

**NADH cytochrome *b*₅ reductase and cytochrome *b*₅ catalyze the
microsomal reduction of xenobiotic hydroxylamines and amidoximes in
humans**

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The abbreviations used are: cyt b₅: cytochrome b₅; NDHR: NADH-dependent hydroxylamine reductase; b₅R: NADH cytochrome b₅ reductase; IPTG: isopropyl β-D-1-thiogalactopyranoside; SMX-HA: sulfamethoxazole hydroxylamine; HLM: human liver microsomes; HPLC: high performance liquid chromatography; HRP: horseradish peroxidase; HSA: human serum albumin; RT: room temperature.

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Abstract

Hydroxylamine metabolites, implicated in dose-dependent and idiosyncratic toxicity from arylamine drugs, and amidoximes, used as pro-drugs, are metabolized by an as yet incompletely characterized, NADH-dependent microsomal reductase system. We hypothesized that NADH cytochrome b₅ reductase and cytochrome b₅ were responsible for this enzymatic activity in humans. Purified human soluble NADH cytochrome b₅ reductase and cytochrome b₅, expressed in *E. coli*, efficiently catalyzed the reduction of sulfamethoxazole hydroxylamine, dapsone hydroxylamine, and benzamidoxime, with apparent K_m values similar to those found in human liver microsomes, and specific activities (V_{max}) 74 to 235 times higher than in microsomes. Minimal activity was seen with either protein alone, and microsomal protein did not enhance activity other than additively. All three reduction activities were significantly correlated with immunoreactivity for cytochrome b₅ in individual human liver microsomes. In addition, polyclonal antibodies to both NADH cytochrome b₅ reductase and cytochrome b₅ significantly inhibited reduction activity for sulfamethoxazole hydroxylamine. Finally, fibroblasts from a patient with Type II hereditary methemoglobinemia (deficient in NADH cytochrome b₅ reductase) showed virtually no activity for hydroxylamine reduction, compared to normal fibroblasts. These results indicate a novel direct role for NADH cytochrome b₅ reductase and cytochrome b₅ in xenobiotic metabolism, and suggest that pharmacogenetic variability in either of these proteins may affect drug reduction capacity.

Hydroxylamine and amidoxime compounds are metabolized in humans by an as yet incompletely characterized NADH-dependent reductase system. Hydroxylamine metabolites have been implicated in dose-dependent and idiosyncratic drug toxicity from sulfamethoxazole, dapsone, procainamide, and other arylamine drugs (Utrecht, 2002). Amidoximes and other hydroxylated amines have been developed as prodrugs to enhance the absorption of a wide range of antihypertensive, antiprotozoal, and antithrombotic drugs (Weller et al., 1996; Hall et al., 1998; Clement, 2002), and the reduction of these compounds is necessary for drug bioactivation. Thus, the reduction of hydroxylamines and amidoximes has important toxicologic and therapeutic implications.

Xenobiotic hydroxylamines and amidoximes are reduced to their parent amines by a microsomal, oxygen-insensitive, NADH-dependent enzyme system, variably termed NADH-dependent N-hydroxy amine reductase, NADH-benzamidoxime reductase, or NADH-dependent hydroxylamine reductase (NDHR) (Kadlubar and Ziegler, 1974; Cribb et al., 1995; Clement et al., 1998; Trepanier and Miller, 2000). NDHR was originally described in the reduction of N-methylhydroxylamine, N-hydroxyamphetamine, and related compounds (Kadlubar et al., 1973; Kadlubar and Ziegler, 1974; Yamada et al., 1988). NDHR has more recently been shown to catalyze the reduction of hydroxylamine metabolites of sulfamethoxazole and dapsone (Cribb et al., 1995; Clement et al., 1998), as well as the reduction of N-hydroxylated derivatives of benzamidine, debrisoquine, guanabenz, metamphetamine, and pentamidine (Clement, 2002) (**Figure 1**).

The complete characterization of NDHR has not been reported in humans, but in the pig, a protein with sequence homology to CYP2D6 has been proposed to be involved in the reduction of hydroxylamines (Clement et al., 1997), along with NADH cytochrome b₅ reductase and cytochrome b₅. NADH-dependent hydroxylamine reduction in humans, however, does not correlate with the activities of CYP2D6, nor with the activities of CYP1A2, 2A6, 2C8, 2C9, 2C19, 2E1, 3A4, or 3A5 (Cribb et al., 1995; Lin et al., 1996). In addition, NDHR activity is insensitive to azide and carbon monoxide (Kadlubar et al., 1973; Trepanier and Miller, 2000), which is inconsistent with the involvement of electron transport through P450 in this reaction.

NADH cytochrome b₅ reductase (EC 1.6.2.2; Diaphorase I; NADH:ferricytochrome b₅ oxidoreductase) and cytochrome b₅ themselves have all of the biochemical characteristics of NDHR, and therefore may be capable of directly mediating NADH-dependent hydroxylamine reduction in humans. These proteins, however, have historically been viewed as “helper” enzymes involved in intermediate electron transfer (Porter, 2002), and have not been shown to have a major direct role in xenobiotic metabolism. In this report, we provide evidence that purified human recombinant NADH cytochrome b₅ reductase (b₅R) and cytochrome b₅ (cyt b₅) without any other microsomal components, are sufficient for the reduction of sulfamethoxazole hydroxylamine, dapsone hydroxylamine, and benzamidoxime to their parent amines/amidines. We show further that the apparent K_m's for reduction by the recombinant system are similar to those seen in human liver microsomes, and that addition of microsomal protein does not enhance activity more than additively. In addition, hydroxylamine and amidoxime reduction activities correlate with

microsomal cyt b5 content, are inhibited by antiserum to either b5R or cyt b5, and are absent in fibroblasts deficient in b5R. We conclude that b5R and cyt b5 together have an important direct role in xenobiotic reduction, and further hypothesize that variability in the expression of either of these proteins may have important therapeutic and toxicologic consequences.

