Geometric Relationship between the Nicotinamide and Isoalloxazine Rings in NADPH–Cytochrome P-450 Oxidoreductase: Implications for the Classification of Evolutionarily and Functionally Related Flavoproteins†

Daniel S. Sem and Charles B. Kasper*

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706

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ABSTRACT: The stereospecificity of hydride abstraction from NADPH and the conformation of the nicotinamide ring around the glycosidic bond have been determined for the flavoprotein NADPH–cytochrome P-450 oxidoreductase (P-450R). The A-side (*pro-R*) hydrogen is abstracted from NADPH, and the nicotinamide ring is in the anti conformation. These results are consistent with the apparently strong correlation between A-side stereospecificity and anti conformation and between B-side stereospecificity and syn conformation [You, K. (1985) *CRC Crit. Rev. Biochem. 17*, 313]. This correlation reveals how the flavin and nicotinamide rings are oriented relative to each other. In P-450R, the flavin is then "on top of " (on the exo side of) the nicotinamide ring. In another flavoprotein dehydrogenase, glutathione reductase, which is a B-side/anti enzyme [Pai, E. F., & Schulz, G. E. (1983) *J. Biol. Chem. 258*, 1752], the flavin is "underneath" (on the endo side of) the nicotinamide ring. We argue that all enzymes that are evolutionarily related to these two flavoproteins should have their respective overall configurations. The overall configuration is defined by the following five properties: (1) relative orientation of the isoalloxazine and nicotinamide rings, (2) stereospecificity of hydride transfer to/from the nicotinamide ring, (3) conformation of the nicotinamide ring around the glycosidic bond, (4) stereospecificity of hydride transfer to/from the flavin, and (5) conformation of the flavin around its N5–N10 axis. There are only eight possible overall configurations, and a knowledge of only three of the five properties is needed to determine which one is present (as long as the combination of properties is not 1, 2, 3 or 1, 4, 5). Furthermore, although there may be evolutionary advantages for enzymes that possess certain values or combinations of values for properties 1–5 (depending on the reaction that is catalyzed), it is the overall configuration, which is defined by all five properties, that should be evolutionarily conserved within a family of related flavoprotein dehydrogenases.

NADPH–cytochrome P-450 oxidoreductase (P-450R)[1] is a 78 225-dalton flavoprotein that contains 1 mol each of FAD and FMN (Iyanagi & Mason, 1973). P-450R is a membrane-bound protein found in the endoplasmic reticulum (Williams & Kamin, 1962; Phillips & Langdon, 1962) and nuclear envelope (Kasper, 1971) of most eukaryotic cells. It is known to catalyze the transfer of two reducing equivalents from NADPH to FAD, then to FMN, and finally to any of a number of cytochromes P-450 or to various alternate electron acceptors such as cytochrome *c*, menadione, or 2,6-dichloroindophenol (Vermilion et al., 1981). The physiologically relevant electron acceptors are the cytochromes P-450.

On the basis of the previously reported amino acid sequence of P-450R (Porter & Kasper, 1985) it was suggested (Porter & Kasper, 1986) that this enzyme arose through the fusion of the ancestral genes of bacterial flavodoxin, which is homologous to the FMN binding domain (residues 77–228 of P-450R), and ferredoxin–$NADP^+$ reductase (FNR), which is homologous to the NADPH and FAD binding domains (residues 267–678 of P-450R). Although no crystal structure is available for P-450R, structures are available for flavodoxin (Watenpaugh et al., 1973) and FNR (Karplus et al., 1991). We are interested in the interactions between the nicotinamide ring of NADPH and the isoalloxazine ring of FAD that allow for hydride transfer. The structural nature of these interactions has been proposed for FNR on the basis of the crystal structure of the 2′-phospho-AMP·FNR complex (Karplus et al., 1991). These authors proposed that the nicotinamide ring in FNR is in the anti conformation and is situated relative to the flavin in such a way that transfer of the A-side (*pro-R*) hydrogen to N5 of FAD can occur. The stereospecificity of hydride transfer has been determined previously (Krakow et al., 1965), although the conformation of the nicotinamide ring has not yet been reported. The exact nature of these interactions will not be clear until the crystal structure of the $NADP^+$·FNR complex is obtained.

Alternatively, one can determine the geometric relationship between the nicotinamide and flavin rings based on a knowledge of the stereospecificity of hydride transfer and the conformation of the nicotinamide ring. This approach was first suggested by Oppenheimer (1984, 1986) and was recently applied to dihydrofolate reductase (Brito et al., 1991). Using this approach, we have determined the geometric relationship between the nicotinamide and isoalloxazine rings in the active

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[1] Abbreviations: P-450R, NADPH–cytochrome P-450 oxidoreductase; FNR, ferredoxin–$NADP^+$ reductase; 2′-phospho-AMP, 2′-phosphoadenosine 5′-monophosphate; WT, wild type; TRNOE, transferred nuclear Overhauser effect; NMR, nuclear magnetic resonance; FID, free induction decay; DMSO, dimethylsulfoxide; DTT, dithiothreitol; TMS, tetramethylsilane; TAPS, *N*-tris(hydroxymethyl)-methyl-3-aminopropanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid.

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site of P-450R. We also suggest that since this relationship should be a conserved property of evolutionarily related enzymes, P-450R and glutathione reductase, which are not evolutionarily related, may be representative members of their respective families in this regard. Glutathione reductase is a member of the carbon–sulfur transhydrogenase family (Massey & Hemmerich, 1980; Pai et al., 1988) (also called the flavoprotein disulfide oxidoreductase family) along with lipoamide dehydrogenase (Williams, 1976), mercuric reductase (Fox & Walsh, 1983), trypanothione reductase (Shames et al., 1986; Krauth-Siegel et al., 1987), thioredoxin reductase (Russel & Model, 1988), and asparagusate dehydrogenase (Yanagawa, 1979). Enzymes in this family catalyze two-electron transfer between nicotinamide nucleotides and disulfide–dithiol pairs. P-450R is a member of the dehydrogenase/electron transferase family (Massey & Hemmerich, 1980) along with FNR, NADH–nitrate reductase, NADH–cytochrome b_5 reductase, and NADPH–sulfite reductase (Karplus et al., 1991; Porter & Kasper, 1986). Enzymes in this family catalyze the transfer of electrons between nicotinamide nucleotides (obligate two-electron or hydride donors/acceptors) and obligate one-electron donors/acceptors such as cytochrome *c*.

