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^{13}C-, ^{15}N- and ^{31}P-NMR studies of oxidized and reduced low molecular mass thioredoxin reductase and some mutant proteins

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Thioredoxin reductase (TrxR) from *Escherichia coli*, the mutant proteins E159Y and C138S, and the mutant protein C138S treated with phenylmercuric acetate were reconstituted with [U-$^{13}C_{17}$,U-$^{15}N_4$]FAD and analysed, in their oxidized and reduced states, by ^{13}C-, ^{15}N- and ^{31}P-NMR spectroscopy. The enzymes studied showed very similar ^{31}P-NMR spectra in the oxidized state, consisting of two peaks at −9.8 and −11.5 p.p.m. In the reduced state, the two peaks merge into one apparent peak (at −9.8 p.p.m.). The data are compared with published ^{31}P-NMR data of enzymes closely related to TrxR. ^{13}C and ^{15}N-NMR chemical shifts of TrxR and the mutant proteins in the oxidized state provided information about the electronic structure of the protein-bound cofactor and its interactions with the apoproteins. Strong hydrogen bonds exist between protein-bound flavin and the apoproteins at C(2)O, C(4)O, N(1) and N(5). The N(10) atoms in the enzymes are slightly out of the molecular plane of the flavin. Of the ribityl carbon atoms C(10α,γ,δ) are the most affected upon binding to the apoprotein and the large downfield shift of the C(10γ) atom indicates strong hydrogen bonding with the apoprotein. The hydrogen bonding pattern observed is in excellent agreement with X-ray data, except for the N(1) and the N(3) atoms where a reversed situation was observed. Some chemical shifts observed in C138S deviate considerably from those of the other enzymes. From this it is concluded that C138S is in the FO conformation and the others are in the FR conformation, supporting published data. In the reduced state, strong hydrogen bonding interactions are observed between C(2)O and C(4)O and the apoprotein. As revealed by the ^{15}N chemical shifts and the N(5)H coupling constant the N(5) and the N(10) atom are highly sp^3 hybridized. The calculation of the endocyclic angles for the N(5) and the N(10) atoms shows the angles to be ≈ 109°, in perfect agreement with X-ray data showing that the flavin assumes a bent conformation along the N(10)/N(5) axis of the flavin. In contrast, the N(1) is highly sp^2 hybridized and is protonated, i.e. in the neutral state. Upon reduction of the enzymes, the ^{13}C chemical shifts of some atoms of the ribityl side chain undergo considerable changes also indicating conformational rearrangements of the side-chain interactions with the apoproteins. The chemical shifts between native TrxR and C138S are now rather similar and differ from those of the two other mutant proteins. This strongly indicates that the former enzymes are in the FO conformation and the other two are in the FR conformation. The data are discussed briefly in the context of published NMR data obtained with a variety of flavoproteins.

Keywords: FAD; flavoprotein; flavin–apoprotein interaction; NMR spectroscopy; thioredoxin reductase.

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Abbreviations: PMA, phenylmercuric acetate; TrxR, thioredoxin reductase; TARF, tetraacetyl riboflavin.

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Thioredoxin reductase (TrxR) (EC 1.6.4.5) catalyses the transfer of reducing equivalents from NADPH to thioredoxin. The substrate, thioredoxin, is a small protein (m = 11 700 Da) which contains a single redox-active disulfide and is involved in ribonucleotide reduction [1], bacteriophage assembly [2], transcription factor regulation [3], and protein folding [4]. TrxR is a member of a class of related flavoenzymes that includes lipoamide dehydrogenase, glutathione reductase, and mercuric ion reductase [5–7]. The homodimeric proteins contain one FAD and one redox-active disulfide per monomer. The flow of electrons in TrxR is from NADPH to FAD, from reduced FAD to the active site disulfide, and from the active site dithiol to thioredoxin.

TrxR is found in two distinct types depending on the source organism [8]. The enzyme from *Escherichia coli* is of

the low molecular mass type, which is also found in other bacteria, fungi and lower plants. The enzyme found in most higher organisms is of a high molecular mass form. The crystal structure of TrxR from *E. coli* revealed that each monomer consists of two globular domains connected by a double-stranded β-sheet. One domain contains the FAD binding site, whereas the other domain comprises the NADPH binding site and the redox-active disulfide [9,10]. In this structure, there is no obvious path for the flow of electrons from NADPH to the active-site disulfide. The active-site disulfide is adjacent to the flavin and is buried such that it cannot interact with the protein substrate thioredoxin; the nicotinamide ring of bound NADPH is located 17 Å away from the FAD. The observed conformation is referred to as FO. Another conformation, referred to as FR, was revealed when the enzyme was crystallized in the presence of aminopyridine adenine dinucleotide [11]. This confirmed the earlier proposal that the enzyme undergoes a large conformational change during catalysis whereby the two domains rotate 67° relative to each other [11]. This rotation and counter-rotation alternatively places the nicotinamide ring of NADPH or the active-site disulfide adjacent to the flavin and allows for the active-site disulfide to move from a buried position to the surface where it can react with the protein substrate thioredoxin. Thus, TrxR must assume two conformational states in catalysis: the form in which the active site disulfide is close to and can oxidize the flavin (FO), and the form in which the nascent dithiol is exposed to solvent and the pyridine nucleotide binding site is close to and can reduce the flavin (FR) (Fig. 1). The structure of the reduced enzyme in the FO conformation shows that the isoalloxazine ring of the flavin assumes a 34° 'butterfly' bend about the N(5)–N(10) axis [12].

The current view is that the FO and FR forms are in a dynamic equilibrium. Several recent studies have provided evidence that is consistent with the enzyme being in the FR form [13–16]. Rapid reaction studies on the reductive half-reaction of wild-type and several active site mutants have led to the hypothesis that the FR form is favoured in the wild-type enzyme and that the mutants have characteristic FO/FR ratios at equilibrium [13]. For example, in the mutant enzyme having one of the cysteine residues comprising the redox active disulfide/dithiol altered to serine, C138S, the equilibrium favours the FO conformation. The fluorescence of the flavin in C138S is quenched, consistent with the flavin being adjacent to Ser138 in the FO conformation. The flavin fluorescence increases 9.5-fold upon reaction of the remaining cysteine residue, Cys135, with phenylmercuric acetate (PMA) as the equilibrium shifts to the FR conformation. The fluorescence is quenched when the nonreducing NADP(H) analogue, 3-aminopyridine adenine dinucleotide, binds in the FR conformation. Reduced, wild-type thioredoxin reductase reacts with phenylmercuric acetate to shift the equilibrium between the FO and FR conformations to more completely favour the FR form. In this conformation, the flavin fluorescence is strongly quenched by the binding of 3-aminopyridine adenine dinucleotide [14].

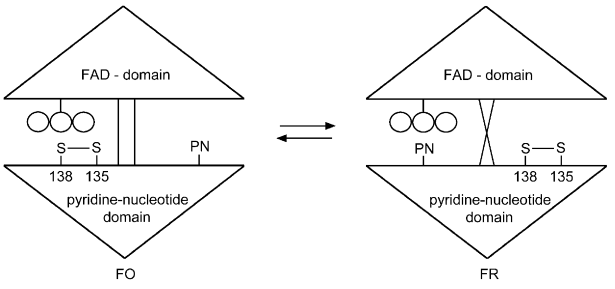

Fig. 1. Representation of TrxR in the FO and FR conformations. The two domains of the enzyme are shown as triangles and the double-stranded β-sheet connecting the domains are shown as lines. The FAD is depicted as three rings, and the pyridine nucleotide binding site indicated by PN. In the FR conformation, the buried flavin is adjacent to the nicotinamide of bound NADPH and the redox active disulfide, Cys135 and Cys138, are on the surface. In the FO conformation, the redox active disulfide is adjacent to the flavin and the pyridine nucleotide binding site has moved away. In the C138S mutant, Cys138 is converted to a serine leaving Cys135 as the remaining active site thiol. Note that if Cys135 in the C138S mutant is reacted with the thiol-specific reagent PMA, the enzyme becomes sterically 'locked' in the FR conformation.

The FAD of thioredoxin reductase is readily replaced by other flavins. This led us to utilize wild-type enzyme and several mutant forms in an NMR investigation of these enzymes in which FAD was replaced by [U-$^{15}N_4$,U-$^{13}C_{17}$]-FAD. As shown previously [17], such data can yield detailed information about the perturbation of the electronic structure of flavin upon binding to the apoprotein and its specific interactions with the apoprotein. In particular, we hoped to further confirm the presence of the FR and FO forms of the enzyme under the appropriate conditions and the differences in the electronic structures of FAD bound to native and mutant proteins.

Experimental procedures

Purification of thioredoxin reductase and reconstitution with [U-$^{15}N_4$,U-$^{13}C_{17}$]FAD

Recombinant wild-type and C138S TrxR from *E. coli* were purified as described previously [18]. The C138S mutant has been described in earlier studies [19,20]. Apo-TrxR was prepared by denaturation of the native enzyme with guanidinium chloride and removal of the unbound FAD by treatment with activated charcoal according to published procedures [21]. Extinction coefficients of 11 300 $M^{-1}\cdot cm^{-1}$ and 11 800 $M^{-1}\cdot cm^{-1}$ at 450 nm were used measuring concentrations of the oxidized native wild-type and C138S enzymes, respectively. The apoenzyme forms were quantified either by measuring absorbance at 280 nm using a calculated extinction coefficient of 22 200 $M^{-1}\cdot cm^{-1}$ [22], or by using the Bio-Rad Protein Assay reagent according to the manufacturer's instructions with BSA as the standard. Protein concentrations measured by these two methods agreed within 5%. Recoveries of protein typically ranged from 60% to 90%.

Reconstitution of apo-TrxR with labelled FAD was typically performed on 1.0–1.5 μmoles of protein in 0.5 mL 10 mM Na/K phosphate, pH 7.6, containing 0.15 mM EDTA (Buffer A). One equivalent of ^{13}C-, ^{15}N-labelled FAD was added and the solution was placed in a

Centricon-10 centrifugal concentrator unit (Millipore). After centrifugation at 5000 ***g*** for 20 min at 4 °C, an absorbance spectrum of the flow-through was taken to observe any unbound FAD. The concentrated protein above the filter was diluted to 2 mL with Buffer A and 0.1 molar equivalent of labelled FAD was added followed by centrifugation. This process was repeated until flavin could be detected in the flow-through solution showing that the enzyme in the filtrate was saturated with labelled FAD. The reconstituted enzyme was then washed with Buffer A by repeated cycles of dilution and concentration in the Centricon unit to give a dilution factor of at least 10^5. In control experiments in which wild-type apo-TrxR was reconstituted with unlabelled FAD, recoveries were 99% (relative to untreated enzyme) according to analysis of absorbance spectra, and 96% as measured by activity assays. Comparable reconstitution results were observed for samples of wild-type and mutant forms which were measured spectroscopically.

