Stereospecificity of Hydride Removal from NADH by Reductases of Multicomponent Nonheme Iron Oxygenase Systems

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The stereospecificity of hydride removal from the 4 position of the pyridine ring of NADH by reductases from all three classes of multicomponent nonheme iron oxygenases was examined. The class I and III reductases, modules of which show significant sequence similarity with and which belong to the ferredoxin-NADP$^+$ reductase family of flavin-dependent oxidoreductases, transferred the *pro-R* hydrogen. By contrast, the class II enzymes, which do not show significant sequence similarity to the class I and III enzymes but modules of which belong to the glutathione reductase family of flavoenzymes, transferred the *pro-S* hydrogen.

Multicomponent nonheme iron oxygenases comprise a growing set of enzyme systems (12, 19), which Batie et al. (1) grouped into three classes, I, II, and III, largely on the basis of their electron transport components. Electron transport involves the transfer of electrons from NADH to a redox center on the oxygenase component, which in turn activates molecular oxygen for attack on the organic substrate of the oxygenase.

The electron transport chain contains a minimum of two redox centers, a flavin and a [2Fe-2S] center; a second [2Fe-2S] center may also be present. These redox centers can be distributed on one or two components, the flavin-containing reductase and, if present, a small iron-sulfur protein. Class I enzymes represent the two-component systems, where the flavin, which is flavin mononucleotide (FMN) in class IA and flavin adenine dinucleotide (FAD) in class IB, and a plant-type ferredoxin [2Fe-2S] are both present on the reductase. In class II enzymes, the flavin, which is always FAD, and the [2Fe-2S] center are found on separate components. Furthermore, class IIA enzymes have a plant-type ferredoxin in the small iron-sulfur protein, whereas class IIB enzymes have a Rieske center. Class III enzymes have FAD and a plant-type ferredoxin in the reductase as well as a small iron-sulfur protein with a Rieske center (1). In all three classes, electrons are abstracted from NADH by the reductase, whose flavin enables both two-electron transfer from NADH and one-electron transfer to the iron-sulfur centers (e.g., see reference 1).

Comparison of sequence alignments of representative reductases from classes I and III has revealed that they share common structural motifs which are involved in binding their respective flavin and pyridine nucleotide cofactors (4, 20, 25). Moreover, both class I and III reductases belong to a larger family of flavin-dependent oxidoreductases whose members include spinach ferredoxin-NADP$^+$ reductase and NADPH–cytochrome P-450 reductase (5). However, since the overall degree of sequence identity between the class I and III enzymes is generally no more than 10%, it was of significant interest to determine whether this limited conservation of amino acid sequence correlated to a conservation of the reaction mechanism. Therefore, we have determined the stereospecificity of hydride removal from the 4 position of the pyridine ring of NADH by at least one enzyme from each (sub)class of classes I and III. In addition, we have determined the stereospecificity of hydride removal by two reductases from class II enzymes since sequence comparisons with the class I enzymes have not revealed significant sequence identity (20). The data indicate that the class II enzymes contain a sterically different conserved reaction mechanism.

Phthalate dioxygenase reductase (class IA) was a kind gift of D. P. Ballou, University of Michigan, Ann Arbor (2); 4-chlorophenylacetate dioxygenase reductase (class IA) and 2-chlorobenzoate dioxygenase reductase (class IB) were kindly provided by S. Fetzner, University of Hohenheim, Stuttgart, Germany (9, 18, 23); dibenzofuran dioxygenase reductase A2 (class IIA) was purified by P. Bünz (3); benzene dioxygenase reductase (class IIB) was a present from J. R. Mason, King's College, London, United Kingdom (10). 4-Toluenesulfonate monooxygenase reductase (class IA) (17) was prepared as described previously (16). Naphthalene dioxygenase reductase (class III) was purified from extracts of salicylate-grown *Pseudomonas putida* NCIB 9816 essentially as described elsewhere (7) with the following exceptions: extracts were prepared as described by Locher et al. (16), we used a Mono Q column (10 by 100 mm; Pharmacia) for anion-exchange chromatography, and the protein was eluted with a linear gradient of sodium sulfate (0 to 150 mM in 50 min; flow, 4 ml min^{-1}; fraction size, 4 ml) in 20 mM Tris-sulfate, pH 7.5, containing 1.0 mM dithiothreitol. A well-separated cytochrome *c* reductase activity (component A) eluted in yellow fractions 23 to 26. The identity of the reductase was confirmed by reconstitution of the naphthalene dioxygenase activity after combining the presumed component A with red-brown fractions B (fractions 33 and 34) and C (fractions 47 and 48) of naphthalene dioxygenase.