Materials and Methods

HEPES, TAPS, DTT, $NADP^+$, NADPH, glucose 6-phosphate, glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, aldehyde dehydrogenase from bakers' yeast, alcohol dehydrogenase from *Thermoanaerobium brokii*, and horse heart cytochrome *c* were from Sigma. Ethanol-d_6 (99.5%), glucose-1-*d* (98%), TMS, and D_2O (99.9% and 100%) were from Aldrich. Dipotassium phosphate was from Mallinckrodt.

The construction of the rat liver NADPH–cytochrome P-450 oxidoreductase expression plasmid, pOR263, and methods for purification of the wild type (Shen et al., 1989) and C566S and C566A mutant P-450Rs (Shen et al., 1991) expressed in *Escherichia coli* have been reported previously, as was the purification of P-450R from rat liver (Yasukochi & Masters, 1976; Vermilion & Coon, 1978).

Preparation of Deuterated Nucleotides. A-side and B-side NADPDs were synthesized enzymatically using slight modifications of the procedure of Viola et al. (1979). A-side NADPD was synthesized at 25 °C from ethanol-d_6 (0.2 M) and $NADP^+$ (5.6 mM) in 20 mM TAPS and 0.1 mM DTT using 5 units/mL alcohol dehydrogenase and 1 unit/mL aldehyde dehydrogenase. The pH was maintained at 9.0 throughout the reaction. The reaction was complete after 6–7 h, at which point protein was removed by extraction with several drops of CCl_4. The aqueous layer was applied to a 2 × 25 cm AG MP-1 column (Bio-Rad). Nucleotide was eluted using a 1-L linear gradient of 0.2–0.6 M LiCl at pH 10.0 (NH_4OH). Fractions with the highest A^{340}/A^{260} were pooled and desalted on a Bio-Gel P-2 column (Bio-Rad). The nucleotide solution was concentrated by rotary evaporation.

B-side NADPD was synthesized from glucose-1-*d* (15 mM) and $NADP^+$ (10 mM) in 50 mM potassium phosphate buffer (pH 8.0) and 40% DMSO using 100 units/mL glucose-6-phosphate dehydrogenase. The reaction was done in 1 h, at which point nucleotide was purified as described above.

NMR Analysis of Stereospecificity. The stereospecificity reaction mixtures contained 2 mM horse heart cytochrome *c* (ferric), 0.5 mM NADPD (either A-side or B-side), 50 mM potassium phosphate buffer (pH 8.0), 100 mM KCl, and 2 units/mL P-450R (purified from rat liver, WT, or C566A or C566S mutants). The reaction was complete after 1 h, at which point enzyme was removed by filtration through a Centricon 30 filter. The filtrate was lyophilized and taken up in 99.9% D_2O. ^{1}H-NMR spectra of these solutions were obtained on a Bruker AM500 spectrometer operating at 500.13 MHz. Chemical shifts were referenced to external TMS in a coaxial tube and D_2O provided the lock.

NMR Analysis of Nicotinamide Conformation. P-450R (WT) was concentrated and taken up in D_2O by repeated ultrafiltration through an Amicon YM10 membrane. The enzyme was concentrated from 8 mL to 1 mL, taken up in 0.1 M potassium phosphate buffer in 99.9% D_2O, pD 7.7 [pD is the meter reading + 0.4 (Perrin & Dempsey, 1974)], and reconcentrated. This process was repeated four times. The enzyme concentration was determined using a Lowry assay (Lowry et al., 1951) and by measuring flavin absorption at 454 nm, based on an extinction coefficient of 21.4 mM^{-1} cm^{-1} (French & Coon, 1979). The $NADP^+$ stock solution was prepared in 0.1 M potassium phosphate buffer in 99.9% D_2O, pD 7.7, lyophilized, and taken up in the same buffer in 100% D_2O. The last step was then repeated. The concentration of $NADP^+$ was then determined using a glucose-6-phosphate dehydrogenase end-point assay in 0.1 M HEPES in the presence of 5 mM glucose 6-phosphate.

^{1}H-NMR spectra were obtained with a Bruker AM500 spectrometer operating at 500.13 MHz. Experiments were performed at 27 °C using a 5-mm tube containing 0.35 mL of sample. Chemical shifts were referenced to a value of 4.8 ppm for residual water. The sample for the transferred nuclear Overhauser effect (TRNOE) experiment contained 0.11 mM P-450R (WT) and 2.3 mM $NADP^+$ in 0.1 M potassium phosphate in D_2O, pD 7.7, which gives a ratio for free to bound $NADP^+$ of ca. 20. The sample for the control TRNOE experiment contained 2.3 mM $NADP^+$ in the same buffer without enzyme.

