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Backbone dynamics of oxidized and reduced *D. vulgaris* flavodoxin in solution

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Summary

Recombinant *Desulfovibrio vulgaris* flavodoxin was produced in *Escherichia coli*. A complete backbone NMR assignment for the two-electron reduced protein revealed significant changes of chemical shift values compared to the oxidized protein, in particular for the flavine mononucleotide (FMN)-binding site. A comparison of homo- and heteronuclear NOESY spectra for the two redox states led to the assumption that reduction is not accompanied by significant changes of the global fold of the protein. The backbone dynamics of both the oxidized and reduced forms of *D. vulgaris* flavodoxin were investigated using two-dimensional ^{15}N-^{1}H correlation NMR spectroscopy. T_1, T_2 and NOE data are obtained for 95% of the backbone amide groups in both redox states. These values were analysed in terms of the 'model-free' approach introduced by Lipari and Szabo [(1982) *J. Am. Chem. Soc.*, **104**, 4546–4559, 4559–4570]. A comparison of the two redox states indicates that in the reduced species significantly more flexibility occurs in the two loop regions enclosing FMN. Also, a higher amplitude of local motion could be found for the N(3)H group of FMN bound to the reduced protein compared to the oxidized state.

Introduction

It is well accepted that the internal mobility of proteins is of importance for their biochemical activity and function. Heteronuclear 2D NMR experiments are powerful tools for investigating the motion of particular molecular parts in proteins (Kay et al., 1989,1992; Clore et al., 1990a,b; Dayie and Wagner, 1994). Furthermore, it is possible to gain insight into the relation between protein dynamics and biochemical activity.

Desulfovibrio vulgaris flavodoxin is a member of a group of small microbial electron-transfer proteins containing a molecule of noncovalently bound flavine mononucleotide (FMN) as the redox active prosthetic group (Mayhew and Ludwig, 1975; Mayhew and Tollin, 1992). It consists of 147 amino acid residues and has a molecular mass of 16.3 kDa (Dubourdieu and Fox, 1977). The protein-bound FMN occurs in three redox states (Fig. 1): an oxidized form, a one-electron reduced form and a fully reduced form (Simondson and Tollin, 1980). In most physiological reactions, the flavodoxins shuttle between the one-electron reduced state and the fully reduced state while mediating exclusively electron transfers between other redox proteins. Upon binding to the flavodoxin apoprotein, the redox potential of FMN is shifted from –172 mV (Draper and Ingraham, 1968) to values between –320 and –518 mV (Deistung and Thorneley, 1986; Vervoort, 1991). Since very few structural changes are observed upon reduction of flavodoxin, we concentrated on investigating the differences of flexibility between the oxidized and the fully reduced forms of *D. vulgaris* flavodoxin.

The 3D solution structure of oxidized *D. vulgaris* flavodoxin (Fig. 2) has been described as a central five-stranded parallel β-sheet, surrounded by two pairs of α-helices on either side (Knauf et al., 1996). The three regions between residues 9–13, 58–70 and 93–107 are considered to be important for the binding of the prosthetic group.

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Materials and Methods

Protein expression, purification and isotopic labelling

Recombinant flavodoxin from *D. vulgaris* was expressed in *E. coli* strain TG2. The bacteria contained the plasmid pDKFl300 which carries the gene coding for flavodoxin controlled by a *tac* promotor sequence (Curley et al., 1991). The growth and purification protocol for nonlabelled flavodoxin has been described elsewhere (Curley et al., 1991; Knauf et al., 1993).

For uniform enrichment of the flavodoxin in the ^{15}N isotope, the bacteria were grown on modified minimal medium with $^{15}NH_4Cl$ as the sole nitrogen source. The modified minimal medium contained per litre: 2 g D-glucose, 8 ml glycerol, 8.6 g $Na_2HPO_4 \cdot 2H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 20 mg kanamycine sulphate, 4 mg thiamine and 1 g $^{15}NH_4Cl$. Also, $CaCl_2$, $FeSO_4$ and $MgSO_4$ were added to final concentrations of 0.1, 0.25 and 2.0 mM, respectively. The bacterial growth was followed by optical spectroscopy at 600 nm. The expression of flavodoxin was induced at $A_{600} = 0.6$ by adding isopropyl-β-D-thiogalactoside to a final concentration of 0.02 mM. The isolation and purification procedure is identical to the protocol for the nonlabelled flavodoxin.

Uniform ^{13}C and ^{15}N labelling of the flavodoxin was obtained by growing the bacteria on ^{13}C- and ^{15}N-enriched protein hydrolysate mixture (Celtone-CN, Martek Corporation, Columbia, MD, U.S.A.). The induction of flavodoxin production as well as the isolation and purification of the protein were performed as described for the ^{15}N enrichment.

Sample preparation and reduction of the protein

Flavodoxin solutions in 130 mM potassium pyrophosphate (KPP_i) buffer, pH = 8.3, were concentrated to a final volume of 450 µl by ultrafiltration. Protein concentrations were 5 and 2.5 mM for the ^{15}N- and ^{13}C-^{15}N-labelled samples, respectively. After filling 450 µl of the concentrated protein into an NMR tube, 20 µl of D_2O was added for locking the magnetic field. To obtain the fully reduced form of the protein, a twofold molar excess of dithionite dissolved in the buffer described was added under anaerobic conditions to 470 µl of protein solution in an NMR tube to a final volume of 500 µl. The reduced sample was sealed immediately with a rubber stopper to keep the protein reduced. To prepare nearly identical protein samples for both redox states, a reduced sample was reoxidized by air and degassed again.