Preparation of mutant forms of TrxR

The C138S form of TrxR was described previously [14,23]. The E159Y mutation was introduced by site-directed mutagenesis. Phagemid pTrR301, which carries the wild-type *trxR* gene cloned into an expression vector [18] was used as the template. Single-stranded DNA was purified and used in mutagenesis reactions according to protocols of the Sculptor mutagenesis kit (Amersham) as described previously [18] using the oligonucleotide 5′-CAGCGCCTATATAAACGGTATTGCC-3′ in which the underlined bases were altered to introduce the desired amino acid change and to make a silent mutation to eliminate the *Sac*II restriction site. Mutagenesis reactions were performed on wild-type single-stranded template DNA according to the manufacturer's instructions and plasmids isolated from mutant candidates were screened for the appropriate change in restriction digest pattern. To verify the correct sequence changes, the resulting mutant plasmid DNA was sequenced across the entire *trxR* gene by using automated methods at the University of Michigan Biomedical Research Core Facility. The new plasmid was designated pTrR311.

Isolation and purification of flavokinase/FAD-synthetase of *Corynebacterium ammoniagenes*

The gene specifying the bifunctional flavokinase/FAD synthetase (accession number D37967) was amplified from bp 248 to bp 1264 by PCR using chromosomal DNA of *C. ammoniagenes* DSM20305 as template and the oligonucleotides FAD(CA)-1 and FAD(CA)-2 as primers (Table 1). The 1006-bp amplification product was digested with *Eco*RI and *Bam*HI and ligated into the vector pMal-c2 (New England Biolabs) which had been treated with the same enzymes. The resulting plasmid pMalribF-(CA) was transformed into *E. coli* XL-1 to create strain *E. coli* [pMalribF-(CA)] which was grown in Luria–Bertani medium containing ampicillin (170 mg·L^{-1}) to an *D* value of 0.6 at 600 nm. Isopropyl-thio-galactoside (Sigma) was added to a final concentration of 2 mM, and incubation was continued for 4 h with shaking at 37 °C. The cells were harvested by centrifugation (5000 r.p.m., 15 min, 4 °C) and stored at −20 °C.

Table 1. Oligonucleotides used for the amplification of flavokinase/FAD synthetase from *C. ammoniagenes*.

Primer FAD(CA)-1	5′-TCAGAATTCCATGGATATTTGGTA CGG-3′
Primer FAD(CA)-2	5′-GGCCAACGCAAAGGGATCCTCGAT ACC-3′

Frozen cell mass was thawed in 50 mM sodium/potassium phosphate buffer, pH 7.0, containing 5 mM EDTA and 5 mM Na_2SO_3 and the suspension was ultrasonically treated and centrifuged. The supernatant (15 mL) was passed through a column of amylose resin (New England Biolabs; 20 × 30 mm), which had been equilibrated with 50 mM sodium/potassium phosphate pH 7.0 containing 5 mM EDTA and 5 mM Na_2SO_3. The column was washed with 80 mL of the equilibration buffer. The immobilized enzyme on amylose resin was stable for 1 week at 4 °C.

Preparation of [U-$^{13}C_{17}$, U-$^{15}N_4$]riboflavin

[U-$^{13}C_{17}$, U-$^{15}N_4$]riboflavin was prepared according to published procedures [24].

Preparation of [U-$^{13}C_{17}$, U-$^{15}N_4$]FAD

A suspension (9 mL) of immobilized flavokinase/FAD-synthetase from *C. ammoniagenes* in 50 mM sodium/potassium phosphate pH 7 containing 5 mM EDTA and 5 mM Na_2SO_3 was added to 30 mL 100 mM sodium/potassium phosphate pH 7.0 containing 40 mM $MgCl_2$, 100 mM Na_2SO_3, 10 mM EDTA, 23.4 μmol [U-$^{13}C_{17}$, U-$^{15}N_4$]riboflavin and 272 μmol ATP. The mixture was incubated at 37 °C with gentle shaking. After 4 h, 9 mL of the immobilized enzyme and 8 mL 1 mM ATP were added and the mixture was incubated for 2 h at 37 °C. Finally, 8 mL 1 mM ATP and 4.5 mL of the immobilized enzyme were added. Incubation was continued for 2 h, and the mixture was centrifuged. The supernatant contained 8.1 μmol [U-$^{13}C_{17}$, U-$^{15}N_4$]FAD as shown by HPLC.

The solution was concentrated under reduced pressure. Aliquots were placed on top of a RP Nucleosil 10C_{18} column (20 × 250 mm) which was developed with an eluent containing 12% methanol, 0.1 M formic acid and 0.1 M ammonium formate. The flow rate was 20 mL·min^{-1}. Fractions containing [U-$^{13}C_{17}$,U-$^{15}N_4$]FAD (retention volume, 160 mL) were combined and concentrated under reduced pressure. The solution was passed through a Sep-Pak-Vac 35 cc (10 g) C_{18} cartridge (Waters) which was then developed with water. Fractions were combined and methanol was removed under reduced pressure. The remaining aqueous solution was lyophilized.

Reverse phase HPLC

HPLC was performed with a RP Hypersil ODS 5-μm column (4.6 × 250 mm) and an eluent containing 5 mM ammonium acetate in 25% methanol. Riboflavin, FMN and FAD had retention volumes of 44 mL, 10 mL and 6 mL, respectively.

NMR spectroscopy

Samples for ^{13}C-NMR measurements contained 10 mM phosphate buffer pH 7.5. Samples for ^{15}N and for ^{31}P-NMR measurements contained 10 mM Hepes, pH 7.8. Protein concentrations ranged from 0.2 to 1.5 mM. The samples contained 10% $^{2}H_2O$ (v/v) for the ^{2}H signal to lock the magnetic field. Precision NMR tubes (5 mm; Wilmad) were used for the acquisition of the spectra. Reduction of the enzyme was conducted by the addition of dithionite solution to the anaerobic protein solutions. Anaerobic conditions were achieved by flushing the NMR tube containing the sample with argon for ≈ 10 min. The NMR tube was sealed with an Omni-Fit sample tube valve (Wilmad).

Measurements were made at 7 °C on a Bruker DRX500 spectrometer (500.13 MHz ^{1}H frequency). Composite pulse decoupling was used for ^{13}C- and ^{31}P-NMR measurements. No ^{1}H decoupling was applied for ^{15}N-NMR measurements unless indicated otherwise. All spectra were recorded using a flip angle of 30° and a relaxation delay of 1.0 s, except for ^{31}P-NMR measurements for which a relaxation delay of 2.0 s was used. Quadrature detection and quadrature phase-cycling were applied in all NMR measurements. The resulting free induction decays were processed by zero filling and exponential multiplication with a line-broadening factor of 2–10 Hz to improve the signal-to-noise ratio. 3-(Trimethylsilyl)-1-propanesulfonate served as an external standard for ^{13}C-NMR measurements. [5-^{15}N]6,7-Dimethyl-8-ribityllumazine was used as an external reference for ^{15}N-NMR measurements. The ^{15}N-NMR signal of [5–^{15}N]6,7-dimethyl-8-ribityllumazine at 327.0 p.p.m. was used as an external reference for ^{15}N-NMR measurements. For ^{31}P-NMR measurements, 85% H_3PO_4 was used as an external reference.

Results

Characterization of the E159Y mutant

The UV/visible absorbance maxima of the E159Y mutant protein were located at 377 nm and 451 nm (compared to 380 nm and 456 nm for wild-type protein) and the fluorescence was only 37% of the intensity observed for the wild-type (456 nm excitation, 540 nm emission). The specific activity of E159Y TrxR measured at 20 μM NADPH was 25% of wild-type controls. It is postulated that the decreased fluorescence of this mutant is due to the proximity of the introduced tyrosine to the isoalloxazine ring of protein-bound FAD: this would only occur in enzyme that is in the FR conformation.

^{31}P-NMR analyses

The ^{31}P-NMR spectra of the native wild-type TrxR from *E. coli* in the oxidized and reduced state are shown in Fig. 2. The ^{31}P-NMR spectrum of the pyrophosphate moiety of FAD bound to oxidized TrxR shows two peaks at −9.2 and −11.5 p.p.m. (Fig. 2A). A sharp line observed at 4.5 p.p.m. originates from inorganic phosphate incompletely removed by dialysis. Reconstitution of wild-type apoprotein with isotope-labelled FAD gave essentially the same ^{31}P-NMR spectrum indicating a high degree of reconstitution which is supported by activity measurements. The ^{31}P-NMR spectrum of the native mutant protein E159Y was virtually identical with that of the native enzyme (Table 2). It is

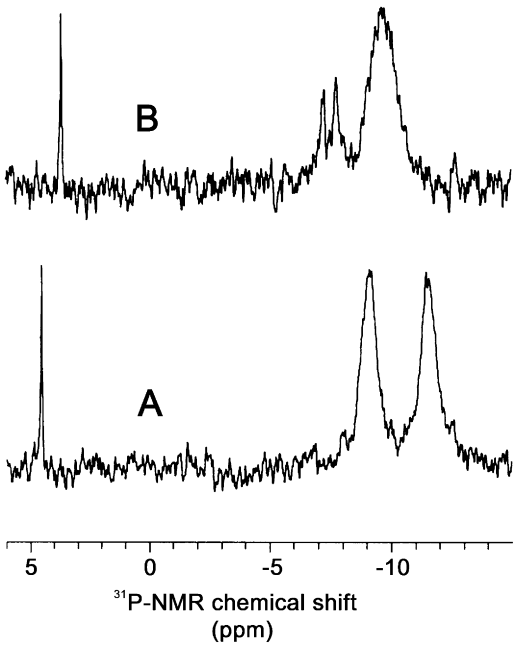

Fig. 2. ^{31}P-NMR spectrum of wild-type TrxR in the oxidized (A) and the reduced state (B).