The theory behind the TRNOE experiment was discussed by Clore and Gronenborn (1982). We used the same pulse sequence used by these authors: $(D_1\text{–}D_2\text{–}\pi/2\text{–AT})_n$, where D_1 is the length of time that the selective irradiation is applied (1 s), D_2 is a fixed delay to allow for electronic switching following the selective irradiation (5 ms), $\pi/2$ is the nonselective observation pulse (7.75 μs) and AT is the acquisition time (0.680 s). Several different TRNOE experiments were carried out simultaneously by selectively irradiating at the desired frequency and collecting 8K data points for that FID, then repeating this with the next selective irradiation frequency and so on for each experiment. This process was repeated 2250 times for each selective irradiation frequency. This procedure minimizes any variations due to magnetic field fluctuations or sample instability over time. Averaged FIDs obtained for each experiment (corresponding to a certain selective irradiation) were then subtracted from that of a control where irradiation was done at 7.65 ppm (the closest resonance to this is at 8.10 ppm). Then, 5-Hz exponential line broadening was applied prior to Fourier transformation. The resulting difference spectra therefore show negative NOEs as having positive intensity. The NOE is the ratio of the change in signal intensity upon selective irradiation to the signal intensity without selective irradiation. A control experiment was done in the absence of enzyme, and the NOEs that were observed were negligible. Spin diffusion effects are also a concern in TRNOE experiments, although they should not affect the qualitative conclusions from these experiments. The proton numbering scheme used in the text refers to the protons on NADPH as shown in Figure 1. Specific NOEs are abbreviated as $f_{UI}(I)$, where I is the selectively irradiated proton and

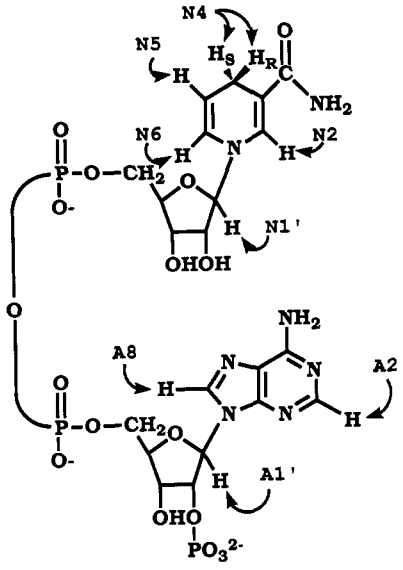

FIGURE 1: Structure of NADPH with the numbering scheme used in the text. The *pro*-R (A-side) and *pro*-S (B-side) hydrogens are labeled H_R and H_S, respectively.

Table I: Nuclear Overhauser Effects

f_{UI}(I)	% NOE
$f_{N1'}$ (N2)	−8.4
$f_{N1'}$ (N6)	−0.92
f_{N2} (N1′)	−9.5
f_{N6} (N1′)	−2.3

UI is the unirradiated proton that is experiencing the NOE from proton I.

Results

Stereospecificity of Hydride Transfer. A-side and B-side NADPDs were oxidized with P-450R (WT) and the ^{1}H-NMR spectra of the reaction products are shown in Figure 2, panels A and B, respectively. When A-side NADPD is the starting material, the ^{1}H-NMR spectrum in the aromatic region is the same as that for undeuterated NADP$^+$, with the N4 resonance present at 8.9 ppm (Figure 2A). But when B-side NADPD is the substrate, the ^{1}H-NMR spectrum no longer shows the presence of the N4 resonance (Figure 2B). These results are consistent with rat liver P-450R (WT) being 100% stereospecific for abstraction of the A-side (*pro-R*) hydrogen. There have been preliminary reports of A-side stereospecificity for P-450R from rabbit and pig liver (Sugiyama & Mason, 1984; Drysdale, 1966), but to our knowledge these observations have not been described in detail. We have also determined that P-450R purified from rat liver and the C566A and C566S mutant enzymes are also 100% stereospecific for abstraction of the A-side (*pro-R*) hydrogen (data not shown).

Conformation of the Nicotinamide Ring. The TRNOE values for some of the protons on the nicotinamide portion of NADP$^+$ are summarized in Table I. NADP$^+$ was used rather than NADPH to avoid problems with reduction of the flavin, leading to a heterogeneous mixture of oxidized and reduced nucleotides. The ^{1}H-NMR spectrum of the P-450R + NADP$^+$ solution, prior to selective irradiation, is shown in Figure 3A. Irradiation of N1′ (Figure 3B) results in a much larger TRNOE on N2 than on N6. This is consistent with N1′ being closer in space to N2 than to N6, which requires the anti conformation around the nicotinamide glycosidic bond.

This selectivity of the TRNOE [$-f_{N2}$(N1′) ≫ $-f_{N6}$(N1′)) rules out a spin diffusion mechanism for these effects (Feeney et al., 1983). Furthermore, in the converse experiment, irradiation of N2 (Figure 3C) gave a much larger TRNOE on N1′ than did irradiation of N6 (Figure 3D). These results are also consistent with only the anti conformation. Further evidence that spin diffusion effects should not affect our qualitative conclusions about conformation comes from the results of Brito et al. (1991). They determined primary and secondary (due to spin diffusion) NOEs by measuring the total NOE as a function of mixing time and fitting their data to a second-order polynomial. Extracting the primary NOEs in this manner allowed for quantitative calculation of interatomic distances but would not have affected their qualitative conclusions about conformation of the nicotinamide ring, since spin diffusion effects were not significant for f_{N2}(N1′) and f_{N6}(N1′) NOE values (that is, their plots of total NOE as a function of mixing time were essentially linear). The NOE values obtained in the control experiment with NADP$^+$ in the absence of enzyme were small (0.7–1.4%), relative to those reported in Table I.

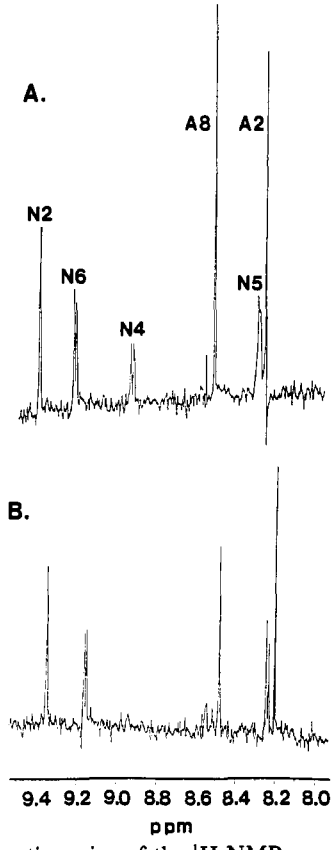

FIGURE 2: Aromatic region of the ^{1}H-NMR spectrum of the stereospecificity reaction mixture with P-450R (WT), using (A) A-side NADPD as substrate and (B) B-side NADPD as substrate.