Tritium-labelled (4*R*)-[4-^{3}H]NADH was prepared enzymatically from D-[1-^{3}H]glucose (Amersham) and NAD$^+$ (28), with the following modifications: acetaldehyde was used for the synthesis of [4-^{3}H]NAD$^+$; (4*R*)-[4-^{3}H]NADH was synthesized by using [4-^{3}H]NAD$^+$, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Sigma). After completion of the enzymatic reactions, the proteins were removed by ultrafiltration (Centricon-10). [4-^{3}H]NAD$^+$ was purified by reversed-phase chromatography (15). The column

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TABLE 1. Reductases from different classes[a] of multicomponent nonheme iron mono- and dioxygenases and the stereospecificity of their removal of hydride ion from NADH[b]

Class	Reductase component	Ratio of recovery of $^3H^+$ ion [^{3}H]NAD$^+$	Recovery of tritium (%)	Stereospecificity
IA	Phthalate dioxygenase	59:1	102	*pro-R*
	4-Chlorophenoxyacetate dioxygenase	58:1	87	*pro-R*
	4-Toluenesulfonate monooxygenase	11:1	102	*pro-R*
IB	2-Chlorobenzoate dioxygenase	85:1	93	*pro-R*
Ix[c]	Xylene monooxygenase			*pro-R*
IIA	Dibenzofuran dioxygenase	1:104	94	*pro-S*
IIB	Benzene dioxygenase	1:65	89	*pro-S*
III	Naphthalene dioxygenase	16:1	106	*pro-R*

[a] The classification of nonheme iron oxygenases (1) is based on the overall electron transport chain in the oxygenase system.
[b] Data from a typical experiment are shown. With the exception of the class Ix enzyme, for which we quote previously published data (24), all experiments were done at least twice with independently prepared solutions.
[c] The xylene monooxygenase system has properties of both classes IA (one protein species in the oxygenase component) and IB (FAD in the reductase), and we have provisionally used the notation "Ix."

was equilibrated with eluent A (2.4% [vol/vol] methanol in H_2O, containing 0.1% [vol/vol] trifluoroacetic acid), and the sample was injected. After 8 min, eluent B (56% [vol/vol] methanol in H_2O, containing 0.1% [vol/vol] trifluoroacetic acid) was applied. Fractions containing [4-^{3}H]NAD$^+$, which eluted at about 14.5 min, were pooled and lyophilized. NADH eluted at about 14.8 min.

To determine the stereospecificity of hydride removal from NADH during catalysis, tritiated (4*R*)-[4-^{3}H]NADH (approximately 170,000 dpm, 0.4 nmol), together with 0.1 μmol of unlabelled NADH and either 0.2 μmol of cytochrome *c* (Boehringer) with class I and III enzymes or 0.2 μmol of dichlorophenolindophenol with class II enzymes, was incubated with enzyme (5 to 50 μg) in 1 ml of 10 mM phosphate buffer, pH 7.0, or 20 mM Tris-sulfate, pH 7.5. After completion of the reaction, which was monitored photometrically at 550 nm (cytochrome *c*) or 600 nm (dichlorophenolindophenol) (16), the proteins were removed by ultrafiltration (Centricon-10) and the filtrate (400 to 800 μl) was separated by reversed-phase chromatography as described above. Fractions (1 ml) were collected, and tritium was quantified in a liquid scintillation counter (Ready-safe cocktail; Beckman). The radioactivity was detected essentially either in the elution front as $^3H^+$ (retention time, about 4 min) or in the NAD$^+$ fraction (retention time, about 14.5 min). The ratio of the radioactivity recovered as $^3H^+$ and as 4-[^{3}H]NAD$^+$ was calculated.