Table 2. ^{31}P-NMR chemical shifts (in p.p.m.) of native and native reconstituted thioredoxin reductase (TrxR), and of the native mutant protein E159Y in the oxidized and reduced states. For comparison the chemical shifts of free FAD and those of other members of the class of pyridine nucleotide-disulfide oxidoreductases are given.

		^{31}P Chemical shift (in p.p.m.)		
Compound	pH	Oxidized	Reduced	Reference
Free FAD	7.5	−10.4, −11.1		[17]
Wild-type native TrxR	7.8	−9.2, −11.5		This report
Wild-type reconstituted TrxR	7.8	−9.4, −11.4	−9.8	This report
Native E159Y TrxR mutant protein	7.8	−9.4, −11.4		This report
Glutathione reductase	7.0	−9.7, −10.5	−9.8	[17]
Lipoamide reductase	7.0	−8.4, −12.4		[17]
Mercuric reductase	7.0	−12.1, −12.9	− 10.1	[17]

interesting to note that lipoamide dehydrogenase, glutathione reductase and mercuric reductase, which belong to a different subclass of redox-active disulfide-containing flavoproteins, show different ^{31}P-NMR spectra (Table 2) [17]. The data indicate different binding modes as well as differences in the direct environment of the pyrophosphate group in the different enzymes.

Upon reduction, the two ^{31}P resonance lines of native TrxR merge into one broader line centred at about −10 p.p.m. (Fig. 2B, Table 2). The change in chemical shift upon reduction suggests that a (small) conformational change occurs in the binding pocket of FAD and/or that the conformation of the pyrophosphate moiety in FAD has changed upon reduction. The spectrum in the reduced state shows again the resonance line of free phosphate, upfield shifted by ≈0.7 p.p.m. due to a small decrease of the pH in the solution, caused by the addition of dithionite. Two additional sharp signals at −7.3 and −7.8 p.p.m. were also present in the spectrum of the reduced enzyme. These are tentatively assigned as signals arising from residual free $FADH_2$ which was present in most preparations, but typically in smaller amounts than seen in Fig. 2B. The reduced form of the native, reconstituted enzyme and that of the native mutant E159Y gave essentially identical results as observed with the native enzyme (Table 2).

^{13}C- and ^{15}N-NMR analyses: general considerations

We studied the wild-type enzyme, two mutants (E159Y, C138S), and a chemically modified mutant protein (C138S treated with PMA) of TrxR reconstituted with uniformly ^{13}C- and ^{15}N-labelled FAD. The wild-type enzyme will be discussed first and these results will then be compared with the mutants in order to describe the possible structural differences between these FAD-containing enzymes. The interpretation and the assignment of the chemical shifts are based on published ^{13}C- and ^{15}N-NMR studies on protein-bound and free flavins [17], the data referring to free flavins are also given in Tables 3 and 4 for comparison. In the latter studies, the flavin was either dissolved in water (FMN, polar environment for the flavin) or in chloroform (tetraacetylriboflavin, TARF) to mimic an apolar environment. These studies have shown that the ^{13}C chemical shifts in the flavin molecule correlate well with the π-electron density at the corresponding atoms [25,26]. This means that any perturbation of the electronic structure of the flavin, e.g. polarization of the molecule by hydrogen bonding, will result in a downfield shift of the atoms involved (π-electron density decrease). On the other hand, a π-electron density increase leads to an upfield shift.

For the ^{15}N chemical shifts, the following should be kept in mind. The flavin molecule contains four nitrogen atoms. In the oxidized state, the N(1) and N(5) atoms of flavin are so-called pyridine- or β-type nitrogen atoms. The chemical shifts of such atoms are rather sensitive to hydrogen bonding and undergo a relatively large upfield shift upon hydrogen bond formation (see [27] and references therein). The N(10) and N(3) atoms are so-called pyrrole- or α-type nitrogen atoms and are much less sensitive to hydrogen bonding leading to a small downfield shift. In reduced flavin all four nitrogen atoms belong to the latter class of nitrogen atoms. When observable, ^{15}N–^{1}H coupling constants were also used for the assignment of nitrogen atoms and to determine the degree of hybridization of the corresponding nitrogen atom.

Table 3. ^{13}C and ^{15}N-NMR chemical shifts (in p.p.m.) of flavins in solution and FAD bound to wild-type thioredoxin reductase (TrxR) and mutant proteins in the oxidized state.

Atom	Free FMN[a]	Free TARF[a]	TrxR wild-type	TrxR E159Y mutant	TrxR C138S mutant	TrxR C138S mutant + PMA
C(2)	159.8	155.2	159.1	159.4	158.8	159.2
C(4)	163.7	159.8	165.2	164.2	164.6	164.5
C(4a)	136.2	135.6	136.1	135.6	137.5	135.7
C(5a)	136.4	134.6	135.4	135.6	137.5	135.7
C(6)	131.8	132.8	130.4	130.1	130.4	130.2
C(7)	140.4	136.6	140.3	139.2	138.5	139.4
C(7α)	19.9	19.4	19.0	19.1	19.6	19.6
C(8)	151.7	147.5	151.2	151.0	149.2	151.0
C(8α)	22.2	21.4	21.6	21.1	22.4	21.6
C(9)	118.3	115.5	119.4	119.3	119.1	119.3
C(9a)	133.5	131.2	130.4	131.5	130.4	131.6
C(10a)	152.1	149.1	151.2	151.0	151.1	151.0
C(10α)	48.8[b]	45.3[d]	51.8	51.5	50.6	51.1
C(10β)	70.7[b]	69.2[d]	71.6	71.5	71.6	71.5
C(10 γ)	74.0[b,c]	69.5[d]	79.3	79.2	80.1	79.3
C(10δ)	73.1[b,c]	70.6[d]	73.0	73.0	74.2	73.0
C(10ε)	66.4[b]	62.0[d]	68.3	67.5	69.2	68.2
N(1)	190.8	200.1	183.0	183.2	185.6	183.7
N(3)	160.5	159.6	156.6	156.2	154.4	156.5
N(5)	334.7	346.0	326.8	328.5	322.6	327.6
N(10)	163.5	151.9	161.5	160.4	157.8	159.5

[a] Taken from [30]. [b] Taken from [31]. [c] Previous assignment revised on the basis of new data. [d] Taken from [26].

Table 4. ^{13}C and ^{15}N-NMR chemical shifts (in p.p.m.) of reduced flavins in solutions and FAD bound to wild-type thioredoxin reductase (TrxR) and mutant proteins in the reduced state.

Atom	$TARFH_2$[a]	$FMNH_2$[a]	$FMNH^-$[a]	TrxR wild-type	TrxR E159Y mutant	TxR C138S mutant	TxR C138S mutant + PMA
C(2)	150.6	151.1	158.2	158.2	158.3	158.4	157.5
C(4)	157.0	158.3	157.7	158.8	158.3	158.4	158.0
C(4a)	105.2	102.8	101.4	105.6	104.5	106.1	104.7
C(5a)	136.0	134.4	134.2	143.3	143.2	142.7	142.9
C(6)	116.1	117.1	117.3	116.1	116.5	115.7	116.1
C(7)	133.6	134.3	133.0	130.6	130.9	131.5	130.2
C(7α)	18.9	19.0	19.0	19.5	19.1	19.5	19.0
C(8)	129.0	130.4	130.3	130.6	130.9	131.5	130.2
C(8α)	18.9	19.2	19.4	19.5	19.1	19.5	19.0
C(9)	118.0	117.4	116.8	117.2	116.5	115.7	117.3
C(9a)	128.2	130.4	130.9	136.0	134.4	134.1	134.8
C(10a)	137.1	144.0	155.5	153.3	153.2	152.9	153.4
C(10α)	47.4[b]	51.1[c]	46.0[c]	53.7	53.5	53.3	53.5
C(10β)	69.7[b]	71.4[c]	71.2[c]	72.2	72.1	72.8	72.4
C(10 γ)	70.0[b]	72.6[c]	73.0[c]	76.3	75.5	75.7	75.7
C(10δ)	70.1[b]	73.3[c]	73.9[c]	73.3	72.1	72.8	72.4
C(10ε)	62.0[b]	67.7[c]	66.5[c]	69.2	69.1	69.1	69.2
N(1)	119.9	128.0	181.3	118.3	–	119.0	119.0
N(3)	149.0	149.7	150.0	146.0	–	148.8	138.0
N(5)	59.4	58.0	58.4	14.4	–	15.1	15
N(10)	76.8	87.2	96.5	52.1	–	50.8	51

[a] Taken from [30]. [b] Taken from [26]. [c] Taken from [31].

Natural abundance ^{13}C resonance lines are observed in the NMR spectra of proteins of this size (Fig. 3B). They originate from peptide carbonyl carbons and carboxylic side-chain carbons (170–180 p.p.m.), Arg C(ζ) atoms (158 p.p.m.), aromatic carbon atoms of Trp, Tyr, Phe, and His (110–140 p.p.m.), C(α) atoms (≈ 60 p.p.m.) and aliphatic carbon atoms of amino acid residues (10–70 p.p.m.). If such resonances interfered with those of protein-bound flavin, difference spectra were recorded (Fig. 4). However in the majority of cases, difference spectra were not needed to identify unambiguously the resonances due to the flavin.

Resonances not due to flavin also appear in the ^{15}N-NMR spectra of the proteins: the (broad) lines at 120 p.p.m. and at ≈310 p.p.m. originate from natural abundance ^{15}N nuclei in the proteins. The former resonance can be assigned to peptide bonds, whereas the latter one remains unassigned but has also been observed in ^{15}N-NMR spectra of other flavoproteins [28].

Magnetic anisotropy and ring current effects have not been taken in account in this paper because they contribute to chemical shift changes usually smaller than 1 p.p.m. and are therefore less important in determining ^{13}C chemical shifts than is the case for ^{1}H shifts. Steric and stereochemical effects, however, can considerably influence the ^{13}C chemical shifts [29].

Oxidized enzymes

The ^{13}C-NMR spectrum and the ^{15}N-NMR spectrum of wild-type TrxR reconstituted with [U-$^{15}N_4$,U-$^{13}C_{17}$]FAD are shown in Fig. 3B and Fig. 5, respectively. The chemical shifts are summarized in Table 3, including those of free FMN and TARF. Even though the enzyme contains FAD as cofactor, FMN is preferred as reference as free FAD forms an internal complex whereas protein-bound FAD acquires generally an open, i.e. extended form. The ^{15}N chemical shifts of the flavin chromophore form the basis for a detailed interpretation of the ^{13}C chemical shifts [27]. Therefore they are discussed first.

