochem. 257, 178−184 (1998)
© FEBS 1998

Identification of an N-hydroxyguanidine reducing activity of xanthine oxidase

Maija DAMBROVA[1], Staffan UHLÉN[1], Christopher J. WELCH[2] and Jarl E. S. WIKBERG[1]

[1] Department of Pharmaceutical Biosciences, Division of Pharmacology, Uppsala University, Uppsala, Sweden
[2] Department of Pharmaceutical Chemistry, Division of Organic Chemistry, Uppsala University, Uppsala, Sweden

(Received 13 May 1998) − EJB 98 0651/4

A guanoxabenz [1-(2,6-dichlorobenzylideneamino)-3-hydroxyguanidine; an N-hydroxyguanidine] reducing enzymatic activity of rat spleen cytosol was investigated. By means of protein purification and N-terminal amino acid sequencing, the reducing activity was shown to reside in xanthine oxidase. The action of the enzyme on guanoxabenz resulted in the formation of guanabenz [1-(2,6-dichlorobenzylideneamino)-3-guanidine]; the product formation could be monitored by HPLC and its identity was confirmed by NMR analysis. The reduction of guanoxabenz required xanthine or NADH as reducing substrates, while the process could be blocked by allopurinol, a selective inhibitor of xanthine oxidase. By using bovine milk xanthine oxidase, the guanoxabenz reducing activity of the enzyme was also verified. We conclude that guanoxabenz is a novel electron acceptor structure for xanthine oxidase.

Keywords: N-hydroxyguanidine; guanoxabenz; reduction; xanthine oxidase.

In the course of our studies of the pharmacology of α_2-adrenoceptors, we discovered that 1-(2,6-dichlorobenzylideneamino)-3-hydroxyguanidine (guanoxabenz), which is an N-hydroxyguanidine derivative, had peculiar properties since, in some tissues, it appeared to bind to α_2-adrenoceptors with low affinity only, whereas in other tissues it appeared to bind also with high affinity [1−3]. In more recent studies, we provided evidence that this apparent bimodal property of guanoxabenz was due to its tissue-specific conversion to a metabolite that possessed high affinity for α_2-adrenoceptors [4].

The present study was initiated to elucidate which enzyme is responsible for the activation of guanoxabenz. We found initially that the majority of the guanoxabenz-converting activity resided in the cytosolic fractions of many tissues. By means of protein purification from the rat spleen cytosol, we show here that the guanoxabenz converting activity resides in xanthine oxidase (XO). Moreover, we show that the activity can also be reproduced with bovine milk XO.

XO is a molybdoprotein present in various tissues of animals [5]. The enzyme is known to catalyse the oxidation and reduction of a wide variety of substances, that also includes N-reductions [6]. The enzyme is naturally involved in oxidative hydroxylation of substrates such as xanthine and hypoxanthine, in which case O_2 is the physiological electron acceptor. With respect to other substrates undergoing reduction by XO, the reduction of nitro compounds can be observed essentially only under anaerobic conditions [7, 8]. In contrast, it has been found that N-reduction of 6-*N*-aminopurine to adenine by XO is just slightly inhibited by oxygen [9]. Our present results show that both xanthine and NADH can serve as electron donators in the reduction of guanoxabenz by XO under anaerobic conditions. The catalytic reduction of guanoxabenz was insensitive to blockade by oxygen when NADH was used as electron donator. However, when xanthine was used, the reduction was partially inhibited in the presence of oxygen.

MATERIALS AND METHODS

Materials. [^{3}H]RX821002 (59 Ci/mmol) was from Amersham. Guanoxabenz was a gift from Rousell. Allopurinol, 1-(2,6-dichlorobenzylideneamino)-3-hydroxyguanidine (guanabenz), xanthine, NADH and bovine XO (grade I from buttermilk) were from Sigma. DEAE-Sepharose® CL-6B and the Superdex™ 200 HR 10/30 column were from Pharmacia Biotech. Hydroxyapatite (Macro-Prep® Ceramic Hydroxyapatite) was from BioRad.

Preparation of HT-29 cell membranes. HT-29 cell membranes were prepared in order to obtain a source of α_2-adrenoceptors for the guanoxabenz/guanabenz-binding assays (e.g. guanoxabenz-converting activity assays). HT-29 cells were grown in Dulbecco's Modified Eagle medium supplemented with 10% foetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2 in an air-ventilated humidified incubator in 55-cm^2 Costar cell-culture dishes. Cell membranes were prepared by rinsing HT-29 cells twice with Hanks' balanced salt solution buffer. The cells were scraped off the plates into 5 ml 2 mM Tris/HCl, 1 mM EDTA, pH 7.5, then homogenised by using an Ultra-Turrax homogeniser (IKA T25, equipped with an 8-mm diameter probe). The homogenate was centrifuged at 600×*g* for 5 min, the supernatant was decanted, and then centrifuged at 30000×*g* for 15 min at 4°C in a Beckman J2−21 centrifuge. The resulting pellet was resuspended at a protein concentration of about 0.6 mg/ml in 50 mM Tris, 1.5 mM EDTA, pH 7.5, and stored frozen at −80°C until further used. Protein was determined according to the method of Lowry et al. [10].

Preparation of rat spleen cytosol. Rat spleens were homogenised (1:10, mass/vol.) in ice-cold 20 mM Tris, 1.5 mM

Correspondence to J. E. S. Wikberg, Pharmaceutical Pharmacology, BMC, Box 591, S-751 24 Uppsala, Sweden
Fax: +46 18 559718.
E-mail: Jarl.Wikberg@farmbio.uu.se

Abbreviations. Guanoxabenz, 1-(2,6-dichlorobenzylideneamino)-3-hydroxyguanidine; guanabenz, 1-(2,6-dichlorobenzylideneamino)-3-guanidine; XO, xanthine oxidase.

Enzymes. Xanthine dehydrogenase (EC 1.1.1.204); xanthine oxidase (EC 1.1.3.22).