NMR experiments for assignment

Unless otherwise stated, NMR spectra were recorded on a Bruker DMX600 spectrometer using a ^{1}H,^{13}C,^{15}N triple-resonance probe, which was equipped with a self-shielded z-gradient coil. The data collection was performed at a sample temperature of 300 K. The spectra were

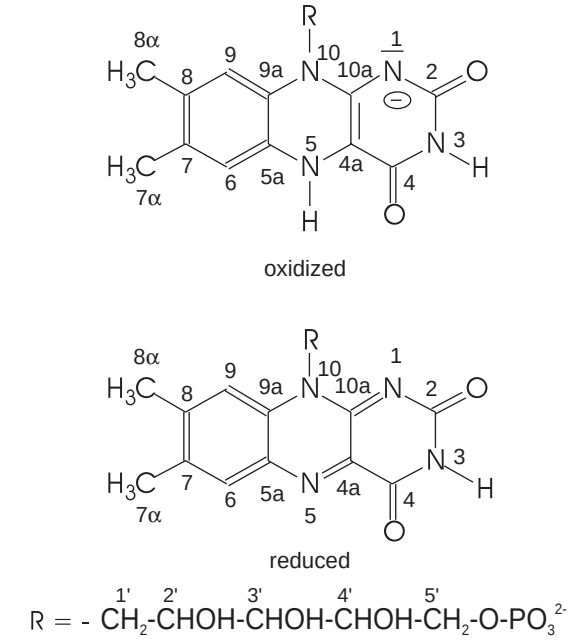

Fig. 1. Structure of the oxidized and reduced prosthetic group FMN at pH = 8.3.

processed and analysed on a Silicon Graphics Personal Iris 4D/35 using the programs UXNMR and AURELIA (Bruker Analytische Messtechnik GmbH, Karlsruhe, Germany) or the TRITON (G.W. Vuister and R. Boelens, Utrecht University, Utrecht, The Netherlands) software package. In order to obtain sequential assignments of the backbone resonances, an HNCA experiment was carried out in the constant-time version described by Grzesiek and Bax (1993). One hundred and twenty-eight t_1 ($^{13}C^{\alpha}$) and 60 t_2 (^{15}N) real increments containing 512 complex data points each were collected. The spectral widths were set to 15.9, 24.5 and 8.0 ppm for F1, F2 and F3, respectively. The carrier frequencies were set to 120 ppm for ^{15}N, and 7.8 ppm for ^{1}H (4.75 ppm for decoupling).

For 3D NOESY-HSQC spectra (Marion et al., 1989; Zuiderweg and Fesik, 1989), time domain data consisted of $256 \times 72 \times 1024$ real data points in t_1, t_2 and t_3, respectively. The acquisition times were 17.6 ms (t_1), 15.4 ms (t_2) and 106.5 ms (t_3) with corresponding spectral widths of 12.25, 31.38 and 8.01 ppm in F1, F2 and F3, respectively. The ^{15}N carrier frequency was placed in the centre of the backbone amide ^{15}N spectrum at 120 ppm. The proton carrier was moved from the frequency of the water resonance to the amide region (8.05 ppm) at the end of the NOESY mixing period. The mixing time of the NOESY-HSQC experiment was set to 140 ms. In the middle of this period, a composite 180° pulse followed by a homospoil pulse was applied to suppress coherence orders other than zero and to improve water suppression. 3D matrices of the NOESY-HSQC measurement were zero-filled to yield a final frequency domain data set of $512 \times 64 \times 1024$ real points in the F1, F2 and F3 dimensions, respectively.

In order to be able to detect the resonances of N3(H) and N5(H) in protein-bound FMN, an HSQC experiment with water suppression by gradient coherence selection was carried out. A total of 1024 t_1 (^{15}N) real increments containing 2048 complex data points each were collected. The spectral widths were set to 117 and 15.4 ppm for F1 and F2, respectively. The carrier frequencies were set to 80 ppm for ^{15}N, and 4.75 ppm for ^{1}H.

NMR experiments for relaxation measurements

In all NMR experiments for ^{15}N relaxation measurements, the ^{1}H carrier frequency was set to 4.75 ppm. Spectral widths were set to 32.8 ppm in the nitrogen dimension and 15.4 ppm in the proton dimension. The spectra were processed and analysed on a Silicon Graphics Personal Iris 4D/35 using the programs UXNMR and AURELIA (Bruker).

T_1 and T_2 measurements

Longitudinal and transverse nitrogen relaxation times were measured using the gradient-selected sensitivity-enhanced pulse sequences described by Dayie and Wagner (1994). These experiments include effective solvent suppression while avoiding saturation of the H_2O resonance with the help of selective 'flip-back' pulses (Grzesiek and Bax, 1993). Series of seven 2D spectra were recorded in each experiment, where the relaxation delays were set to 12.0, 84.4, 181.0, 301.5, 458.3, 663.3 and 904.5 ms for the T_1 measurements, or 9.4, 28.2, 66.3, 170.0, 217.8, 274.6 and 331.4 ms in the experiments designed to measure nitrogen transverse relaxation.

Nuclear Overhauser effect

In order to evaluate the (^{1}H)-^{15}N nuclear Overhauser effect on the amide nitrogen spin, 2D spectra were recorded with and without NOE enhancement by presaturation of the amide protons. ^{1}H presaturation in the NOESY experiments was achieved by applying 120° pulses, spaced at 5 ms intervals, during 3 s prior to the first ^{15}N pulse (Grzesiek and Bax, 1993).

Data evaluation

In the analysis of the oxidized and reduced forms of *D. vulgaris* flavodoxin, 130 and 126 amide groups have been considered, respectively. Information from the remaining resonances was unreliable due to spectral overlap.