Methods

Expression of soluble human NADH cytochrome b5 reductase (b5R) -The full length cDNA of human soluble b5R was kindly provided by Dr. Komei Shirabe, Oita Medical University, Japan. Initial attempts to purify the protein using DEAE and 5'AMP-Sepharose column chromatography lead to poor yields, due to weak affinity of b5R for 5'AMP-Sepharose under a variety of conditions. The cDNA was inserted into an N-terminal histidine tag expression system (pCR T7/NT-TOPO®; Invitrogen; Carlsbad, CA) to allow purification from *E. coli* lysate using nickel affinity chromatography. Induction with 1 mM IPTG was performed for 4 hours at room temperature to prevent over-expression and compartmentalization of the protein in lipid vacuoles. Stepwise elution of lysate contaminants followed by His-tagged b5R was accomplished with three elution buffers: 20, 100 and 350 mM imidazole, each at pH 8.0 in binding buffer. The eluent was collected in 1 ml fractions, which were tested for purity by gel electrophoresis and subsequent silver staining, for identity using immunoblotting, and for activity using potassium ferricyanide reduction (Arinc and Cakir, 1999). The fractions containing a single 36 kD band on silver stain were pooled and dialyzed against binding buffer, pH 8.0, through 10,000 MW cut-off cassettes (Pierce Biotechnology, Rockford IL) to remove

imidazole. The dialyzed sample was again bound to a nickel column and re-purified and pooled as described above. The purified b5R was dialyzed against PBS pH 7.4 and concentrated to a final protein concentration of 2-3 mg/ml using 10,000 MW cut-off filtration devices (Centricon®, Millipore). Purified protein retained maximal activity at 4⁰C for approximately 3 weeks.

Expression of soluble human cytochrome b₅ (cyt b₅): The full length cDNA of human soluble cyt b₅ (Lloyd et al., 1994), kindly provided by Dr. Grant Mauk, University of British Columbia, was expressed in the pCR T7/CT-TOPO® system, using methods as described for b5R, with some modifications. Briefly, stepwise elutions using 20, 40 and 100 mM imidazole, each at pH 8.0 in binding buffer, were collected in 1 ml fractions and tested for purity by gel electrophoresis and subsequent silver staining. Identity was verified with immunoblotting. Fractions containing a single 16.5 kD band on silver stain were pooled, dialyzed, and re-purified as described for b5R. Purified protein retained maximal activity at 4⁰C for approximately 3 weeks.

Hydroxylamine reduction by human b5R and cyt b5- Sulfamethoxazole-hydroxylamine (SMX-HA; Dalton Chemical Laboratories, Toronto, Canada) in 50% DMSO/ 3 mM ascorbic acid was incubated at varying concentrations with purified soluble human recombinant b5R and cyt b5. Commercially available cyt b5 protein (Panvera Corporation, Madison WI), was used for preliminary experiments; the soluble form of cyt b5, purified to homogeneity as described above, was used for all subsequent experiments. Reactions were performed in PBS pH 7.4 with 1 mM NADH; glutathione and ascorbic

acid (1mM each) were included to maximally stabilize the SMX-HA against further oxidation (Trepanier and Miller, 2000). The final DMSO concentration in all reactions was 1.25%. The optimal stoichiometry of b5R: cyt b5 was determined by measuring reduction activity using different ratios of b5R to cyt b5 while keeping constant the total nmoles of protein (Huang, 1982). Sulfamethoxazole hydroxylamine reduction activities were determined using isocratic HPLC with UV detection as previously described (Trepanier and Miller, 2000). Negative controls included human serum albumin (HSA) or protein purified from sham-transformed *E. coli*, and recombinant systems lacking NADH or substrate.

Dapsone hydroxylamine was a gift from Dr. Reginald Frye, School of Pharmacy, University of Pittsburgh. Reduction of dapsone hydroxylamine was determined using HPLC as described for sulfamethoxazole. Retention times for dapsone and its hydroxylamine were 6.6 and 5.2 minutes, respectively. In addition, the reduction of benzamidoxime (a prototypical oxime substrate also subject to NADH-dependent microsomal reduction (Clement et al., 1997)), to benzamidine was measured using a modification of a previously published HPLC method (Clement et al., 1988). Briefly, the mobile phase consisted of water / methanol / phosphoric acid 85% / triethylamine / 1-octanesulfonic acid sodium salt (68/ 32/ 0.0036/ 0.0050/ 0.0012, V/V/V/V/M), with a flow rate of 1.5 ml/min. UV detection was at 229 nm, with retention times for benzamidoxime of 13.5 min and benzamidine of 15.1 min.

Estimates for apparent K_m and V_{max} of the expressed protein system for the three substrates were determined by non-linear curve fitting using commercial software (Prism 3, GraphPad Software Inc., San Diego CA). For comparison of specific activity and apparent K_m values between recombinant and microsomal systems, kinetics were also determined in pooled human liver microsomes (Gentest Corp., Bedford, MA).