EDTA, pH 7.5, with a glass/Teflon homogeniser. The homogenate was centrifuged at 30 000×*g* for 15 min at 4°C in a Beckman J2−21 centrifuge and the supernatant stored frozen at −80°C. After thawing, the sample was centrifuged at 70 000×*g* for 1 h in an ultracentrifuge at 4 °C and the supernatant decanted and used for the enzyme purification.

Enzyme activity test by α_2-adrenoceptor-binding assay. Detection of guanoxabenz-converting activity during protein purification was performed by incubating HT-29 cell membranes containing approximately 40 pM α_2-adrenoceptors (about 60 µg membrane protein) with 1 nM [^{3}H]RX821002, 1 µM guanoxabenz, 1 mM NADH, 1 mM EDTA, 33 mM Tris/HCl, pH 7.5, with 25 µl of the fractions eluted from the different purification steps, for 1 h at 25°C in a total volume of 150 µl. When the activity of bovine milk XO was evaluated, varying concentrations of guanoxabenz were added into 150 µl assays that contained 0.004 unit/ml bovine milk XO, HT-29 cell membranes (≈40 pM α_2-adrenoceptors), 1 nM [^{3}H]RX821002, 1 mM EDTA and 33 mM Tris/HCl, pH 7.5. Xanthine (100 µM) or allopurinol (20 µM) was also added when indicated in the text. The assays were stopped by filtration and washing with 20 ml ice-cold 50 mM Tris/HCl, pH 7.5, on GF/C glass-fibre filters. The filters were then subjected to scintillation radioactivity counting. When the activity of the enzyme was followed routinely during the chromatographic purification, the activity was calculated relative to the maximal inhibition of [^{3}H]RX821002 binding obtained in a particular experiment. However, for the tests where competition curves had been obtained, the data were analysed using the BindAid radioligand-binding analysis package (Wan System), essentially as described [11−13], the purpose being to obtain the apparent K_i values of the guanoxabenz/guanabenz mixtures. The degree of conversion of guanoxabenz to guanabenz could then be approximated from the formula:

$$q = \frac{K_a (K_x - K_r)}{K_r (K_x - K_a)},$$

where q is the fraction of guanabenz in a guanoxabenz/guanabenz mixture, K_a the known K_i of guanabenz, K_x the known K_i of guanoxabenz and K_r the apparent K_i of the reaction mixture containing both guanoxabenz and guanabenz. The following K_i values were used in the calculations: K_x = 7200 nM (K_i value of guanoxabenz for the HT-29 cell α_2-adrenoceptors) and K_a = 25 nM (K_i value of guanabenz for α_2-adrenoceptors) [1].

Purification procedure. The rat spleen cytosol (210 ml) was loaded onto a DEAE-Sepharose® CL-6B column (115 cm×2.6 cm) equilibrated with 20 mM Tris, 1.5 mM EDTA, pH 7.5. The column was then washed with the same buffer until no absorbance at 280 nm was detectable in the eluate. The protein was then eluted with 500 ml linear gradient of 20−400 mM NaCl in the same buffer. Fractions containing guanoxabenz-converting activity were pooled and directly loaded onto a BioRad Macro-Prep® Ceramic hydroxyapatite column (9 cm×1.5 cm) equilibrated with 100 mM potassium phosphate, pH 7.5. The protein was eluted with 140 ml linear gradient of 100−400 mM potassium phosphate, pH 7.5. The fractions containing guanoxabenz converting activity were pooled and concentrated in a Millipore Ultrafree®-15 Centrifugal filter device (Biomax 30 membrane) to a final volume of about 1 ml. Aliquots of 200 µl of the concentrate were then applied to a Superdex™ 200 HR 10/30 column and eluted with 20 mM Tris, 1.5 mM EDTA, pH 7.5. The fractions containing guanoxabenz-converting activity were pooled, concentrated ≈10X in Millipore Ultrafree®-15 Centrifugal filter device, then reapplied to the Superdex™ 200 HR 10/30 column. After the second Superdex™ 200 run, the active fractions were again pooled, concentrated and stored at −80°C until analysed further.

Gel electrophoresis and protein sequence analysis. The purity of the final enzyme preparation was checked by non-denaturing 6% PAGE, and 8% SDS/PAGE according to the method of Laemmli [14]. Molecular-mass standards used were Pharmacia high molecular-mass electrophoresis calibration kit containing thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and albumin (67 kDa) as standards and Pharmacia high molecular-mass SDS electrophoresis calibration kit containing myosin (212 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa) and glutamic dehydrogenase (53 kDa). Gels were stained for proteins with silver nitrate according to the method of Nesterenko et al. [15], or with Coomassie brilliant blue G250. For the protein sequence analysis, the purified enzyme was electrophoresed by an 8% SDS/PAGE, whereafter the protein bands were transferred to a poly(vinylidene difluoride) protein sequencing membrane (BioRad) by electroblotting. The poly(vinylidene difluoride) membrane was stained with Coomassie brilliant blue, bands cut out and subjected to N-terminal amino acid sequence analysis using an automated Edman degradation sequence analyser.

Reduction of guanoxabenz by milk xanthine oxidase. *Aerobic incubations.* Incubations were carried out at room temperature in the presence of atmospheric oxygen. The standard incubation mixture contained 250 µM guanoxabenz, 250 µM NADH and/or 250 µM xanthine, 0.1 unit/ml bovine milk XO (according to producers definition one unit will convert 1.0 µmol/min xanthine to uric acid at pH 7.5 at 25°C) in 25 mM Tris/HCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.5, in a total volume of 1 ml. In some incubations, 20 µM allopurinol was also included. Reaction was started by the addition of enzyme and terminated after 30 min by boiling for 5 min. The samples were then centrifuged at 20 000×*g* at 4°C and 50 µl aliquots of the supernatant were directly analysed by HPLC.