In a ^{1}H-(^{15}N) HSQC spectrum, the cofactor FMN in the oxidized form gives rise to one signal due to N3(H) and an additional signal for the reduced species due to N5(H). The remaining noise band from the water signal prevents the N5(H) signal from integration. ^{15}N longitudinal (T_1) and transverse (T_2) relaxation times were evaluated by fitting the intensity decay of corresponding resonances in the series of 2D spectra to a single exponential in dependence of relaxation delays. The data were fitted by a least-squares minimization procedure based on a downhill-simplex algorithm (Press et al., 1988).

Spin relaxation and the model of motion

The values of T_1, T_2 and the heteronuclear NOE for flavodoxin in both redox states are derived by averaging the results from three (T_1, T_2) and two (NOE) independent experiments, respectively. The dynamical parameters of flavodoxin were evaluated using standard expressions (Abragam, 1961; Lipari and Szabo, 1982). The interactions that were taken into account for the nuclear spin relaxation of the amide nitrogen under consideration are the ^{15}N chemical shift anisotropy (CSA) and the dipole–dipole interaction between the amide nitrogen and its directly bound hydrogen. The relevant correlation function of the N-H bond reorientations is taken as a product of the corresponding correlation functions describing the overall tumbling of the protein and the internal motions, $C_i(t)$. The form of $C_i(t)$ used in the present work is the one introduced by Lipari and Szabo (LS) (1982) within the so-called 'model-free' approach, representing the decay of the correlation of internal motion by a one-exponential process:

$$C_i(t) = S^2 + (1 - S^2)\exp(-t/\tau_i)$$

In this approach, the internal dynamics is described in terms of the correlation time τ_i, which is assumed to be much shorter than the correlation time of overall tumbling, τ_c, and the order parameter S characterizing the amplitude of local motion: $S = 1$ for completely restricted motion, while $S = 0$ for unrestricted internal reorienta-

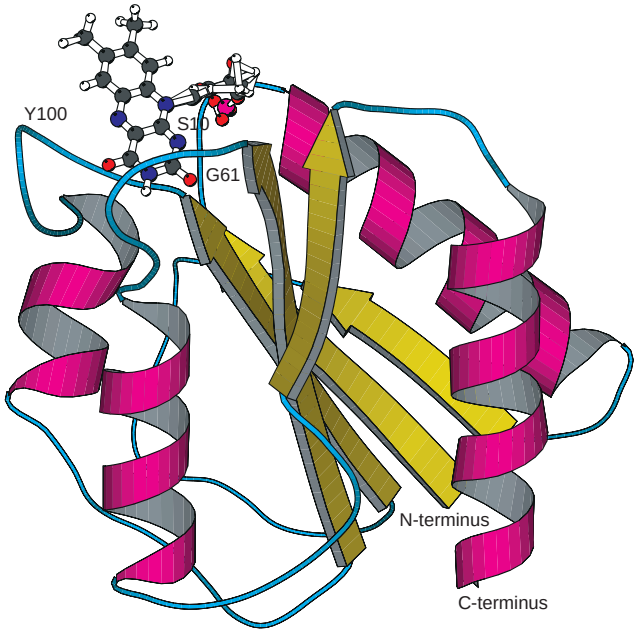

Fig. 2. MOLSCRIPT plot representing the solution structure of *D. vulgaris* flavodoxin obtained from NMR data using the distance geometry algorithm DIANA.