^{15}N-NMR. The N(1) and N(5) atoms of protein-bound FAD in the wild-type enzyme resonate at higher field than those of FMN in water and TARF in chloroform (Table 3, Fig. 6A). With respect to FMN, the chemical shifts of the N(5) and N(1) atoms are upfield shifted by 7.9 p.p.m and 7.8 p.p.m., respectively.

The N(3) atom of protein-bound FAD resonates at 156.6 p.p.m., which is upfield from that of free FMN (−3.9 p.p.m.) and TARF (−3.0 p.p.m.). For the N(3)H group a coupling constant of 87 Hz was estimated. The observation of an NH coupling indicates that the exchange of the N(3)-H proton in the protein is slow on the NMR time scale. Apparently solvent access to this group is prevented by binding of FAD to the apoprotein.

Model studies have shown that the N(10) atom in free flavin exhibits an unexpected large downfield shift on going from apolar to polar solvents [27]. This pyrrole-like nitrogen atom cannot form a hydrogen bond. Therefore, this observation was explained by an increase of sp^2 hybridization of the N(10) atom. The resulting mesomeric structures are preferentially stabilized by hydrogen bonds to the carbonyl functions at position 2 and 4, as supported by ^{13}C-NMR data [27]. The ^{15}N chemical shift

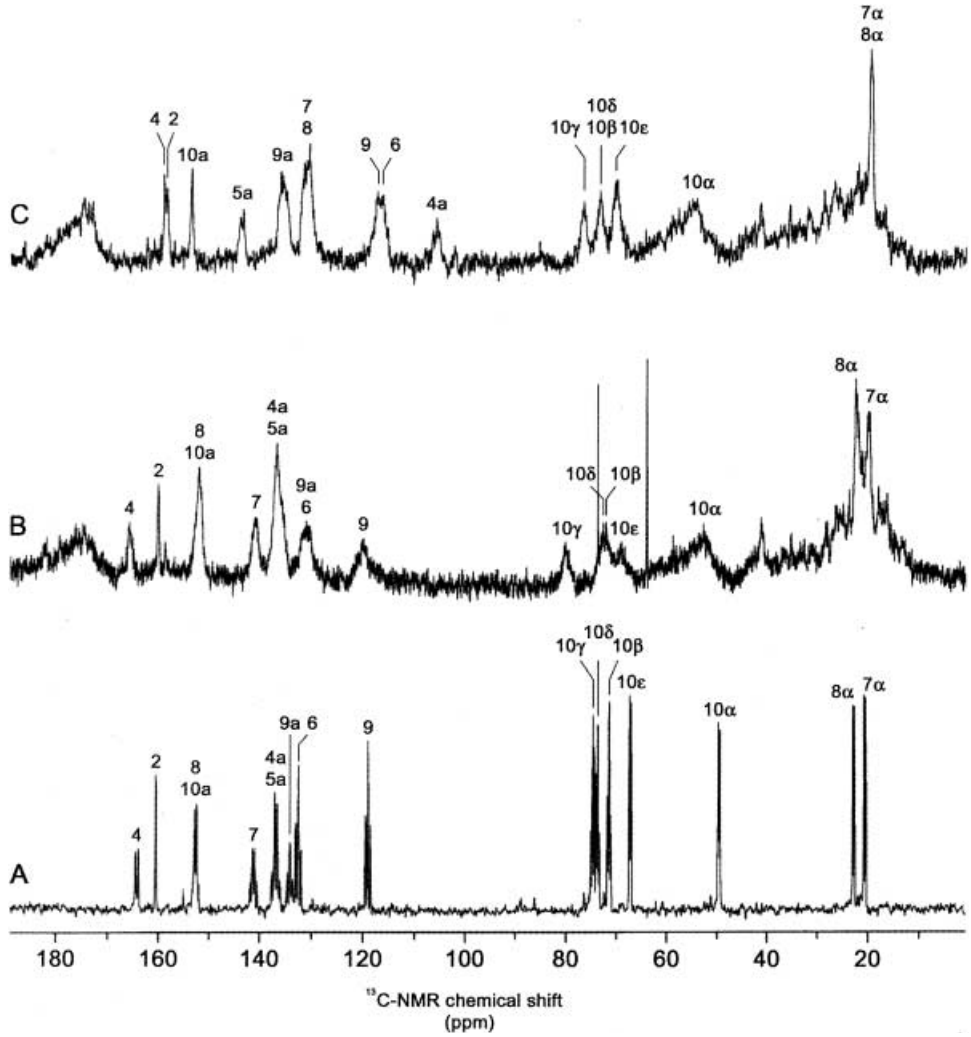

Fig. 3. ^{13}C-NMR spectrum of free FMN (A), wild-type TrxR reconstituted with [U-$^{13}C_{17}$, U-$^{15}N_4$]FAD in the oxidized (B) and reduced state (C).

of the N(10) atom of TrxR-bound FAD appears at 161.5 p.p.m., 2.0 p.p.m. upfield from that of free FMN (Table 3, Fig. 6A) implicating an increase of π-electron density at the N(10) atom.

With respect to the ^{15}N chemical shifts of the mutants, it is interesting to note that they are variably affected by modification of the protein. In comparison to the native protein, the N(5) atom resonates at higher field in C138S (−4.2 p.p.m.) but at lower field in C138S + PMA (+0.8 p.p.m.), and in E159Y (+1.7 p.p.m.). The ^{15}N chemical shifts of the N(10) atom in the mutants are considerably more influenced in comparison with that of the native enzyme, they are upfield shifted by −1.1 p.p.m. in E159Y, by −3.7 p.p.m. in C138S and by −2.0 p.p.m. in C138S + PMA, indicating a further increase in π-electron density at the N(10) atom with respect to that observed in the wild-type enzyme (Fig. 6A). The resonances due to the N(3) atom in the mutant proteins appear at slightly higher fields in E159Y (−0.4 p.p.m.) and in C138S + PMA (−0.1 p.p.m.) than those of wild-type protein, whereas that in C138S is considerably more shifted (−2.2 p.p.m.). The estimated coupling constants for the mutant proteins are the same (85–87 Hz) as that observed in the wild-type enzyme. The N(1) atom in the mutant proteins resonates at 183.7 p.p.m. in C138S + PMA, at 185.6 p.p.m. in C138S and at 183.2 p.p.m. in E159Y.

^{13}C-NMR. The ^{13}C chemical shift due to the C(2) atom of FAD in the wild-type enzyme is shifted slightly upfield (−0.7 p.p.m.) and that of the C(4) atom is downfield shifted (+1.5 p.p.m.) in comparison with that of FMN in aqueous solution (Table 3, Fig. 7A), indicating a strong polarization of the C(2) and the C(4) carbonyl groups. In the mutant proteins, the corresponding ^{13}C chemical shifts are still at lower field than that of FMN but at slightly higher field than that of protein-bound FAD in wild-type enzyme.

As shown by model studies, polarization of the isoalloxazine ring of flavin through hydrogen bonding at the C(2)O and the C(4)O groups (FMN in water) influences the π-electron density on C(8), C(9a), N(5) and C(10a) through conjugative effects leading to a downfield shift of the corresponding ^{13}C chemical shifts, and to an upfield shift of that of C(6) [32], as compared with

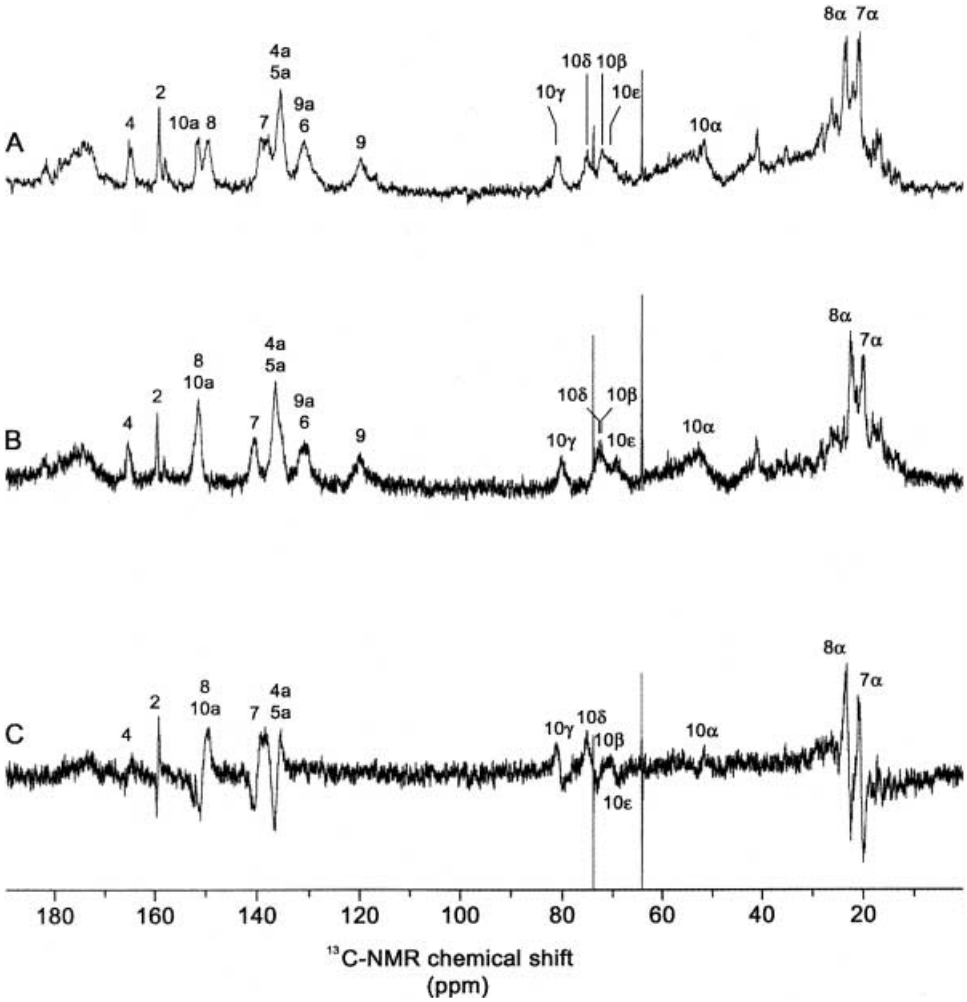

Fig. 4. ^{13}C-NMR spectrum of TrxR C138S mutant protein reconstituted with [U-$^{13}C_{17}$, U-$^{15}N_4$]FAD in the oxidized state (A), TrxR wild-type reconstituted with [U-$^{13}C_{17}$, U-$^{15}N_4$]FAD in the oxidized state (B), and difference spectrum (C) = (A) – (B).