Immunocorrelation studies - For preliminary studies, rabbit polyclonal antibody to rat b5R was kindly provided by Dr. Jose Villalba, Universidad de Cordoba, Spain. For subsequent studies, purified human b5R and cyt b5 were used to immunize individual rabbits using standard protocols. Immune rabbit anti-b5R and anti-cyt b5 sera were used to probe immunoblots prepared from microsomal protein (40 μ g) from individual human liver microsomes (Gentest Corp.; Human Biologics International, Scottsdale AZ). PVDF membranes (Immobilon-P, Millipore Corp., Billerica MA) were blocked with 2.5% non-fat dry milk in PBS/0.1% Tween-20, and washed with PBS/0.1% Tween-20. Primary antisera were diluted 1:10,000 for immunoblotting; HRP-linked secondary antibody (donkey anti-rabbit IgG, Amersham BioSciences, Piscataway NJ) was diluted 1:10,000. An enhanced chemiluminescence system was used for signal detection (Amersham), and was quantified using a Storm 840 imaging system with ImageQuANT software (Molecular Dynamics, Amersham). For each of the individual human liver microsome samples, b5R and cyt b5 immunoreactivity were correlated with microsomal reduction of each substrate, as determined by HPLC as described above, using a correlation z test (Statview, Abacus Concepts, Berkeley CA).

Immunoinhibition of reduction – Antisera (150 μ l each) to human b5R and cyt b5 as described above was pre-incubated with human liver microsomes (0.5 mg) for 30 mins. at RT, followed by quantitation of reduction of sulfamethoxazole hydroxylamine. Preimmune rabbit serum or HSA instead of serum were used as negative controls.

Hydroxylamine reduction in fibroblasts deficient in b5R - Fibroblasts from a patient with Type II hereditary methemoglobinemia, a disorder in which systemic b5R activity is deficient or completely lacking, were generously provided by Drs. Wilfried Kugler and Arnulf Pekrun at Universitats-Kinderklinik, of Goettingen, Germany (Kugler et al., 2001). Normal human dermal fibroblasts were a gift from the laboratory of Drs. Gregory MacEwen and David Vail (University of Wisconsin-Madison). Cells were cultured in Eagle's Minimum Essential Media (without phenol red, calcium, or magnesium; Mediatech Inc., Herndon VA), supplemented with 1mM sodium pyruvate, 2 mM L-glutamine, penicillin (100 IU/ml), streptomycin (100 μ g/ml), and amphotericin (0.25 μ g/ml), and incubated at 37 °C in a humidified atmosphere with 5% CO₂. Cells were harvested with trypsin containing 670 μ M EDTA, washed with media followed by HANKS buffer, and resuspended in 15 mM HEPES pH 7.4. Reduction activity reactions contained 1.5×10^6 cells, along with 500 μ M of SMX-HA. Glutathione and ascorbate (1 mM each) were included in SMX-hydroxylamine reactions to prevent further oxidation of the hydroxylamine. Cells were incubated at 37 °C for 40 mins. Reactions were stopped on ice with half the reaction volume of methanol, then filtered through 30kD filters (Millipore) prior to HPLC analysis. Fibroblasts were also subjected to immunoblotting using both α -cyt b5 and α - b5R.

Results

b5R protein expression and purification - Expression of histidine-tagged human soluble b5R in *E. coli* and purification using nickel affinity chromatography yielded an average of 3-4 mg of purified protein per liter of culture. A single 36 kD protein (32 kD protein plus 4 kD tag) was obtained, with consistent purity and strong immunoreactivity with anti-b5R antibody (**Figure 2**). Preliminary studies showed no difference in activity between tagged and untagged protein (as others have shown; (Bewley et al., 2001)), and since enterokinase cleavage and processing to remove the His tag resulted in a less pure product (data not shown), all subsequent studies were performed with histidine-tagged protein.

Cyt b5 protein expression and purification - Expression and purification of histidine-tagged human soluble cyt b5 yielded an average of 5 mg of purified protein per liter of culture. A single 16.5 kD protein (12.5 kD protein plus 4 kD tag) was obtained, with consistent purity and strong immunoreactivity with anti-cyt b5 antibody (**Figure 3**).

Reduction of hydroxylamine and amidoxime substrates - Expressed b5R and cyt b5 catalyzed the efficient reduction of sulfamethoxazole hydroxylamine (500 μ M), at rates 160 times greater than pooled human liver microsomes (HLM; **Figure 4**). No activity was seen with protein purified with sham transformed *E. coli*, or in the absence of NADH, and minimal activity was observed with either b5R or cyt b5 alone. No enhancement of activity other than an additive effect was seen with the addition of 10,

100, 300, or 1000 μg of pooled human liver microsomes to the recombinant system (**Figure 5**).

The optimal stoichiometry of cyt b5 to b5R was between 8:1 and 10:1 as determined by a Job plot (Huang, 1982) (data not shown). The kinetics of sulfamethoxazole hydroxylamine reduction in the expressed system (**Figure 6**) and in HLM were each fit to a one site model with similar apparent K_m values (**Table 1**). Dapsone hydroxylamine and benzamidoxime were also reduced by the recombinant system, with 163 and 74 times higher specific activities, respectively, and similar apparent K_m values for each substrate compared to HLM.

Immunocorrelation - Microsomal b5R content (as measured by immunoreactivity to α -b5R) did not correlate with SMX-HA reduction activity (500 μM substrate) in 27 individual human liver microsomes (**Figure 7A**). However, b5R content in these 27 commercially available samples only differed by 2.2-fold. On the other hand, microsomal cyt b5 content (as measured by immunoreactivity to α -cyt b5) correlated strongly with SMX-HA reduction activity in 19 individual human liver microsomes. ($r = 0.75$; $P = 0.0001$; **Figure 7B**), with 9.3-fold variability in cyt b5 immunoreactivity found in this small sample. In addition, microsomal cyt b5 content also correlated with benzamidoxime reduction ($r = 0.64$; $P = 0.025$; **Figure 8**), and with dapsone hydroxylamine reduction ($r = 0.90$; $P = 0.015$; only 7 individual microsomes were tested because of limited substrate supply).