Anaerobic incubations. Anaerobic incubations were performed essentially as described above for the aerobic incubations with the exception that, prior to incubation, all solutions were gassed for 10 min with argon, whereafter incubations were performed in test-tubes air-tightened with rubber stoppers. In order to obtain enough product for NMR analysis, 2.5 ml 1 mM guanoxabenz, 1 mM xanthine and 0.2 units/ml XO in 25 mM Tris/HCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.5, was incubated for 1 h at 25°C. The reactions were terminated by boiling the reaction mixtures for 5 min. The samples were then centrifuged at 20000×*g* at 4°C and the supernatants were directly analysed by HPLC.

HPLC analysis. 50 µl aliquots of the supernatants were injected onto a Waters 2690 Separation Module equipped with a 10×250 Vydac™ RP C_{18}, 90A, 201HS52 column, equilibrated with methanol/20 mM ammonium acetate (30:70, by vol.), pH 4.5. The flow rate was 0.15 ml/min and a photo diode array detector was used to record chromatograms. Standard curves for guanoxabenz and guanabenz (5−200 µM) were constructed by introducing known amounts of substance into the incubation buffer and treating in the same way as the experimental incubation mixtures. Standard curves were linear over the range used, with correlation coefficients being better than 0.999. The retention time was 25.5 min for guanoxabenz and 31.5 min for guanabenz. The amounts of guanabenz in the incubation mixtures were determined using the Millenium® Chromatography Manager PDA software package.

NMR recordings. The HPLC fractions corresponding to the product peak were collected, lyophilized, dissolved in dimethylsulfoxide and used for NMR analysis. Sample concentration for the enzymatically prepared sample was estimated to be 200 µM. Reference spectra were obtained for guanabenz and guanoxa-

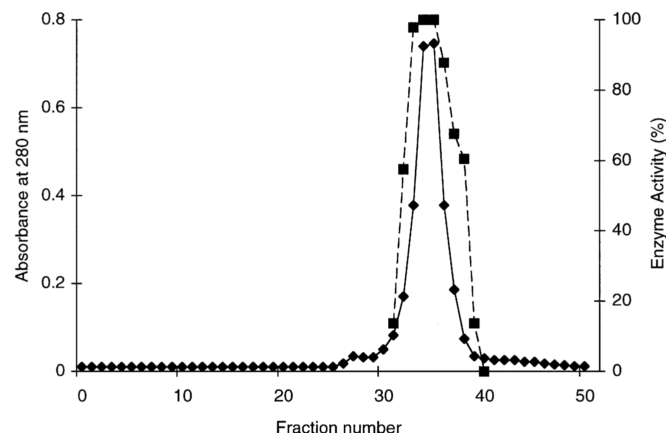

Fig. 1. Elution of the guanoxabenz-reducing enzyme of rat spleen cytosol from a Superdex™ 200 HR 10/30 column. The chromatogram represents a second Superdex™ run (see Materials and Methods for details). Shown is the protein content of the eluate monitored by the absorbance at 280 nm (◆——◆), and the enzyme activity (■– – –■) measured as the inhibition induced by addition of 25 µl eluate to a binding assay containing [^{3}H]RX821002, HT-29 cell α_2-adrenoceptors and 1 µM guanoxabenz (see Materials and Methods for details). The enzyme activity is expressed in relative to of the activity obtained in the fraction giving the maximal inhibition of [^{3}H]RX821002 binding (fraction 35).

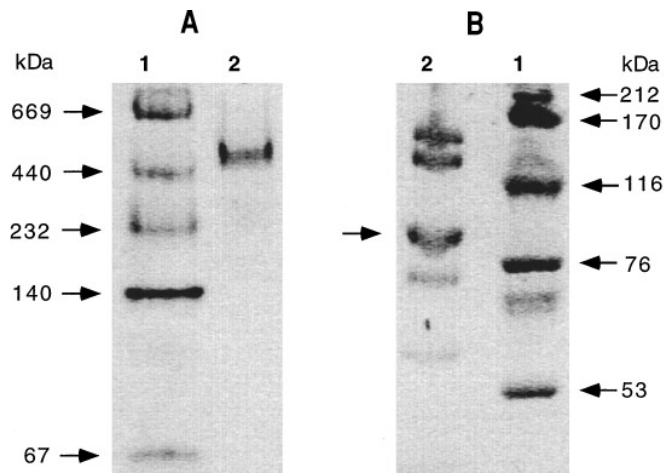

Fig. 2. Analysis of the protein product obtained from the last Superdex 200HR 10/30 step by PAGE. (A) Non-denaturing 6% PAGE; (B) 8% SDS/PAGE gel stained with Coomassie blue. For (A) and (B): lane 1, marker proteins; lane 2, enzyme preparation. The arrow indicates the sequenced protein band corresponding to XO.

benz samples at 10 mM. Spectra were recorded at 270.17 MHz, using a JEOL EX 270 spectrometer equipped with a 5-mm inversely configured probe, 2000 transients were collected with a spectral width of 3000 Hz consisting of 65 k data points, and at 399.78 MHz using a JEOL EX 400 spectrometer equipped with a standard 5-mm probe. 2000 transients were collected with a spectral width of 5000 Hz consisting of 65 k data points. The processed data were compared with spectra obtained for the reference compounds.