Discussion

P-450R catalyzes the oxidation of NADPH to NADP$^+$ along with the subsequent reduction of various one- or two-electron acceptors. We have determined that it is the A-side (*pro-R*) hydrogen that is abstracted from NADPH, making P-450R an A-stereospecific dehydrogenase/electron transferase. Also, since the C566A and C566S mutants are likewise 100% stereospecific for the A-side (*pro-R*) hydrogen, the cysteine at position 566, which is thought to play some role in binding NADPH (Haniu et al., 1989; Shen et al., 1991), must not be important for determining the stereochemistry of hydride transfer.

There have been numerous hypotheses put forth to explain why some dehydrogenases are A-stereospecific and some are B-stereospecific. Benner (1982) has proposed that there is

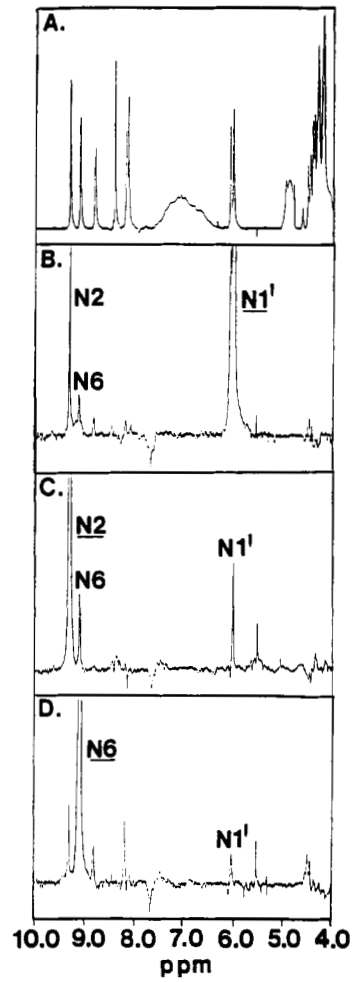

FIGURE 3: ^{1}H-NMR spectra of NADP$^+$ (2.3 mM) and P-450R (0.11 mM): (A) spectrum without any selective irradiation; (B) difference spectrum with selective irradiation of the N1′ resonance; (C) difference spectrum with selective irradiation of the N2 resonance; (D) difference spectrum with selective irradiation of the N6 resonance. Elimination of the residual HDO signal and the calculation of difference spectra was accomplished using the processing package, FELIX-PC (Hare Research, Woodinville, WA). Spectra corresponding to the individual saturation frequencies were first circularly shifted such that the residual solvent was moved exactly to the center of the spectrum. The data were then inverse Fourier transformed, and the resulting FID was smoothed by taking the average of the 16 closest data points (weighted by a sine bell). The resulting FID contains only zero and very low frequency components. This FID was then subtracted from the original FID to obtain a "solvent free" FID corresponding to each selective saturation frequency. The difference spectra (B, C, and D) were obtained by taking the difference between the "solvent free" selective saturation FIDs and the control FID corresponding to spectrum A (which was also processed to remove the residual solvent) where the selective saturation was done at 7.65 ppm.

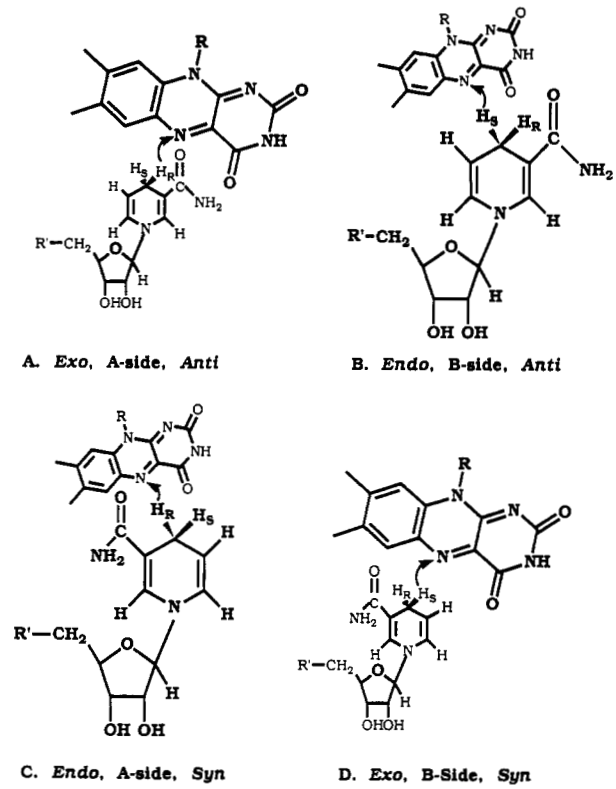

FIGURE 4: The four nicotinamide configurations along with corresponding designations for relative orientation of the nicotinamide and FAD rings (endo or exo), stereospecificity of hydride transfer from NADPH (A-side or B-side) and conformation around the nicotinamide glycosidic bond (anti or syn). In A and D the flavin is "on top of" the nicotinamide ring, while in B and C the flavin is "underneath" the nicotinamide ring.

evolutionary pressure on dehydrogenases to balance redox potentials. Thus, since NADH in the anti conformation (which is usually observed with A-side stereospecificity) is thought to be a weaker reducing agent, this conformation (and stereospecificity) would be seen when the hydride acceptor is a relatively strong oxidizing agent. The converse would be true for NADH in the syn conformation. The feasibility of this hypothesis has been questioned, though, since there are numerous exceptions (Oppenheimer, 1984). Srivastava et al. (1985) have shown that there is direct transfer of nucleotides via enzyme–enzyme complexes between some metabolically coupled dehydrogenases and that this transfer can only occur from A-stereospecific to B-stereospecific dehydrogenases, and vice versa. Thus, there would be evolutionary pressure on these metabolically coupled dehydrogenases to be of opposite stereospecificity. Garavito et al. (1977) suggested that early dehydrogenases merely carried out a reaction with A or B stereospecificity initially, and all enzymes that evolved from these primordial enzymes retained this original stereospecificity. This argues that regardless of whether there is some evolutionary advantage for one stereospecificity over the other, all evolutionarily related enzymes must have the same stereospecificity. Why some enzymes are A-stereospecific and some are B-stereospecific is still an unresolved issue, although this last proposal, which is only a partial explanation, is generally accepted.