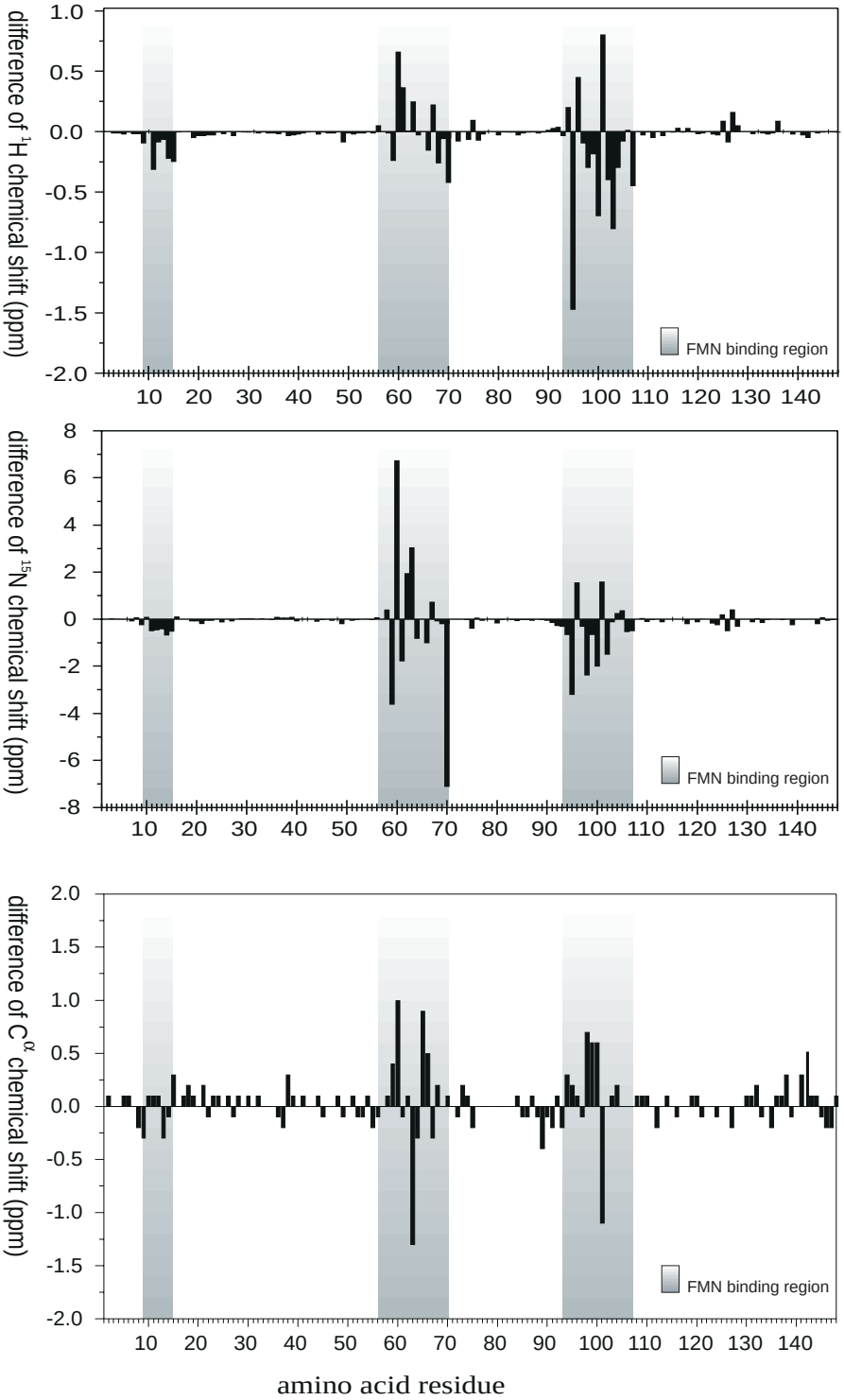

Fig. 3. Diagrams showing the differences of ^{1}H, ^{15}N and $^{13}C^{\alpha}$ chemical shifts between oxidized and reduced *D. vulgaris* flavodoxin. Significant changes occur throughout the FMN-binding region, which is marked in grey.

tions. All calculations were performed under the assumption of isotropic overall tumbling of the protein which may be inferred from the ratio of the three principal components of the inertia tensor as calculated from the high-resolution NMR structure of *D. vulgaris* flavodoxin (1:1.2:1.4).

Evaluation of microdynamical parameters

The microdynamical parameters τ_c, τ_i and S^2 were derived from the minimization of a target function (Fushman et al., 1994):

$$\chi^2 = \sum_{k=1}^{n} ((T_{1k}^{o} - T_{1k}^{c})^2/\sigma_{1k}^2 + (T_{2k}^{o} - T_{2k}^{c})^2/\sigma_{2k}^2 + (NOE_k^{o} - NOE_k^{c})^2/\sigma_{NOE\,k}^2)$$

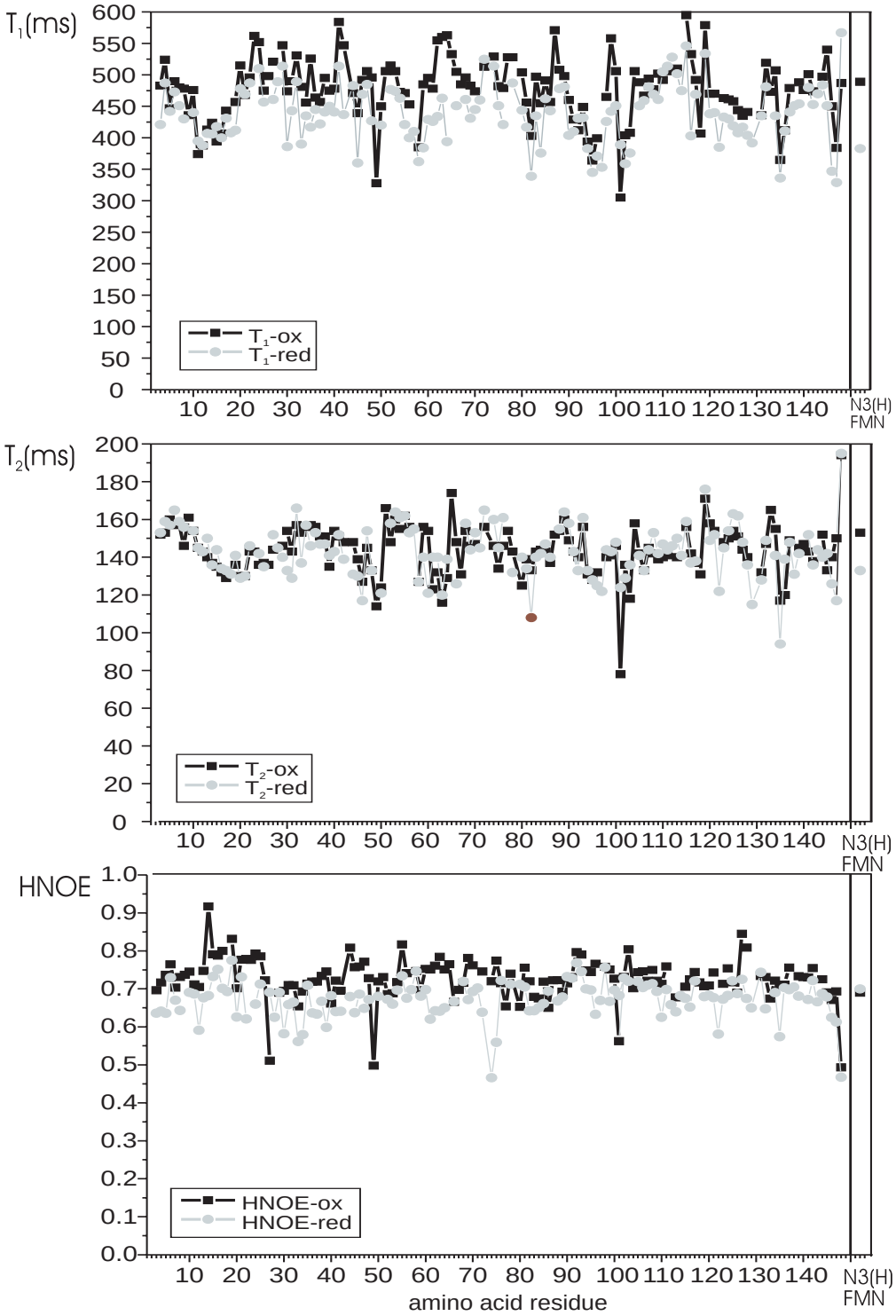

Fig. 4. Experimental T_1, T_2 and (^{1}H)-^{15}N NOE values for oxidized and reduced *D. vulgaris* flavodoxin as a function of residue number.