Spectrophotometric assays. Guanoxabenz-reducing activity of bovine XO was characterized under anaerobic conditions using a spectrophotometric method. The standard incubation mixture contained 50 µM xanthine or 250 µM NADH, 0.05 units/ml or 0.15 units/ml XO, and varying concentrations of guanoxabenz in 25 mM Tris/HCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.5, in a total volume of 1 ml. As anaerobic controls served incubation mixtures where guanoxabenz was excluded. Prior to incubation, all solutions were gassed for at least 10 min with argon. The reaction was started by the injection of milk XO to a Helma® sealable cuvette for anaerobic applications. The rate of xanthine oxidation was monitored by observing the rate of formation of uric acid by measuring the increase in absorbance at 295 nm ($\varepsilon_{\text{uric acid}}$ = 12.6 mM^{-1} cm^{-1}), while the rate of NADH oxidation was followed at 340 nm ($\varepsilon_{\text{NADH}}$ = 6.2 mM^{-1} cm^{-1}). K_m and V_{max} values for the catalysis of guanoxabenz reduction by xanthine and NADH were determined by fitting the data to the Michaelis-Menten equation, $V = V_{max}$ [S]/([S] + K_m), using non-linear least squares regression. Statistical testing for the eventual deviation of the data from the Michaelis-Menten equation was performed by once again fitting the same data to the logistic function, ($V = V_{max}$ [S]^n/([S]^n + K^n), and comparing the significance of the reduction in sums of squares with the sums of squares for the Michaelis-Menten fit using the extra sums of squares principle [16]. An f value indicating a reduction of the sums of square at $P < 0.05$ was taken to indicate a significant deviation from the hyperbolic Michaelis-Menten relation.

RESULTS

Purification of spleen guanoxabenz-converting activity. The enzyme activity was monitored during the purification by evaluating the guanoxabenz-reducing capacity of the fractions using 1 mM NADH as cofactor. The activity could be detected as an increase in the ability of 1 µM guanoxabenz to compete with the binding of the α_2-adrenoceptor radioligand [^{3}H]RX821002 to α_2-adrenoceptors in HT-29 cell membranes. A single peak of guanoxabenz-reducing activity was eluted from the DEAE-Sepharose® column at about 100 mM NaCl of a linear gradient of 20–400 mM NaCl, pH 7.5. After applying the active fraction from the DEAE-Sepharose® to a hydroxyapatite column, it was eluted as a single peak close to 400 mM potassium-phosphate using a potassium-phosphate gradient. The fractions with enzymatic activities were loaded twice onto Superdex™ 200 HR and, in the second Superdex run, a single symmetric ultraviolet-absorbing peak was obtained (Fig. 1). The purification procedure resulted in a final purification of the enzyme of about 1000-fold (data not shown). The absorbency ratio A_{280}/A_{450} (the ratio of protein to flavin) of the purified enzyme was 6.0. The purified enzyme was analysed by 6% native PAGE, where one major band of molecular mass 450±10 kDa (mean±SEM, $n = 3$) was visualized by both silver and Coomassie blue staining (Fig. 2A).

The subunit structure of the final enzyme preparation was further examined by 8% denaturing SDS/PAGE, whereupon several bands were seen (Fig. 2B). The subunits separated by SDS/PAGE were blotted onto a poly(vinylidene difluoride) membrane and bands were subjected to N-terminal amino acid sequencing. A SwissProt database search revealed that the N-terminal amino acid sequence of the arrow-marked band (Fig. 2B, molecular mass 85±7 kD, $n = 3$) was ANVQLFQE, which matched eight amino acid residues of rat xanthine dehydrogenase and XO, starting at residue 543 of the published sequence [20].

Conversion of guanoxabenz by bovine milk XO under aerobic conditions. A guanoxabenz-reducing activity of commercially available bovine milk XO was demonstrated using an α_2-adrenoceptor-binding assay. In this assay, competition curves of guanoxabenz were obtained using the α_2-adrenoceptor containing HT-29 cell membranes and the α_2-adrenoceptor radioligand [^{3}H]RX821002, in the presence or absence of the bovine enzyme

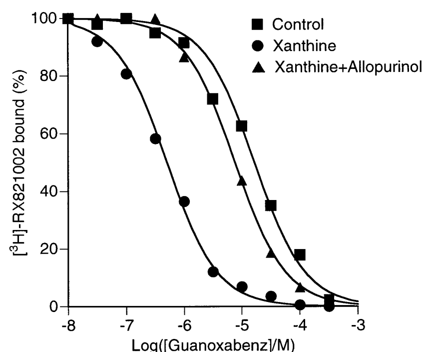

Fig. 3. Guanoxabenz-reducing activity of bovine XO demonstrated by an α_2-adrenoceptor assay. Shown in the figure are competition curves of guanoxabenz using HT29 cell membranes in an assay supplemented with 0.004 units/ml bovine XO and using ≈1.1 nM [^{3}H]RX821002 as radioligand. Additions were (as indicated) 100 μM xanthine and 20 μM allopurinol (n = 2).

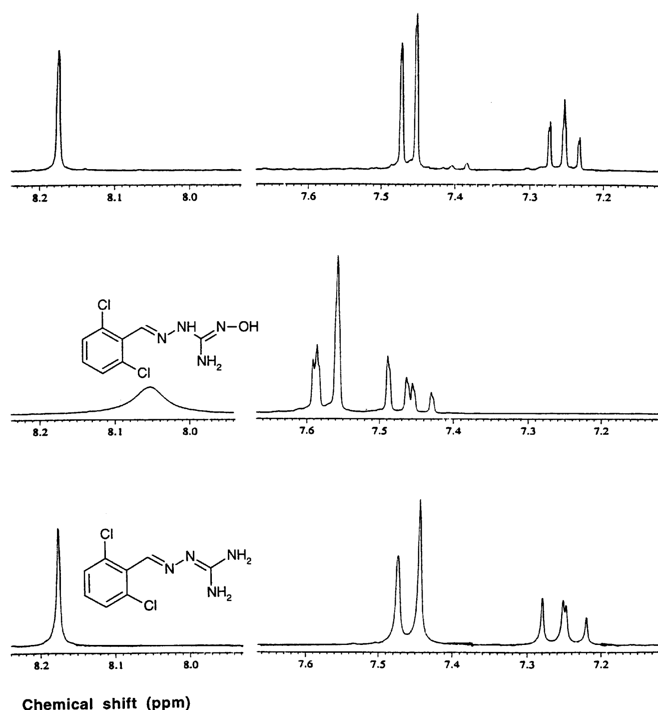

Fig. 4. 270-MHz ^{1}H-NMR spectra of guanabenz, guanoxabenz and the reduction product obtained from incubation of guanoxabenz together with XO in the presence of xanthine. Top panel, compound isolated after bovine milk XO treatment of guanoxabenz, approximately 200 μM. Mid panel, authentic sample of guanoxabenz, (approximately 10 mM) (centre). Bottom panel, authentic sample of guanabenz (approximately 10 mM). Scale shows chemical shift values with solvent dimethylsulfoxide as reference.