Immunoinhibition– Antisera to b5R and cyt b5 inhibited SMX-HA reduction in human liver microsomes by 69% and 58% respectively, compared to preimmune serum (**Figure 9**). This was comparable to the inhibition of SMX-HA reduction by these antisera in the recombinant b5R/cyt b5 system, with 63% and 51% inhibition, respectively, compared to preimmune serum.

Reduction activity in b5R-deficient fibroblasts: Immunoreactive b5R was present in normal human fibroblasts, but was absent, as expected, in fibroblasts from a patient with Type II hereditary methemoglobinemia (**Figure 10**). Similar cyt b5 content was present in both cell types. Reduction of sulfamethoxazole hydroxylamine was virtually undetectable in the b5R-deficient fibroblasts compared to normal human dermal fibroblasts.

Discussion

NADH cytochrome b₅ reductase is an FAD-containing protein with soluble and membrane-bound forms, each the product of the same gene through alternative promoters and alternative splicing (Tomatsu et al., 1989; Leroux et al., 2001). Both soluble (approximately 32 kD) and membrane-bound (approximately 35 kD) forms of b5R have identical catalytic domains, and differ only in a hydrophobic domain at the N-terminus of the membrane-bound form. This 3 kD N-terminal sequence is myristoylated, and allows insertion of the membrane-bound form into endoplasmic reticulum and outer mitochondrial membranes of somatic cells (Ozols et al., 1984). The hemoprotein cytochrome b₅ is also expressed in soluble and membrane-bound forms, although the

hydrophobic anchor of membrane-bound cyt b5 is at the C-terminus (Borgese et al., 1993). As for b5R, the microsomal and soluble isoforms of cyt b5 are the product of a single gene (Giordano and Steggles, 1991). Cyt b5 and b5R together mediate electron transfer from NADH to fatty acid desaturases (Oshino et al., 1971) and P450 oxidases (Hildebrandt and Estabrook, 1971) and, in erythrocytes, play a primary role in the maintenance of hemoglobin in its reduced state (Hultquist and Passon, 1971). In addition, b5R mediates the reduction of the partially oxidized form of ascorbate, ascorbyl free radical, back to ascorbate (Ito et al., 1981; Shirabe et al., 1995).

The results of our studies indicate that b5R and cyt b5 also have a direct role in xenobiotic metabolism. This enzyme complex efficiently catalyzed the reduction of the xenobiotic hydroxylamines of dapsone and sulfamethoxazole, as well as the reduction of the prototypical amidoxime pro-drug, benzamidoxime. The apparent K_m 's for reduction by the purified system were nearly identical to those found in human liver microsomes, and microsomal activity was inhibited by antibodies to either b5R or cyt b5 to the same degree as the recombinant system. The microsomal content of cyt b5 correlated strongly with both microsomal hydroxylamine and amidoxime reduction, and human dermal fibroblasts that lack b5R (Kugler et al., 2001) showed virtually no activity for the reduction of sulfamethoxazole hydroxylamine. Addition of microsomal protein to the recombinant system did not enhance activity more than additively, suggesting that additional microsomal proteins are not important for optimal activity. These data together provide strong evidence that the enzyme complex of b5R and cyt b5 is responsible for the reduction of hydroxylamines and amidoximes in humans.

The reduction of hydroxylamines and amidoximes represents a novel direct role for this enzyme complex in xenobiotic metabolism. Although b5R has been shown to reduce some chemotherapeutic agents to their cytotoxic forms (Mahmutoglu and Kappus, 1988; Hodnick and Sartorelli, 1993), other reports of direct biotransformation by this enzyme complex are lacking. Clement *et al.* reported that b5R and cyt b5 were cofactors in amidoxime and hydroxylamine reduction (Clement *et al.*, 1997; Clement *et al.*, 1998); however, they concluded that a third protein of P450 origin was necessary for efficient reduction. Our data are in disagreement with these studies, in that our purified recombinant system containing only b5R, cyt b5, and NADH was capable of efficient and complete reduction of one amidoxime and two hydroxylamine substrates. Some reasons for the differences between our data and previous studies could be the species studied (human for our studies compared to pig in previous studies), the source of enzyme complex (recombinant expression in our studies vs. biochemical purification in previous studies), or the pH of the reactions (pH 7.4 in our studies vs. pH 6.3 previously). In addition, we found an optimal stoichiometry of 8 to 10:1 for cyt b5: b5R, while previous studies used b5R in excess of cyt b5 (Clement *et al.*, 1998).

The optimal stoichiometry of 8-10 moles cytochrome b₅ to one mole b5R for hydroxylamine reduction is interesting, given that the reaction presumably entails two successive single-electron reductions from b5R to cyt b5 (Iyanagi *et al.*, 1984). A 10:1 stoichiometry of cyt b5 to b5R has also been found in native liver microsomes (Yang and Cederbaum, 1996), and may reflect the amount of cyt b5 required to maintain a high

turnover rate of b5R for cyt b5 reduction (Meyer et al., 1995; Arinc and Cakir, 1999). Because our recombinant system had an optimal stoichiometry similar to that reported for native liver microsomes, the higher activity seen with the recombinant system was most likely due to enrichment of these two proteins, rather than a difference in the ratio of cyt b5: b5R content between recombinant and native systems.