CLUSTAL, FASTA, FETCH, GAP, SEQED, STRINGSEARCH, and TOCLUSTAL software packages (11) were used to search for homologies between the known sequences and sequences in databases.

To verify our methodology, we first examined phthalate dioxygenase reductase, which has been shown previously to exhibit *pro-R* stereospecificity (4). As can be seen from Table 1, when phthalate dioxygenase reductase was incubated in the presence of (4*R*)-[4-^{3}H]NADH, the ratio of total radioactivity recovered as $^3H^+$ and [4-^{3}H]NAD$^+$ was 59:1, indicating that the enzyme removed the tritium from the *pro-R* position of (4*R*)-[4-^{3}H]NADH. The two other reductases in class IA (4-chlorophenylacetate dioxygenase reductase and 4-toluenesulfonate dioxygenase reductase), the class IB enzyme (2-chlorobenzoate dioxygenase reductase), and naphthalene dioxygenase reductase (class III) all showed *pro-R* specificity as observed with phthalate dioxygenase reductase (Table 1). By contrast, both the reductases from the class II enzymes, dibenzofuran dioxygenase reductase A2 (class IIA) and benzene dioxygenase reductase (class IIB), were *pro-S* specific (Table 1).

Comparison of the stereospecificity of hydride removal from NADH by eight reductases covering all three classes of multicomponent nonheme iron oxygenases indicates that class I and III reductases remove the *pro-R* hydrogen (as a hydride ion) whereas the class II enzymes remove the *pro-S* hydrogen (Table 1).

The common removal of the *pro-R* hydrogen by the class I and III reductases mirrors the conservation of amino acid residues which have been identified in the phthalate dioxygenase reductase crystal structure as being important for the binding of the [2Fe-2S] center, the flavin, and the NADH cofactors (4). Indeed, 5 of the 8 residues which are absolutely conserved in the [2Fe-2S] domain of phthalate dioxygenase reductase, xylene monooxygenase reductase, and naphthalene dioxygenase reductase and in benzoate 1,2-dioxygenase reductase and toluate 1,2-dioxygenase reductase, for which the stereospecificity of hydride removal has yet to be determined, form part of an iron-sulfur binding loop (C-X-X-G-X-C-G-X-C) which, in phthalate dioxygenase reductase, packs against FMN and plays an important role in the interface between the [2Fe-2S] domain and the FMN domain (4). Similarly, only 18 residues in the flavin and pyridine nucleotide-binding domains of phthalate dioxygenase reductase are absolutely conserved. While some of these residues, including those corresponding to Arg-55, Tyr-57, and Ser-58, form a motif involved in flavin binding (4), others, including Gly-119, Gly-120, Gly-122, and Pro-125, form a consensus sequence (G-G-X-G-X-X-P), the core region of which (X-G-X-G) is a variant of the nucleotide phosphate loop commonly found in NAD$^+$- and FAD-binding proteins (13, 29). In addition, Tyr-197, Cys-199, and Gly-200, which lie close to the nucleotide binding site, are absolutely conserved, as is Phe-225, which is known to stack against the pyridine ring of NADH (4). Although Phe-225 lies between the pyridine ring of NADH and the FMN molecule, thereby preventing direct stacking of the cofactors, model-building studies suggest that the side chain of Phe-225 undergoes a displacement to allow the direct transfer of electrons from NADH to the flavin moiety (4). Pro-201, which is conserved in phthalate dioxygenase reductase (4), benzoate 1,2-dioxygenase reductase (20), toluate 1,2-dioxygenase reductase (20), and xylene monooxygenase reductase (26) but not in naphthalene dioxygenase reductase (25), stacks on the opposite side of the pyridine ring. Since Pro-201 is conserved in four of the five class I and III enzymes and Phe-225 is absolutely conserved, it is likely that a similar stacking mechanism occurs in the other reductases. Moreover, this stacking may account, at least in part, for ori-

enting the pyridine ring such that the *pro-R* hydrogen is removed.