(see Materials and Methods) (Fig. 3). In the presence of bovine milk XO, but without the addition of cofactors, the K_i value of guanoxabenz for the HT-29 cell α_2-adrenoceptors was 7200±1100 nM, which corresponds to an essentially total absence of conversion of guanoxabenz to guanabenz. However, when both XO and 100 μM xanthine was added to the assays, the apparent K_i value of guanoxabenz decreased to 180±47 nM, a value which corresponds to an approximately 14% conversion of guanoxabenz to guanabenz. When the XO was incubated with 20 μM allopurinol (an XO inhibitor), the decrease in the K_i value was abolished, its value being 5200±500 nM (mean±SEM,

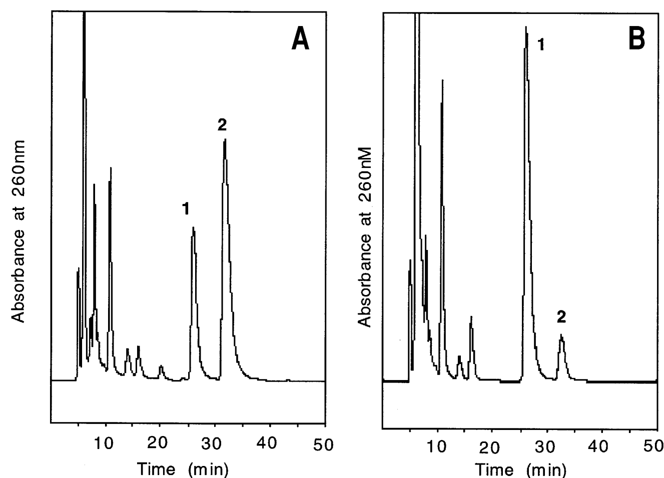

Fig. 5. Representative HPLC chromatogram of the anaerobic incubation of guanoxabenz with the bovine milk XO. Shown in the figure is the run of a 30-min incubation of 250 μM guanoxabenz with 0.1 unit/ml bovine milk XO in the presence of 250 μM xanthine (A) and 250 μM xanthine and 20 μM allopurinol (B). Indicated in the figure are guanoxabenz (1) and guanabenz product (2).

n = 4), corresponding to only 0.13% conversion of guanoxabenz to guanabenz. Thus, the results indicate that bovine XO can reduce guanoxabenz, using xanthine as reducing substrate. Moreover, allopurinol seems to be capable of completely blocking the xanthine-activated conversion of guanoxabenz.

Analysis of the product by NMR. To obtain enough of the N-hydroxyguanidine-derived product for NMR analysis, 1 mM guanoxabenz was incubated under anaerobic conditions with 1 mM xanthine and milk XO (see Materials and Methods for details). The reaction mixture was then purified by HPLC and the fractions containing the product peak were collected, pooled, lyophilised, and subjected to NMR analysis (Fig. 4). The spectrum of the enzymatic reaction product (Fig. 4) corresponded exactly to that of guanabenz, most notably only the spectra of the sample and guanabenz showing a sharp singlet at 8.18 ppm.

Characterisation of the guanoxabenz-reducing activity of milk XO. In the further characterisation of the influence of cofactors, oxygen and allopurinol on the N-reduction of guanoxabenz by milk XO the formation of guanabenz was assayed under different conditions by HPLC analysis. The retention time of guanoxabenz was 25.5 min and that of guanabenz 31.5 min (Fig. 5A). For the chromatogram where the corresponding incubation had been performed with the addition of 20 μM allopurinol, the guanabenz peak was very small, indicating essentially total blockade of the conversion of guanoxabenz (Fig. 5B). Using the same system, the N-reduction of guanoxabenz was followed in the absence and presence of xanthine (250 μM), NADH (250 μM) and allopurinol (20 μM), both under aerobic and anaerobic conditions (Table 1). The N-reduction of guanoxabenz was sensitive to oxygen if xanthine was used as cofactor (i.e. aerobically produced guanabenz amounts to less than 20% of that produced under anaerobic conditions). However, in strong contrast to these results, in the presence of 250 μM NADH the conversion of guanoxabenz to guanabenz was not inhibited by oxygen. The effects of xanthine and NADH were not additive (Table 1). Moreover, allopurinol blocked both the xanthine-driven and the NADH-driven reduction of guanoxabenz (Table 1). If neither xanthine or NADH were added to the

Table 1. Electron donor and enzyme inhibitor specificities for N-reduction of guanoxabenz to guanabenz by bovine milk XO under anaerobic and aerobic conditions. Shown in the table is the influence of xanthine (250 µM), NADH (250 µM) and allopurinol (20 µM) on the initial rate of reduction of 250 µM of guanoxabenz by 0.1 unit/ml bovine milk XO under aerobic and anaerobic conditions. The amount of guanabenz formed was measured by HPLC. Values represent mean ± standard deviation from three separate determinations.