We had previously reported a model with two apparent K_m 's for the reduction of SMX hydroxylamine by HLM (Trepanier and Miller, 2000). However, this was based upon an Eadie Hofstee transformation of reactions performed in HEPES buffer. Closer examination of that data revealed an artifact (a hump in the velocity vs. substrate concentration curve at low concentrations) that was resolved with a switch to PBS buffer, and which then yielded a single apparent K_m in both HLM and the recombinant system.

Polyclonal antibodies to b5R and cyt b5 inhibited hydroxylamine reduction activity, but not completely (**Figure 9**). Although this could indicate the involvement of other proteins in this pathway, it is more likely to reflect the imperfect inhibitory capacity of our antisera, since our antisera inhibited the purified b5R/cyt b5 system to the same incomplete degree (63% inhibition by α -b5R, and 51% inhibition by α -cyt b5, towards the purified system). One possible mechanism for incomplete inhibition by our sera towards even the recombinant system is steric interference by antibodies directed towards non-inhibitory epitopes, preventing the complete binding of antibodies directed at inhibitory epitopes.

One unexpected finding was the lack of correlation between b5R immunoreactive protein and hydroxylamine reduction activity in individual human liver microsomes (**Figure 7A**). This was most likely due to the relatively narrow range of b5R content (only 2.2-fold variability) in the 27 individual human liver microsomes evaluated, since it is clear from the lack of activity in reductase-deficient fibroblasts that b5R is necessary for hydroxylamine reduction. It is possible that sampling a much larger group of individual microsomes would reveal outliers in b5R content that would correlate with reduction activity. It is worth noting that cyt b5 content appears to vary more widely in human livers (9.3-fold in only 19 samples) than does its reductase, and therefore cyt b5 may be a more significant source of pharmacogenetic variability for this pathway.

Markedly decreased to absent b5R expression has been reported in hereditary methemoglobinemia, a rare homozygous recessive disorder in which mutations in the b5R gene lead to deficiencies in either soluble b5R alone (Type I methemoglobinemia, associated with persistent cyanosis) or in both soluble and membrane-bound b5R (Type II methemoglobinemia, associated with cyanosis and mental retardation) (Leroux et al., 1975; Jaffe, 1986). Subjects heterozygous for this disorder show decreased activity of b5R (Reghis et al., 1983), without clinical cyanosis. In contrast to relatively rare mutations causing hereditary methemoglobinemia, less catastrophic polymorphisms in the b5R gene (*DIAI*) have been observed but have not been well characterized. One genetic polymorphism in *DIAI* (T116S) has been reported in African Americans (Jenkins and Prchal, 1997), and slightly higher mean b5R activities have been observed in African Americans compared to Caucasians (Mansouri and Nandy, 1998), although a causal

relationship between these two findings has not been established. Absolute cyt b5 deficiency has been reported very rarely, primarily in association with congenital methemoglobinemia (Giordano et al., 1994). Given that b5R and cyt b5 together catalyze the efficient reduction of hydroxylamines and amidoximes, further work is needed to establish the relationship between polymorphisms in this system, variability in hydroxylamine and amidoxime metabolism, and therapeutic and toxicologic outcome.

In conclusion, the results of these studies indicate that human recombinant NADH cytochrome b₅ reductase and cytochrome b₅ efficiently reduce hydroxylamines and amidoximes without the need for other proteins, and support the conclusion that this enzyme complex comprises the previously reported but poorly characterized NADH-dependent hydroxylamine reductase system.

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Footnotes

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Legends for Figures

Figure 1: Reported substrates of NADH-dependent, oxygen-insensitive, microsomal hydroxylamine reductase (NDHR).

Figure 2: Purified human soluble cytochrome b₅ reductase. Panel A: Lane 1: Marker; Lane 2: *E. coli* lysate (50 µg) containing expressed human soluble NADH cytochrome b₅ reductase; Lane 3: purified b₅ reductase (b₅R; 25 µg), as purified by an N-terminal histidine tag expression system and nickel affinity chromatography. 4-20% polyacrylamide gel with Coomassie stain. Panel B: Purified b₅ reductase (as shown in panel A), immunoblotted with rabbit anti-human b₅R antisera. The 36 kD molecular weight reflects the 32 kD soluble protein with a 4 kD histidine tag.

Figure 3: Purified human soluble cytochrome b₅. Panel A: Lane 1: Marker; Lane 2: *E. coli* lysate (50 µg) containing expressed human soluble cytochrome b₅; Lane 3: purified cytochrome b₅ (25 µg), as purified by a C-terminal histidine tag expression system and nickel affinity chromatography. 4-20% polyacrylamide gel with Coomassie stain. Panel B: Purified cytochrome b₅ (as shown in panel A), immunoblotted with rabbit anti-human cytochrome b₅ antisera. The 16.5 kD molecular weight reflects the 12.5 kD soluble protein with a 4 kD histidine tag.

Figure 4: Specific activity for reduction of SMX-HA is 160-fold higher by expressed NADH cytochrome b₅ reductase (b5R) with cytochrome b₅ (10:1 stoichiometry, cyt b₅:b5R), compared to pooled human liver microsomes (HLM; 0.5 mg). Negative controls include b5R alone, cyt b₅ alone, and protein purified from sham transformed *E. coli*. Reactions included SMX-HA (500 μM) with 1 mM NADH, GSH, and ascorbate, in PBS pH 7.4. Data is expressed as mean ± SD for two separate experiments, each performed in duplicate.

Figure 5: Activity for reduction of SMX-HA (500 μM) by the recombinant system (Recomb; b5R and cyt b₅) compared to the recombinant system plus pooled human liver microsomes (HLM; 0.1 mg) is not more than additive. Note that activity is expressed per minute and is not normalized to mg of protein. All reactions are as described in Figure 4; similar results were seen with 0.01 mg, 0.3 mg, and 1.0 mg of additional microsomal protein.