We have determined the conformation of the nicotinamide ring in P-450R to be anti. Oppenheimer (1984, 1986) pointed out that the stereospecificity of reaction along with the conformation of the nicotinamide ring indicates whether the nicotinamide ring resides "on top of" the hydride donor/acceptor or vice versa. We would like to propose a more meaningful nomenclature where endo refers to the orientation with the hydride donor/acceptor on the same side of the nicotinamide ring as the endocyclic oxygen of the ribose ring (or "underneath" the nicotinamide ring) and exo refers to the orientation with the hydride donor/acceptor on the opposite side of the nicotinamide ring (or "on top of" it). All A-side/anti and B-side/syn enzymes must have the exo orientation, while all A-side/syn and B-side/anti enzymes must have the endo orientation. The orientation and nicotinamide stereospecificity and conformation are separate properties of a dehydrogenase, but together they specify one of the four nicotinamide configurations shown in Figure 4.

One can construct a more detailed picture of the active site of flavoprotein dehydrogenases if one knows the flavin stereospecificity (see below), and if one assumes that hydride

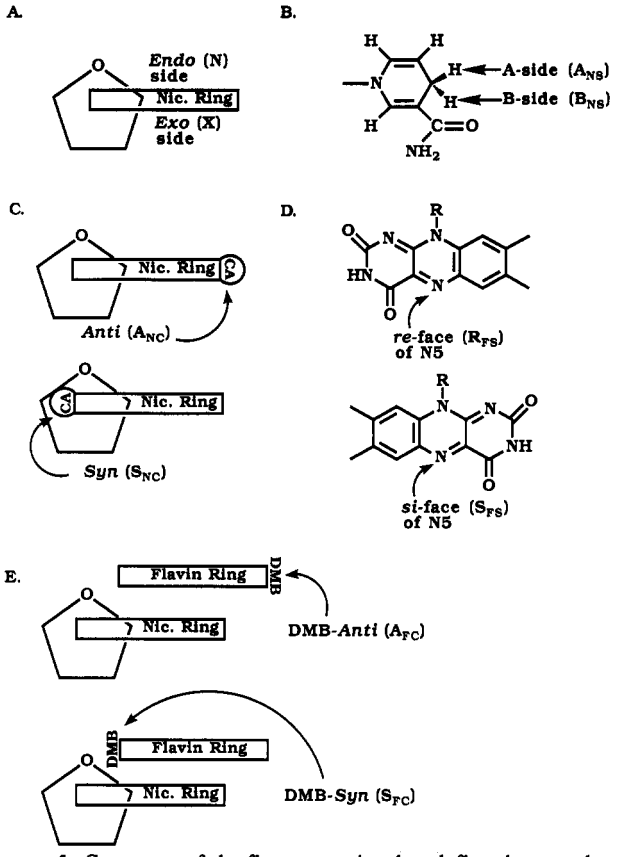

FIGURE 5: Summary of the five properties that define the complete configuration. Nicotinamide stereospecificity and conformation are abbreviated with subscripts NS and NC, respectively, while flavin stereospecificity and conformation are abbreviated with subscripts FS and FC, respectively. (A) Orientation: End-on view of the nicotinamide ring showing the two sides of the nicotinamide ring on which the flavin can reside. If the flavin is on the same side of the nicotinamide ring as the endocyclic oxygen of the ribose ring, the two rings are in the endo orientation (N), while if it is on the opposite side, the two rings are in the exo orientation (X). (B) Nicotinamide stereospecificity (NS): The two prochiral hydrogens on C4 of the nicotinamide ring that can be transferred are the A-side (A_{NS}) and B-side (B_{NS}) hydrogens. (C) Nicotinamide conformation (NC): End-on view of the nicotinamide ring showing the two conformations around the glycosidic bond. If the carboxamide group (abbreviated CA) on C3 of the nicotinamide ring points away from the ribose ring, it is in the anti conformation (A_{NC}), while a 180° rotation around the glycosidic bond puts it in the syn conformation (S_{NC}). (D) Flavin stereospecificity (FS): The two faces of the N5 nitrogen of the flavin ring to which the hydride can be transferred are the *re* face (R_{FS}) and the *si* face (S_{FS}). (E) Flavin conformation (FC): End-on view of the parallel flavin and nicotinamide rings, shown in the endo orientation. If the dimethylbenzene ring of the flavin ring system points away from the ribose, the flavin is in the DMB-anti conformation (A_{FC}), while a 180° rotation around the N5–N10 axis puts it in the DMB-syn conformation (S_{FC}). The same applies with the exo orientation of the flavin and nicotinamide rings.

transfer is to N5 of the flavin and that the central ring of the flavin is essentially parallel to the nicotinamide ring. These assumptions are based on what is known from the crystal structure of the NADPH·glutathione reductase complex (Pai & Schulz, 1983), and what seems chemically reasonable (Walsh, 1979; Karplus et al., 1991). This refined picture of the active-site arrangement of the nicotinamide and flavin rings is called the complete configuration. The complete configuration is defined by the following five properties:

(1) *Orientation* (Figure 5A). Looking end-on at the essentially planar nicotinamide ring (down the C4–N1 axis), the side of the nicotinamide ring where the endocyclic oxygen of the ribose ring is located is the endo side. If the flavin resides on this side of the nicotinamide ring, it is called the endo orientation (N). If the flavin is on the opposite side, it is called the exo orientation (X).