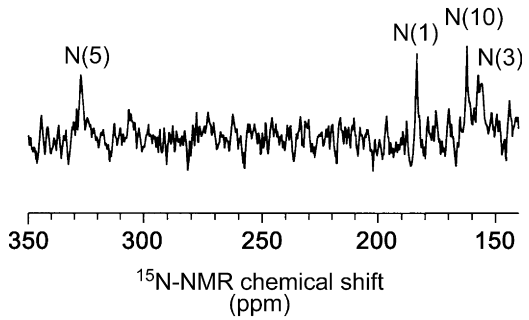

Fig. 5. ^{15}N-NMR spectrum of wild-type TrxR reconstituted with [U-$^{15}N_4$]FAD in the oxidized state.

TARF. These effects are observed in the wild-type enzyme, except for C(9a) which is upfield shifted (−0.8 p.p.m.). The chemical shifts of these atoms exhibit a similar trend in the mutant proteins as observed in the wild-type enzyme, except that the chemical shift due to C(9a) in E159Y and in C138S + PMA is slightly downfield from that of TARF. The ^{13}C chemical shifts of C(5a), C(7) and C(9) in all enzyme preparations are downfield shifted in comparison to those of TARF. The ^{13}C chemical shift of C(4a) of wild-type protein resonates at the same field as that of FMN. In the mutant proteins the chemical shift of the C(4a) atom appears at a higher field in E159Y and in C138S + PMA, and at lower field in C138S. In addition, the resonances due to C(8) and C(10a) are well separated in the latter enzyme, whereas overlap occurs in the other preparations (Fig. 4A and Fig. 7A).

The state of hybridization of the N(10) atom is also reflected by the chemical shift of the methylene group [C(10α)] directly bound to this atom (Table 3). The assignment of the chemical shifts due to the carbon atoms of the ribityl side chain has been done following the trends of the chemical shifts observed in FMN and FAD. With respect to FMN, the ^{13}C chemical shifts of C(10α), C(10γ) and C(10ε) are downfield shifted in all enzymes, and the resonances due to C(10β) and C(10δ) are practically unaffected (Table 3).

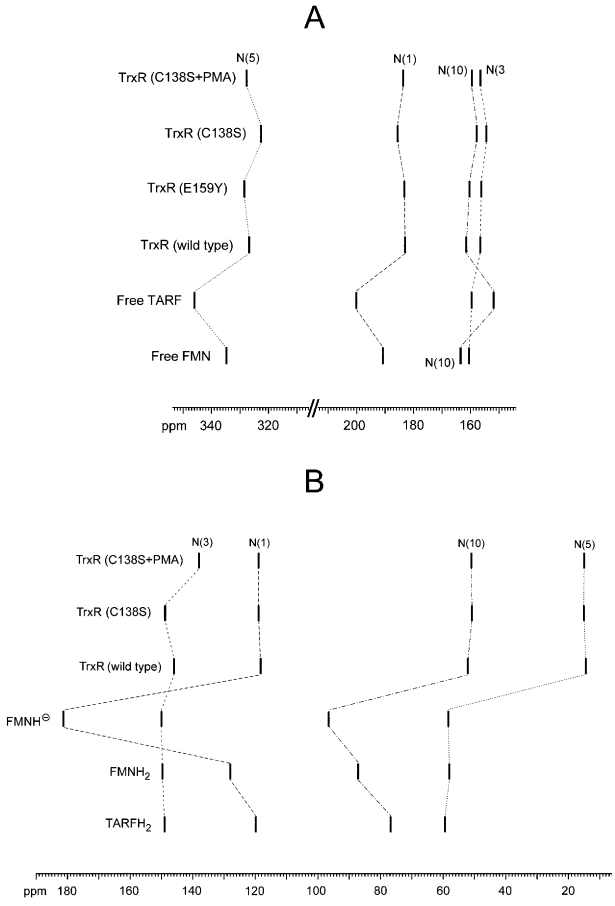

Fig. 6. Correlation diagrams of ^{15}N-NMR chemical shifts of flavins in solution and FAD bound to TrxR in the oxidized (A) and reduced state (B).

Reduced enzymes

As the fundamental concept for the interpretation of the ^{13}C and ^{15}N chemical shifts of reduced flavin is the same as that used above for oxidized flavin, the description of the data will be confined to the most relevant findings. Typical ^{13}C and ^{15}N-NMR spectra are given in Fig. 3C and Fig. 8, respectively. The results are summarized in Table 4 and Fig. 6B and Fig. 7B, together with the chemical shifts of free flavin in different environments.

^{15}N-NMR. Upon two-electron reduction of free flavin, the ^{15}N chemical shift of the N(3) atom is the least affected of the four nitrogen atoms and is shifted by only $\approx$10 p.p.m. to higher field, reflecting its relatively high isolation from the remaining π-electron system of the molecule. The resonance frequencies of N(3) and N(1) in reduced flavin indicate that these two atoms are predominately pyrrole-type, i.e. sp^2 type nitrogens. However, the chemical shift of the N(1) atom is much more affected upon reduction and upfield shifted by $\approx$80 p.p.m. As shown in Fig. 6B, these two atoms of the enzyme preparations studied in this paper resonate at about the same field as those of free flavin. Whereas the N(5) and N(10) atom of free reduced flavin resonate in the region of aniline-type N atoms, the N(10) atom of the enzyme preparations is also an aniline-type nitrogen atom, but shifted further upfield, while the N(5) atom of the enzymes resonates in the region of aliphatic amino groups, and therefore resonates at a much higher field than that of free reduced flavin.

The N(1) atom of the wild-type enzyme resonates at 118.3 p.p.m., 1.6 p.p.m. upfield from that of $TARFH_2$ in chloroform (119.9 p.p.m.) (Fig. 6B). The similarity of the ^{15}N chemical shifts in the two molecules strongly indicates that N(1) in the enzyme is protonated. This is further supported by the fact that a coupling constant of $\approx$100 Hz has been determined for the N(1)H function of protein-bound $FADH_2$. Therefore, the chemical shifts of the enzyme preparations must be compared with those of neutral, reduced flavin ($TARFH_2$ and $FMNH_2$). In fact, ionization of the N(1)H group would lead to a large downfield shift of $\approx$53 p.p.m. of the chemical shift due to N(1), caused by the negative charge (field effect, see also below), as observed in $FMNH^-$ (Fig. 6B) [27]. Introducing point mutations in the enzyme leads to a small downfield shift of the ^{15}N chemical shifts of N(1) in the mutant proteins as compared to that of wild-type enzyme, but they are still $\approx$0.9 p.p.m. upfield from that of $TARFH_2$.

The ^{15}N chemical shift of the N(5) atom of wild-type enzyme is upfield shifted by 45 p.p.m and 43.6 p.p.m. in comparison to those of $TARFH_2$ and $FMNH_2$, respectively (Fig. 6B). The coupling constant of the N(5)H group is $\approx$77 Hz. The chemical shifts of this atom are slightly downfield shifted by introducing mutations in the enzyme.

The chemical shift of the N(10) atom of wild-type enzyme shows the same trend as observed for that of N(5), a drastic upfield shift of 35.1 p.p.m and 24.7 p.p.m. in comparison to $FMNH_2$ and $TARFH_2$, respectively. The ^{15}N chemical shifts of this atom in the mutants are slightly further downfield shifted in comparison to that of the wild-type enzyme (Fig. 6B).

With respect to $TARFH_2$ and $FMNH_2$ the ^{15}N chemical shift of the N(3) atom of the wild-type enzyme is upfield shifted by $\approx$3 p.p.m. No reliable coupling constant could be determined for the N(3)H group (broad line) in all preparations studied. In the mutants the N(3) atom in C138S is practically unaffected and that in C138S + PMA is upfield shifted in comparison to that of $TARFH_2$.

^{13}C-NMR. In wild-type enzyme the ^{13}C-NMR resonance due to C(2) is downfield shifted by 7.6 p.p.m and 7.1 p.p.m. in comparison with $TARFH_2$ and $FMNH_2$, respectively, and resonates at about the same position as C(2) in $FMNH^-$ (Table 4, Fig. 7B). The large downfield shift of C(2) in $FMNH^-$ is caused by the negative charge on N(1) (electric field effect which also holds for the C(10a) atom, see $FMNH^-$ in Table 5): this effect plays no role in wild-type enzyme because N(1) exists in the neutral form. Therefore, the downfield shift must be caused by other effects (see below). In the mutant proteins the chemical shifts resemble those in the wild-type enzyme, except that in C138S + PMA which is upfield shifted. In contrast, the ^{13}C chemical shifts due to C(4) in all enzymes are very similar to those in $FMNH_2$.

Of the remaining carbon atoms constituting the isoalloxazine ring of protein-bound $FADH_2$ the most