Figure 6: Velocity versus substrate concentrations for reduction of SMX hydroxylamine by purified human recombinant cytochrome b₅ and NADH cytochrome b₅ reductase (optimal 10:1 stoichiometry) in PBS pH 7.4, with 1 mM NADH.

Figure 7A: Microsomal content of NADH cytochrome b₅ reductase (as measured by immunoreactivity to α-b5R) could not be correlated with SMX-hydroxylamine reduction

activity (500 μ M substrate) in 27 individual human liver microsomes ($r = 0.05$). However, there was only 2.2-fold range in b5R content in these available microsomes.

Figure 7B: Microsomal cytochrome b₅ content (as measured by immunoreactivity to α -cytochrome b₅) correlates strongly with SMX-hydroxylamine reduction activity (500 μ M substrate) in 19 individual human liver microsomes ($r = 0.75$; $P = 0.001$). Activity results are from three experiments each performed in duplicate.

Figure 8: Microsomal cyt b₅ content correlates strongly with benzamidoxime reduction activity (100 μ M benzamidoxime) in 12 individual human liver microsomes ($r = 0.64$; $P = 0.025$). Activity results are from two experiments each performed in duplicate.

Figure 9: Antisera to b5R and cyt b₅ (150 μ l each) significantly inhibited the reduction of sulfamethoxazole hydroxylamine in human liver microsomes (by 69% and 58%, respectively) compared to pre-immune serum. This was comparable to inhibition seen with these antibodies towards the purified system containing b5R and cyt b₅ (63% and 51% inhibition, respectively; data not shown). Microsomes (500 μ g) or b5R and b₅ (20.6 μ g total protein) were pre-incubated with antisera for 30 mins. at RT prior to initiation of the reaction. Data shown are the results of 2-3 experiments each performed in duplicate.

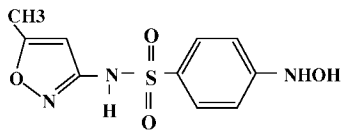
Figure 10: Activity for hydroxylamine reduction is virtually absent in b5R-deficient fibroblasts. Immunoreactivity to b5R (**Panel A**; 35 kD protein represents microsomal b5R that predominates in fibroblasts) and cyt b₅ (**Panel B**; 17 kD protein represents

microsomal cyt b5) antisera in normal human dermal fibroblasts (lane 1) and fibroblasts derived from a patient with Type II hereditary methemoglobinemia (lane 2 (Kugler et al., 2001); 40 μ g of protein loaded in each lane). **Panel C:** Reduction of sulfamethoxazole hydroxylamine by normal and b5R-deficient fibroblasts.

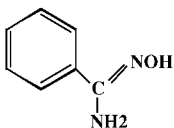
Table 1: Kinetic data for the reduction of two hydroxylamines (HA) and an amidoxime by human recombinant NADH cytochrome b₅ reductase (b5R) and cytochrome b₅ (cyt b5), compared to that seen in pooled human liver microsomes (HLM). All reactions were performed in PBS pH 7.4 with 1 mM NADH, under conditions approximating linear kinetics. Velocity data are expressed in nmole/total mg protein/min. Data represent 2-3 experiments performed in duplicate for each condition, and are given as means ± standard error.

Substrate	Apparent K _m (μM)		V _{max} (nmole/mg/min)	
	HLM	b5R/cyt b5	HLM	b5R/ cyt b5
Sulfamethoxazole-HA	363 ± 31	392 ± 45	1.93 ± 0.07	455 ± 23
Dapsone-HA	357 ± 62	395 ± 95	1.26 ± 0.09	205 ± 24
Benzamidoxime	629 ± 116	515 ± 149	3.05 ± 0.36	226 ± 31

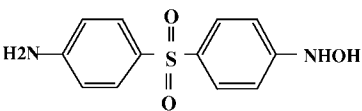
Figure 1



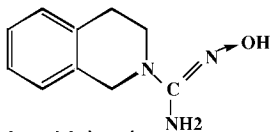
Sulfamethoxazole hydroxylamine



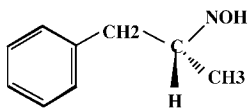
Benzamidoxime



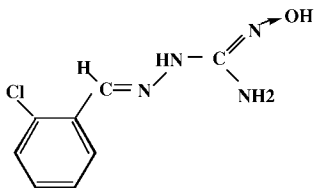
Dapsone hydroxylamine



N-hydroxydebrisoquine



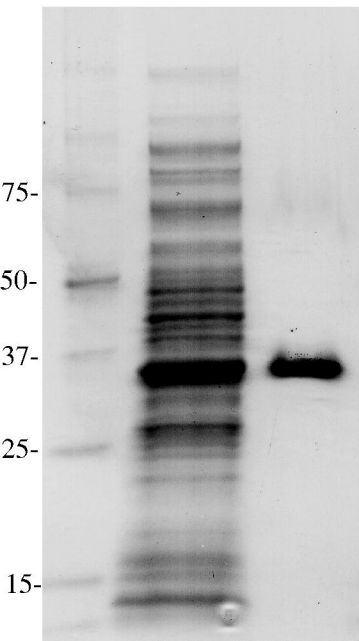
N-hydroxyamphetamine



Guanoxabenz

Figure 2

A



B

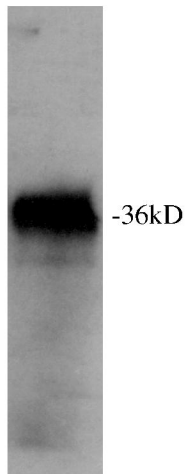
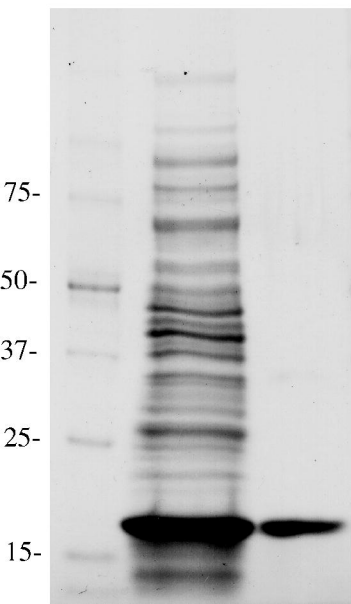