Cofactors and inhibitor	Guanabenz formed	
	aerobic	anaerobic
	$nmol \cdot min^{-1} \cdot unit^{-1}$ XO	
Xanthine	7.1 ± 2.0	38.0 ± 6.0
Xanthine + Allopurinol	3.1 ± 0.2	2.2 ± 0.3
NADH	9.8 ± 2.4	8.9 ± 0.9
NADH + Allopurinol	1.6 ± 0.2	1.6 ± 0.2
Xanthine + NADH	11.4 ± 1.9	39.3 ± 2.4
Xanthine + NADH + Allopurinol	3.0 ± 0.5	2.1 ± 0.2

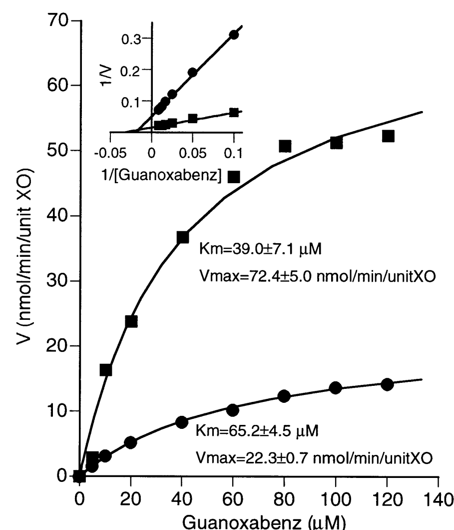

Fig. 6. Analysis of the kinetics of guanoxabenz as cofactor in the anaerobic oxidation of xanthine and NADH by 0.05 units/ml and 0.15 units/ml bovine milk XO, respectively. Shown is the effect of varying concentrations of guanoxabenz on the initial rate of the oxidation of 50 µM xanthine or 250 µM NADH under anaerobic conditions. The lines represent the best fit of the data to the Michaelis-Menten equation. The insert shows the Lineweaver-Burk plot of the data. Each data point represent the mean of three independent measurements.

incubations, no formation at all of guanabenz was observed (data not shown).

Studies of the kinetics of the anaerobic N-reduction of guanoxabenz. The efficiency of xanthine and NADH as electron donating cofactors for the anaerobic N-reduction of guanoxabenz by bovine milk XO was characterised by light spectrophotometry, using xanthine and NADH as electron-donating cofactors. Varying concentrations of guanoxabenz (5−120 µM) were incubated with bovine milk XO in the presence of 50 µM xanthine or 250 µM NADH. The reaction was followed by registering the increase in the absorbance at 295 nm (i.e. the maximum absorbance for uric acid), using xanthine as cofactor, or by the decrease in absorbance at 340 nm when the reaction was driven by NADH. The initial reaction rate was linear for at least 2.5 min for the xanthine and 15 min for the NADH incubations. To obtain blank control readings, an equal volume of the solvent for guanoxabenz was added to the reaction mixture and the initial rate of the absorbance change was subtracted from the rate readings of the test samples (Fig. 6). The N-reduction of guanoxabenz by bovine milk XO followed Michaelis-Menten kinetics, as was indicated by essentially linear Lineweaver-Burk plots (Fig. 6, inset), as well as an insignificat deviation from unity of the logistic function slope factors in the curve-fitting tests (see Materials and Methods for details). The apparent K_m and V_{max} values for guanoxabenz were determined to be, for xanthine as cofactor $K_m = 39.0 \pm 7.1$ µM and $V_{max} = 72.4 \pm 5.0$ nmol $\cdot min^{-1} \cdot unit^{-1}$, and for NADH as cofactor $K_m = 65.2 \pm 4.5$ µM and $V_{max} = 22.3 \pm 0.7$ $nmol \cdot min^{-1} \cdot unit^{-1}$ XO.

DISCUSSION

In the present study, a guanoxabenz metabolising enzyme was purified from the rat spleen cytosol using DEAE-Sepharose, hydroxyapatite and Superdex-200 HR chromatography. The enzymatic activity was monitored by evaluating the ability of the chromatographic fractions to enhance the ability of guanoxabenz to compete with the binding of an α_2-adrenoceptor ligand at α_2-adrenoceptors in HT-29 cell membranes. This assay, which is simple and convenient to perform, is founded on the basis that the reduction product of guanoxabenz (i.e. guanabenz) shows about 100-fold higher affinity for the α_2-adrenoceptors than does guanoxabenz itself. We also established the identity of the reaction product since it showed both identical chromatographic properties and an identical NMR spectrum to that of guanabenz.

Although the final purified protein product appeared as a single band on non-denaturing PAGE it was revealed to be composed of several subunits on SDS/PAGE. The electrophoresis pattern indicated that the enzyme was extensively proteolyzed during the purification procedure [17]. XO is known for its ability to undergo extensive cleavage without concomitant reduction in its molecular mass [18]. The enzyme remains catalytically active because disulphide linkages and non-covalent forces maintain the tertiary structure of the protein [19], thus explaining why the enzymatic activity was not destroyed during the purification procedure, even though we did not add protease inhibitors to the chromatographic buffers. We found that the N-terminal amino acid sequence of one of the purified subunits was identical to the eight N-terminal amino acid portion of an 85-kDa fragment resulting from tryptic digestion of XO [20], thus giving unequivocal evidence for the identity of the purified protein product. However, although the protein of the final preparation appeared to be essentially pure in a native polyacrylamide gel, the absorbency ratio A_{280}/A_{450} (the ratio of protein to flavin) of the sample was about 6.0, which may indicate that our preparation contained some additional protein since, for highly purified bovine XO the ratio is reported to be 5.1 [17, 21]. However, as also commercially available bovine XO reproduced the guanoxabenz-reducing activity of the purified rat preparation, it seems clear beyond doubt that XO is the major enzyme responsible for reducing guanoxabenz to guanabenz in the rat spleen cytosol.

XO and xanthine dehydrogenase are two functional forms of the xanthine oxidoreductase enzyme, also called type O and type D. The distinction is determined by the preference of each enzyme for a different electron acceptor (oxygen and NAD^+, respectively), otherwise both forms have the same molecular mass and similar structure of a homodimeric protein. Each subunit contains one molybdenum centre, one FAD and two iron/sulphur centres [22]. The natural reducing substrates xanthine and hypoxanthine are oxidised at the molybdenum centre, and from there

the electrons are passed further via intramolecular electron transfer to the iron-sulphur centres, and finally to the FAD centre where the reducing equivalents are transferred to molecular oxygen (type-O enzyme) or NAD^+ (type-D enzyme) [23].