Table II: Summary of Overall Configurations[a]

nicotinamide conformation	nicotinamide stereospecificity	
	A_{NS}	B_{NS}
A_{NC}	X:__/$R_{FS}A_{FC}$ X:__/$S_{FS}S_{FC}$	N:__/$R_{FS}S_{FC}$ N:__/$S_{FS}A_{FC}$
S_{NC}	N:__/$R_{FS}S_{FC}$ N:__/$S_{FS}A_{FC}$	X:__/$R_{FS}A_{FC}$ X:__/$S_{FS}S_{FC}$

[a] For any given combination of nicotinamide stereospecificity (columns A_{NS} and B_{NS}) and nicotinamide conformation (rows A_{NC} and S_{NC}), there are only two possible overall configurations. These are shown for each of the four possible combinations of nicotinamide stereospecificity and conformation.

(2) *Nicotinamide Stereospecificity* (*NS*) (Figure 5B). The two prochiral hydrogens on C4 of the nicotinamide ring are designated A-side (A_{NS}) or B-side (B_{NS}) hydrogens, and only one of them is transferred to N5 of the flavin (Levy et al., 1962).

(3) *Nicotinamide Conformation* (*NC*) (Figure 5C). Looking end-on at the nicotinamide ring as described above, the carboxamide group on C3 of the nicotinamide ring points away from the ribose ring in the anti conformation (A_{NC}) or, upon rotating 180° around the glycosidic bond, it resides over the ribose ring, in the syn conformation (S_{NC}) (IUPAC–IUB Joint Commission on Biochemical Nomenclature, 1983).

(4) *Flavin Stereospecificity* (*FS*) (Figure 5D). The N5 atom of the flavin ring can receive the hydride ion on either the *re* face (R_{FS}) or the *si* face (S_{FS}) (Manstein et al., 1986).

(5) *Flavin Conformation* (*FC*) (Figure 5E). Looking end-on at the nicotinamide ring as described above and also end-on at the parallel flavin ring (down the N5–N10 axis), one observes that there are two conformations for the flavin ring. The dimethylbenzene ring of the flavin ring system can point away from the ribose ring to give the DMB–anti conformation (A_{FC}) or be rotated 180° around the N5–N10 axis to give the DMB–syn conformation (S_{FC}). The flavin ring in Figure 5E is shown on the endo side of the nicotinamide ring, but the same nomenclature applies with the exo orientation.

The overall configuration is specified using the above abbreviations for the five properties, in the order presented, that is, orientation (N or X), followed by nucleotide stereospecificity (A_{NS} or B_{NS}) and conformation (A_{NC} or S_{NC}), followed by flavin stereospecificity (R_{FS} or S_{FS}) and conformation (A_{FC} or S_{FC}). Since only three of these five properties are independent variables, there are only $2^3 = 8$ possible overall configurations, which are X:$A_{NS}A_{NC}/R_{FS}A_{FC}$, X:$A_{NS}A_{NC}/S_{FS}S_{FC}$, N:$B_{NS}A_{NC}/R_{FS}S_{FC}$, N:$B_{NS}A_{NC}/S_{FS}A_{FC}$, N:$A_{NS}S_{NC}/R_{FS}S_{FC}$, N:$A_{NS}S_{NC}/S_{FS}A_{FC}$, X:$B_{NS}S_{NC}/R_{FS}A_{FC}$, X:$B_{NS}S_{NC}/S_{FS}S_{FC}$. Knowledge of any two of properties 1, 2, or 3 automatically defines the third, and the nicotinamide configuration. Knowledge of any two of properties 1, 4, or 5 automatically defines the third, and the flavin configuration. Finally, knowledge of any three of the five properties defines the remaining two properties, and the overall configuration (as long as the combination of three properties is not 1, 2, 3 or 1, 4, 5). In the absence of a crystal structure, there are currently no techniques available to determine properties 1 and 5, so the complete configuration must be specified in terms of flavin stereospecificity and nicotinamide stereospecificity and conformation. Table II shows the possible overall configurations if one knows the nicotinamide stereospecificity and conformation. These two properties define the orientation and narrow the overall configuration to two possibilities, which can be distinguished if the flavin stereospecificity is known.

There is known to be a strong correlation between A-side stereospecificity and the anti conformation and between B-side stereospecificity and the syn conformation (You et al., 1978). It has been suggested that this correlation could be due to a stereoelectronic effect (Benner, 1982). Specifically, only in the above two nicotinamide configurations (which have the exo orientation), is the lone electron pair on the nicotinamide nitrogen able to interact with the C–O antibonding orbital of the ribose ring. Glutathione reductase is the only enzyme that deviates from this correlation, since it is a B-side/anti enzyme (Stern & Vennesland, 1960; Pai & Schulz, 1983) and therefore has the endo orientation and the $N:B_{NS}A_{NC}$ nicotinamide configuration. The four possible nicotinamide configurations (Figure 4A–D) should require very specific interactions with the surrounding protein and would therefore be expected to fit into enzyme active sites with very different local tertiary structure. Hence, it is this more global property of nicotinamide configuration, rather than just one of the three general properties that define this configuration, that should be completely conserved among enzymes that are evolutionarily related. So, another explanation for this correlation between nicotinamide stereospecificity and conformation is that, for some mechanistic reason, there has been an evolutionary selection for the exo orientation over the endo orientation. Alternatively, primordial enzymes may have used the exo orientation initially, and the selective pressure on subsequent enzymes was not great enough to justify the large number of mutations that would have been needed to switch to the endo orientation.

Oppenheimer (1984, 1986) has pointed out that the correlation of A-side stereospecificity with anti conformation or B-side stereospecificity with syn conformation makes the tacit assumption that the nucleotide binds first to the enzyme with the hydride donor/acceptor binding next, on top. This corresponds to the exo orientation (Figure 4A,D). He also points out that in one instance, glutathione reductase, the nucleotide binds second, on top of the hydride donor/acceptor (Figure 4B). Here, the assumption has been made that what binds second binds on top of what binds first. With flavoproteins, the hydride donor/acceptor (FAD or FMN) is always bound first, so the nicotinamide ring would always be on top, and the orientation would always be endo. But, P-450R has the exo orientation. Thus, the notion of top or bottom is an ambiguous one, so we recommend the endo/exo nomenclature.