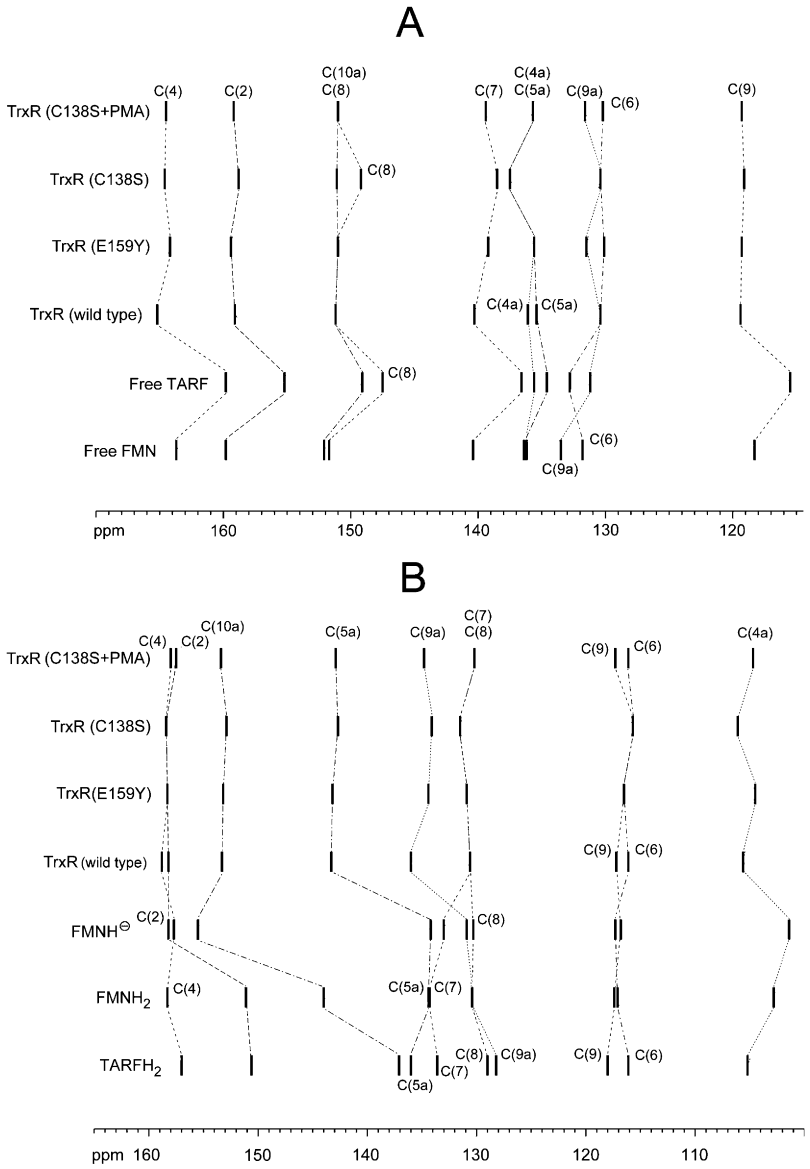

Fig. 7. Correlation diagrams of ^{13}C-NMR chemical shifts of flavins in solution and FAD bound to TrxR in the oxidized (A) and reduced state (B).

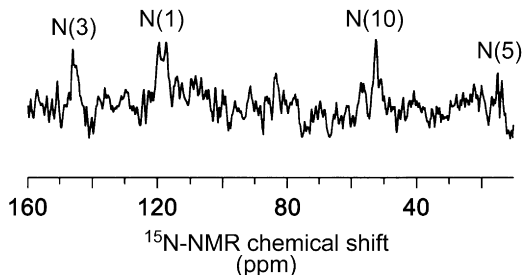

Fig. 8. ^{15}N-NMR spectrum of wild-type TrxR reconstituted with [U-^{15}N$_4$]FAD in the reduced state.

affected carbon atoms, in comparison with both $TARFH_2$ and $FMNH_2$, are C(5a), C(7), C(9a) and C(10a), and as already mentioned above, C(2) (Fig. 7B). With respect to the ^{13}C chemical shifts of $FMNH_2$, C(2), C(5a), C(9a) and C(10a) of wild-type and mutant enzymes are considerably downfield shifted (π-electron density decrease), whereas C(7) is upfield shifted (π-electron density increase). The resonances due to C(8), C(6) and C(9) are similar to those of $FMNH_2$ except for the mutant C138S where C(8) is downfield shifted, and C(6) and C(9) are upfield shifted. The signal of C(4a) is downfield shifted in all preparations.

The chemical shifts of the methylene group at the N(10) atom of the enzyme preparations are further downfield shifted compared with those of the oxidized enzymes and follow the trend of the nitrogen atom, already mentioned above. In comparison with the oxidized enzyme preparations, the chemical shifts due to the C(10δ) atoms are practically unaffected by reduction of the enzymes, and those of the C(10ε) atoms are little affected. In contrast, the chemical shifts of the C(10β) and the C(10γ) atoms are downfield and upfield shifted, respectively (Table 4, Fig. 7B).

Table 5. Relevant chemical shift differences (Δδ, p.p.m.) between ^{13}C and ^{15}N-NMR signals of FAD bound to wild-type TrxR and mutant proteins in the oxidized and reduced states.

	Chemical shifts of wild-type TrxR (in p.p.m.)		Δδ (p.p.m.)					
			E159Y		C138S		C138S + PMA	
Atom	Oxidized	Reduced	Oxidized	Reduced	Oxidized	Reduced	Oxidized	Reduced
C(4a)	136.1	105.6	−0.5	−1.1	+1.4	+0.5	−0.4	−0.9
C(5a)	135.4	143.3	+0.2	−0.1	+2.1	−0.6	+0.3	−0.4
C(8)	151.2	130.6	−0.2	+0.3	−2.0	+0.9	−0.2	−0.4
C(10α)	51.8	53.7	−0.3	−0.2	−1.2	−0.4	−0.7	−0.2
C(10γ)	79.3	76.3	−0.1	−0.8	+0.8	−0.6	0	−0.6
C(10δ)	73.0	73.3	0	−1.2	+1.2	−0.5	0	−0.9
C(10ε)	68.3	69.2	−0.8	−0.1	+0.9	−0.1	−0.1	0
N(1)	183.0	118.3	+0.2	–	+2.6	+0.7	+0.7	+0.7
N(3)	156.6	146.0	−0.4	–	−2.2	+2.8	−0.1	−8.0
N(5)	326.8	14.4	+1.7	–	−4.2	+0.7	+0.8	+0.6
N(10)	161.5	52.1	−1.1	–	−3.7	−1.3	−2.0	−1.1

Discussion

^{31}P-NMR chemical shifts

The ^{31}P-NMR spectra of oxidized TrxR (70.6 kDa) and its mutants show two resonance lines for the pyrophosphate moiety of FAD and are practically identical considering the broad lines. The broad lines (line width 150 Hz) implicate a tight binding of FAD to the apoprotein. Compared with free FAD, the resonance at low field is downfield shifted by ≈ 1 p.p.m., whereas the high field peak is only slightly upfield shifted (0.4 p.p.m.). Kainosho and Kyogoku [32] have assigned the low field peak in the spectrum of free FAD to the 5′-AMP and that at high field to the FMN moiety. It is tempting to assign the two peaks in TrxR to these moieties, however, it is conceivable that these assignments may be reversed due to specific interactions between the apoprotein and the FAD pyrophosphate group. The chemical shifts of the mutants are practically identical to those of the wild-type enzyme indicating an identical configuration of the pyrophosphate group in these enzymes. However, within the class of pyridine nucleotide-disulfide oxidoreductases, the ^{31}P-NMR chemical shifts differ considerably (Table 2). The ^{31}P-NMR spectrum of glutathione reductase shows some similarity with that of TrxR: the low field peak is upfield shifted by 0.5 p.p.m. and the high field peak is downfield shifted by 1 p.p.m. In contrast with conclusions drawn recently from X-ray data on TrxR [10] (resolution 0.2 nm) that the conformation of FAD in oxidized TrxR and in glutathione reductase is virtually identical (r.m.s. deviation between the two structures of 0.036 nm), the NMR data imply that the configuration of the pyrophosphate moiety of FAD in the two enzymes differs. It is known that ^{31}P chemical shifts are very sensitive towards differences in the O-P-O bond angles [33]. It can therefore be expected that small differences in the interaction between the pyrophosphate group of FAD and the apoprotein, thereby influencing the dihedral angle of the O-P-O bond, lead to (relatively large) differences in the ^{31}P-NMR spectra. Such subtle differences can probably not be detected in the X-ray data of TrxR at the current resolution.

In reduced TrxR the ^{31}P-NMR spectrum shows one apparent resonance peak with a line width of ≈ 300 Hz that is twice that observed in the spectrum of oxidized enzyme. Compared with the spectrum of oxidized TrxR, the low field peak in the spectrum of reduced TrxR is upfield shifted by 0.6 p.p.m. and the high field peak is downfield shifted by 1.7 p.p.m. (Table 2). Although a relatively large number of FMN- and FAD-containing flavoproteins have been studied by ^{31}P-NMR [17], only glucose oxidase [34] and wild-type as well as mutant electron transfer flavoproteins from different sources [35] have been investigated in the oxidized and two-electron reduced state. These spectra are affected only slightly in the transition from oxidized to reduced enzyme, suggesting little structural change of the pyrophosphate moiety between the two redox states. Therefore, the large change observed in the ^{31}P spectrum of TrxR in the reduced state, as compared with that of the oxidized state, indicates a considerable configurational change of the pyrophosphate group of FAD in TrxR, associated with a conformational change in this binding region. In keeping with the above mentioned (tentative) assignment of the FMN and AMP moieties of FAD in the ^{31}P spectrum, one could then conclude that the FMN moiety of FAD is affected much more than the AMP moiety upon reduction. The data also demonstrate that mutations, as introduced in the current mutants, have practically no influence on the pyrophosphate configuration as compared to that of native protein.

^{13}C-NMR chemical shifts of the ribityl side chain

Compared with FMN, the ^{13}C chemical shifts of the side-chain carbon atoms are variably affected in oxidized protein-bound FAD. The ^{13}C chemical shifts of free oxidized FAD have been determined recently: 49.2 p.p.m. for C(10α), 71.3 p.p.m. for C(10β), 74.5 p.p.m. for C(10γ), 73.0 p.p.m. for C(10δ) and 69.3 p.p.m. for C(10ε) (unpublished results). The chemical shifts of the carbon atoms C(10α,β,γ,δ) in FAD resemble those of free FMN (Table 3, Fig. 3A). Only the chemical shift due to C(10ε) (downfield shift) differs considerably from that of FMN, because

C(10ε) is under the influence of the pyrophosphate group in FAD. The resonances of the C(10β,δ) atoms in FAD are either unaffected or are affected very little upon binding to the protein, indicating similar hydrogen bonding interaction with the hydroxyl groups and/or configuration of the carbon atoms as in free flavin. The chemical shift due to C(10ε) is upfield shifted by 1 p.p.m. in comparison to that of free FAD, ascribed to a (small) configurational change of the O-P-O-P bonds in the protein-bound state. The C(10γ) atom undergoes a large downfield shift of ≈ 5 p.p.m., implicating strong interactions with the apoprotein by hydrogen bonding. The downfield shift of the C(10α) methylene group reflects the specific configuration of this atom in protein-bound FAD. Among the enzymes investigated the chemical shifts of the C(10γ,δ,ε) atoms (downfield shift) and the C(10α) atom of C138S are the most influenced. This could be due to a change in the hydrogen bonding pattern at the C(10γ,δ) atoms and a configurational change at the two other atoms, as compared with the wild-type and the other mutant enzymes.