where the sum runs over all the residues, σ^2_{1k}, σ^2_{2k} and $\sigma^2_{NOE\ k}$ are standard deviations in T_1, T_2 and NOE for the *k*th residue, respectively, and superscripts o and c refer to the observed and calculated values of the relaxation parameters. The overall tumbling time, τ_c, was changed stepwise, being taken to be the same for all the residues, while the parameters of internal motion varied from one residue to the other.

Since the T_2 values observed in CPMG-type experiments (Carr and Purcell, 1954; Meiboom and Gill, 1958) may be subject to an apparent shortening due to processes of conformational or chemical exchange on time

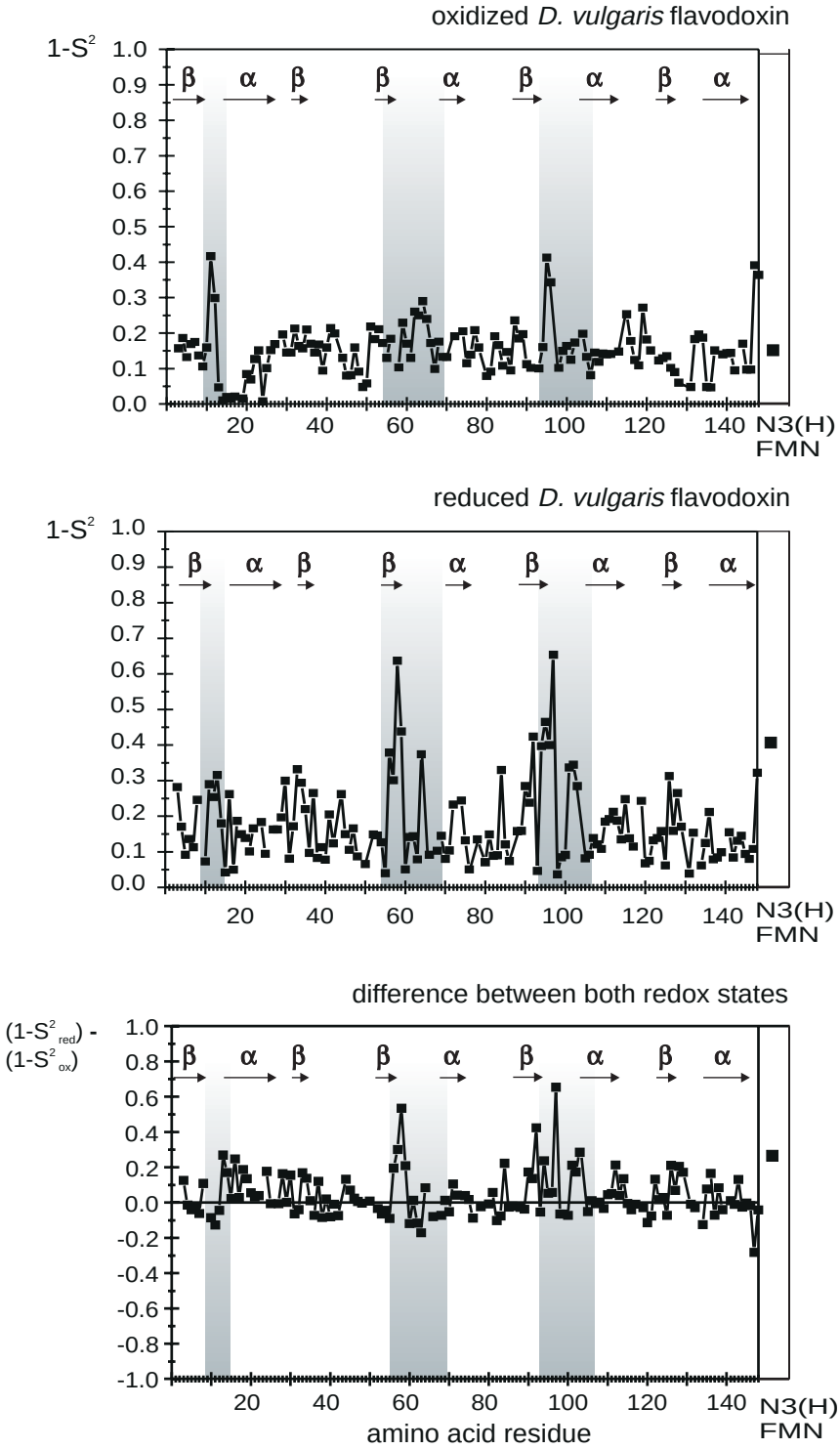

Fig. 5. The calculated $1 - S^2$ values of the amide nitrogens for oxidized *D. vulgaris* flavodoxin and reduced *D. vulgaris* flavodoxin, as well as the differences of calculated $1 - S^2$ values for both redox states.

scales smaller than or comparable to the delay between successive 180° pulses, not all measured T_2 values may be used for calculation of the parameters of internal motion (Bloom et al., 1965; Kay et al., 1989). In order to determine the exchange contributions to the apparent transverse relaxation rate of individual nitrogen spins, a 'selection criterion' based on the analysis of T_1/T_2 ratios was applied (Clore et al., 1990b). Following this criterion, 13 amide groups in the oxidized flavodoxin and nine in the reduced protein showed T_1/T_2 ratios more than one standard deviation larger than the mean value ($\langle T_1/T_2 \rangle$). For these residues, only T_1 and NOE data

were used for the determination of the dynamical parameters.