Since the nicotinamide configuration should be evolutionarily conserved, one would expect that all enzymes related to P-450R should be A-side/anti enzymes and therefore have the exo orientation and the $X:A_{NS}A_{NC}$ nicotinamide configuration. P-450R is functionally characterized as a dehydrogenase/electron transferase since it carries out the "transformation of two-electron transfer into one-electron transfer" (Massey & Hemmerich, 1980). Several other enzymes in this functional family have been shown to have some sequence homology to P-450R (Karplus et al., 1991; Porter & Kasper, 1986) and therefore are thought to be evolutionarily related (see Table III).

Likewise, enzymes related to glutathione reductase should be B-side/anti enzymes and therefore have the endo orientation and the $N:B_{NS}A_{NC}$ nicotinamide configuration. This is an intriguing prospect since glutathione reductase is the only enzyme known to have the endo orientation. Perhaps the endo orientation is only possible with enzymes that have the hydride donor/acceptor bound first to the enzyme, which is the case for all flavoproteins. This orientation is not a requirement for all flavoproteins, though, since P-450R binds the nicotinamide nucleotide and flavin in the exo orientation. Glutathione reductase is functionally characterized as a carbon–sulfur transhydrogenase (or disulfide oxidoreductase) since it carries out the "two-electron transfer between nicotinamide nucleotide and a disulfide–dithiol pair" (Massey & Hemmerich, 1980). Lipoamide dehydrogenase (Williams, 1976; Caruthers et al., 1991), mercuric reductase (Fox & Walsh, 1983), and trypanothione reductase (Shames et al., 1988), which are also members of this functional family, have been shown to have sequence homology with glutathione reductase and therefore are thought to be evolutionarily related (see Table III). Thioredoxin reductase, another member of this functional family, shows much less sequence homology (Russel & Model, 1988; Kuriyan et al., 1991).

Table III: Summary of Known Stereochemical and Conformational Properties of Nicotinamide Nucleotides in Two Functionally and Evolutionarily Related Families of Flavoproteins

enzyme	stereospecificity	nicotinamide conformation
C–S Transhydrogenase (Disulfide Oxidoreductase) Family		
glutathione reductase	B[a]	anti[b]
thioredoxin reductase	B[c]	nd[d]
lipoamide dehydrogenase	B[e]	nd
mercuric reductase	nd	nd
trypanothione reductase	nd	nd
asparagusate dehydrogenase[f]	nd	nd
Dehydrogenase/Electron Transferase Family		
NADPH–cytochrome P-450 oxidoreductase	A	anti
ferredoxin–$NADP^+$ reductase	A[g]	(anti)[h]
NADH–cytochrome b_5 reductase	A[i]	ND
NADH–nitrate reductase	A[j]	ND
NADPH–sulfite reductase	nd	nd

[a] Stern & Vennesland, 1960. [b] Pai & Schulz, 1983. [c] Larsson & Thelander, 1965. [d] Not determined. [e] Ammeraal et al., 1965. [f] Asparagusate dehydrogenase is functionally related to enzymes in the glutathione reductase family, but since a sequence is not yet available for this enzyme, it is not clear what its evolutionary relationship is to other enzymes in this family. [g] Krakow et al., 1965. [h] The nicotinamide conformation in FNR is not yet known, although an anti conformation has been proposed (Karplus et al., 1991) on the basis of the crystal structure of the 2′-phospho-AMP·FNR complex and the known stereochemistry of hydride transfer. [i] Drysdale et al., 1961. [j] Guerrero & Vennesland, 1975.

Just as with the nicotinamide configuration, one would expect the complete configuration to be conserved within families of structurally and functionally related flavoprotein dehydrogenases.[2] In the case of glutathione reductase, the nicotinamide stereospecificity is B-side (B_{NS}), and the nicotinamide conformation is anti (A_{NC}), which defines the orientation as endo and narrows the overall configuration to $N:B_{NS}A_{NC}/R_{FS}S_{FC}$ or $N:B_{NS}A_{NC}/S_{FS}A_{FC}$ (see Table II). But, since the flavin stereospecificity is known to be *re* (R_{FS}), the overall configuration is therefore $N:B_{NS}A_{NC}/R_{FS}S_{FC}$, consistent with the crystal structure of the NADPH·glutathione reductase complex (Stern & Vennesland, 1960; Pai & Schulz, 1983). In the case of P-450R, the nicotinamide stereospecificity is A-side (A_{NS}), and the nicotinamide conformation is anti (A_{NC}), which defines the orientation as exo and narrows the overall configuration to $X:A_{NS}A_{NC}/R_{FS}A_{FC}$ or $X:A_{NS}A_{NC}/S_{FS}S_{FC}$ (see Table II). It was suggested, on the

[2] The complete configuration could be conserved within a family because the enzymes have divergently evolved from a primordial enzyme. This would be a case of structure begetting function. Another possibility is that the complete configuration, which may have functional significance, is a result of convergent evolution. This would also yield some sequence homology within a family of functionally related enzymes and would be a case of function begetting structure.