The xanthine oxidoreductase enzyme exists in rat tissues predominantly in the dehydrogenase form (type-D form) [22]. However, most purification procedures, as well as storage, change it gradually to the type-O form [24]. It is possible that the fresh spleen supernatant, as well as the first purification steps, contained some of the type-D enzyme, which was later converted to the type-O form. However, the possibility that xanthine dehydrogenase also can participate in guanoxabenz N-reduction is still to be tested.

Several alternative oxidising substrates to oxygen have been reported for XO, e.g. nitro compounds [7, 8, 25], methylene blue [26], 6-*N*-hydroxylaminopurine [9] and other substances [6]. The reducing equivalents in the partially reduced XO distribute among the four redox-active centres and the sites of the enzyme where the above mentioned compounds are reduced are not entirely clear [26, 27]. In our study, the N-reduction of guanoxabenz was essentially completely blocked by the xanthine oxidoreductase inhibitor allopurinol. The blockade was observed when xanthine was used electron donor, something which is easy to understand since the oxidised form of allopurinol is known to form a tight complex with the molybdenum centre of XO, thus blocking the oxidation site for xanthine [28, 29]. NADH can also be used as a source of reducing equivalents, but NADH is known to be oxidised at the FAD site of XO [30]; the fact that the reduction of guanoxabenz was blocked by allopurinol also when NADH was used indicates that the state of the molybdenum centre itself is important for the reduction of guanoxabenz by XO.

Since oxygen is a competitor in N-reduction reactions catalysed by XO, we measured the formation of guanabenz under aerobic and anaerobic conditions in the presence of xanthine or NADH. The reduction of guanoxabenz was considerably more sensitive to the interference by oxygen when xanthine was used as electron donor, compared to using NADH as donor. However, kinetic parameters indicated that xanthine was a more effective cofactor than NADH for the anaerobic reduction of guanoxabenz. Our study shows that XO mediates the N-reduction of guanoxabenz with a K_m value in the micromolar range (65.2 ± 4.5 μM with NADH and 39.0 ± 7.1 μM with xanthine as electron donor). Interestingly, Clement et al. [31] have reported that rabbit microsomes can also catalyze N-reduction of guanoxabenz in the presence of NADH, the K_m value for the reaction being about 34 μM. Moreover, these authors reported recently that the microsomal N-reduction is catalyzed by an enzyme system composed of cytochrome b_5, NADH cytochrome b_5-reductase and benzamidoxime reductase [32]. Thus, it seems that guanoxabenz can be biologically N reduced by at least two different enzyme systems, XO and cytochromes, both of which are active at about the same concentration ranges for guanoxabenz.

Current interest in XO comes from its ability to activate prodrugs [33] and its proposed role in postischemic tissue damage due to oxygen-derived radicals [34−36]. Guanoxabenz has been used as a centrally active antihypertensive whose action is presumed to be mediated by activation of α_2-adrenoceptors [37]. In view of the here described metabolic conversion of guanoxabenz to guanabenz, the latter which is also a centrally active antihypertensive agent known to be mediating its effect via stimulation of α_2-adrenoceptors, and the about 100-fold higher affinity of guanabenz for the α_2-adrenoceptors compared to guanoxabenz, it seems very plausible that guanoxabenz acts as a prodrug to guanabenz. Whether or not guanoxabenz could affect the generation of oxygen-derived radical by XO remains to be established. Nevertheless, in conclusion, guanoxabenz is a novel electron-acceptor structure for XO, and our results thus broaden the range of drugs whose reduction is shown to be catalyzed by XO.

We thank Dr P.-I. Ohlsson (Umeå University, Sweden) for carrying out the N-terminal amino acid sequencing. This work was supported by the Swedish MRC (04X-05957) and the Wiberg foundation. Staffan Uhlén was supported from the *Berth von Kantzow* foundation.

REFERENCES

1. Uhlén, S. & Wikberg, J. E. S. (1991) Delineation of three pharmacological subtypes of α_2-adrenoceptors in the kidney, *Br. J. Pharmacol. 104*, 657−664.
2. Uhlén, S., Xia, Y., Chhajlani, V., Lien, E. J. & Wikberg, J. E. S. (1993) Evidence for the existence of two forms of α_{2A}- adrenoceptors in the rat, *Naunyn-Schmiedebergs Arch. Pharmacol. 347*, 280−288.
3. Xia, Y., Uhlén, S., Chhajlani, V., Lien, E. J. & Wikberg, J. E. S. (1993) Further evidence for the existence of two forms of α_{2B}-adrenoceptors in the rat, *Pharmacol. Toxicol. 72*, 40−49.
4. Wikberg, J. E. S., Chhajlani, V., Xia, Y., Muceniece, R. & Uhlén, S. (1995) Newer subtypes of the α_2? in *Adrenoceptors* (Ruffolo, R. R., ed.) pp. 109−118, Harwood Academic Publishers, Luxembourg.
5. Kooij, A. (1994) A re-evaluation of the tissue distribution and physiology of xanthine oxidase, *Histochem. J. 26*, 889−915.
6. Rajagopalan, K. V. (1980) Xanthine oxidase and aldehyde oxidase, in *The enzymatic basis of detoxication* (Jakoby, W. B., ed.) pp. 295−309, Academic Press, New York.
7. Tatsumi, K., Kitamura S., Yoshimura H. & Kawazoe Y. (1978) Susceptibility of aromatic compounds to xanthine oxidase-catalyzed reduction, *Chem. Pharm. Bull. 26*, 1713−1717.
8. Tatsumi, K., Inoue A. & Yoshimura, H. (1981) Mode of reactions between xanthine oxidase and aromatic nitro componds, *J. Pharm. Dyn. 4*, 101−108.
9. Clement, B. & Kunze, T. (1992) The reduction of 6-N-hydroxylaminopurine to adenine by xanthine oxidase, *Biochem. Pharmacol. 44*, 1501−1509.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) Protein measurement with the folin phenol reagent, *J. Biol. Chem. 193*, 265−275.
11. De Lean, A., Hancock, A. M. & Lefkowitz, R. J. (1982) Validation and statistical analysis of radioligand binding data for mixtures of pharmacological receptor subtypes, *Mol. Pharm. 21*, 5−16.
12. Bergström, A. & Wikberg, J. E. S. (1986) Guanine nucleotides regulates both agonist and antagonist binding to cod brain α_1-adrenoceptors, *Acta Pharmacol. Toxicol. 59*, 270−278.
13. Uhlén, S. & Wikberg, J. E. S. (1991) Delineation of rat kidney α_{2A}- and α_{2B}-adrenoceptors with [^{3}H]RX821002 radioligand binding: computer modelling reveals that guanfacine is an α_{2A}-selective compound, *Eur. J. Pharmacol. 202*, 235−243.
14. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature 227*, 680−685.
15. Nesterenko, M. V., Tilley, M. & Upton, S. J. (1994) A simple modification of Blum's silver stain method for 30 minute detection of proteins in poliacrylamide gels, *J. Biochem. Biophys. Methods 28*, 239−242.
16. Druper, N. R. & Smith, R. (1966) *Applied regression analysis*, John Willey & Sons, NY.
17. Bray, R. C. (1975) Molybdenum hydroxylases, in *The enzymes*, (Boyer, P. D., ed.) vol. 12B, pp. 304−326, Academic Press, NY-San Francisco-London.
18. Nagler, L. G. & Vartanyan, L. S. (1976) Subunit structure of bovine milk xanthine oxidase, effect of limited cleavage by proteolytic enzymes on activity and structure, *Biochim. Biophys. Acta 427*, 78−90.
19. Krenitsky, T. A., Spector, T. & Hall, W. W. (1986) Xanthine oxidase from human liver: purification and characterization, *Arch. Biochem. Biophys. 247*, 108−119.