Figure 3

A



B

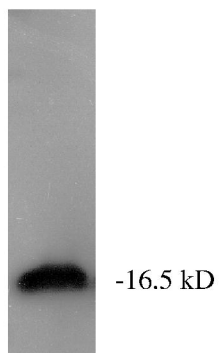


Figure 4

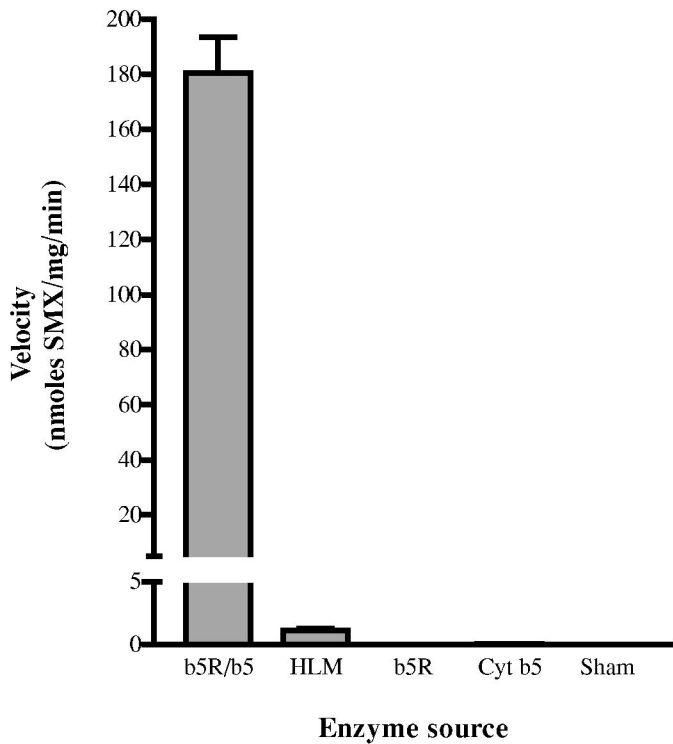


Figure 5

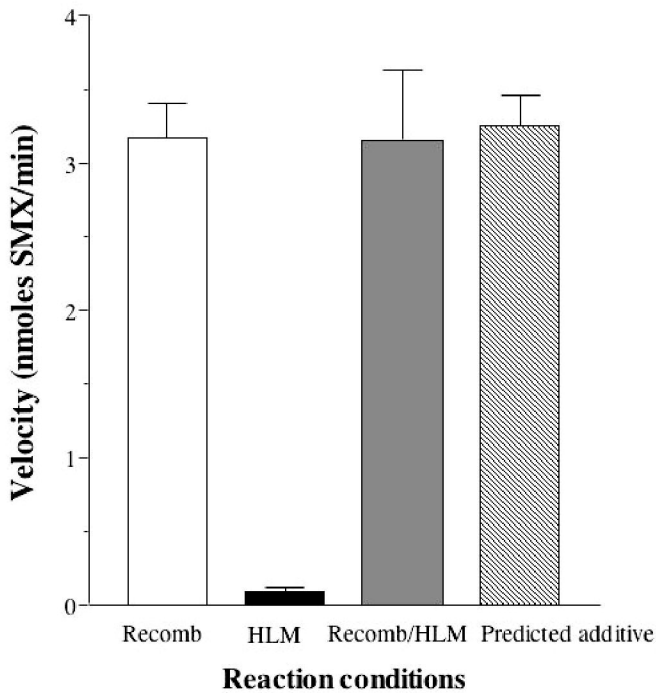


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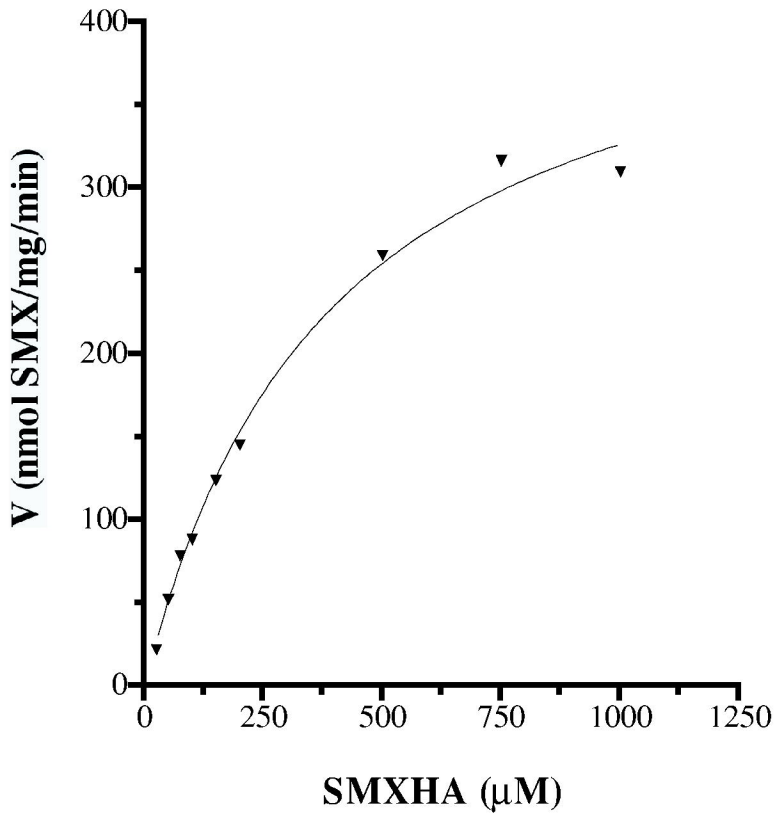