The overall rotational correlation time τ_c for residues with T_1/T_2 ratios falling within one standard deviation of the mean can be estimated by (Fushman et al., 1994):

$$\tau_c = 1/2\omega_N \times ((6T_1/T_2) - 7)^{1/2}$$

ω_N being the Larmor frequency of ^{15}N. The values of τ_c thus derived are 4.5 ns for the oxidized form of the protein as well as for reduced flavodoxin.

Results and Discussion

Reduction of the protein

The reduction of the protein sample with dithionite is associated with a decrease in the pH value. After complete reoxidation of the protein, the pH was measured to be 7.1. The experimental determination of the pH value for the reduced sample is not possible. Thus, for the pH of the reduced sample $7.1 < pH < 8.3$ has been concluded. This change of pH eases a comparison of chemical shift values for both redox states of *D. vulgaris* flavodoxin, since the assignment of the oxidized protein had been carried out at pH = 7.0 (Knauf et al., 1993). The decrease of the reduction potential of dithionite at lower pH values leads to reoxidation of the reduced protein sample within several days. Thus, it is important to check whether the protein is still reduced after the experiments by visual inspection. The presence of the blue semiquinone form requires a complete purification of the sample, as it is not possible to reduce the flavodoxin again by adding more dithionite.

Comparison of chemical shift values for oxidized and reduced D. vulgaris flavodoxin

A standard assignment procedure comprising a ^{15}N HSQC experiment, a TOCSY-HSQC experiment and an HNCA experiment led to a complete assignment of ^{1}H, ^{15}N and $^{13}C^{\alpha}$ resonances in reduced *D. vulgaris* flavodoxin. Significant changes occur solely for the chemical shift values of ^{15}N and ^{1}H resonances of residues 9–15, 58–70 and 94–106. These areas are loop regions close to the prosthetic group FMN (Fig. 3). A table containing the assignments of the reduced *D. vulgaris* flavodoxin is available from the authors upon request.

Investigation of the N(3)H and N(5)H groups of FMN

The gradient coherence selection technique for water suppression and a ^{15}N spectral width of 117 ppm allowed the detection of the N3(H) and N5(H) resonances of FMN in the reduced form. Figure 1 shows the constitution of the oxidized and reduced FMN. The chemical shift values for oxidized/reduced flavodoxin are $^{15}N(3)$ 159.7/148 ppm, $^{1}H_{(N3)}$ 10.44/8.65 ppm, $^{15}N(5)$ 340/61.5 ppm and $^{1}H_{(N5)}$ –/4.81 ppm.

As in the oxidized state the protein-bound FMN is not protonated at N(5), the nitrogen chemical shifts were obtained from a directly detected ^{15}N spectrum. The N(3)H group of oxidized FMN forms a weak hydrogen

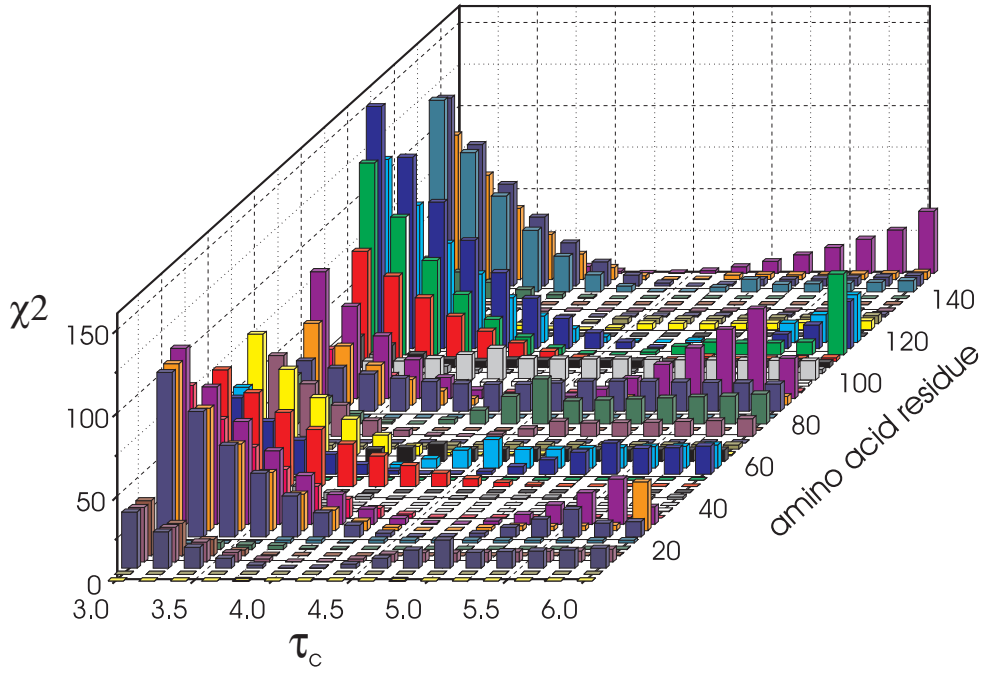

Fig. 6. Diagram showing the error χ^2 as derived from calculations of the order parameter S^2 with different values of the overall correlation time τ_c as a function of residue number. χ^2 is minimal for $\tau_c = 4.5$ ns, the overall correlation time of *D. vulgaris* flavodoxin.

bond with the apoflavodoxin (Vervoort et al., 1985). The upfield shift for the N(3) resonance in the reduced state upon binding to the flavodoxin apoprotein is similar to the one observed for the oxidized form. It was suggested that the hydrogen bond pattern formed with the apoprotein is similar for both reduced and oxidized FMN.