20. Amaya, Y., Yamazaki, K.-I., Sato, M., Noda, K., Nishino, T. & Nishino, T. (1990) Proteolytic conversion of xanthine dehydrogenase from the NAD-dependent type to the O_2-dependent type, *J. Biol. Chem. 265*, 14170−14175.
21. Nishino, T., Nishino, T. & Tsushima, K. (1981) Purification of highly active milk xanthine oxidase by affinity chromatography on Sepharose 4B/folate gel, *FEBS Lett. 131*, 369−372.
22. Nishino, T. (198694) The conversion of xanthine dehydrogenase to xanthine oxidase and the role of the enzyme in reperfusion injury, *J. Biochem. 116*, 1−6.
23. Hille, R. & Massey, V. (1981) Studies on the oxidative half-reaction of xanthine oxidase, *J. Biol. Chem. 256*, 9090−9095.
24. Stirpe, F. & Della Corte, E. (1968) The regulation of rat liver xanthine oxidase, *J. Biol. Chem. 244*, 3855−3863.
25. Kutcher, W. W. & McCalla, D. R. (1984) Aerobic reduction of 5-nitro-2-furaldehyde semicarbazone by rat liver xanthine dehydrogenase, *Biochem. Pharmacol. 33*, 799−805.
26. Salaris, S. C., Babbs, C. F. & Voorhees, W. D. (1991) Methylene blue as an inhibitor of superoxide generation by xanthine oxidase, *Biochem. Pharmacol. 42*, 499−506.
27. Olson, J. S., Ballou, D. P., Palmer, G. & Massey, V. (1974) The mechanism of action of xanthine oxidase, *J. Biol. Chem. 249*, 4363−4382.
28. Hille, R. & Massey, V. (1981) Tight binding inhibitors of xanthine oxidase, *Pharmac. Ther. 14*, 249−263.
29. Massey, V. & Harris, C. M. (1997) Milk xanthine oxidoreductase: the first one hundred years, *Biochem. Soc. Trans. 25*, 750−755.
30. Bray, R. C. (1975) Molybdenum hydroxylases, in *The enzymes* (Boyer, P. D., ed.) vol. 12B, pp. 344−389, Acad Press, NY-San Francisco-London.
31. Clement, B., Demesmaeker, M. & Line, S. (1996) Microsomal catalyzed N-hydroxylation of guanabenz and reduction of the N-hydroxylate metabolite: Characterization of the two reactions and genotoxic potential of guanoxabenz, *Chem. Res. Toxicol. 9*, 682−688.
32. Clement, B. & Demesmaeker, M. (1997) Formation of guaoxnoxabenz from guanabenz in human liver. A new metabolic metabolic marker for CYP1A2, *Drug Metab. Disp. 25*, 1266−1271.
33. Krenitsky, T. A., Hall, W. W., De Miranda, P., Beauchamp, L. M., Schaeffer, H. J. & Whiteman, P. D. (1984) 6-Deoxyacyclovir: a xanthine oxidase-activated prodrug of acyclovir, *Proc. Natl Acad. Sci. USA 81*, 3209−3213.
34. White, R. C., Darley-Usmar, V., Berrington, W. R., McAdams, W. R., Gore, J. Z., Thompson, A. J., Parks, D. A., Tarpey, M. M. & Freeman, B. A. (1996) Circulating plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterolemic rabbits, *Proc. Natl Acad. Sci. USA 93*, 8745−8749.
35. McCord, J. M. (1985) Oxygen-derived free radicals in postischemic tissue injury, *New Engl. J. Med. 312*, 159−163.
36. Saugstad, O. D. (1996) Role of xanthine oxidase and its inhibitor in hypoxia:reoxygenation injury, *Pediatrics 98*, 103−107.
37. Ledoux, F., Welsc, M., Steimer, C. & Schwartz, J. (1981) A clinical trial of guanoxabenz: a hypotensive agent with central and hypertensive action, *Therapie 36*, 187−191.