In the reduced enzymes the chemical shifts of C(10γ,δ) are upfield shifted and those of C(10α,β,ε) are downfield shifted. C(10α) and C(10γ) undergo the largest shifts. The data indicate a rearrangement of the hydrogen bonding pattern at the C(10γ) atom. The largest shifts are exhibited by the side-chain carbon atoms of C138S, except for the C(10ε) atom which is not affected at all.

The conclusion drawn from the ^{31}P-NMR spectra with regard to the conformational change induced by reduction of the oxidized enzyme is further corroborated by certain ^{13}C chemical shift changes of the ribityl moiety of FAD.

The above interpretations are fully supported by X-ray data. In oxidized wild-type enzyme [10] the hydroxyl group of the C(10δ) atom is hydrogen bonded to one water molecule whereas five hydrogen bonds are formed with that of the C(10γ) atom. In the reduced enzyme [12] the hydrogen bonding pattern at the C(10γ) atom is altered and a hydrogen bond is formed at the C(10β) atom. In addition the torsion angle about the N(10)–C(10α) bond is changed.

^{13}C- and ^{15}N-NMR chemical shifts of the isoalloxazine ring

The ^{15}N chemical shifts of the N(1) and the N(5) atoms (β-type nitrogen) in oxidized enzymes are upfield shifted with respect to TARF. The upfield shifts exceed those observed in FMN. This indicates the presence of strong hydrogen bonding interactions between these atoms of protein-bound FAD and the apoprotein. If the N(3)H group (α-type nitrogen) would also be involved in hydrogen bonding then a small downfield shift of the corresponding resonance is expected. This is not the case, instead the resonance is even further upfield shifted than that of TARF. At a first glance this would indicate the absence of a hydrogen bond at this position in protein-bound FAD. The upfield shift could, however, be explained by a transfer of π-electron density from the highly sp^2 hybridized N(1) atom onto the N(3) atom. This interpretation is supported by the coupling constant of ≈ 87 Hz for the N(3)H group. It has been shown that ^{1}J-coupling constants between ^{15}N and ^{1}H are generally governed by the Fermi-contact term [36]. Based on this fact a semiempirical relationship between the experimental ^{1}J(^{15}N–^{1}H) constants and the hybridization of the corresponding nitrogen atom has been deduced [36,37] (%s = −0.43 × ^{1}J(N − H) − 6 [37]). From the coupling constant of 87 Hz the s-character of the hybrid orbital is calculated to be ≈ 31% which is less than that expected for a fully sp^2 hybridized nitrogen atom. Since a slight increase in π-electron density at N(3) causes a larger upfield shift than a downfield shift by a hydrogen bond, the probable hydrogen bond between N(3)H and the apoprotein is masked (opposing effects).

The N(10) atom in oxidized protein-bound FAD exhibits a rather high degree of sp^2 hybridization, somewhat less than that in FMN, i.e. the N(10) atom in FMN is somewhat more in the molecular plane than that of FAD in TrxR. An excellent correlation (R^2 = 0.993) exists between the ^{15}N chemical shift of the N(10) atom of protein bound FAD in all enzymes studied in this paper and the corresponding ^{13}C chemical shift of the C(10α) atom (data not shown), demonstrating structural differences between native and mutant enzymes, as also manifested by the ^{13}C chemical shifts due to the ribityl side chain (see above). A similar correlation (R^2 = 0.993) has been noticed previously in flavin model studies (data not shown) [27]. Of special interest is the fact that almost parallel lines are obtained for the two sets of data, separated by ≈ 3.5 p.p.m., owing to a downfield shift of the ^{13}C resonances due to the methylene group in protein-bound FAD. This downfield shift reflects the different configuration of the methylene group between free and protein-bound flavin. It would be of interest to see if a similar correlation exists within a certain group of flavoproteins or among flavin-dependent proteins. However, such data are currently not available.

The carbonyl groups at C(2) and C(4) are strongly polarized, that at C(4) exceeding that of FMN. This indicates strong hydrogen bonding interactions between these groups of protein-bound FAD and the apoprotein. The hydrogen bond at C(2)O is most probably stronger than indicated by the corresponding chemical shift, because the partial positive charge on N(1), originating from the strong hydrogen bonding interaction with the apoprotein, leads to an upfield shift of the neighbouring carbon atoms (α-effect [38]), of C(2) and C(10a).

The hydrogen bonding interactions between the prosthetic group and the apoprotein in oxidized TrxR are, as deduced from the NMR data, shown in Fig. 9. It should,

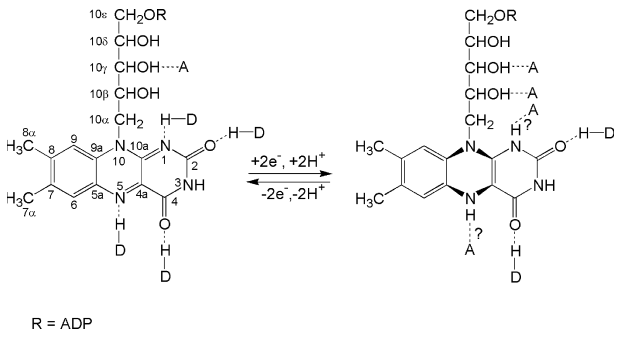

Fig. 9. Structure of FAD bound to TrxR in the oxidized and reduced state. D, proton donor amino acid of TrxR; A, proton acceptor amino acid of TrxR.

however, be noted that the strength of the hydrogen bonding interactions in native and mutant proteins show subtle differences. This is especially manifested by the chemical shifts of the atoms C(2), N(1), N(3), N(5) and N(10) in C138S. The hydrogen bonding pattern observed by NMR are in good agreement with the X-ray data [10], except for the interaction between N(1) of FAD and the apoprotein where the crystal structure revealed a marginal interaction and the NMR data a strong one.

Polarization of the two carbonyl groups by hydrogen bonding leads to a downfield shift of the resonances due to C(8), C(9a) and C(10a) and an upfield shift of that due to C(6) [38]. The lesser than expected downfield shift of C(10a) can be explained by the α-effect caused by the partial positive charge on N(1). The unexpected upfield shift of C(9a) in native enzyme and the mutant C138S (Table 3) could be ascribed to a slightly out-of-molecular-plane of this atom. Such an effect has been observed in free flavins [25] and in *Anabaena 7120* flavodoxin [39] by ^{13}C-NMR.

The chemical shift changes of the C(4a), C(5a), C(7) and C(9) atoms with respect to TARF can be explained by the high degree of sp^2 hybridization of the N(10) atom. The partial positive charge on N(10) is delocalized mainly onto C(9) in all enzyme preparations, whereas the chemical shifts of C(4a) and C(5a) and C(7) parallel that of N(10), i.e. follow the degree of increasing sp^2 hybridization of N(10) in the order C138S, C138S + PMA, E159Y and native protein. However, C(7) receives increasingly more of the partial positive charge (downfield shift) with increasing sp^2 hybridization, whereas π-electron density is transferred onto C(4a) and C(5a) (upfield shift) with increasing sp^2 hybridization of N(10).

The C(2), C(4), C(4a) and C(10a) atoms in oxidized lipoamide dehydrogenase, glutathione reductase and mercuric reductase have been studied by ^{13}C-NMR [17]. In comparison with wild-type TrxR, hydrogen bonding interactions with the C(2)O and C(4)O functions are also observed in the three related enzymes. The hydrogen bond to C(4)O in TrxR is considerably stronger than that in the other enzymes, whereas that with the C(2)O group is about the same as that in glutathione reductase and mercuric reductase, but weaker in lipoamide dehydrogenase. Moreover, the π-electron density at the C(10a) centre is slightly decreased in the three related enzymes in comparison with TrxR, whereas that at the C(4a) atom is decreased in lipoamide dehydrogenase and in mercuric reductase, and increased in glutathione reductase with respect to TrxR.

Compared to $FMNH_2$ and $TARFH_2$ the ^{15}N chemical shifts of all four nitrogen atoms of reduced enzymes are upfield shifted. The two pyrrole-like nitrogen atoms, N(1) and N(3), are the least affected and resonate at fields comparable to those of $TARFH_2$. The ^{15}N chemical shift of the N(1) atom differs greatly from that of ionized reduced flavin (Table 5). This fact already proves that the N(1) atom carries a proton which is supported by the observation of a coupling constant of ≈ 100 Hz. The protonation of the N(1) group in TrxR has also been implicated by a previous potentiometric study [40]. The s-character calculated from the coupling constant for the N(1) atom yields a value of 34%, demonstrating the high degree of sp^2 hybridization of the N(1) atom. The upfield shift of the resonance due to N(1) in the enzymes, as compared to that of $TARFH_2$, could be ascribed to a small decrease in the sp^2 hybridization of the atom. An alternative explanation is that N(1)H in $TARFH_2$ is (partially) hydrogen bonded, because reduction has been achieved in the NMR tube by vigorously shaking a chloroform solution of TARF with an aqueous solution containing dithionite, yielding a water-saturated chloroform solution. This interpretation explains the fact that the coupling constant for N(1)H in $TARFH_2$ could not be observed at room temperature but required lower temperatures [41]. Therefore, it is concluded that no hydrogen bond exists between the N(1)H group of protein-bound $FADH_2$ and the apoprotein. With respect to the protonated N(1) atom, the reduced enzymes studied in this paper represent so far exceptional cases in published NMR data on flavoproteins [17], where it was found that the N(1) atom in all flavoproteins studied is ionized, with the exception of riboflavin binding protein [42].

The N(3) atom of reduced enzymes resonates at higher field than that of $TARFH_2$. A coupling constant could not be determined with certainty (broad line). From the resonance position of the N(3) atom one could conclude the absence of a hydrogen bond at this position, as is probably also the case in the oxidized enzyme, except it would be masked by extra π-electron density delocalized from N(1) (highly sp^2 hybridized) to N(3).

The drastic upfield shift of the chemical shift due to N(5) in the reduced enzymes (≈ 44 p.p.m.) reflects a π-electron density increase at this position, and a corresponding drastic increase in sp^3 hybridization. From the coupling constant of 77 Hz the s-character of N(5) is calculated to be 26%, in agreement with the high sp^3 hybridization of the atom. From these data, no conclusion can be drawn regarding the presence or absence of a hydrogen bond to the N(5)H group of $FADH_2$ bound to the apo TrxR.

Like the N(5) atom, the chemical shift of the N(10) atom in reduced enzymes is also upfield shifted further (≈ 20 p.p.m.) than that of $TARFH_2$. This fact can be ascribed to an increased π-electron density at the N(10) atom in comparison to that of free flavin, and, consequently, an increased sp^3 hybridization of the N(10) atom results.