Figure 7A

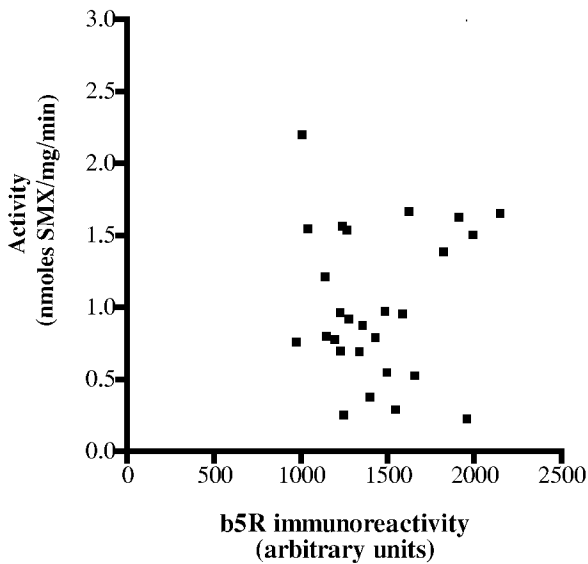


Figure 7B

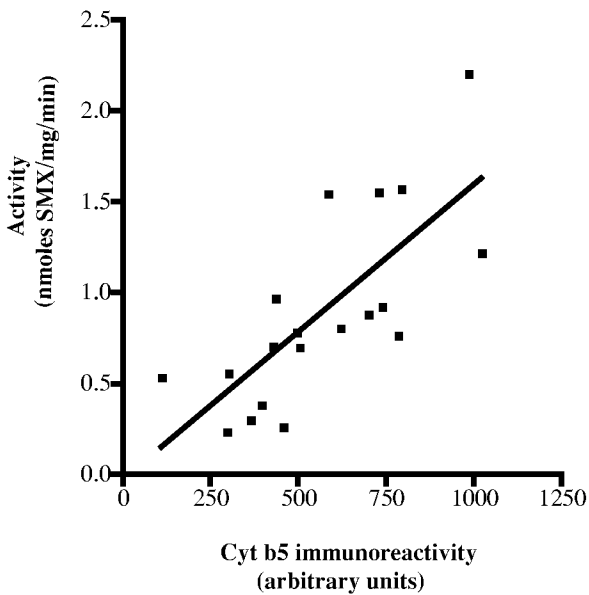


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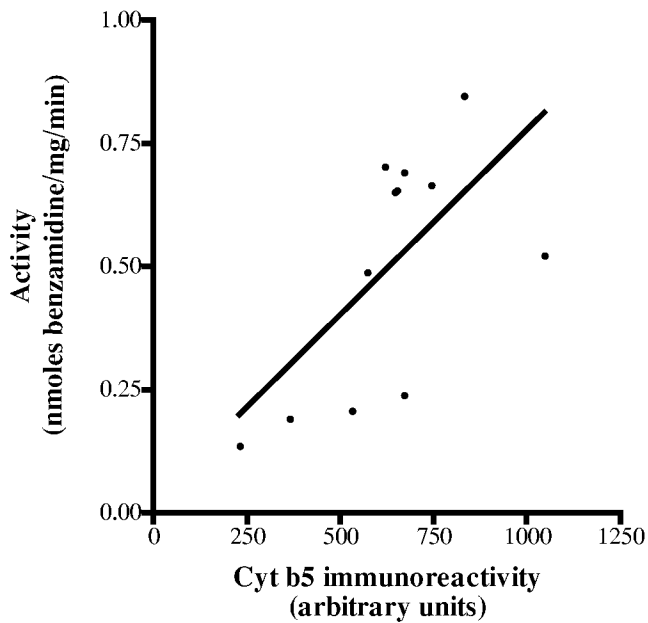


Figure 9

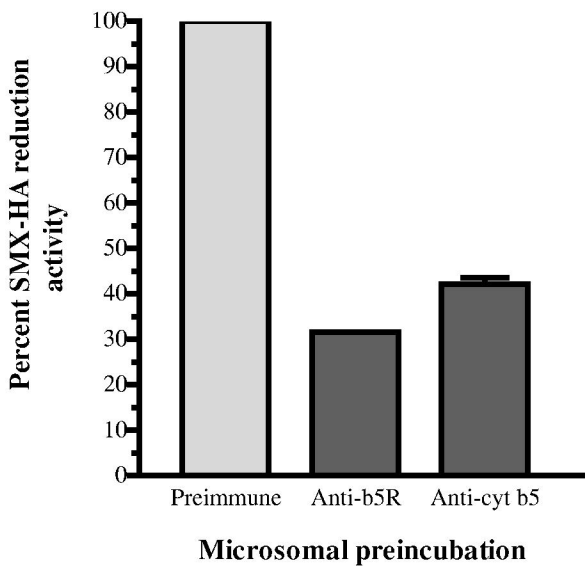


Figure 10