Relaxation data and dynamical parameters

The longitudinal and transverse ^{15}N relaxation times as well as the heteronuclear NOEs are presented in Fig. 4 for both the oxidized and the reduced flavodoxin, respectively. The average level of the NOE values for oxidized flavodoxin is higher than for the reduced protein. This behaviour represents the higher flexibility of reduced flavodoxin compared to the oxidized protein. The similarity of the T_2 values for both redox states shows that there were no impurities due to partial reoxidation present, as the paramagnetic FMN semiquinone would lead to dramatic decreases in the T_2 values (data not shown).

In Fig. 5, the order parameters $(1 - S^2)$ obtained for *D. vulgaris* flavodoxin in both redox states are plotted versus the amino acid sequence. They represent the deviation of S^2 from its complete restriction limit value and therefore increase with increasing degrees of local motion.

Figure 6 presents the overall rotational correlation time τ_c as derived from calculations of the order parameter S^2 with different values of τ_c. The value of τ_c as calculated from T_1/T_2 ratios can be confirmed as the minimum of the error χ^2 can be observed for $\tau_c = 4.5$ ns.

For N(3)H, analysis of the changes in chemical shift values led to the assumption of an unchanged pattern of hydrogen bonding. The analysis of the order parameters $1 - S^2$ (Fig. 5) indicates a higher mobility of N(3)H in the picosecond time scale, instead.

Internal mobility in oxidized flavodoxin

Differences in the internal mobility are found between secondary structure elements and loop regions (Fig. 5). The smallest amplitudes of local motion were obtained for residues belonging to the first α-helix, indicating highly restricted local mobility of these parts of the protein backbone. For the strands of the β-sheet as well as for the remaining α-helical regions, the local mobility is still more restricted than for the loop regions. The local mobility of the FMN-binding site as well as for the C-terminal region is less restricted as follows from rather high $1 - S^2$ values, up to 0.4 for Thr^{11} and Asp^{95}. The B-factors derived from X-ray crystallographic data (Watenpaugh et al., 1972) show higher values for the regions between residues 32–50, 55–63, 74–87 and 92–103 (Fig. 7). In solution no evidence can be found for an increased mobility for residues in the region between residues 32–50. The high B-factor values are probably caused by static disorder or different rigid conformations.

Internal mobility in reduced flavodoxin

The mean value and the amplitudes of local motion for this redox state are higher than those obtained for oxidized flavodoxin (Fig. 5). The main changes in local mobility, compared to the oxidized protein, were observed for the FMN-binding site. Generally, the reduced form of the protein is more flexible, in particular throughout the active site. It can be suggested that increased internal mobility might be important for either the redox reaction or the interaction with other proteins.

A comparison of the order parameters $(1 - S^2)$ with the B-factors from the X-ray structure of the reduced protein (Fig. 7) shows a similar behaviour for the B-factors: the values, typically, are higher for the reduced flavodoxin compared to the oxidized form, especially throughout the

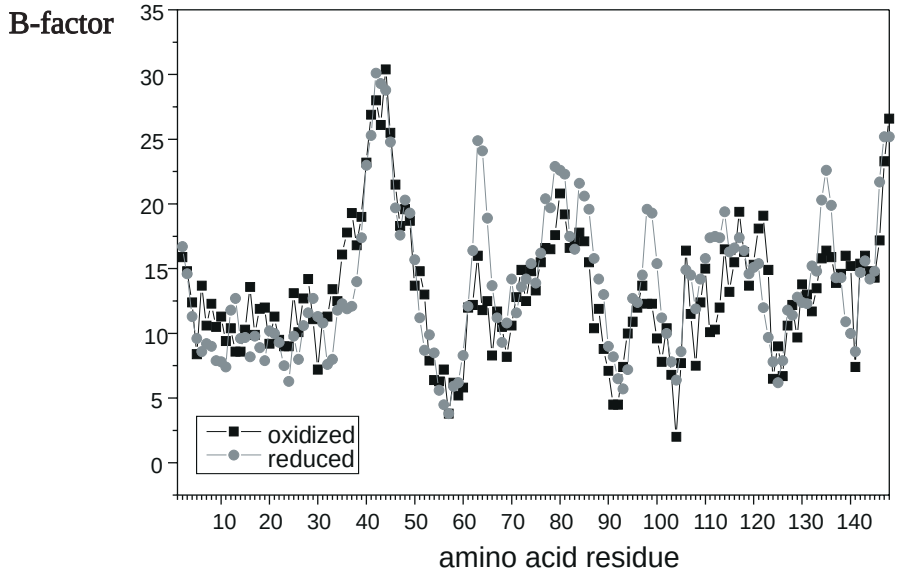

Fig. 7. B-factors of the amide nitrogens (from the crystal X-ray data; Watenpaugh et al., 1972).