Analysis of the X-ray structure of phthalate dioxygenase reductase also reveals that the NADH molecule is held by two prominent charged residues. While Arg-148, which interacts with the phosphate of the AMP group (4), is conserved in xylene monooxygenase reductase (26) and naphthalene dioxygenase reductase (25), it is not conserved in benzoate 1,2-dioxygenase reductase (20) or toluate 1,2-dioxygenase reductase (20), which both have threonine at the corresponding position. Furthermore, Asp-173, which in phthalate dioxygenase reductase forms hydrogen bonds with the alcohol groups of the adenosine ribose (4), is replaced by valine in benzoate 1,2-dioxygenase reductase (20), xylene monooxygenase reductase (26), and naphthalene dioxygenase reductase (25) and by cysteine in toluate 1,2-dioxygenase reductase (20). It is likely, therefore, that while residues involved in binding certain portions of the NADH molecule such as the adenosine ring and the phosphate of AMP may have diverged more significantly during evolution, those residues involved in binding and orienting the pyridine ring have been constrained in order to maintain the *pro-R* stereospecificity characteristic of the class I and III enzymes.

By contrast, the reductases of the class II enzymes remove the *pro-S* hydrogen from NADH (Table 1) and share little or no sequence similarity with representative reductases from classes I and III. However, three structural motifs have been identified in the sequence of the class IIB enzyme benzene dioxygenase reductase which have been predicted to interact with FAD (residues 4 to 35 and 265 to 275) and NADH (residues 145 to 173) (27). These motifs, which are also found in toluene dioxygenase reductase (31), are also found in a number of enzymes, including rubredoxin reductase (6), putidaredoxin reductase (21), biphenyl dioxygenase reductase (8), 4-hydroxybenzoate hydroxylase (22), thioredoxin reductase (6), dihydrolipoamide dehydrogenase (6), and glutathione reductase (14), which are members of the larger glutathione reductase family of flavin-dependent oxidoreductases (6). Interestingly, the enzymes of this family, including thioredoxin reductase, dihydrolipoamide dehydrogenase, and glutathione reductase, all remove the *pro-S* hydrogen from NADH (30), which is the same as for benzene dioxygenase reductase and dibenzofuran dioxygenase reductase A2 (Table 1). The common transfer of the *pro-S* hydrogen, coupled to the sequence similarities between benzene dioxygenase reductase and members of the glutathione reductase family, supports the inclusion of these class IIB reductases in the latter family.

Integration of the class IIA reductases into the glutathione reductase family can be argued via the putidaredoxin component of the known class IIA enzymes (1, 3), which presumably require a putidaredoxin reductase similar to that characterized by Peterson et al. (21) and classified in the glutathione reductase family (6). The putidaredoxin reductase examined by Peterson et al. (21) is a component of a major class of multicomponent oxygenases, the heme iron cytochromes P-450, of which cytochrome P-450cam is the best-characterized example (e.g., see reference 21). Correll et al. (5) complement these data by showing that human microsomal NADPH–cytochrome P-450 reductase, in contrast, belongs to the spinach ferredoxin-$NADP^+$ reductase family.

In summary, the stereospecificity of hydride removal by the reductases of class I and III enzymes has been conserved during their evolution, as has the stereospecificity of hydride removal by the class II enzymes. Moreover, this information, coupled to the available sequence data, indicates that the reductase modules associated with both the multicomponent nonheme iron oxygenases and the multicomponent heme iron oxygenases were recruited from at least two different sources.

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