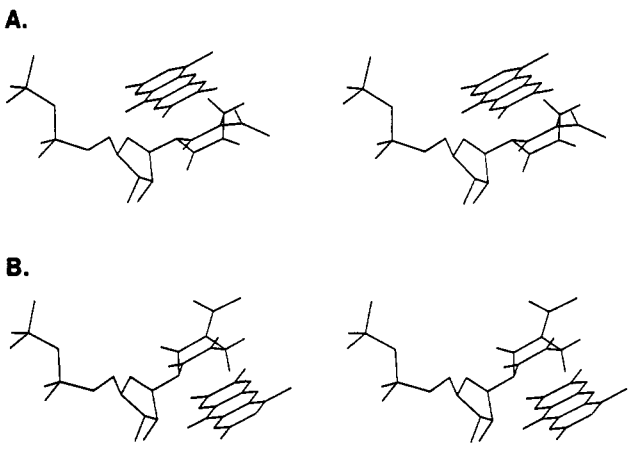

FIGURE 6: The predicted overall configuration for enzymes in the (A) glutathione reductase ($N:B_{NS}A_{NC}/R_{FS}S_{FC}$) and (B) P-450R ($X:A_{NS}A_{NC}/R_{FS}A_{FC}$) families. The two overall configurations differ in that the nicotinamide ring has switched to the other boat conformation, the FAD has moved to the other side of the nicotinamide ring, and the FAD has rotated 180° around its N5–N10 axis so that the dimethylbenzene ring has switched from projecting out of the page in (A) to projecting into the page in (B). All protons have been omitted, except for those on the nicotinamide ring.

basis of the crystal structure of the 2'-phospho-AMP·FNR complex (Karplus et al., 1991), that the nicotinamide ring resides on the *re* face of the flavin in the actual $NADP^+$·FNR complex. Since FNR is homologous to P-450R, and since all flavoprotein dehydrogenases tested to date show hydride transfer to the re face of the flavin (Manstein et al., 1986), we propose that P-450R has the $X:A_{NS}A_{NC}/R_{FS}A_{FC}$ overall configuration.

Thus, enzymes in the glutathione reductase family (Table III) are predicted to have the $N:B_{NS}A_{NC}/R_{FS}S_{FC}$ overall configuration with the active-site structure shown in Figure 6A, while enzymes in the P-450R family (Table III) are predicted to have the $X:A_{NS}A_{NC}$ nicotinamide configuration, and are likely to have the $X:A_{NS}A_{NC}/R_{FS}A_{FC}$ overall configuration with the active-site structure shown in Figure 6B. The nicotinamide ring is shown in the boat conformation, although it is not entirely clear whether this is usually the case or whether it has a more planar conformation (You, 1985; Rotberg & Cleland, 1991). These predictions assume an evolutionary relatedness of the enzymes in question. Lipoamide dehydrogenase (Mattevi et al., 1991) and mercuric reductase (Schiering et al., 1991) are known to have similar active-site tertiary structures to glutathione reductase, while the FAD and NADPH domains in thioredoxin reductase are very different, so that it may bind NADPH differently (Kuriyan et al., 1991). Enzymes such as thioredoxin reductase, which show more functional than structural relatedness, may adopt a different complete configuration. Indeed, a different complete configuration would indicate a lack of evolutionary relatedness between enzymes. It is interesting that adrenodoxin reductase, which is functionally related to enzymes in the P-450R family since it is also a dehydrogenase/electron transferase, has B-side stereospecificity (Sih et al., 1968). This would argue that adrenodoxin reductase is not evolutionarily related to P-450R, consistent with the lack of sequence homology observed between it and FNR or P-450R (Karplus et al., 1991; Hanukoglu & Gutfinger, 1989).

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Cholesterol Modulation of Lipid Intermixing in Phospholipid and Glycosphingolipid Mixtures. Evaluation Using Fluorescent Lipid Probes and Brominated Lipid Quenchers†

John R. Silvius
Department of Biochemistry, McGill University, Montréal, Québec, Canada H3G 1Y6
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ABSTRACT: Carbazole- and indole-labeled phospholipids have been used to monitor the homo- or heterogeneity of lipid mixing in several types of lipid bilayers combining a brominated and a nonbrominated lipid with varying amounts of cholesterol. Experimental quenching curves (relating the normalized probe fluorescence intensity to the mole fraction of brominated lipid) show a characteristic smooth, monophasic form for homogeneous liquid–crystalline lipid mixtures. However, for mixtures exhibiting lipid lateral segregation, such curves show marked perturbations in form over the region of composition where segregation occurs. Using this approach, it is found that high mole fractions of cholesterol (40–50 mol %) promote the formation of apparently homogeneous solutions in mixtures of disaturated and monounsaturated phosphatidylcholines (PCs) that exhibit extensive thermotropic phase separations in the absence of sterol. At only slightly lower levels of cholesterol, however, these systems exhibit inhomogeneous lipid mixing over a wide range of relative proportions of the two PC components. Mixtures of cerebroside and monounsaturated PCs, even at high bilayer cholesterol contents, exhibit significant inhomogeneity in lipid mixing over a wide range of cerebroside/PC ratios. Phase-separating PC/PC and PC/cerebroside mixtures can readily form long-lived metastable solutions when the level of the higher-melting component in the liquid–crystalline phase exceeds its equilibrium solubility by as much as 20–30 mol %; this tendency is significantly increased by cholesterol. Cholesterol shows no significant ability to enhance lipid intermixing in a third type of phase-separating lipid system, combining a monounsaturated PC with a monounsaturated phosphatidic acid–calcium complex. Experiments using cleavable phospholipid conjugates, linking a fluorescent lipid to a brominated lipid, suggest that each fluorescent molecule probes a local lipid domain comprising $\lesssim$40–50 nearby acyl chains.

The lateral organization of different lipid species in complex lipid mixtures, including those found in biological membranes, remains an important issue in membrane research. While various thermodynamic and spectroscopic methods have been used to characterize the organization of a number of binary lipid mixtures [for reviews, see Lee (1977), Silvius (1982), Thompson and Tillack (1985), Cullis et al. (1985), Knoll et al. (1991), and Caffrey et al. (1991)], such methods are often much more complicated to apply to ternary or higher-order lipid mixtures. This problem can be still more acute for systems containing cholesterol [reviewed in Yeagle (1985), Presti (1985), Vist and Davis (1990), and Finean (1990)], which reduces the cooperativity of lamellar phase transitions and may potentially reduce the sizes of individual domains in phase-separated lipid mixtures.

In spite of the limitations just noted, the properties of ternary and higher-order systems containing cholesterol are of considerable interest to model and to characterize better the

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