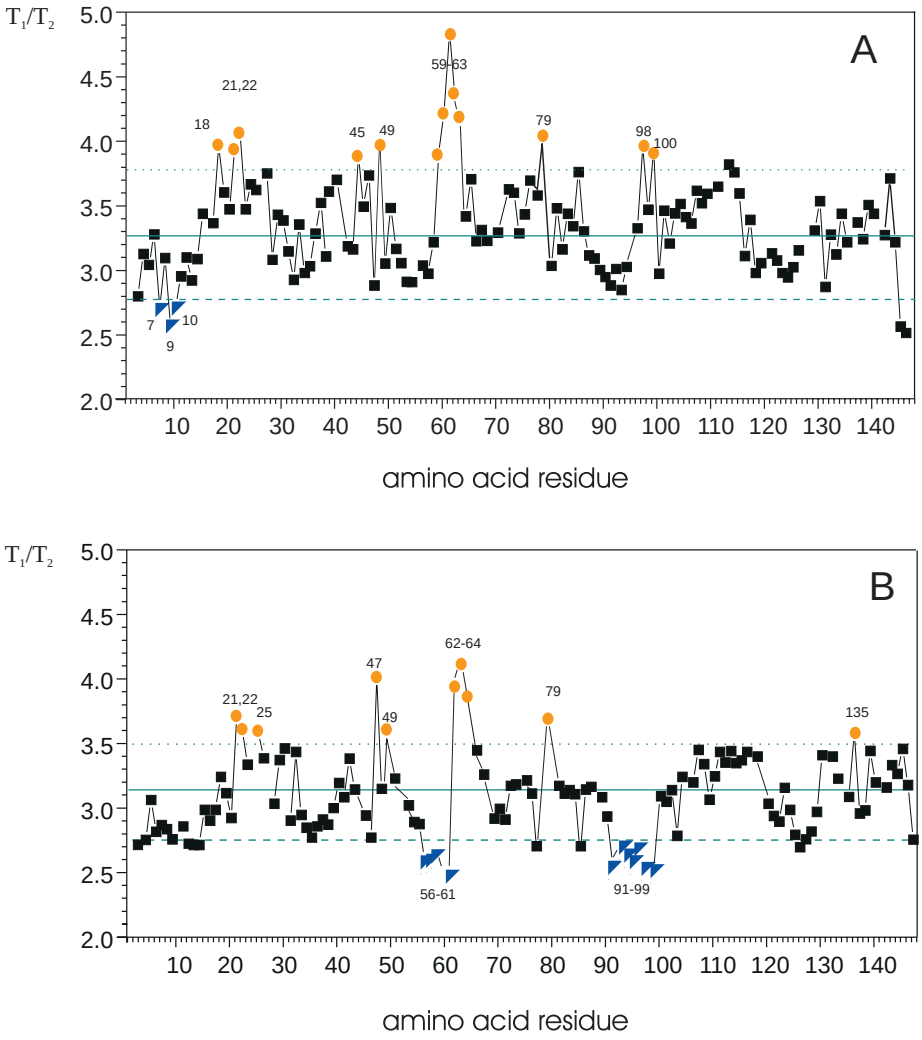

Fig. 8. Ratio of T_1 and T_2 relaxation times for oxidized (A) and reduced (B) *D. vulgaris* flavodoxin as a function of residue number. Circles indicate values higher than one standard deviation above the average whereas triangles indicate values lower than one standard deviation below the average.

loop regions between residues 55–63 and 92–103. As found for the oxidized form of the protein, in solution there should be no higher flexibility for the backbone of reduced flavodoxin for the regions between residues 32–50 and 74–87.

Discussion of T_1/T_2 ratios for oxidized and reduced flavodoxin

Thirteen amide groups in the oxidized flavodoxin (Thr^{18}, Thr^{21}, Glu^{22}, Gly^{45}, Gly^{49}, Thr^{59}–Asp^{63}, Glu^{79}, Tyr^{98} and Tyr^{100}) and nine in the reduced protein (Thr^{21}, Glu^{22}, Gln^{25}, Phe^{47}, Gly^{49}, Asp^{62}–Ser^{64} and Glu^{79}) show T_1/T_2 ratios more than one standard deviation larger than the mean value ($\langle T_1/T_2 \rangle$) and hence may be influenced by chemical exchange processes (Fig. 8).

T_1/T_2 ratios more than one standard deviation below the mean value can be observed for 15 amide groups in the reduced flavodoxin (Gly^{56}–Gly^{61} and Phe^{91}–Glu^{99}) and only for three amide groups in the oxidized protein (Val^7, Gly^9 and Ser^{10}). The presence of local motions on a pico-second time scale for the FMN-binding site of the reduced flavodoxin is responsible for these findings. Thus, the reduction of the flavodoxin leads to significantly higher mobility for the backbone amide groups of the FMN-binding site.

Conclusions

In the present study, an assignment for ^{1}H, ^{15}N and $^{13}C^{\alpha}$ resonances for reduced *D. vulgaris* flavodoxin was performed and compared to the chemical shift values of the oxidized protein. Significant changes in the chemical shift values for ^{1}H, ^{15}N as well as ^{13}C nuclei were observed throughout the FMN-binding region, namely in the loop regions between residues 9–13, 58–70 and 93–107. A comparison of homonuclear NOESY and 3D ^{15}N-edited NOESY-HSQC spectra for the two redox states led to the assumption that reduction is not accompanied by significant changes of the global fold of the protein.

Furthermore, the backbone dynamics of oxidized *D.*

vulgaris flavodoxin as well as the change of the microdynamical parameter S^2 for the reduced form of the protein were investigated by a consistent NMR methodology. From the S^2 values obtained for both the oxidized and reduced forms of *D. vulgaris* flavodoxin, it is evident that reduction changes the mobility of the FMN-binding region of the protein, namely in the regions between residues 56–62 and 91–103. The increase of the parameter $(1 - S^2)$ in these regions for reduced flavodoxin indicates significantly higher mobility compared to the oxidized form of the protein. Furthermore, the analysis of T_1/T_2 ratios suggests the presence of local motions on a time scale comparable to the time of overall tumbling for the flexible FMN-binding region in reduced flavodoxin.

A detailed analysis of the FMN-binding region for both redox states shows that the more flexible regions of the protein in the reduced state are located along the ribityl side chain of the prosthetic group FMN. It can be assumed that flexibility of *D. vulgaris* flavodoxin around the active site plays an important role for electron transfer from the reduced protein to an acceptor molecule.

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