As compared with $FMNH_2$, the ^{13}C chemical shifts due to C(4), C(8) and C(9) of the enzymes are least affected by reduction, and those of C(6) and C(7) are upfield shifted by a π-electron density increase coming from N(5) and N(10), respectively. In contrast the chemical shifts of C(2), C(4a), C(5a), C(9a) and C(10a) experience a large downfield shift upon reduction. The downfield shift of the C(2) atom cannot be ascribed solely to hydrogen bonding, but is related to the drastic π-electron density decrease at C(10a) (see below) which is partially compensated by π-electron density withdrawal from C(2). As the N(5) and the N(10) atoms are highly sp^3 hybridized, these atoms withdraw π-electron density from the pairs C(10a)/C(9a) and C(5a)/C(4a), respectively, leading to parallel downfield shifts of the corresponding ^{13}C chemical shifts, as observed in free reduced reduced flavins [27]. The chemical shift due to C(4a) is downfield shifted less than expected because it is under opposing effects, i.e. π-electron donation from N(10) and withdrawal from either N(1) or N(3).

The chemical shifts due to C(2) and C(4) indicate strong hydrogen bonding interactions of protein-bound $FADH_2$ with the apoprotein (Fig. 9). The chemical shift of N(1)

suggests the absence of a hydrogen bond at this position in the protein. With regard to the N(3)H group the NMR data are ambiguous: Furthermore, current NMR data on flavins do not yield any information about the possible involvement of the N(5)H group in hydrogen bonding in reduced TrxR. This is a consequence of the fact that no reference value is available for a (non)-hydrogen bonded N(5)H group of free reduced flavin possessing a high degree of sp^3 hybridization. However, according to the X-ray structure [12], a water molecule (# 668), already present in oxidized enzyme, is still present in reduced enzyme. The hydrogen bonding observed for the two carbonyl groups by NMR are in agreement with the X-ray data.

Based on the above described correlation between the chemical shifts of the N(5) and the N(10) atoms with those of the pairs C(10a)/C(9a) and C(4a)/C(5a) in free reduced flavin, a concept was developed to calculate the degree of hybridization of the two nitrogen atoms from the ^{13}C chemical shifts of the carbon pairs [27]. This concept works well for free reduced neutral flavins but did not prove useful in the application to reduced flavoproteins. The reason for this became obvious as the number of flavoproteins investigated by NMR increased and it became evident that most reduced flavoproteins are ionized at N(1) [17]. Nevertheless, the concept should also hold for flavoproteins, although it must be considered that the interactions between the cofactor and the apoflavoproteins are much more complex than those between free flavin and solvent, leading to a different perturbation of the π-electronic structure of protein-bound flavin. Plotting the corresponding ^{13}C chemical shifts of the available reduced flavoproteins [17] against the corresponding ^{15}N chemical shifts yields a fair correlation ($R^2 = 0.8$–0.9) for C(5a) and C(9a), except for the C(10a) and the C(4a) atom. The latter atom is under opposing effects in flavoproteins as outlined above and is therefore not a useful parameter for such exercises. As the N(5) atom is practically independent of the ionization state of N(1) in reduced flavin [27], the available ^{13}C chemical shifts of the C(10a) atom of various flavoproteins have been corrected for the influence of the negative charge of N(1) (electric field effect), i.e. by −11.5 p.p.m. (155.5 p.p.m. − 144.0 p.p.m. for $FMNH^-$ minus $FMNH_2$, respectively, see Table 5). Although this approach should be used cautiously, the result (data not shown) is very satisfactory ($R^2 = 0.97$): it demonstrates the correlation between the chemical shift of the C(10a) atom with that of the N(5) atom. Therefore, the data could be used to estimate the ^{15}N chemical shift of the N(5) atom where only the ^{13}C chemical shift of the C(10a) atom is known, as is the case for several flavoproteins [17]. The correlations found now allows us to determine, with some confidence, the endocyclic angle ϕ of the N(5) and the N(10) atom of reduced flavoproteins by the method developed by Moonen *et al.* for free reduced flavins [27]:

$$\varphi = \frac{\delta(C_{i,sp^3}) - \delta_{obsd}(C_i)}{\delta(C_{i,sp^3}) - \delta(C_{i,sp^2})} \times 10.5^\circ + 109.5^\circ \qquad (1)$$

where, C_i is C(9a) or C(10a) for the calculation of the endocyclic angle of N(5), $\delta(C_{i,sp^3})$ and $\delta(C_{i,sp^2})$ are the corresponding limit chemical shifts of carbon atom C_i for a sp^3 and sp^2 hybridized nitrogen atom, respectively. The endocyclic angle for a sp^3 hybridized nitrogen atom is 109.5°. The difference in angles between a sp^2 and sp^3 hybridized nitrogen atom is 10.5°. For the calculation of the endocyclic angle of N(5) in reduced TrxR, the published chemical shifts [27] of the C(10a) atom of 1,3,7,8,10-pentamethyl-1,5-dihydroisoalloxazine [compound 5b in [27] (138.4 p.p.m)] and of 1,3,7,8,10-pentamethyl-5-acetyl-1,5-dihydroisoalloxazine [compound 5e in [27] (150.8 p.p.m)] were used as limit chemical shifts for a sp^2 and sp^3 hybridized nitrogen atom, respectively, and 153.4 p.p.m. for C_i [C(10a) of TrxR, Table 5]. The calculation yields an endocyclic angle of 107.3° for the N(5) atom in reduced TrxR. Similarly, the endocyclic angle of the N(10) atom was calculated from the chemical shifts of the C(5a) atom using the following limit chemical shifts [27]: 3,7,8-trimethyl-1,10-ethano-5-acetyl-1,5-dihydroisoalloxazine [compound 5f in [27] (124.9 p.p.m.)] and 1,3,5,7,8,10-hexamethyl-1,5-dihydroisoalloxazine [compound 5c in [27] (141.6 p.p.m.)] for a sp^2 and sp^3 hybridized nitrogen atom, respectively, and 143.5 p.p.m. for C_i [C(5a) of TrxR, Table 5]. The calculated endocyclic angle ϕ for the N(10) atom was found to be 108.3°. These calculations are in excellent agreement with the high degree of sp^3 hybridization of both nitrogen atoms as exhibited by the corresponding ^{15}N chemical shifts, and the coupling constant of the N(5)-H group yielding a calculated s-character of ≈26%. These data imply that reduced $FADH_2$ in TrxR is bent along the N(5) and N(10) axis. Using Eqn 1 and the limit ^{13}C chemical shifts of reduced TrxR (153.4 p.p.m) and of salicylate hydroxylase (151.7–11.5 = 140.2 p.p.m. [17]), for the C(10a) atom, the endocyclic angle of N(5) for a number of flavoproteins was calculated (data not shown). A good correlation ($R^2 = 0.97$) is found so that the method can also be used for flavoproteins, albeit with caution. For the N(10) atom less satisfactory results were obtained. From the X-ray structure endocyclic angles of 115.7° for N(5) and 115.8° for N(10) have been calculated [12]. These values are probably less accurate than those deduced from NMR data, owing to the current resolution of the X-ray data. However, from the X-ray data it was concluded that the flavin in reduced TrxR is bent along the N(5)–N(10) axis of the molecule. This conclusion is confirmed by the current NMR data.

This study was also undertaken to explore the possibility of identifying the two earlier proposed conformations of the wild-type TrxR, i.e. the FR and FO conformations (see Introduction) [9,11]. In subsequent studies the possible existence of the two conformations was indicated by (time-resolved) fluorescence studies on oxidized TrxR [14,20]. It has been mentioned several times in this paper that the chemical shifts of some atoms due to flavin in the mutant C138S show larger deviations with respect to wild-type TrxR and the other mutants studied. For an easy comparison the relevant chemical shift differences (Δδ) of the mutants with respect to wild-type TrxR are collected in Table 5. The data demonstrate that the chemical shift differences of several atoms of protein-bound flavin in C138S deviate considerably more from wild-type enzyme and more than the mutants E159Y, and C138S + PMA. The four nitrogen atoms in C138S are the most affected, but also some side-chain carbon atoms and the π-electron

distribution in the isoalloxazine ring of flavin are altered. The data indicate that a change of the conformation of TrxR from FR (wild-type TrxR, E159Y and C138 + PMA) to FO (C138S) affects a large part of the protein-bound FAD moiety. The current NMR data confirm the previous proposal and reveal further details on the sub-molecular events in the conformational changes. The data do not, however, provide any evidence for an equilibrium between the two conformations, as suggested previously [20]. If an equilibrium did exist then the minor species would have to be present in excess of 20% to be observed owing to the width of the resonance lines. In addition the downfield, and respectively, the upfield shifted resonances of the N(5) and N(10) atoms in E159Y and C138S + PMA can probably be ascribed to the presence of the additional aromatic moieties in the two mutants, located close to the protein-bound FAD, or alternatively to a small structural difference between the two mutants and wild-type enzyme.

In the four-electron reduced (flavin and disulfide) state of TrxR the pattern of the differences in chemical shifts between wild-type TrxR and those of the mutants has changed somewhat in comparison to those of the oxidized state (Table 5). All, except for N(3), chemical shift differences from wild-type TrxR are considerably decreased in C138S whereas some chemical shift differences are increased in the two other mutants. From this one could conclude that the structures of the wild-type enzyme and that of C138S have become similar in the reduced state, but not identical. Since C138S remains in the FO conformation in the reduced state, the NMR data strongly suggest that the conformation of wild-type enzyme has changed from FR to FO upon reduction of the enzyme. This conclusion is in agreement with X-ray data showing that wild-type enzyme is in the FO conformation [12].

It has been proposed [12] that the high sp^3 hybridization of the N(10) and the N(5) atoms in reduced TrxR could be ascribed to the close proximity of the dithiol groups to the reduced flavin. This is a reasonable explanation. However as the four-electron reduced state of TrxR is catalytically inactive [1] and has therefore to be considered as an artefact, it would be interesting to study the catalytically competent intermediate (reduced flavin with the disulfide group still intact) in order to evaluate the proposal.

In conclusion it can be stated that NMR spectroscopy using TrxR with stable-isotope labelled cofactor provides unique insights into the electronic structure of the cofactor and its interactions with the apoprotein. The present study can be taken as a model for further characterization of wild-type and mutant TrxR from other organisms, as well as a model for biophysical studies on flavoproteins in general.

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